Identification and Characterization of HIPK2 Interacting with p73 and Modulating Functions of the p53 Family in Vivo*

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To study the biological role of p73, a member of the p53 tumor suppressor family, we performed a yeast two-hybrid screen of a human cDNA library. Using a p73α fragment consisting of amino acids 49–636 as bait, we found that p73α is functionally associated with the human homologue of mouse and hamster homeodomain-interacting protein kinase 2 (HIPK2). The hamster homologue, also known as haHIPK2 or PKM, was used for further characterization of interactions between HIPK2 and members of the p53 protein family. Systematic yeast two-hybrid assays indicated a physical interaction between the oligomerization domains of p73α and p53 (amino acid regions 345–380 and 319–360, respectively) and amino acid region 812–907 of haHIPK2. This region of haHIPK2 includes a PEST sequence, an Ubc9-binding domain, and a partial speckle retention sequence and is identical to amino acid residues 846–914 of human HIPK2 (hHIPK2). The interaction was confirmed by glutathione S-transferase pull-down assays in vitro and immunoprecipitation assays in vivo. HIPK2 colocalized with p73 and p53 in nuclear bodies, as shown by confocal microscopy. Overexpression of HIPK2 stabilized the p53 protein and greatly increased the p73- and p53-induced transcriptional repression of multidrug-resistant and collagenase promoters in Saos2 cells but had little effect on the p73- or p53-mediated transcriptional activation of synthetic p53-responsive and p21WAF1 promoters. Stable expression of HIPK2 in U2OS cells enhanced the cispalatin response of sub-G1 and G2/M populations, and it also increased the apoptotic response to cispalatin and adriamycin as demonstrated by fluorescence-activated cell sorter and 4′,6-diamidino-2-phenylindole-staining analyses. HIPK2 potentiated the inhibition of colony formation by p73 and p53. These results suggest that physical interactions between HIPK2 and members of the p53 family may determine the roles of these proteins in cell cycle regulation and apoptosis.

The tumor suppressor protein p53 is one of the most important regulators of cellular growth functions, such as cell cycle arrest, DNA repair, and apoptosis, and is mutated in about 50% of all human tumors (1). Some human tumors containing a normal p53 gene nonetheless exhibit functional inactivation of p53 at the protein level by cellular MDM2 or by viral oncoproteins, such as human papilloma virus E6, SV40 T antigen, and adenovirus E1B. As a transcriptional regulator, p53 modulates expression of various genes in response to cellular genotoxic stresses including DNA damage, oncogene activation, and hypoxia (2).

The effect of p53 on transcription is dependent upon the promoter context, type of stimulus, and cellular environment, so that it can function both as an activator and as a repressor (3). One gene activated by p53 is p21WAF1, which encodes an inhibitor of cyclin-dependent kinases and thus can initiate cell cycle arrest (4). Other positively regulated targets include the GADD45, MDM2, cyclin G, KILLER/D5, IGF-BP3, and Bax genes, whose products function as regulators of several aspects of cell growth (5). Overexpression of p53 represses transcription of the MDR1 gene, which encodes a transmembrane glycoprophoprotein that mediates resistance to chemotherapeutic agents (6). Certain serum-inducible genes (7), cell cycle regulators (8–10), viral enhancers (11, 12), apoptotic regulators (13–17), and tumor progression genes (18–22) are also negatively regulated by p53. The mechanism of transcriptional repression by p53 may involve direct binding to response elements, indirect interactions with other transcriptional regulators, or both. The C-terminal oligomerization domain (OD) of p53 has been reported to be necessary for p53-mediated repression (23–25), and histone deacetylases (HDACs) and mSin3a also appear to be involved (26, 27).

Recently, several groups identified p73 and p63 (also known as p51, KET, p40, p73L, and p53CP) as members of the p53 tumor suppressor family (28–33). Like p53, both p73 and p63 can form homo-oligomers, bind to canonical p53 DNA binding sites, modulate transcription of p53-responsive genes, and suppress growth or induce apoptosis when overexpressed in certain human tumors (34–37). Furthermore, their polypeptide sequences appear to contain the three principal domains of p53: (a) an N-terminal transcriptional activation domain (AD), (b) a sequence-specific DNA-binding domain (DBD), and (c) an OD that mediates tetramerization.

The p73 and p63 proteins are unlike p53 in certain notable ways, however. First, p73 and p63 undergo alternative splicing,
giving rise to a family of isoforms of unknown physiological significance (38, 39). Second, the C-terminal regions of the p73α and p63α isoforms harbor a sterile α motif (SAM) domain that is not found in p53 and may be involved in protein-protein interactions or developmental regulation (30). Differences in the SAM domains of the different family members may reflect significant divergence in signaling and function (40, 41). Third, distinct developmental abnormalities were observed in mice lacking either p73 or p63 (42, 43) and in humans with germ-line p63 mutations (44). Finally, p73 and p63 are rarely mutated in tumors and are thus unlikely to be classical tumor suppressors (45). Thus, although certain characteristics of p73 and p63 are similar to those of p53, they cause some different physiological responses to extracellular signals and developmental cues. Here, we report our investigation into the molecular bases for the differences in function among these three proteins of the p53 family.

To study the biological role of p73, we first performed yeast two-hybrid screening of a human cDNA library with a p73 and found that p73 is functionally associated with the human homologue of mouse and hamster homeodomain-interacting protein kinase 2 (HIPK2). This protein has been identified previously as a nuclear serine/threonine kinase that interacts with the NK homeodomain transcription factor (46), acts as a corepressor for the NK homeodomain, and cooperates with Groucho and HDAC-1 in enhancing transcriptional repression (47). Furthermore, SUMO-1 modification of HIPK2 correlates with its localization to nuclear speckles (dots) or nuclear bodies (48). Yeast two-hybrid screens have shown that HIPK2 also interacts with various other proteins including interferon type I-induced MxA (49), Pas/CD95 (50), and HMGI(Y) (51). The biological significance of these interactions has not been determined.

The human homologue of HIPK2 (hHIPK2) was recently cloned and mapped to chromosome 7q32-q34 (52).

HIPK2 has very recently been reported to activate p53-mediated transcription through down-regulation of MDM2, which may result in enhanced p53 protein levels (53). The two-hybrid results of our two-hybrid described here provide strong evidence for a functional association between other p53 family proteins and HIPK2. We have examined the sites of interaction on these proteins, the biological significance of these interactions, and whether association with HIPK2 stabilizes p53 family proteins. Using the hamster HIPK2 homologue (haHIPK2), also known as PKM, for further characterization both in vivo and in vitro, we have found evidence for a physical interaction between the OD of p53 family proteins and a region of haHIPK2 near a PEST sequence. These proteins colocalize in nuclear bodies, and the repressor functions of p73 and p53 are enhanced by HIPK2 in a dose-dependent manner. The corepressor activity of HIPK2 is abrogated in the presence of tri-chostatin A (TSA), an inhibitor of HDAC-1. Finally, modulation of transcription of p53 family proteins by HIPK2 correlates with inhibition of colony formation, suppression of the cell cycle, and induction of apoptosis.

MATERIALS AND METHODS

Cell Lines and Cell Culture—All cells used in our experiments were routinely maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum and penicillin/streptomycin (all from InVitrogen). Stock solutions of G418 (neomycin) (InVitrogen), adriamycin (Sigma), and cisplatin (Amersham Biosciences) were 2 mg/ml in H2O.

cDNA Constructions—All cDNAs were made according to standard methods (54) and verified by sequencing. The multicycop yeast expression plasmids used in the two-hybrid assays were pB7TM116 (a Trp1-LexA DBD vector) and pASV3 (a Lex2-VP16 acidic transactivation domain vector). In these plasmids the target fusion genes are expressed under control of the ADH1 and phosphoglycerate kinase promoters, respectively (55). Deletion and point mutants of the desired genes were created by PCR amplification and subcloned into the pB7TM116 or pASV3. Hemagglutinin (HA)-tagged p73α, p73β, and p53 expression plasmids were described previously (56) and modified by subcloning into yeast vectors for two-hybrid assays and pEGFP-N1 vector (CLONTECH, Palo Alto, CA) for colocalization assays, respectively. Expression plasmids for p53 point and mutants were provided by Drs. K. Roemer (Hamburg, Germany) and C. C. Harris (Bethesda, MD). HA-tagged p63α (or p51B) and p63γ (or p51A) expression plasmids were kindly provided by Dr. Y. Ikawa (Tokyo, Japan) and modified by subcloning into yeast vectors for two-hybrid assays. haHIPK2 cDNA (a kind gift from Otto Haller, Freiburg, Germany) was PCR-amplified to construct the tagged haHIPK2 in pCDNA-HA, a derivative of vector pCDNA3 encoding HA tag, and GFP-tagged haHIPK2 in pEGFP-N1 vector (CLONTECH) for colocalization assays. MDR1-CAT reporter plasmid was kindly provided by Dr. S. L. Dong (Taiwan, Taipei Republic of China). An expression plasmid for a glutathione S-transferase (GST)-haHIPK2 fusion protein was created by the subcloning of PCR-amplified haHIPK2 into vector pGEX2T (Amersham Biosciences). Details of plasmid constructions are available upon request.

Yeast Two-Hybrid Assays—A human liver cDNA library (Matchmaker, CLONTECH) in the prey plasmid pGAD10 (CLONTECH) was screened for proteins that interact with p73 using yeast reporter strain L40 (MATa, his3Δ200, trp1-901, leu2–3, 112, ade2, LYS::(lexAop)2-HIS3, URA3::(lexAop)2-zei) (55). The transactivation domain LexA was deleted so that amino acids 1–52 of p73α was deleted and was expressed as a fusion with LexA on the bait plasmid pBTM116. The prey and bait plasmids were cotransformed into L40 using lithium-acetate. Transformed cells were spread directly on minimal medium lacking histidine, leucine, and tryptophan and supplemented with 5 μg 3-aminotriazole. Positive clones were isolated and then retested for β-galactosidase activity on permeabilized cells. Library plasmids from positive isolates were transformed into and recovered from Escherichia coli strain HB101 (leu2–) and were then analyzed by restri ctive digests. Unique inserts were sequenced and analyzed by comparison to the GenBank sequence data bank. The longest insert was systematically tested for interactions for transfections containing the LexA DBD fused to p73α (49–936) or to other p53 family proteins by β-galactosidase assays (56). Two p73 mutants were fused with a VP16 AD by subcloning into vector pASV3. The resulting VP16-AD-haHIPK2 fusion vectors were cotransformed with a pB7TM116 derivative encoding the LexA DBD-p73α (49–936) fusion protein.

Glutathione S-Transferase Pull-down Assays—GST fusions of p53 (319–395) and haHIPK2 (812–907) were purified by cloning the appropriate sequence into pGEX2T. Puriﬁed proteins were expressed in E. coli and puriﬁed on glutathione-Sepharose beads (Amersham Biosciences) by standard methods. Full-length haHIPK2, p53, p73α, p73β, p63α, and p63γ proteins were in vitro translated in rabbit reticulocyte lysate (Promega, Madison, WI) supplemented with [35S]methionine (Amersham Biosciences). In vitro binding experiments were performed as described previously (56).

Confocal Immunofluorescence Microscopy—After transfection with plasmid DNA, U208 osteosarcoma cells were washed in phosphate-buffered saline (PBS), and cell lysates were prepared by adding 1 ml of ice-cold RIPA buffer (50 mM HEPES, pH 7.0, 250 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA) supplemented with protease inhibitors. Lysates were precleared by pre-incubation with protein A-Sepharose beads for 1 h and then incubated overnight at 4 °C with protein A-Sepharose beads and a 1:200 dilution of either a mouse anti-HA monoclonal antibody (mAb) 12CA5 (Roche Molecular Biochemicals) or rabbit anti-GFP polyclonal antibody sc-8334 (Santa Cruz Biotechnology, Santa Cruz, CA). The beads were washed five times in RIPA buffer and twice with PBS, and the immune complexes were released from the beads by boiling in sample buffer for 5 min. Following electrophoresis on 8% SDS-polyacrylamide gels, immunoprecipitation products were analyzed by Western blotting using either rabbit polyclonal anti-GFP polyclonal antibody sc-8334 (1:200), mouse anti-p53 mAb NCL-p53P (Novocastra, Burlington, CA) (1:100), or mouse anti-p73 mAb Ab-2 (Neomarkers, Fremont, CA) (1:100).

Confocal Microscopy—Confocal imaging was performed on a Leica TCS-SP (Beverly, MA). U208 cells were cultured on glass cover slips in 6-cm plates and transfected with pCDNA-HA- or pCDNA-HA-GFP vectors. Two days later, cells were washed with PBS, fixed with 4% paraformaldehyde in PBS for 10 min at room temperature, and permeabilized for 4 min at 4 °C in PBS containing 0.3% Triton X-100 and 10% goat serum. Subsequent incubations were performed at room temperature. After washing, cells were blocked for 60 min in PBS containing 3% bovine serum albumin and then incubated for 2 h with mouse anti-HA mAb 12CA5.
HIPK2 Interacts with Members of the p53 Family—The yeast two-hybrid system was used to identify proteins that interact with p73α (49–636), which lacks the transactivation domain of p73α (Fig. 1A). We isolated 285 His gene-positive clones from a screen of a human liver cDNA library fused to the GAL4 AD. When tested for activation of a GAL1-lacZ reporter gene, which contains eight LexA-binding sites in the promoter region, 73 of the clones exhibited high-level expression of lacZ. We isolated and amplified the plasmids from these clones and subjected them to agarose gel electrophoresis. The insert sequences from a total of 42 unique library plasmids were used in a GenBank search, and five of these clones were found to be human homologues of mouse HIPK2 (mHIPK2) (49) and haHIPK2 (49). The longest insert corresponded to amino acids 1–636) but not with the unrelated IRF-2 protein in a yeast two-hybrid assay (Fig. 2B). Subsequent yeast two-hybrid assays indicated that hHIPK2 interacts with other members of the p53 family including p73α, p53, and p63α, and p63γ, with the unrelated IRF-2 protein in a yeast two-hybrid assay (Fig. 2A). Subsequent yeast two-hybrid assays indicated that hHIPK2 interacts with other members of the p53 family including p73α, p53, p63α, and p63γ, all of which were truncated to remove their transactivation domains for these assays (Fig. 2B).

To map the HIPK2-interaction domain on p53 family proteins, genes for LexA DBD fusions of these proteins and their deletion derivatives were placed on pBTM116. The full-length LexA DBD-p73α clone showed transcriptional activity when cotransformed into yeast along with an expression plasmid encoding a GAL4 AD-hHIPK2 fusion protein. Assays with a series of C-terminal deletion mutants of p73α indicated that a region near the OD is required for interaction with HIPK2 (Fig. 3A). Further analysis with p73α (49–380) and p73α (434–400) revealed that the minimal interaction domain is the region bounded by amino acids 345–380. Interestingly, another, inde-
A pendent interaction region was found near the C terminus and includes the SAM domain of p73/H9251. Further deletions in those regions abolished interactions with HIPK2. Like p73, p53 strongly associated with HIPK2 in two-hybrid assays. A region between amino acid residues 319 and 360 of p53, which harbors the OD, was found to be sufficient for mediating an interaction with HIPK2 (Fig. 3B). Likewise, the interaction domain in p63 was the amino acid segment 352–388, which includes the OD (Fig. 3C). A second interaction domain was mapped to near the C terminus of p63/H9251 that includes the SAM domain. Thus far, no functional domains have been identified near the C termini of p73/H9251 and p63/H9251.

Mapping of the p73-interacting Domain of haHIPK2—To map the p73-interaction domain on haHIPK2 proteins, genes for VP16 AD-haHIPK2 fusions of haHIPK2 and its deletion derivatives were placed on pASV3. These plasmids were co-transformed into yeast along with a LexA DBD-p73/H9251-(49–636) expression plasmid, and levels of interaction were determined by β-galactosidase assays in permeabilized yeast (Fig. 4). In control assays, the LexA DBD-p73/H9251-(49–636) fusion and the LexA DBD alone were assayed for activation in the presence of the VP16 AD. No increase in reporter activity was detected when LexA DBD-p73/H9251-(49–636) was coexpressed with the VP16 AD. In contrast, a strong interaction was observed between LexA DBD-p73/H9251-(49–636) and VP16 AD-haHIPK2. Analysis of a series of N- and C-terminal truncation mutants of HIPK2 revealed that a C-terminal amino acid segment 812–907 of haHIPK2 is required for interaction with p73α. Further

![Fig. 2. Human HIPK2 interacts with p53 family proteins. A, identification of hHIPK2 by yeast two-hybrid screening of genes encoding the GAL4 AD fused to a human liver cDNA library. B, interaction of p53-family proteins with hHIPK2 as analyzed by yeast two-hybrid and β-galactosidase assays. The genes for LexA DBD-fused p53-family proteins were carried on pBTM116, and the GAL4 AD-fused hHIPK2 gene was carried on pGAD10, as described under “Materials and Methods.”](image)

![Fig. 3. Specific mapping of the domains of p73 (A), p53 (B), and p63 (C) responsible for association with hHIPK2. Yeast two-hybrid and β-galactosidase assays were performed using genes for LexA DBD-fused truncations of the p53-family proteins carried on pBTM116 and the gene for GAL4 AD-fused hHIPK2 carried on pGAD10 as described under “Materials and Methods.”](image)
deletions in that region abolished interaction with p73α, and assays with three other mutants indicated that haHIPK2-(812–907) is sufficient for the interaction. Thus, this haHIPK2 region, which includes a PEST sequence, appears to be required and sufficient for the interaction with p73α. Notably, the corresponding region of hHIPK2 has been shown to interact with several proteins including Ubc9 (48), Fas/CD95 (50), and HMGI(Y) (51).

Members of the p53 Family Associate with haHIPK2 in Vitro and in Mammalian Cells—To further examine whether a direct physical interaction between haHIPK2 and p53 family proteins is present, we performed in vitro binding assays. GST-p53 and GST-HIPK2 fusion proteins were expressed in E. coli, purified, and mixed separately with in vitro-translated [35S]methionine-labeled haHIPK2, p53, p73α, p73β, p63α, or p63γ in a rabbit reticulocyte lysate. In agreement with the results of our yeast two-hybrid experiments, [35S]labeled HIPK2 was retained by GST-p53 immobilized on glutathione-Sepharose beads (Fig. 5A). In a reciprocal experiment, [35S]labeled p53 was retained by immobilized GST-HIPK2. Further assays with labeled p73 and p63 showed that HIPK2 associates with other members of the p53 family, although these interactions are somewhat weaker (Fig. 5B). Overall, these data confirm that HIPK2 directly interacts with members of the p53 family in vitro.

To demonstrate that the interaction between HIPK2 and the p53 family also occurs in vivo, p53-null Saos2 mammalian cells were cotransfected with HA-tagged p73α or p53 and GFP-tagged haHIPK2 constructs. Immunoprecipitation with an anti-HA antibody and subsequent Western blotting with an anti-GFP antibody revealed that both p73α and p53 interact with HIPK2 in vivo (Fig. 6A). In other experiments in which untagged p73α or p53 was cotransfected with GFP-tagged haHIPK2, both p73α and p53 were detected in precipitates obtained with an anti-GFP antibody. The interaction of p73α with haHIPK2 was weaker than that of p53 (Fig. 6, B and C). The kinase-defective HIPK2 mutant K221W (49) was also able to interact with p73α and p53 as expected from two-hybrid mapping experiments (data not shown).

HIPK2 and p53 Family Proteins Colocalize in Nuclear Body-like Structures—We next sought to determine the subcellular localization of p73α, p53, and HIPK2. For this purpose, p53-null Saos2 cells were transiently cotransfected with plasmids encoding HA-tagged p73α or HA-tagged p53 and GFP-tagged HIPK2. Immunofluorescence and confocal laser microscopy showed that the majority of HIPK2 localized to nuclear speckles in agreement with previous reports (49). Texas Red staining of cells transfected singly with a p73α or p53 plasmid yielded a diffuse nuclear red fluorescence (data not shown). However, when p53 or p73α and HIPK2 plasmids were cotransfected, p53 and p73α were localized to distinct spots within the nucleus (Fig. 7, A and B). These findings are reminiscent of previous reports that PML targets p53 into PML speckles or nuclear bodies (57, 58). Taken together, our in vitro and in vivo inter-

![Image](308x337 to 552x729)

**Fig. 4.** Mapping of the haHIPK2 domain responsible for interaction with p73α—636. Yeast two-hybrid and β-galactosidase assays were performed using a pBTM116 derivative carrying the gene for a LexA DBD-p73α(49–636) fusion proteins and pASV3 derivatives carrying genes for VP16 AD-fused truncations of haHIPK2.

![Image](50x453 to 294x728)

**Fig. 5.** GST pull-down assays. In vitro translated [35S]labeled proteins were incubated for 2 h with GST-p53(319–393) or GST-haHIPK2(812–907) fusion proteins immobilized on glutathione-Sepharose beads. Beads were washed five times with 20 mM HEPES, pH 7.5, 50 mM KCl, 2.5 mM MgCl2, 10% glycerol, 1 mM dithiothreitol, 1% Nonidet P-40, and 200 mM phenylmethylsulfonyl fluoride. Bound proteins were eluted with SDS sample buffer, resolved by electrophoresis on 10% SDS-polyacrylamide gels, and visualized by autoradiography.

![Image](50x293 to 272x314)

**Fig. 6.** Immunoprecipitation assay for in vivo interactions between haHIPK2 and p73α or p53. U2OS cells were cotransfected with genes for fusion proteins GFP-haHIPK2 and HA-tagged p73α or p53. Cells were lysed in RIPA buffer supplemented with protease inhibitors, and lysates were precleared by incubation with protein A-Sepharose for 1 h. Immunoprecipitation was carried out with anti-HA or anti-GFP antibodies. Precipitated proteins were eluted with SDS sample buffer, resolved by electrophoresis on 10% SDS-polyacrylamide gels, and visualized by Western blotting using anti-GFP (A), anti-p73α (B), or anti-p63 (C) antibodies.
expression of a GAL4 DBD-HIPK2 fusion protein inhibited transcriptional repression, the HDAC inhibitor TSA in a dose-dependent manner. We also observed that p53 in the absence and presence of HIPK2 was mitigated by TSA in a dose-dependent manner. 

To further examine the role of HIPK2 in p73- and p53-mediated apoptosis, we carried out FACS and DAPI analyses of HIPK2-U2OS and U2OS cells treated with DNA-damaging agents such as cisplatin or adriamycin. After cisplatin treatment, the DNA content of propidium iodide-stained cells was determined by FACS. In FACS analysis, a distinct quantifiable region below the G1 phase (the sub-G1 peak; designated as M1) was used to treat cells cotransfected with p53 and HIPK2 expression vectors and the Coll-CAT reporter plasmid. As shown in Fig. 8A, the down-regulation of the Coll-CAT gene by p53 in the absence and presence of HIPK2 was mitigated by TSA in a dose-dependent manner. We also observed that expression of a GAL4 DBD-HIPK2 fusion protein inhibited transcription of the 5× GAL-TATA-CAT reporter gene, and that this inhibition was relieved by TSA (data not shown). Overall, our transfection data indicate that haHIPK2 modulates the transcriptional regulatory activity of p53 family proteins.

Stable Expression of HIPK2 Retards the Growth of U2OS Cells—To correlate transcriptional modulation by HIPK2 with its in vivo function, we first transiently transfected p53-positive U2OS cells with a haHIPK2 expression vector. Western blotting showed that HIPK2 increased the amount of p53 protein produced in these cells (data not shown). Next, we generated U2OS cells stably overexpressing HIPK2 (HIPK2-U2OS cells) and examined the effect of HIPK2 on the growth of these cells and on the levels of p53 and p21WAF1 proteins. As shown in Fig. 9A, stable overexpression of HIPK2 retarded U2OS cell growth during 5 days in a modest level (Fig. 9B). As in our transient transfection experiments, stable overexpression of HIPK2 also increased the amount of p53 in the cell. These observations raise the possibility that transiently or stably expressed HIPK2 stabilizes p53 in U2OS cells through direct protein-protein interactions, leading to enhanced p21WAF1 protein production and subsequent growth arrest. We did not detect any differences in levels of p73 protein in the presence or absence of stably expressed HIPK2, however (data not shown).

To further examine the role of HIPK2 in p73- and p53-mediated apoptosis, we carried out FACS and DAPI analyses of HIPK2-U2OS and U2OS cells treated with DNA-damaging agents such as cisplatin or adriamycin. After cisplatin treatment, the DNA content of propidium iodide-stained cells was determined by FACS. In FACS analysis, a distinct quantifiable region below the G1 phase (the sub-G1 peak; designated as M1)
is indicative of apoptotic cells. As shown in Fig. 10A, cisplatin treatment increased the sub-G1 fraction (hypodiploid) by 2-fold compared with control cells (from 7.87 to 13.83%). Cisplatin treatment caused a further 2-fold increase (from 13.83 to 24.13%) in HIPK2-U2OS cells.

Morphological markers of apoptosis, such as cell shrinkage, nuclear segmentation, and chromatin condensation, were investigated by fluorescence microscopy following DAPI staining. These morphological changes were more evident in DAPI-stained nuclei of HIPK2-U2OS cells after treatment with cisplatin or adriamycin (Fig. 10B). Like our FACS data, these studies showed that HIPK2 increases cell death induced by cisplatin or adriamycin. These overall data suggest that HIPK2 may be a critical mediator for the cellular functions of p73 and p53 such as cell cycle arrest and apoptosis.

**HIPK2 Potentiates the Inhibition of Colony Formation by p53 Family Proteins**—The roles of HIPK2 in mediating cellular functions of p53 family proteins were further investigated by colony formation assays. Saos2 and U2OS cells were transfected with expression vectors for HIPK2 and/or p53 or p73α with a negative control vector (pCDNA3) and selected for resistance to G418. As shown in Fig. 11A, overexpression of p53 or HIPK2 alone in p53-null Saos2 cells dramatically reduced the number of G418-resistant colonies to only 17 or 15%, respectively, of those observed for the negative control. Overexpression of p73α only mildly suppressed the growth of Saos2 cells (65% of control), whereas p73α was undetectable (data not shown). When HIPK2 was cotransfected with p53 or p73α, the number of colonies formed decreased to 1.5% (HIPK2 and p53) or 2.2% (HIPK2 and p73α) of the control value. As was also observed in p53-positive U2OS cells, overexpression of p73α or HIPK2 resulted in a significant reduction in the number of colonies to 35 and 8% of the control value, respectively. Coexpression of p73α and HIPK2 severely inhibited colony formation in U2OS cells as well (Fig. 11B). Together, these observations suggest that HIPK2 significantly suppresses tumor cell growth independent of p53 or p73α and that the suppressing ability of HIPK2 increases in the presence of members of p53 or p73α. However, it remains to be conclusively shown whether this synergistic effect is due to a direct interaction between HIPK2 and p53 family proteins.

**DISCUSSION**

The tumor suppressor protein p53 is one of the most important regulators of cellular growth functions such as cell cycle arrest, DNA repair, and apoptosis. Recently, two p53 family proteins, p73 and p63, were discovered that appeared to have functions similar to those of p53. To begin further investigations of the differential roles of these proteins, we performed yeast two-hybrid screens of a human liver cDNA library using p73α as the bait protein. These screens identified the human homologue of mouse and hamster HIPK2 as a p73α-interacting protein. Fine mapping of the interaction domains indicated that the OD of the p53 family proteins and the amino acid
wild-type p53, p73α, and/or haHIPK2. After selection of stably transfected cells for 2 weeks using G418, G418-resistant colonies were stained with Coomassie Blue, photographed, and counted. Numerical results represent the mean of three independent experiments. A photograph of one representative result is shown.

segment 812–907 of hHIPK2, which includes PEST and SRS sequences and Ubc9 binding regions, are required for the interaction between p53 family proteins and HIPK2. The p53 OD is essential for the tetramerization that is required for the tumor-suppressive activity of p53 (59) and is also required for p53-mediated transcriptional repression (23–25). Interestingly, an additional interacting region was found in the C-terminal region of p73α and p63α, which includes a SAM domain of undetermined function.

The amino acid sequence of the haHIPK2 interaction domain is identical to the sequences of amino acids 846–941 and 839–934 of human and mouse HIPK2, respectively. This region may be an interaction hot spot, as was described for the Mx interaction domain (49), the Ubc9-binding domain (48), and the CD95 binding site (50) that are all located near PEST/SRS sequences. However, this region is different from the NK homedomain binding region (46). Because we have shown that the SRS of HIPK2 is involved in the interaction with p53 family proteins, it is not surprising that p73α and p53 colocalize to nuclear speckles.

The above findings are reminiscent of previous reports that PML targets p53 to PML speckles or nuclear bodies (57, 58). It has been suggested that SUMO-1 modification of PML increases the formation of nuclear bodies and thus augments recruitment of p53 into these structures. Although the sumoylation of p53 family proteins has been reported (60–62), it is unlikely that sumoylation is required for nuclear body localization (63). Because the sumoylation of HIPK2 directs its localization to the nuclear speckles, we expected that a physical association with HIPK2 might target p53 family proteins to the nuclear speckles as well. However, whether SUMO-1 modification of HIPK2 is required for localization of p53 family proteins to the nuclear speckles has yet to be determined. Other modifications of p53, such as phosphorylation by HIPK2 and/or acetylation by p300/CBP, may promote its colocalization into nuclear speckles with HIPK2. Because p300/CBP has also been demonstrated to accumulate in PML bodies (64), the network of interactions between p73/p53, PML, HIPK2, and p300/CBP may be important for various modifications of p73 and p53 that result in differential regulation of transcription in PML bodies. Clearly, more work is required to establish the impact of modifications of p73/p53 by HIPK2 and p300/CBP in these subnuclear structures.

Our in vitro and in vivo binding assays revealed that HIPK2 directly associates with p53 family proteins, suggesting that this association modulates the transcriptional regulatory activity of p73α and p53. Expression of HIPK2 in p53-null Saos2 cells increased the transactivation function of p53 and p53 family proteins as shown by the response of a CAT reporter gene. This response was dependent upon the kinase function of HIPK2 because it was abrogated when the kinase-defective HIPK2 mutant K221W was used (data not shown). Other transfection experiments indicated that HIPK2 enhances the transrepression activity of p73α and p53 with negatively p53-responsive MDR and Coll-CAT reporter genes. Because this transrepression was prevented by treatment with TSA, this result supports the conjecture that an HDAC is critical for the corepressor function of HIPK2 (47). When the amount of mutant K221W in the cells was increased, the corepressor activity of HIPK2 diminished (data not shown), suggesting that K221W may function as a dominant-negative mutant.

Because HIPK2 kinase activity is required for modulation of the transactivation and transrepression functions of p73α and p53, the phosphorylation states of p73α and p53 and the mechanism for switching HIPK2 function between coactivation and corepression are of considerable interest. We have found that the interaction between HIPK2 and CBP involves amino acid residues 1–520 of HIPK2 and 662–1095 of CBP (data not shown). This region of HIPK2 includes the kinase domain. We speculate that interactions with CBP and HDAC are required for the coactivation and corepression functions of HIPK2, respectively. Two recent works provided some clues to how HIPK2 associates and cooperates with p53 and CBP in the p53-dependent transactivation by demonstrating that HIPK2 phosphorylates p53 at Ser-46 and that the phosphorylation promotes the CBP-mediated acetylation of p53 at Lys-382 in the nuclear bodies (65, 66). However, it was not determined whether HIPK2 affects the functions of p73 and p63 and how HIPK2 mediates the p53-dependent transrepression. Therefore, precise determination of the acetylation/deacetylation levels of p53, p73, and p63 in the presence of CBP- or HDAC-associated HIPK2 will provide further insight into the regulation of p53 family proteins in nuclear bodies.

The biological significance of the interactions between HIPK2 and p73α or p53 was examined using HIPK2-U2OS cells, which stably express HIPK2. In agreement with other studies (53), p53 levels in these cells were greater than in the parental U2OS cells. Levels of p53 were also greater in Saos2 cells transiently expressing HIPK2 than in parental Saos2 cells. In HIPK2-U2OS cells, the p53-responsive gene product p21WAF1 was up-regulated, and cell growth was slowed compared with U2OS cells. These cells also exhibited increased apoptosis after cisplatin or adriamycin treatment. Up-regulation of p53 family protein levels via protein stabilization is not likely to be a common mechanism for the effect of HIPK2, however, because p73 levels appeared to be unaffected by stable or transient expression of HIPK2. In other experiments,
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tumor cell growth was significantly inhibited by expression of either HIPK2 or p53 family proteins.

The p53-independent function of HIPK2 may reflect the presence of various cellular phosphorylation targets of HIPK2 that are involved in growth regulation independent of p53. This supposition is supported by the existence of other targets of HIPK2 including NK homedomain transcription factor (46), interferon type I-induced MxA (49), Fas/CD95 (50), STAT3 (67), and HMGI(Y) (51). Alternatively, HIPK2 may suppress interferon type I-induced MxA (49), Fas/CD95 (50), STAT3 (52) that are involved in growth regulation independent of p53. This tumor cell growth was significantly inhibited by expression of either HIPK2 or p53 family proteins.

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