Tumor and Stem Cell Biology

Cancer Research

The p53/p21 Complex Regulates Cancer Cell Invasion and Apoptosis by Targeting Bcl-2 Family Proteins

Eun Mi Kim, Chan-Hun Jung, Jongdoo Kim, Sang-Gu Hwang, Jong Kuk Park, and Hong-Duck Um

Abstract

The tumor suppressor p53 binds prosurvival Bcl-2 family proteins such as Bcl-w and Bcl-X_L to liberate Bax, which in turn exerts proapoptotic or anti-invasive functions depending on stress context. On the basis of our previous finding that p53 interacts with p21, we investigated the possible involvement of p21 in these functions. Here, we report that although p53 can bind Bcl-w alone, it requires p21 to liberate Bax to suppress cell invasion and promote cell death. p21 bound Bcl-w, forming a p53/p21/Bcl-w complex in a manner that maintained all pairwise p53/p21, p21/Bcl-w, and p53/Bcl-w interactions. This allowed Bax liberation from the complex. Accordingly, a p53 derivative incapable of binding p21 failed to mediate radiotherapy-induced tumor cell death in mice. Bcl-X_L also served as a target of the cooperative action of p53 and p21. Overall, our findings indicate that the p53/p21 complex rather than p53 itself regulates cell invasion and death by targeting Bcl-2 proteins. We propose that the p53/p21 complex is a functional unit that acts on multiple cell components, providing a new foundation for understanding the tumor-suppressing functions of p53 and p21. *Cancer Res; 77(11); 3092–100. ©2017 AACR.*

Introduction

The p53 tumor suppressor/transcription factor regulates various cell functions, including the promotion of apoptosis and senescence, and the suppression of cell growth, migration, and invasion (1). Most studies of the mechanisms of p53 action have focused on the transcriptional targets of p53, which mediate p53 functions. $p21^{WAF1}$ represents one such target, which plays important roles in cell growth arrest and senescence (2). p21 can also suppress cell invasion. Notably, we have recently shown that p21 does not simply serve as a downstream mediator of p53 but cooperates with p53 to suppress cell invasion (3). Direct interactions between p53 and p21 were indeed observed, and both proteins were able to bind Mdm2, an E3 ligase, and its substrate Slug, forming a p53/p21/Mdm2/Slug complex that facilitated Mdm2-dependent Slug ubiquitination and degradation. Because Slug enhances cancer cell invasion, its degradation could contribute to the anti-invasive p53/p21 cooperative activity. The cooperative function of p53 and p21 might also affect other cellular targets. However, this hypothesis has not yet been explored, despite the new insights into the mechanisms underlying p53 tumor suppression that it can provide.

Upon apoptotic stimulation, p53 translocates from the cell nucleus to the mitochondria, where it interacts with Bcl-2 family proteins to regulate apoptosis (4, 5). This family comprises prosurvival (e.g., Bcl-2, Bcl-w, and Bcl-X_L), multidomain proapoptotic (e.g., Bax and Bak), and BH3-only (e.g., Bid and Bad) groups. In heathy cells, the prosurvival members bind to and inhibit the multidomain proapoptotic members. However, in response to apoptotic stimulation, the BH3-only members, as well as mitochondrial p53, bind to the prosurvival members, liberating the multidomain proapoptotic members that then trigger the mitochondrial pathway of apoptosis. Thus, p53 also has the ability to regulate cell function by regulating protein–protein interactions.

In nonapoptotic cells, Bcl-2 proteins regulate cell migration and invasion, and thus, cancer metastasis (6). These features are promoted by Bcl-2, Bcl-X_L, and Bcl-w and suppressed by Bax and Bak. Bax and Bak reduce the ability of mitochondria to produce reactive oxygen species (ROS), key inducers of cell invasion and cancer metastasis, whereas Bcl-w and Bcl-X_L promote ROS production by binding to and inhibiting Bax and Bak (6–8). Notably, p53 and its mutant derivatives (e.g., $p53^{K305N}$) frequently accumulate in the cytoplasm and mitochondria of normal and cancer cells even without apoptotic stimulation (9, 10), suggesting the existence of cytoplasmic/mitochondrial functions of p53 in healthy cells. Furthermore, studies using $p53^{K305N+R175H}$, a cytoplasmic mutant of p53 that does not bind to prosurvival Bcl-2 proteins, showed that cytoplasmic p53 suppressed cell invasion by binding to Bcl-w and Bcl-X_L, liberating Bax and Bak, and thus reducing ROS production (7). Therefore, prosurvival Bcl-2 proteins appear to be key targets of cytoplasmic p53 in the regulation of cell death, migration, and invasion.

Here, we investigated whether prosurvival Bcl-2 proteins interact with p21 as well as with p53 and thus, serve as targets of these two proteins. Data obtained using Bcl-w support this possibility.

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Subsequent functional analyses using cellular and animal models revealed that the p53/p21 complex rather than p53 alone regulates cell invasion and death by targeting Bcl-2 proteins. Therefore, the p53/p21 complex appears to represent a functional unit that can bind to multiple cellular components and regulate their biological activities.

Materials and Methods

Antibodies

Santa Cruz Biotechnology supplied all the antibodies used in this study except anti-p53 (Dako); anti-Bcl-w, anti-Bcl-X_L, and anti-Bax (Cell Signaling Technology); and anti-cytochrome *c* (BD Biosciences).

Cell culture, transfection, and treatment

Cells were authenticated by ATCC via STR and growth/morphologic analyses and maintained in our laboratory for fewer than 6 months after purchase (acquired between 2014 and 2016). Cells were regularly screened for mycoplasma contamination using MycoAlert Mycoplasma Detection Kits (Lonza). Cells were cultured in McCoy's 5A (Calu-1 and HCT116 cells) or RPMI1640 medium (all other cells) supplemented with 10% heat-inactivated FBS. Deletion constructs were prepared by PCR and cloned into pcDNA3 (Invitrogen) or pFLAG-C1 vectors (3). Transfection using expression vectors and/or siRNAs (Ambion) was performed using Lipofectamine 2000 (Invitrogen). Transfectants were used for cell invasion assays following a 24-hour recovery period or were selected using G418 sulfate (0.5 mg/mL) for γ-irradiation (3).

Invasion assay

Cells were seeded onto the upper surfaces of Matrigel-coated Transwell chambers (BD Biosciences) and analyzed for their invasiveness (11).

Analysis of cellular viability and ROS levels

Propidium iodide (5 μg/mL) and 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA; 10 μmol/L; Molecular Probes) were used to analyze cellular viability and ROS levels, respectively, by flow cytometry (12).

Western blot analysis

Whole-cell lysates (8) and subcellular fractions (13) were prepared as described previously. Proteins in the samples were separated by SDS-PAGE, electrotransferred to Immobilon membranes (Millipore), and analyzed using the specified antibodies and an ECL detection system (Thermo Fisher Scientific; ref. 3).

Coimmunoprecipitation assay

Cells lysates were subjected to immunoprecipitation using the indicated antibodies and protein G-Sepharose beads (Amersham; ref. 8). The precipitates were analyzed by Western blotting.

In vitro binding assay

Bcl-w and p21 genes and deletion constructs were cloned into pcDNA3 and pCITE-4a(+) vectors (Novagen), respectively, and translated *in vitro* using the TNT Quick Coupled Transcription/Translation Systems (Promega). Where indicated, GST-coupled p53 protein (3) was added to the reaction mixtures. Protein binding was analyzed by coimmunoprecipitation and subsequent Western blotting.

Two-step coimmunoprecipitation assay

Lysates of H1299 cells cotransfected with the expression vectors for Flag-tagged p21 and p53 (or $p53^{K305N}$) were immunoprecipitated with anti-Flag M2-agarose beads (Sigma-Aldrich). The Flag–p21 protein complex was then eluted using the Flag peptide (300 μg/mL; Sigma-Aldrich). A fraction of the eluted proteins was analyzed by Western blotting for Bcl-w, p53, and p21; the remainder was subjected to a second immunoprecipitation with an anti-Bcl-w antibody and protein G-Sepharose beads. The precipitated proteins were analyzed by Western blotting for p53 and p21.

Analysis of tumor growth in mice

Animal studies were conducted in accordance with the guidelines of our Institutional Animal Care and Use Committee. Tumor cells (10^7/mouse) were subcutaneously injected into the hind legs of six-week-old female BALB/cAnNCrj-nu/nu mice (Charles River Laboratories). Tumors that reached approximately 200 mm^3 (14) were locally irradiated using a ^{60}Co γ-ray source (AECL). Tumor volumes were measured every other day, and the mice were sacrificed 2 weeks postirradiation for tumor excision.

IHC and TUNEL assay

Excised tumors were embedded in paraffin. Sections were deparaffinized, rehydrated, and stained with hematoxylin according to standard protocol. Alternatively, the sections were treated with proteinase K (20 μg/mL) for 15 minutes and employed for TUNEL staining using the ApopTag Peroxidase *In Situ* Apoptosis Detection Kit (Millipore). The samples were counterstained with methyl green and analyzed under a light microscope.

Statistical analysis

Experiments shown in graphic form were performed three times to obtain the means and SDs. Statistical significance ($P < 0.05$) was determined by one-way ANOVA (GraphPad software).

Results

Cytoplasmic p53 and p21 cooperate to inhibit cell invasion

To investigate whether p21 is required for cell invasion suppression mediated by cytoplasmic p53 targeting of Bcl-2 proteins, we introduced $p53^{K305N}$, which localizes in the cytoplasm and mitochondria of healthy cells (7), into H1299 lung cancer cells ($p53^{null}$). $p53^{K305N}$ expression reduced cellular ROS levels and invasiveness (7). These effects were completely abolished by p21 knockdown using two sets of p21-targeting siRNAs (Fig. 1A), suggesting that $p53^{K305N}$ requires p21 for suppressing cell invasion. Moreover, p21 overexpression, which did not significantly influence H1299 cell invasiveness (3), reduced that of $p53^{K305N}$/H1299 transfectants (Fig. 1B), further supporting the functional cooperation between $p53^{K305N}$ and p21. Therefore, cell invasion appears to be suppressed by the presence of both $p53^{K305N}$ and p21, but not by each individually.

Bcl-w is a target of the p53/p21 complex

To investigate whether p21 interacts with the prosurvival Bcl-2 proteins as p53 and $p53^{K305N}$ do (4, 7), we performed Bcl-w coimmunoprecipitation assays with *in vitro* translated proteins or in cell lysates. The results support an interaction between p21 and Bcl-w (Fig. 2A), which was almost completely abrogated when the 26 N-terminal residues of p21 (Fig. 2B, left) or the 10 C-terminal

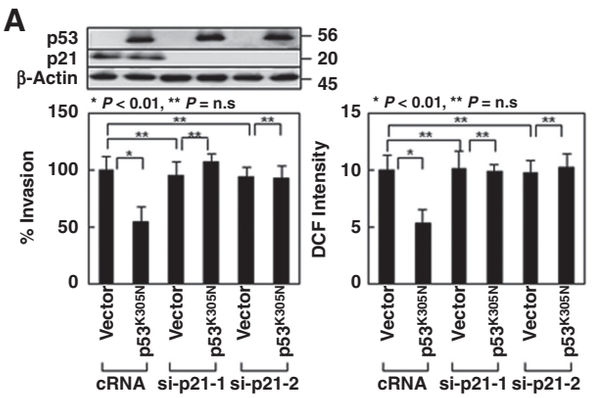

Figure 1.
Cytoplasmic p53 and p21 are both required for suppressing cell invasion. **A** and **B,** Empty, p53^{K305N}-, and p21-encoding pcDNA3 vectors (**A** and **B**), as well as control and two sets of p21-targeting siRNAs (**A**), were introduced into H1299 cells in the indicated combinations. After 24 hours, cellular levels of p53 and p21 were compared by Western blotting with β-actin as a loading control. Cellular invasiveness and ROS levels were analyzed using Matrigel-coated filters and DCF fluorescence, respectively. n.s., nonsignificant.

residues of Bcl-w (Fig. 2B, middle) were deleted. In contrast, these deletions did not significantly influence p21/p53 or Bcl-w/p53 interactions, suggesting that the terminal residues mediate the p21/Bcl-w, but not the p21/p53 or Bcl-w/p53 interactions. This is consistent with our previous findings that p21 binds to p53 via its 12 C-terminal residues (3). Bcl-w/p53 interactions were prevented by deleting the 40 N-terminal residues of Bcl-w, but they were not affected by a deletion of only the 27 N-terminal residues (Fig. 2B, right), suggesting that the Bcl-w N-terminal residues 28–40 mediate p53 binding. Conversely, Bcl-w$^{\Delta N40}$ efficiently bound to p21, confirming that the N-terminal residues of Bcl-w are not involved in this interaction. These results suggest that

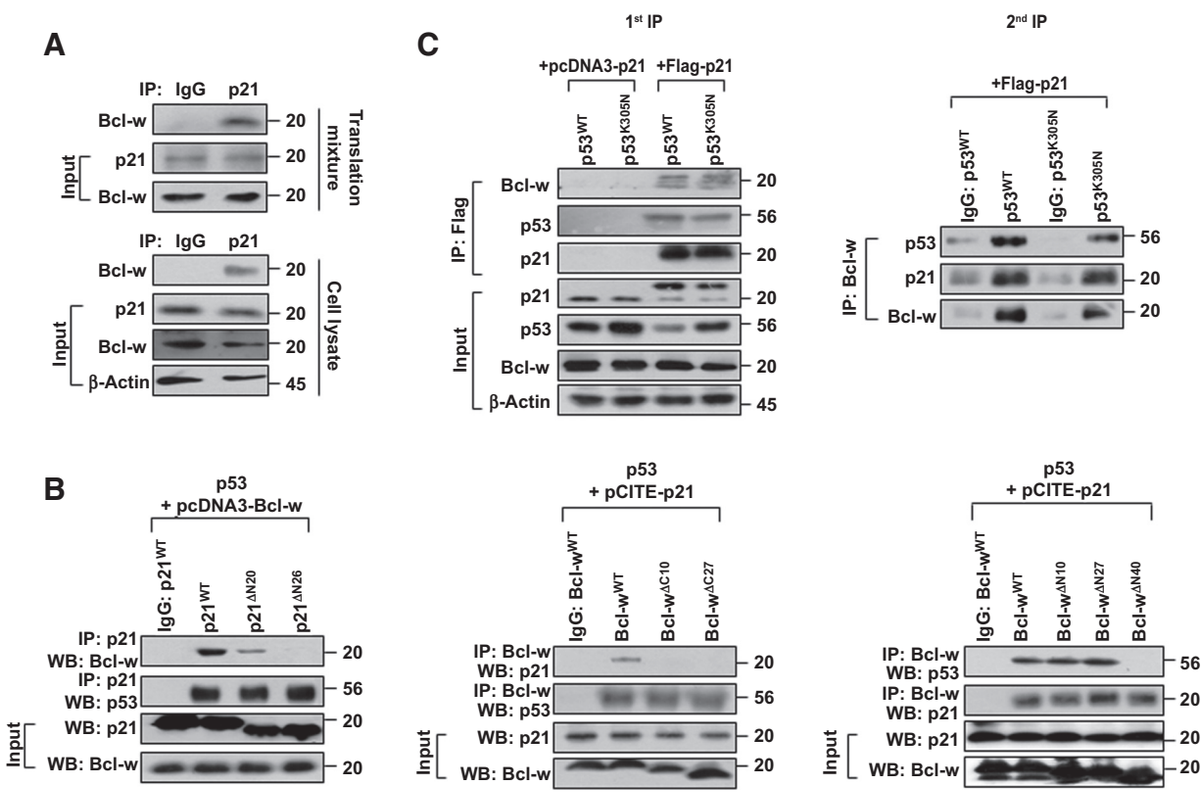

Figure 2.
p21 forms a complex with p53 and Bcl-w. **A,** *In vitro* translation mixtures of p21 and Bcl-w (top) or H1299 cell lysates (bottom) were immunoprecipitated (IP) with an anti-p21 antibody or control IgG. Precipitates and input controls were analyzed by Western blotting (WB) with anti-p21 and anti-Bcl-w antibodies. **B,** *In vitro* translation was performed to generate the following proteins: p21, its N-terminal deletion derivatives, and Bcl-w (left), or p21 and Bcl-w with its C-terminal (middle) or N-terminal (right) deletion derivatives. All reaction mixtures received recombinant p53 protein. Immunoprecipitation was carried out using anti-p21 (left) or anti-Bcl-w antibodies (middle and right). Precipitates and inputs were analyzed for the levels of p21, Bcl-w, and p53. **C,** p53/H1299 and p53^{K305N}/H1299 transfectants were additionally transfected with pFLAG-C1-p21 or pcDNA3-p21 as a negative control vector for Flag. Cell lysates were prepared and immunoprecipitated with an anti-Flag antibody (1st IP). Precipitated proteins were then eluted using the Flag peptide and immunoprecipitated again with an anti-Bcl-w antibody (2nd IP). The first and second precipitates and inputs were analyzed for the levels of Bcl-w, p53, and p21.

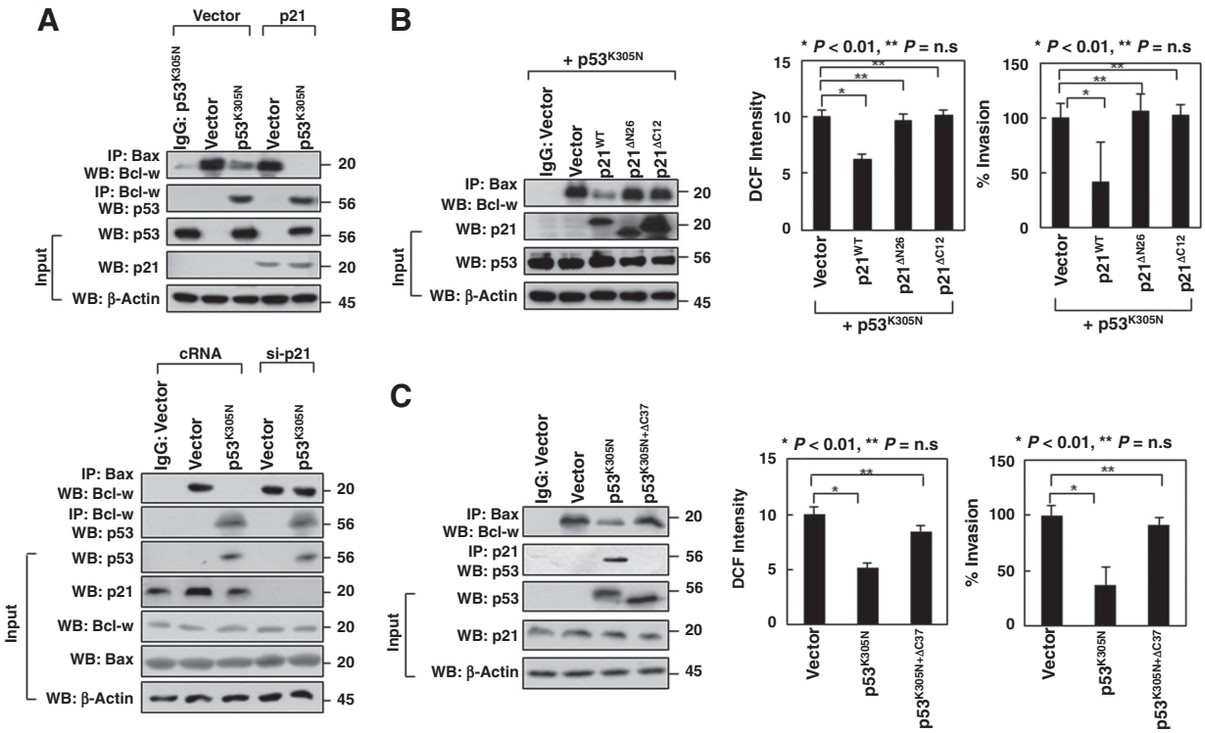

Figure 3.

Formation of the p53/p21/Bcl-w complex is required for releasing Bax from Bcl-w and suppressing cell invasion. **A,** H1299 cells were transfected with the specified expression vectors and siRNAs. Cell lysates were analyzed by immunoprecipitation and subsequent Western blotting using the indicated antibodies. **B** and **C,** $p53^{K305N}$-H1299 (**B**) or H1299 (**C**) cells were transfected with the indicated expression vectors. Bcl-w/Bax interaction in cell lysates, cellular ROS levels, and invasiveness was analyzed. n.s., nonsignificant.

Bcl-w binds to p53 and p21 through different areas of its structure. Furthermore, we have previously reported that p53 interacts with Bcl-w via the arginine residue 175 (7) and p21 via the 37 C-terminal residues (3). Taken together, these findings illustrate that p53, p21, and Bcl-w interact with each other via nonoverlapping residues, allowing the formation of a tripartite complex. This was directly demonstrated by a two-step coprecipitation assay using lysates from H1299 cells expressing Flag-tagged p21 and p53 (or $p53^{K305N}$; Fig. 2C). Therefore, our results indicate that Bcl-w serves as a binding target of the p53/p21 (or $p53^{K305N}$/p21) complex.

Notably, Bcl-w failed to coprecipitate with $p21^{\Delta N26}$, despite the ability of $p21^{\Delta N26}$ to interact with their shared binding partner p53 (Fig. 2B, left). Similarly, p21 (Fig. 2B, middle) and p53 (Fig. 2B, right), despite their direct interaction (3), failed to coprecipitate with the p53/Bcl-$w^{\Delta C10}$ and the p21/Bcl-$w^{\Delta N40}$ complexes, respectively. Overall, it appears that whereas any two of these components can form a complex, the p53/p21/Bcl-w complex is formed only when all of the individual interactions (p53/p21, p53/Bcl-w, and p21/Bcl-w) are maintained.

p53 requires p21 to release Bax from Bcl-w

Consistent with the report that the binding of cytoplasmic p53 to Bcl-w triggers dissociation of the Bcl-w/Bax complex (7), expression of $p53^{K305N}$ in H1299 cells reduced Bcl-w/Bax complex levels (Fig. 3A, top). This effect was enhanced by simultaneous p21 overexpression, whereas p21 overexpression alone did not substantially alter the complex levels. This suggests that p21 was only able to reduce Bcl-w/Bax interactions in cooperation with $p53^{K305N}$. Therefore, Bcl-w/Bax complex dissociation by $p53^{K305N}$ expression might reflect the cooperative action of $p53^{K305N}$ and endogenous p21. In support of this possibility, $p53^{K305N}$ expression failed to reduce Bcl-w/Bax interactions upon p21 knockdown (Fig. 3A, bottom). Conversely, $p53^{K305N}$/Bcl-w interactions were not substantially influenced by either p21 overexpression or knockdown (Fig. 3A), supporting the irrelevance of p21 to the $p53^{K305N}$/Bcl-w interaction itself. Collectively, the data suggest that, although $p53^{K305N}$ can bind to Bcl-w alone (7), it requires p21 for liberating Bax from Bcl-w.

Formation of the p53/p21/Bcl-w complex is required for the release of Bax from Bcl-w and for suppressing cell invasion

To investigate whether dissociation of the Bcl-w/Bax complex required the interactions between $p53^{K305N}$, p21, and Bcl-w, we first used $p21^{\Delta N26}$ (Fig. 2B) and $p21^{\Delta C12}$ (3), which do not bind to Bcl-w and p53, respectively. In contrast to p21, neither $p21^{\Delta N26}$ nor $p21^{\Delta C12}$ cooperated with $p53^{K305N}$ to reduce Bcl-w/Bax interactions, ROS production, or cell invasion (Fig. 3B), suggesting that p21 must bind to both Bcl-w and p53 to promote Bax release and its anti-invasive activity. We have previously reported that $p53^{K305N+R175H}$, a Bcl-w binding–deficient cytoplasmic p53 mutant, also fails to liberate Bax and suppress the ROS-dependent invasion pathway (7). Similar results were obtained using $p53^{K305N+\Delta C37}$, a p21 binding–deficient cytoplasmic p53 mutant (Fig. 3C; ref. 3). Overall, our data suggest that each of the p21/

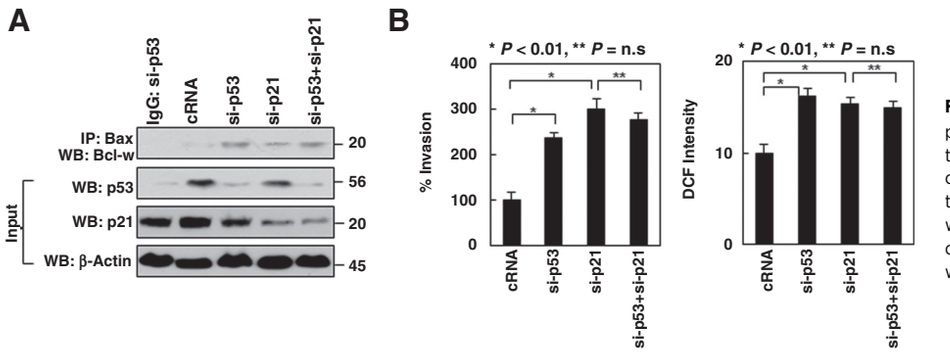

Figure 4.
p21 cooperates with cytoplasmic $p53^{wt}$ to suppress Bcl-w/Bax interaction and cell invasion. **A** and **B,** IMR-32 cells were treated with the indicated siRNAs. Bcl-w/Bax interaction in cell lysates (**A**), cellular ROS levels, and invasiveness (**B**) was analyzed. n.s., nonsignificant.

$p53^{K305N}$, p21/Bcl-w, and $p53^{K305N}$/Bcl-w interactions and the formation of the whole $p53^{K305N}$/p21/Bcl-w complex are required for dissociating Bax from Bcl-w, thus suppressing ROS production and cell invasion.

p21 cooperates with cytoplasmic $p53^{wt}$ to liberate Bax and suppress cell invasion

To validate the cooperation between p21 and cytoplasmic $p53^{wt}$, we used human IMR-32 neuroblastoma cells, which exhibit $p53^{wt}$ cytoplasmic accumulation (9). p53 knockdown in these cells increased Bcl-w/Bax interactions, ROS production, and cell invasion (Fig. 4), confirming the ability of cytoplasmic $p53^{wt}$ to suppress these features by releasing Bax from Bcl-w (7). Similar results were obtained following p21 knockdown. This contrasts with the finding that in $p53^{null}$ cells, p21 knockdown does not affect Bcl-w/Bax interaction (Fig. 3A), ROS production, or cell invasion (Fig. 1A) and thus suggests that p21 cooperates with cytoplasmic $p53^{wt}$ in IMR-32 cells to inhibit these activities. Furthermore, knockdown of both p21 and p53 in IMR-32 cells, compared with knockdown of the individual proteins, did not have an additive effect (Fig. 4), consistent with a model whereby both p21 and cytoplasmic $p53^{wt}$ are required for Bax liberation and its anti-invasive functions.

p21 is required for the apoptotic action of cytoplasmic p53

To investigate whether p21 is also required for the apoptotic function of cytoplasmic p53, we irradiated H1299 cells with γ-rays (20 Gy) inducing approximately 50% cell death (Fig. 5A), with a concomitant release of mitochondrial cytochrome *c* to the cytosol (Fig. 5B), reflecting a p53-independent apoptosis mechanism. Following p53 introduction, irradiation resulted in approximately 80% of cell death without a further increase in cytochrome *c* release. Similarly, under other experimental conditions, the levels of released cytochrome *c* did not correlate with the extent of apoptosis (15). We also confirmed p53 translocation from the nucleus to the cytosol upon irradiation of p53/H1299 transfectants. Therefore, the increase (~30%) in cell death following p53 expression appears to reflect the apoptotic action of translocated cytoplasmic p53. The introduction of $p53^{K305N}$ into H1299 cells also increased γ-irradiation–induced cell death to approximately 80%, suggesting similar efficiencies of $p53^{K305N}$ and p53 to mediate cell death.

The apoptotic actions of p53 and $p53^{K305N}$ were further confirmed by their ability to dissociate the Bcl-w/Bax complex upon γ-irradiation (Fig. 5C). In contrast, $p53^{K305N+R175H}$ failed to induce p53-dependent cell death (Fig. 5A) or reduce the Bcl-w/Bax interaction upon γ-irradiation (Fig. 5C), suggesting that $p53^{K305N}$, similar to cytoplasmic $p53^{wt}$, is able to bind to survival Bcl-2 proteins, liberate Bax, and induce apoptosis. Notably, upon p21 knockdown, both p53 and $p53^{K305N}$ failed to dissociate the Bcl-w/Bax complex (Fig. 5C) and induce p53-dependent cell death upon irradiation (Fig. 5D). These data support the requirement of p21 for the apoptotic actions of cytoplasmic p53 in response to γ-irradiation. Furthermore, similar results were obtained when the experiments shown in Fig. 5A–D were repeated using an alternative apoptotic stimulus (500 μmol/L H_2O_2 treatment; Supplementary Fig. S1). Therefore, the role of p21 in cytoplasmic p53-dependent apoptosis appears to be similar in multiple types of apoptotic stimuli.

Cytoplasmic p53 binding to p21 is required for inducing apoptosis

To further validate the role of p21 in apoptosis, we introduced into H1299 cells $p53^{\Delta C37}$, which does not bind to p21 (3). Similar to p53, $p53^{\Delta C37}$ localized to the nucleus and was translocated to the cytosol upon γ-irradiation; however, it was much less effective than p53 in dissociating the Bcl-w/Bax complex (Fig. 5E) and inducing cell death in response to γ-irradiation (Fig. 5F). This suggests that p53 binding to p21 is required to induce apoptosis.

Cooperation between p53 and p21 supports tumor radiotherapy

To investigate whether p53 and p21 cooperate to facilitate cell death *in vivo*, xenograft tumors were established in mice using H1299, p53/H1299, or $p53^{\Delta C37}$/H1299 transfectants and subjected to radiotherapy. In untreated mice, the tumors grew at similar rates, whereas after radiotherapy, their growth was differentially retarded; the growth of p53/H1299 tumors was affected to a substantially higher degree than that of H1299 and $p53^{\Delta C37}$/H1299 tumors (Fig. 6A), which displayed similar growth retardation rates, suggesting that p53 mediates the therapeutic effect of radiation by binding to p21. To confirm that the radiation effects reflected tumor cell death, the tumors were excised and analyzed by the TUNEL assay. Radiotherapy increased cell death in H1299 and $p53^{\Delta C37}$/H1299 tumors to a similar extent, but it had a greater effect on p53/H1299 tumors (Fig. 6B). The *in vivo* results are consistent with those observed *in vitro* (Fig. 5F) and show a tight correlation between cell death and radiotherapy-induced growth retardation (Fig. 6A), suggesting that radiotherapy-induced tumor cell death contributes to the observed tumor growth retardation. In addition, the differential responses of p53/H1299 and $p53^{\Delta C37}$/H1299 tumors to radiotherapy support the view that the cooperation between p53 and p21 is required for realizing radiation-induced cell death *in vivo*.

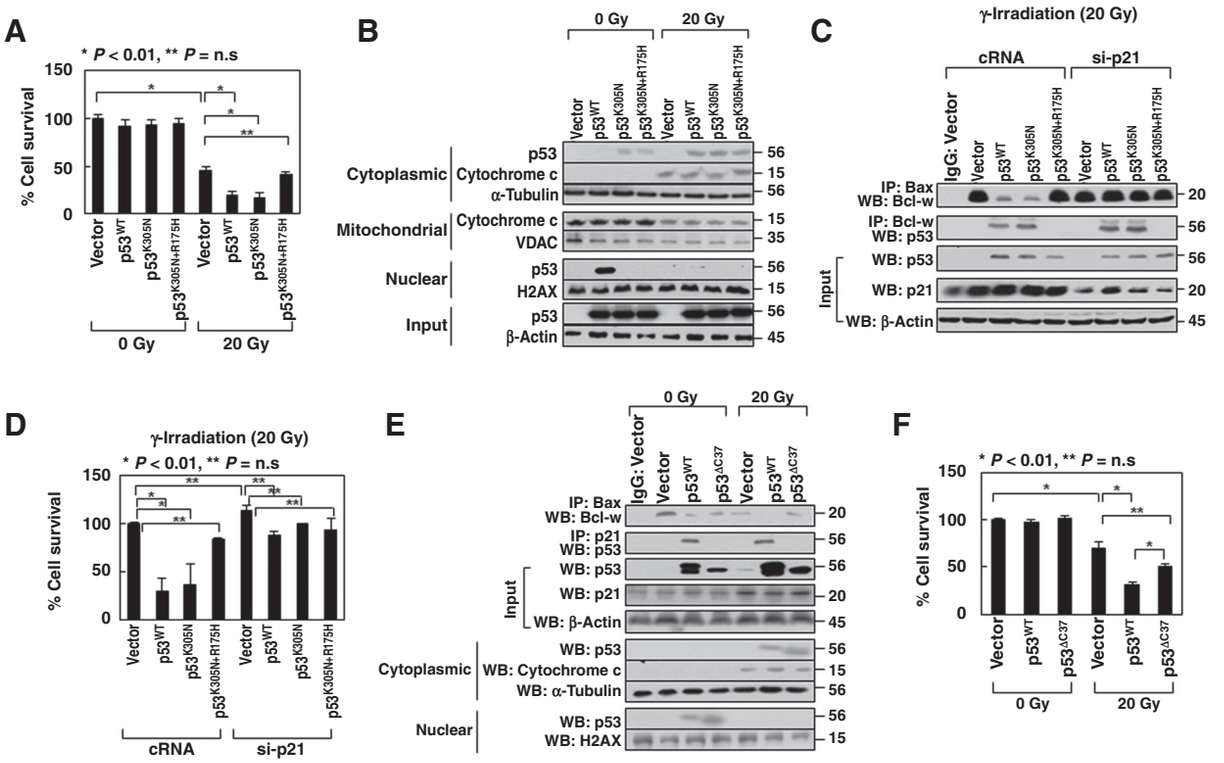

Figure 5.

p53/p21 cooperation is required for inducing apoptosis. **A,** H1299 cells stably transfected with the indicated expression vectors were subjected to 20 Gy γ-irradiation. After a 72-hour incubation, cell viability was analyzed by flow cytometry. **B,** The transfectants were lysed 24 hours postirradiation. Nuclear, mitochondrial, and cytosolic fractions were prepared and analyzed by Western blotting with the indicated antibodies. H2AX, VDAC, and α-tubulin were used as nuclear, mitochondrial, and cytosolic markers, respectively. **C,** The transfectants were treated with control or p21 siRNA and then subjected to 20 Gy γ-irradiation. After a 24-hour incubation, cell lysates were analyzed for Bcl-w/Bax and Bcl-w/p53 interactions by coimmunoprecipitation. **D,** Cell viability measured 72 hours postirradiation. **E,** The specified transfectants were subjected to 20 Gy γ-irradiation. After 24 hours, Bcl-w/Bax and Bcl-w/p53 interactions in cell lysates as well as p53 and cytochrome *c* levels in nuclear and cytosolic fractions were analyzed. **F,** Cell viability measured 72 hours postirradiation is shown. n.s., nonsignificant.

Bcl-X_L represents a target of p53/p21 cooperative function

As prosurvival Bcl-2 proteins commonly interact with p53, proteins other than Bcl-w might also serve as targets of the p53/p21 cooperative action. Bcl-X_L indeed interacted with p21 (Supplementary Fig. S2A). Moreover, Bcl-X_L/Bax interactions were prevented by the presence of both p53 (or $p53^{K305N}$) and p21 but not by that of either protein individually (Supplementary Fig. S2B). Similarly, Bcl-X_L/Bax interactions in H1299 cell lysates were reduced by $p53^{K305N}$ expression but not p21 overexpression (Supplementary Fig. S2C, left). The effects of $p53^{K305N}$ were enhanced or abolished upon p21 coexpression (Supplementary Fig. S2C, left) or knockdown (Supplementary Fig. S2C, right), respectively. Similar effects of p53 (or $p53^{K305N}$) expression and/or p21 knockdown on Bcl-X_L/Bax interactions were observed in cells irradiated with lethal doses of γ-rays (Supplementary Fig. S2D). These results are consistent with those obtained with Bcl-w, suggesting that the cooperative action between p53 and p21 applies to other prosurvival Bcl-2 proteins, such as Bcl-X_L.

Cytoplasmic p53 cooperates with p21 in multiple cell types

To investigate whether cytoplasmic p53 could cooperate with p21 in cells other than H1299, the $p53^{null}$ Calu-1 lung and HCT116 colon cancer cells were selected. Similar to H1299 cells, $p53^{K305N}$ expression in Calu-1 cells reduced cellular ROS levels and invasiveness, and these effects were abrogated by p21 knockdown (Supplementary Fig. S3A). In addition, p21 overexpression reduced the invasiveness of $p53^{K305N}$-expressing Calu-1 cells but not that of control cells (Supplementary Fig. S3B). Furthermore, the introduction of p53 or $p53^{K305N}$ but not of $p53^{K305N+R175H}$ increased radiation-induced cell death, which was abolished by p21 knockdown (Supplementary Fig. S3C). Similar results were obtained when the experiments were repeated in HCT116 cells (Supplementary Fig. S4). Notably, analysis of HCT116 tumors in mice revealed that only p53, and not $p53^{\Delta C37}$, facilitated the therapeutic effects of γ-irradiation *in vivo* (Supplementary Fig. S5). These data indicate that cytoplasmic p53 cooperates with p21 to suppress invasion and promote death of Calu-1 and HCT116 cells. Furthermore, γ-irradiation killed H460 lung cancer cells ($p53^{wt}$) with dissociation of the Bcl-w/Bax complex (Supplementary Fig. S6). This latter event was reversed, albeit partially, by p21 knockdown. This effect of p21 knockdown was not observed in H1975 lung cancer cells harboring $p53^{R273H}$ that does not bind to Bcl-2 proteins (16). Overall, it is clear that p53/p21 cooperation is a general phenomenon occurring in multiple cell types, which can be influenced by p53 mutations.

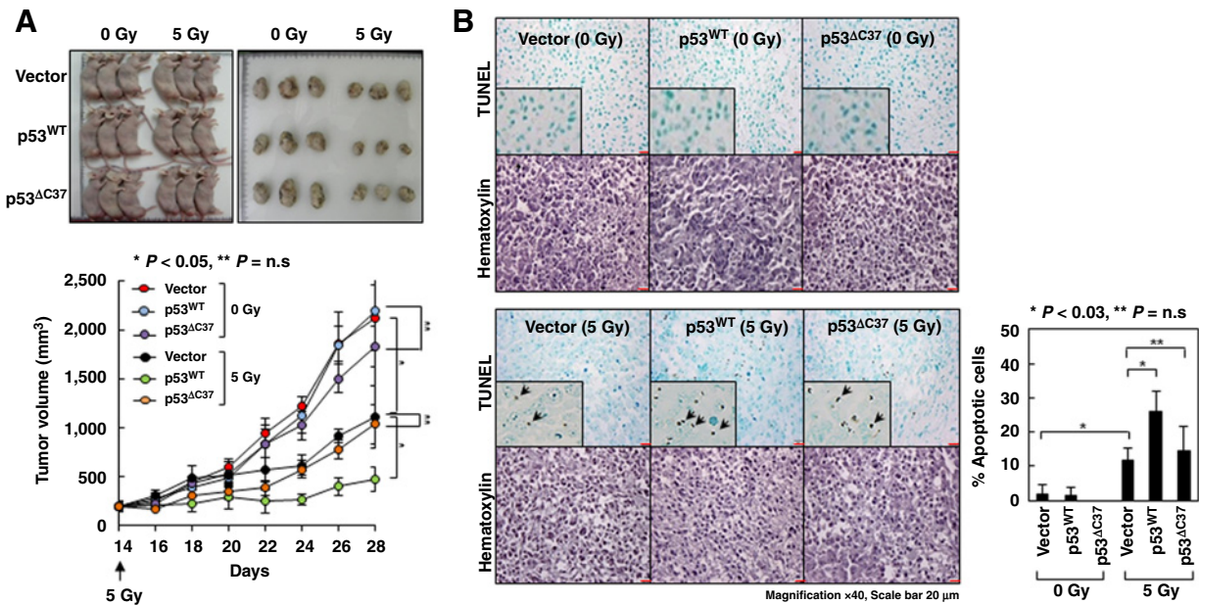

Figure 6.

p53/p21 cooperation is required for tumor radiotherapy response and cell death *in vivo*. **A,** H1299, H1299/p53, and H1299/p53$^{\Delta C37}$ transfectants were implanted in mice to form xenograft tumors. After 14 days, the tumors were locally irradiated with γ-rays (5 Gy), and their volumes were measured every other day. Mice were sacrificed and the tumors were excised 2 weeks postirradiation. **B,** Sections of the excised tumors were analyzed by the TUNEL assay or stained with hematoxylin. The percentage of TUNEL-positive relative to hematoxylin-positive total cells (% apoptotic cells) was determined. Arrows, representative TUNEL-positive cells, which appeared as black dots.

Discussion

Here, we demonstrated that the previously proposed functions of p53 to regulate cell invasion and death by targeting Bcl-2 proteins actually reflect those of the p53/p21 complex, as supported by various lines of both *in vitro* and *in vivo* evidence obtained by the analyses of protein–protein interactions, cell invasion, and death, as well as radiotherapy-induced tumor retardation in mice.

We first showed direct interactions between p21 with Bcl-w. Considering that p53 binds to both p21 (3) and Bcl-w (7), we subsequently analyzed the interactions between the three molecules and found that they interact with each other via distinct sites, forming a p53/p21/Bcl-w complex. By preventing each of the p53/p21, p53/Bcl-w, or p21/Bcl-w interactions by mutation or deletion, we further showed that all three interactions are required to form the p53/p21/Bcl-w complex and that only the trimeric complex, but not any of the individual dimers, facilitates the release of Bax from Bcl-w. We also analyzed the ability of p53^{K305N}, which we used as a model for cytoplasmic p53, to form a complex with p21 and Bcl-w and to liberate Bax and did not find a noticeable difference from the results obtained with p53^{wt}.

Liberated Bax promotes apoptosis or suppresses healthy cell invasion (4–8). We found that cytoplasmic p53 and p21 are both required to manifest these anti-invasive and proapoptotic activities. By using derivatives of p53^{K305N} (or p53) and p21, we further verified that formation of the p53/p21/Bcl-w complex is essential for the regulation of cell functions by p53 and p21. Notably, our finding that p53$^{\Delta C37}$ fails to recapitulate the effects of p53 with respect to radiotherapy-induced tumor retardation and cell death in mice supports the *in vivo* functions of the p53/p21 complex and its contribution to tumor therapy.

The combined biochemical and functional data obtained in this study suggest that the p53/p21 complex rather than p53 alone binds to Bcl-w and liberates Bax to promote its anti-invasive and proapoptotic functions (Fig. 7). This model appears to represent a general phenomenon, as supported by our consistent results in diverse types of cancer cells (lung, colon, and neuroblastoma cells), using two types of death stimuli (γ-irradiation and H_2O_2 treatment). The subsequent verification that p21 binds to Bcl-X_L and cooperates with p53 to release Bax from Bcl-X_L further suggests that the p53/p21 complex might act on other prosurvival Bcl-2 proteins as well.

There is evidence to support the clinical relevance of our model. First, chemoradiotherapy in patients with esophageal cancer resulted in worse therapeutic responses in p53$^+$/p21$^-$ cases than in p53$^+$/p21$^+$ cases (17). The latter consistently showed higher survival rates than the former did for B-cell lymphoma (18), and gastric (19) and lung cancer (20). The association of p21 expression with better prognosis of p53-positive cancer patients is consistent with the requirement of p21 for the tumor-suppressing functions of cytosolic p53. It should also be mentioned that p53 frequently undergoes mutations in cancer, which are concentrated at particular residues such as R175, R248, and R273 (21). Notably, these hotspot mutations abolish the interaction of p53 with prosurvival Bcl-2 proteins (7, 16) and thus may contribute to tumor progression and therapy resistance, as suggested by our finding using the R175H mutation and H1975 cells. Indeed, such hotspot mutations in gastric cancer were significantly associated with poor prognosis, such as worse survival of patients and higher recurrence of cancer in other organs, particularly the liver,

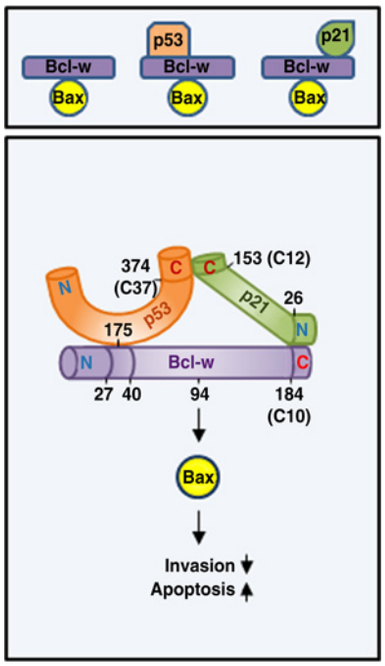

Figure 7.

Schematic model of the cooperative action of cytoplasmic p53 and p21. Although p53 and p21 can independently bind to Bcl-w, this does not induce the dissociation of Bax from the Bcl-w/Bax complex (top). However, the binding of both p53 and p21 to Bcl-w or potentially other prosurvival Bcl-2 proteins, such as Bcl-X_L, and thus the concomitant formation of the p53/p21/Bcl-w complex induces Bax liberation, promoting its anti-invasive and proapoptotic functions (bottom). p53-, p21-, and Bcl-w–interacting residues or regions are numbered from their N-termini; residue numbers from the C-termini are indicated in parentheses.

compared with that reported for wild-type p53 and other mutations (22). The association of p53 hotspot mutations with decreased survival was also reported for patients with B-cell lymphoma (18) and ovarian cancer (23). The high frequency of such p53 mutations that prevent p53/Bcl-2 protein interactions in many cancer types as well as the association of such mutations with poor prognosis further support the view that our model (Fig. 7) represents an important tumor suppression mechanism.

To our knowledge, this is the first report demonstrating that the prosurvival Bcl-2 proteins are direct targets of the p53/p21 complex. In addition to cell invasion and death, prosurvival Bcl-2 proteins can regulate various other cellular functions, including autophagy (24–27), senescence (28–30), and the epithelial–mesenchymal transition (31–34). Notably, these cellular functions are also regulated by p53 and p21 (1, 2, 30, 35–39). In particular, quinacrine, an antimalarial agent, requires both p53 and p21 to induce autophagy in colon cancer cells (35), suggesting that cooperation between p53 and p21 may be necessary for autophagy induction. On the contrary, the prosurvival Bcl-2 proteins bind to the autophagy protein Beclin 1 and inhibit autophagy (24). The functional antagonism between p53/p21 cooperation and prosurvival Bcl-2 proteins for the regulation of autophagy is reminiscent of their antagonism for the regulation of cell invasion and death, raising the possibility that the antiautophagic functions of prosurvival Bcl-2 proteins might also be regulated by the p53/p21 complex.

Our previous (3) and current results indicate that the p53/p21 complex suppresses cell invasion by targeting multiple cellular components, including Mdm2/Slug and the Bcl-2 proteins, which suggests that the p53/p21 complex comprises a functional unit that can act on various cellular targets. Interestingly, both p53 and p21 are required for sustaining the G_2 checkpoint after cell treatment with DNA-damaging agents (40). It is thus possible that the p53/p21 complex targets cellular components involved in the regulation of cell-cycle checkpoints. Further studies on such additional targets of the p53/p21 complex will significantly advance our understanding of the mechanisms whereby p53 and p21 regulate cell function and exert their tumor-suppressive functions.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: E.M. Kim, H.-D. Um
Development of methodology: E.M. Kim, C.-H. Jung
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): E.M. Kim, H.-D. Um
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): E.M. Kim, H.-D. Um
Writing, review, and/or revision of the manuscript: E.M. Kim, H.-D. Um
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J. Kim, S.-G. Hwang, J.K. Park
Study supervision: H.-D. Um

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