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A Truncated Form of p23 Down-regulates Telomerase Activity via Disruption of Hsp90 Function*

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The Hsp90-associated protein p23 modulates Hsp90 activity during the final stages of the chaperone pathway to facilitate maturation of client proteins. Previous reports indicate that p23 cleavage induced by caspases during cell death triggers destabilization of client proteins. However, the specific role of truncated p23 (Δp23) in this process and the underlying mechanisms remain to be determined. One such client protein, hTERT, is a telomerase catalytic subunit regulated by several chaperone proteins, including Hsp90 and p23. In the present study, we examined the effects of p23 cleavage on hTERT stability and telomerase activity. Our data showed that overexpression of Δp23 resulted in a decrease in hTERT levels, and a down-regulation in telomerase activity. Serine phosphorylation of Hsp90 was significantly reduced in cells expressing high levels of Δp23 compared with those expressing full-length p23. Mutation analyses revealed that two serine residues (Ser-231 and Ser-263) in Hsp90 are important for activation of telomerase, and down-regulation of telomerase activity by Δp23 was associated with inhibition of cell growth and sensitization of cells to cisplatin. Our data aid in determining the mechanism underlying the regulation of telomerase activity by the chaperone complex during caspase-dependent cell death.

Telomerase is a specialized reverse transcriptase responsible for the maintenance and preservation of telomere ends in germ cells, immortalized cells, and cancer cells (1). hTERT,[3] the reverse transcriptase subunit of telomerase, possesses catalytic activity, whereas the associated RNA component, human telomerase RNA, serves as a template for the synthesis of telomeric sequences (2). Expression analyses of hTERT and human telomerase RNA components in heterologous systems have enhanced our understanding of the biochemical features of telomerase. Human telomerase activity has been reconstituted in a variety of *in vitro* systems, including yeast, baculovirus, rabbit reticulocyte, wheat germ, and human cell extracts (3–5). In each system, the essential roles of hTERT and human telomerase RNA in active telomerase complexes have been confirmed. Recent studies have identified other proteins associated with the telomerase holoenzyme. For instance, Hsp90 and its co-chaperone, p23, bind hTERT and contribute to telomerase activity (6). The Hsp90 chaperone complex, which includes Hsp90, p23, Hsp70, p60, and Hsp40/ydj, is required for the assembly of human telomerase both *in vitro*, in a cell-free rabbit reticulocyte lysate system, and *in vivo*, in human cells.

Among the Hsp90 partners, the acidic protein p23 is the smallest and has a relatively simple structure (7, 8). p23 is ubiquitously expressed in all eukaryotes, from yeast to humans. Initially discovered as part of the Hsp90 complex with the progesterone receptor (9), p23 has since been identified in complexes containing a variety of Hsp90-associated proteins, including other steroid receptors (10), the heme-regulated kinase HRI (11), Fes tyrosine kinase, heat shock transcription factor, aryl hydrocarbon receptor (12), and polymerases such as telomerase (6) and hepatitis B-reverse transcriptase (13). Although it is known that p23 binds directly to Hsp90 in an ATP-dependent manner, it is unclear whether it interacts with client proteins or solely with Hsp90. Recent studies have confirmed that Hsp90 undergoes a conformational change upon binding to ATP, which promotes formation of additional dimer contacts near the N terminus (14). At this stage, ATP becomes trapped by Hsp90 and is committed to hydrolysis (15, 16). p23 then binds Hsp90 and stabilizes this conformational state. Early studies on steroid receptor complexes indicate that p23 stabilizes the mature complex with Hsp90 in a state in which the receptor is active and able to bind hormones (17–19).

Intensive studies using biochemical and crystallographic analyses revealed that p23 contains two domains: a stable, folded core domain and an unstructured, highly acidic C-terminal tail of ~30–50 amino acids (8). Human p23 is a 160-amino acid protein that contains eight β-strands (residues 1–110) and a C-terminal tail (residues 111–160). The tail is required for chaperone activity but does not participate in binding to Hsp90 (7). Nevertheless, C-terminal truncation of p23 decreases hormone-binding activity to the receptor (8). Inter-

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[3] The abbreviations used are: hTERT, human telomerase reverse transcriptase; CKII, casein kinase II; Hsp90, heat shock protein 90; PP5, protein phosphatase 5; GFP, green fluorescent protein; z, benzyloxycarbonyl; fmk, fluoromethyl ketone; TRAP, telomeric repeat amplification protocol; GA, geldanamycin.

estingly, recent reports have shown that several apoptotic stimuli induce p23 caspase-mediated cleavage at the C-terminal tail but do not directly alter interactions with Hsp90 (20–22). Active caspases 3, 7, and 8 cleave specifically at aspartic acid 142, generating a truncated form of p23 (Δp23) with 18 fewer amino acids than the wild-type protein. Recombinant Δp23 displays an affinity for Hsp90 similar to full-length protein but loses its own chaperone activity *in vitro*. However, it remains to be determined whether cleavage of p23 alters the chaperone activity of Hsp90.

In the present study, we found that p23 cleavage was associated with a reduction in hTERT expression and telomerase activity during apoptosis. Moreover, overexpression of Δp23 induced a decrease in the amount of hTERT protein without affecting hTERT mRNA levels, suggesting that the loss of p23 function destabilizes the hTERT protein. Furthermore, Δp23 suppressed phosphorylation at specific serine residues in Hsp90, and phosphoserine-defective mutants of Hsp90 failed to enhance telomerase activity. These data indicate that down-regulation of telomerase activity during apoptosis correlates with hTERT destabilization via loss of Hsp90 chaperone activity. To our knowledge, this is the first report of caspase-mediated regulation of the Hsp90 chaperone complex. These results further our understanding of the mechanisms that regulate telomerase activity in apoptotic cells.

EXPERIMENTAL PROCEDURES

Cell Lines and Constructs—HeLa and 293 cells were cultured in minimum essential medium or Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Invitrogen, Carlsbad, CA) in 5% CO_2. Human p23 cDNA was kindly provided by Dr. David Toft (Mayo Clinic, Rochester, MN). p23 cDNA was subcloned into pEGFP-C1 expression vector (BD Biosciences Clontech, San Diego, CA) and pcDNA4/HisMax vector (Invitrogen), following amplification. The human Hsp90α cDNA fragment (a kind gift from Dr. Takayuki Nemoto, Nagasaki, Japan) was removed from pGEX-2T by digestion with BamHI/SmaI and subcloned into pcDNA4/HisMax for expression in human cells. Two potential Hsp90 phosphorylation site mutants (S231A/S263A and S231E/S263E) were generated using the QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA). All constructs were verified by restriction enzyme digestion and DNA sequencing.

Immunoprecipitation—To assess protein-protein interactions, we performed co-immunoprecipitation of endogenous or vector-expressed proteins. Cells were lysed in M-PER® Reagent (Pierce) containing 4 mM ATP, 20 mM Na_2MoO_4, 1 mM $MgCl_2$, and 1× protease inhibitor mixture (Sigma) for 30 min on ice. After discarding cell debris, background proteins were pre-absorbed with 40 μl of protein A/G PLUS-agarose (Santa Cruz Biotechnology, Santa Cruz, CA). Anti-p23 (Affinity BioReagents, Golden, CO), anti-GFP (Santa Cruz Biotechnology), anti-phosphoserine (Chemicon, Temecula, CA), anti-Hsp90 (D7a, Upstate Millipore, Temecula, CA), and anti-ubiquitin (Santa Cruz Biotechnology) antibodies were added, followed by 40 μl of protein A/G PLUS-agarose. Immunoprecipitation was performed for 2 h or overnight at 4 °C. The resin was washed three times with co-immunoprecipitation buffer (Pierce) containing 4 mM ATP, 20 mM Na_2MoO_4, and 1 mM $MgCl_2$. Proteins were separated from the resin by boiling for 5 min in 2× SDS sample buffer (100 mM Tris-HCl (pH 6.8), 20% (v/v) glycerol, 4% (w/v) SDS, 200 mM 2-mercaptoethanol, 0.2% (w/v) bromphenol blue).

Protein Purification and in Vitro Binding Assay—To generate p23 depleted of the 18 C-terminal amino acids (Δp23), we constructed point mutants in which translation was terminated at 142 residues. T7-inducible pET-p23 vectors encoding full-length human p23 or Δp23 were produced. Bacterial cultures were grown according to the manufacturer's instructions. After 3-h induction with 0.2 mM isopropyl-β-D-thiogalactopyranoside, bacteria were pelleted and washed with phosphate-buffered saline. Next, bacterial cells were resuspended in 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 10 mM monothioglycerol (Sigma), and sonicated. The soluble extract was applied to a DEAE-cellulose column. Elution with 0–0.5 M KCl resulted in the isolation of highly purified p23 and Δp23. Protein-containing fractions were identified by immunoblotting. Fractions containing p23 or Δp23 were dialyzed into 10 mM Tris-HCl (pH 7.5), 50 mM KCl, 3 mM $MgCl_2$, and 10 mM monothioglycerol. Purified proteins were concentrated using an Amicon filtration unit to ~1 mg/ml, flash frozen, and stored at −70 °C.

Hsp90 binding was measured by combining 2 μg of native purified Hsp90 (Assay Designs Stressgen) with 2 μg of p23 in a final volume of 200 μl binding buffer (10 mM Tris-HCl, 50 mM KCl, 8 mM $MgCl_2$, 2 mM dithiothreitol, 4 mM ATP, 10 mM Na_2MoO_4, 0.01% Nonidet P-40, pH adjusted to 7.5), including an ATP-regeneration system consisting of 10 mM phosphocreatine and 7 units of creatine phosphokinase, as described previously (23). After incubation for 60 min at 30 °C, samples were chilled on ice and subjected to immunoprecipitation with anti-p23, as described above.

In Vitro Phosphorylation Assay—Rabbit reticulocyte lysate (40 μl, Promega, Madison, WI) was incubated with 10 μg of recombinant p23 or Δp23 in the presence of 50 mM HEPES (pH 7.4) and 2 μl of [γ-^{32}P]ATP (3000 Ci/mmol, 10 mCi/ml, PerkinElmer Life Sciences, Boston, MA) for 60 min at 37 °C. The reaction was chilled on ice and subjected to immunoprecipitation with anti-Hsp90, as described above. Immunoprecipitates were subjected to SDS-PAGE and autoradiography. The quantity of immunoprecipitated Hsp90 was monitored by Coomassie Blue staining and Western blotting.

Metabolic Labeling with [^{32}P]Orthophosphate—HeLa cells were grown to near confluency in 10-cm tissue culture dishes in minimum essential medium with 10% fetal bovine serum at 37 °C. Dishes were washed once with phosphate-free minimum essential medium and incubated for 15 min at 37 °C in phosphate-free minimum essential medium with 2% dialyzed bovine serum (Invitrogen). The medium in each dish was replaced with 5 ml of phosphate-free medium containing 0.5 mCi of carrier-free [^{32}P]orthophosphate (8500–9120 Ci/mmol, 10 mCi/ml, PerkinElmer Life Sciences). Cells were incubated for 60 min at 37 °C, washed in cold phosphate-buffered saline, and lysed in M-PER reagent containing 1× protease inhibitor mixture for 30 min on ice. Immunoprecipitation with anti-Hsp90 was performed as described above. Immunoprecipitates were subjected to SDS-PAGE and autoradiography. The quantity of

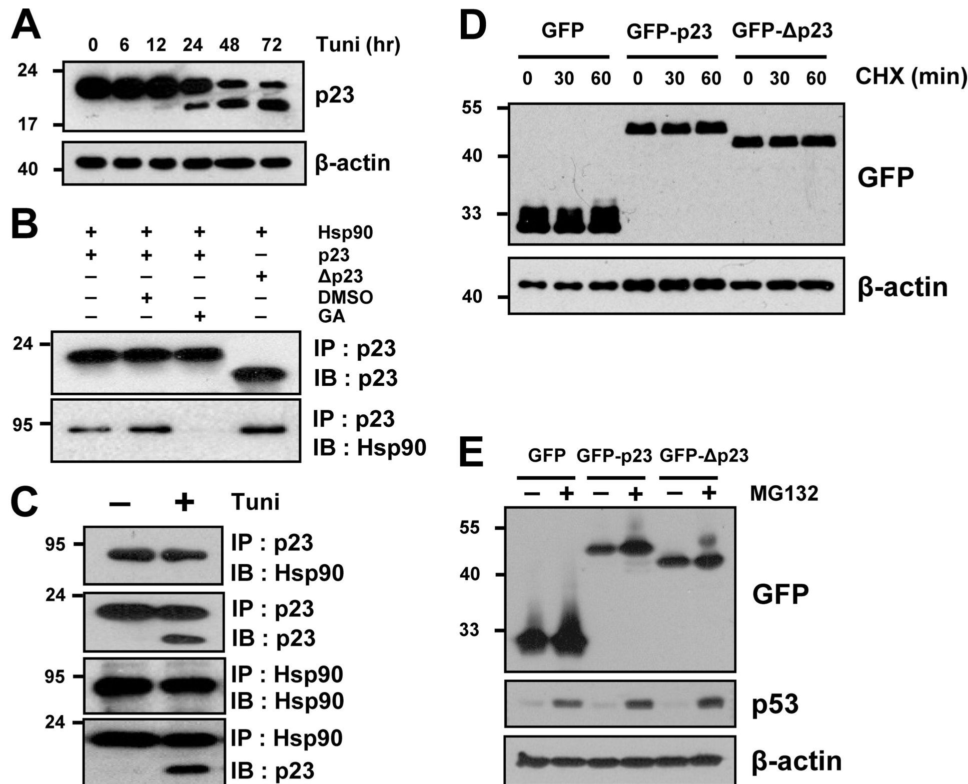

FIGURE 1. **Truncated p23 is stable and retains the ability to bind Hsp90.** *A*, HeLa cells were treated with 40 μM tunicamycin (*Tuni*) for the indicated times. p23 and its truncated form were analyzed by Western blotting with an anti-p23 antibody. *B*, Hsp90 (2 μg) was incubated with p23 (2 μg) or Δp23 (2 μg) in the presence or absence of 1 μM GA. DMSO was used as a vehicle control for GA treatment. Proteins in complexes were immunoprecipitated with anti-p23, and Hsp90 in immunoprecipitates was analyzed by Western blotting. *C*, HeLa cells were treated with 60 μM tunicamycin for 24 h, and cell lysates were immunoprecipitated with anti-p23 or anti-Hsp90 antibodies. Hsp90 and p23 in immunoprecipitates were analyzed by Western blotting. *D*, HeLa cells transfected with GFP, GFP-p23, or GFP-Δp23 were treated with 100 μg/ml cycloheximide (*CHX*), and protein turnover at the indicated times was analyzed by Western blotting with an anti-GFP antibody. *E*, HeLa cells transfected with GFP, GFP-p23, or GFP-Δp23 were treated with 10 μM MG132 for 8 h or left untreated. Cell lysates were analyzed by Western blotting. p53 accumulation was assessed as a positive control for MG132 activity. *β*-Actin was used as the loading control.

immunoprecipitated Hsp90 was monitored by Coomassie staining and Western blotting.

Evaluation of Apoptosis—Apoptosis was determined by staining cells with annexin V-phycoerythrin (BD Biosciences Pharmingen, San Diego, CA), as described previously (24). Briefly, detached cells were washed with phosphate-buffered saline, and resuspended in binding buffer (BD Biosciences Pharmingen) at a concentration of 1×10^6 cells/ml. After incubation with annexin V-phycoerythrin, cells were analyzed with FACScan flow cytometer (BD Biosciences, San Jose, CA).

Western Blot Analysis—Cell lysis and Western blot analyses were performed according to a previous report (24). Membranes were probed with the following antibodies: anti-caspase-3 and anti-caspase-7 antibodies (BD Biosciences Pharmingen); anti-Hsp70 antibody (Assay Designs Stressgen, Ann Arbor, MI); anti-p53 antibody (Novacastra Laboratories, Newcastle-upon-Tyne, UK); anti-MMP2, anti-TERT, and anti-casein kinase II (CKII)-α antibodies (Calbiochem); anti-β-actin antibody (Sigma); and anti-CDK4, anti-CDK6, anti-c-Src, anti-Flk-1, anti-His, anti-survivin, anti-IkB-α, anti-eNOS, anti-Wee1, anti-Akt, and anti-Bid (Santa Cruz Biotechnology). The pan-caspase inhibitor, z-VAD-fmk, was purchased from Calbiochem, whereas tunicamycin was obtained from Sigma.

Telomerase Activity Assay—Telomerase activity was determined with the telomeric repeat amplification protocol (TRAP, TRAP-eze Telomerase Detection Kit (Chemicon), including a 36-bp internal standard to facilitate quantitation of activity), as described previously, with minor modifications (6). After telomerase extension for 30 min at 30 °C, products were amplified by three-step PCR (94 °C for 30 s, 59 °C for 30 s, and 72 °C for 60 s) for 30 cycles in a thermocycler. Amplified products were separated by electrophoresis on a 10% polyacrylamide gel and stained with ethidium bromide.

RESULTS

Stability and Binding Activity of Δp23—The p23 protein is cleaved by caspases during apoptotic cell death induced by several stimuli (20–22). The caspases responsible for cleavage of p23 are dependent on the cell type and the nature of the apoptotic stimulus. The C-terminal Asp-142 residue of p23 is predicted to be the most susceptible site for cleavage. To determine whether p23 cleavage affects the regulation of Hsp90 client proteins, we confirmed p23 cleavage and caspase dependence under our experimental conditions. Tunicamycin, an inhibitor of protein glycosylation, induced p23 cleavage in HeLa cells (Fig. 1*A*). Preincubation of HeLa cells with the pan-caspase inhibitor z-VAD-fmk abolished tunicamycin-induced p23 cleavage following tunicamycin treatment (Fig. 2), indicating that caspases are responsible for cleavage. In *in vitro* cleavage assays, p23 was most effectively cleaved by caspase-7, but was also cleaved, although to a lesser extent, by caspases 3, 8, and 9 (data not shown). Treatment with the caspase inhibitor z-DEVD-fmk, and replacement of Asp-142 with glutamate abolished p23 cleavage by caspase-7 (data not shown). Previous studies have shown that both native and truncated p23 bind to Hsp90 (20, 22). Here, we confirmed that truncated p23 interacts with Hsp90 both *in vitro* and *in vivo*. For *in vitro* binding assays, we purified recombinant full-length p23 and Δp23, as described under "Experimental Procedures." As expected, both wild-type p23 and Δp23 co-immunoprecipitated with purified human Hsp90 (Fig. 1*B*). Geldanamycin (GA), a benzoquinone ansamycin antibiotic, is a known Hsp90 inhibitor that specifically disrupts Hsp90-p23 interactions (25). Hsp90 did not co-immunoprecipitate with p23 in the presence of GA. To demonstrate that Δp23 and Hsp90 interact *in vivo*, we performed reciprocal co-immunoprecipitation experiments on HeLa cell lysates using p23- and

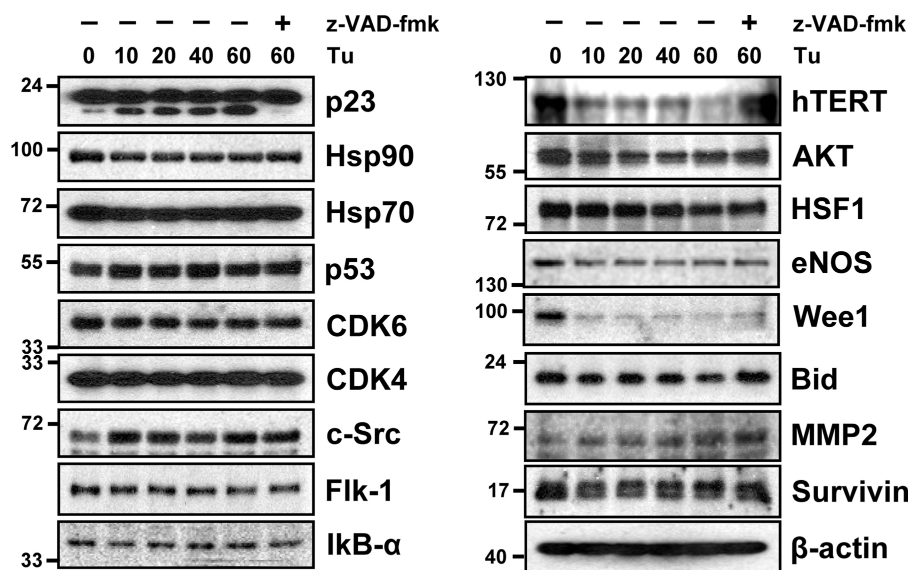

FIGURE 2. **Expression of Hsp90 client proteins during cell death.** HeLa cells were preincubated with z-VAD-fmk (100 μM) for 1 h and exposed to various concentrations of tunicamycin (*Tu*, in μM) for 24 h. Cell extracts were prepared and analyzed by Western blotting. β-Actin was used as the loading control.

Hsp90-specific antibodies. Δp23, as well as full-length p23, co-immunoprecipitated with Hsp90 in tunicamycin-treated HeLa cells (Fig. 1*C*). Notably, endogenous Hsp90 co-immunoprecipitated with both full-length and Δp23 in tunicamycin-treated cells, suggesting that Δp23-Hsp90 complex stably exists in the cells.

Next, we tested if Δp23 is destabilized and degraded through the ubiquitin-proteasome proteolytic pathway. HeLa cells were treated with tunicamycin for 6–72 h, and the amount of Δp23 was monitored over time. As shown in Fig. 1*A*, Δp23 was first detected in lysates prepared from cells treated with tunicamycin for 12 h, and displayed a time-dependent increase during the treatment period. Decreases in Δp23 were not observed for at least 72 h after exposure to tunicamycin. After blocking protein synthesis with cycloheximide, no significant differences in stability were observed between native and Δp23 (Fig. 1*D*). A recent report by Mollerup and Berchtold suggested that Δp23 is targeted to the proteasome (21). However, following treatment of HeLa cells expressing green fluorescent protein (GFP), GFP-tagged p23 (GFP-p23), or GFP-tagged Δp23 (GFP-Δp23) with the proteasome inhibitor MG132, GFP-p23 and GFP-Δp23 levels were similar to those of control GFP (Fig. 1*E*). Thus, it seems unlikely that Δp23 is more sensitive to the proteasomal degradation system than its native counterpart, although we cannot exclude the possibility that the GFP tag in our experiments compromised accessibility of Δp23 to the proteasome. Because Δp23 was not immediately degraded and remained stable in the cytosol, we speculated that it has roles in the regulation of Hsp90 chaperone machinery that are distinct from those of full-length p23.

Down-regulation of Telomerase Activity by Δp23—It has been suggested that Hsp90 confers stability to client proteins through interactions between the heterocomplex and the client protein, which are further stabilized by p23 (17, 23). Disruption of these heterocomplexes by Hsp90 inhibitors, such as GA, promotes proteolytic degradation of client proteins (25–27). Thus, if p23 loses its co-chaperone activity following caspase cleavage, depletion of client proteins may occur, regardless of Hsp90-binding activity. To examine this possibility, we determined the levels of several client proteins by Western blot analysis using lysates from HeLa cells treated with tunicamycin for 24 h (Fig. 2). Treatment with 10 μM tunicamycin for 24 h was sufficient to induce p23 cleavage, whereas z-VAD-fmk completely abolished p23 cleavage, even after exposure to concentrations as high as 60 μM. Notably, after tunicamycin treatment, hTERT levels were markedly reduced in association with p23 cleavage (Fig. 2). In addition, a greater degree of p23 cleavage was observed in cells exposed to 60 μM tunicamycin, coincident with a depletion of cellular hTERT. The decrease in hTERT was completely prevented by z-VAD-fmk. Because expression of hTERT is tightly linked to the regulation of telomerase function in normal and tumor cells (2), we next examined telomerase activity in tunicamycin-treated HeLa cells using a TRAP assay. As shown in Fig. 3*A*, telomerase activity was inhibited in tunicamycin-treated cells but was completely restored upon z-VAD-fmk pretreatment. Consistent with previous results, changes in hTERT expression levels paralleled changes in telomerase activity. Previous reports have demonstrated that full-length p23 is an essential component of the Hsp90 chaperone complex and is necessary to establish the active telomerase holoenzyme, both *in vitro* and *in vivo* (6). Accordingly, we determined whether Δp23 performs a similar telomerase-activity regulatory function. Expression of GFP-p23 in HeLa cells led to enhanced telomerase activity, as expected. In contrast, expression of GFP-Δp23 significantly reduced telomerase activity and hTERT expression compared with control GFP-expressing cells (Fig. 3*B*). Because GFP-Δp23-transfected HeLa cells express endogenous p23 (data not shown), it is possible that GFP-Δp23 functions as a dominant-negative telomerase inhibitor. The observed dose-dependent GFP-Δp23-mediated inhibition of telomerase activity and decreased hTERT expression support the specificity of the inhibitory effect (Fig. 3*C*). Our data collectively indicate that Δp23 induces hTERT depletion, and thereby inhibits telomerase activity.

Ubiquitination-mediated Degradation of hTERT by Δp23—To examine whether Δp23 regulates hTERT protein expression through transcriptional regulation of the *hTERT* gene, we used reverse transcription-PCR analysis to estimate hTERT mRNA levels in GFP-Δp23-transfected HeLa cells or in cells treated with tunicamycin to induce formation of Δp23. Expression of GFP-Δp23 did not affect hTERT mRNA levels compared with those in cells transfected with GFP only (Fig. 4*A*), indicating that the effect of Δp23 on hTERT levels was not mediated at the transcriptional level. Interest-

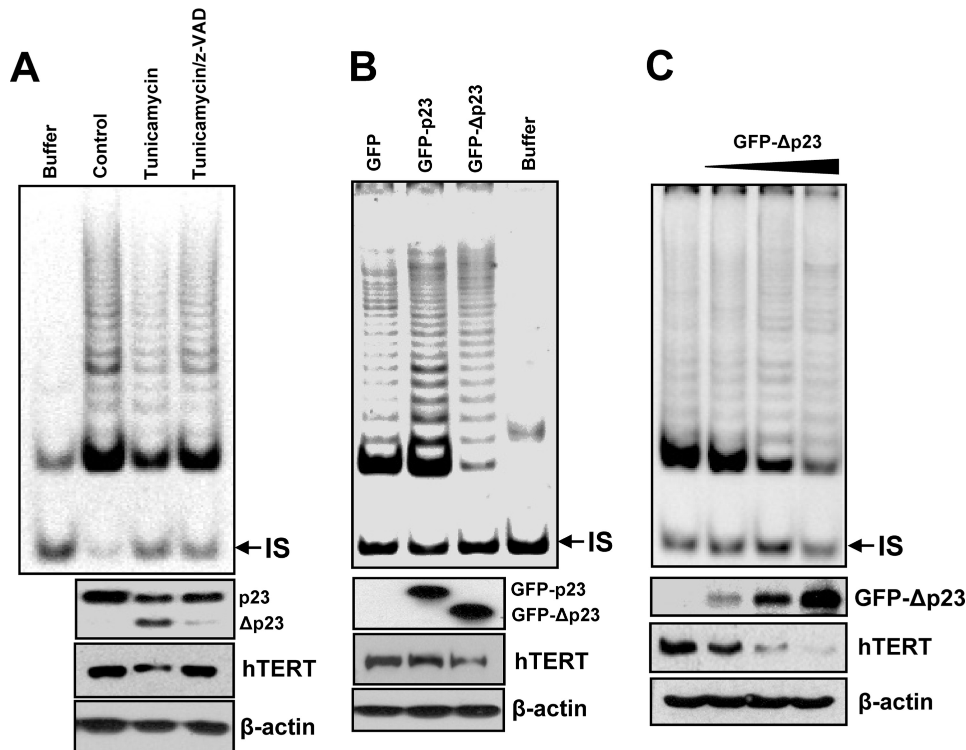

FIGURE 3. **Truncated p23 inhibits telomerase activity via down-regulation of hTERT.** *A*, HeLa cells were treated with 40 μM tunicamycin for 24 h in the presence or absence of z-VAD-fmk (100 μM). *B*, cells were transfected with GFP, GFP-p23, or GFP-Δp23. *C*, increasing concentrations (0.1, 0.2, or 0.4 μg/ml) of GFP-Δp23 were introduced into HeLa cells. Each set of cell lysates was assayed for telomerase activity by TRAP analysis and analyzed for protein expression by Western blotting. β-Actin was used as the loading control. *IS* represents the 36-bp internal standard. All experiments were repeated at least twice, yielding similar results.

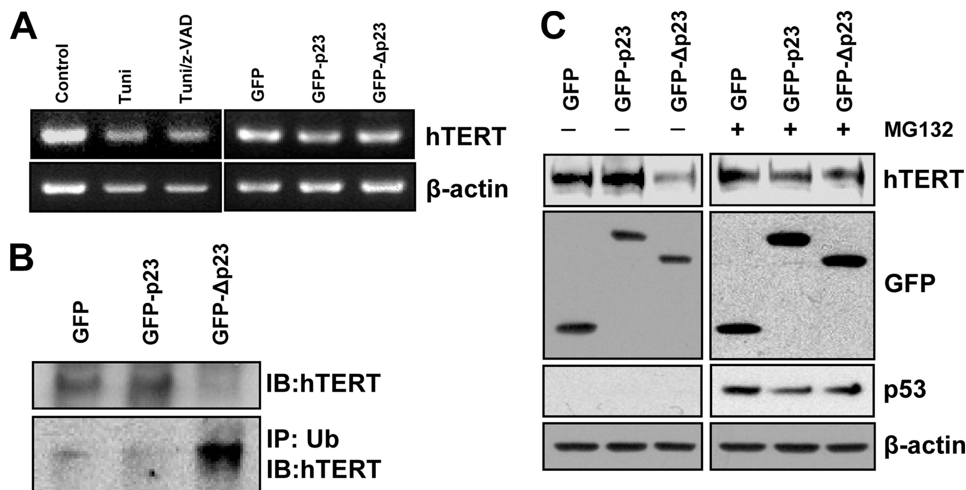

FIGURE 4. **Truncated p23 destabilizes hTERT protein by ubiquitination.** *A*, HeLa cells were treated with tunicamycin (40 μM) for 24 h in the presence or absence of z-VAD-fmk (100 μM), or were transfected with GFP, GFP-p23, or GFP-Δp23. RNA was isolated, and hTERT mRNA levels were analyzed by reverse transcription-PCR using specific primers (5′-TGAACTTGCGGAAGACAGTGG-3′ and 5′-ATGCGTGAAACCTGTACGCCT-3′). *B*, cells were transfected with GFP, GFP-p23, or GFP-Δp23. After 24 h, cell lysates were immunoprecipitated with an anti-ubiquitin (*Ub*) antibody. hTERT in immunoprecipitates was analyzed by Western blotting. *C*, HeLa cells transfected with GFP, GFP-p23, or GFP-Δp23 were treated with 10 μM MG132 for 8 h or left untreated. Cell lysates were analyzed by Western blotting. p53 accumulation was assessed as a positive control for MG132 activity. β-Actin was used as the loading control.

ingly, tunicamycin treatment induced suppression of hTERT mRNA levels. However, pretreatment with z-VAD-fmk, which prevents tunicamycin-induced reduction in hTERT protein level, did not restore hTERT transcript levels (Figs. 3*A* and 4*A*), suggesting that tunicamycin effects on hTERT transcription are functionally unrelated to the mechanism under investigation. To extend these observations, we next tested whether Δp23 inhibits its telomerase activity by destabilizing the hTERT protein. Prior studies have suggested that disruption of Hsp90 chaperone function induces ubiquitination and proteasome-mediated degradation of hTERT (28). To determine whether hTERT is ubiquitinated prior to degradation, we immunoprecipitated lysates from HeLa cells transfected with GFP, GFP-p23, or GFP-Δp23 using an anti-ubiquitin antibody, and evaluated immunoprecipitates by immunoblot with an anti-hTERT antibody. Overexpression of Δp23 markedly increased the level of ubiquitinated hTERT protein (Fig. 4*B*). To confirm that the observed reduction in hTERT levels was due to proteasome-dependent degradation, we treated GFP-Δp23-expressing cells with the proteasome inhibitor MG132. As shown in Fig. 4*C*, treatment with MG132 restored hTERT levels in GFP-Δp23-expressing cells.

Hsp90 Phosphorylation Is Enhanced by p23, but Not by Δp23— Hsp90 is constitutively phosphorylated at serine residues, although phosphorylation of threonine and tyrosine residues has also been reported (29–31). Prior studies have suggested that the pool of phosphorylated Hsp90 plays an important role in the functional regulation of client proteins (32, 33). On the basis of this finding, we examined the phosphorylation status of Hsp90 in GFP-Δp23-expressing HeLa cells. Serine phosphorylation of Hsp90 was significantly decreased in GFP-Δp23-expressing cells compared with cells expressing GFP or GFP-p23 (Fig. 5*A*). Neither phosphothreonine nor phosphotyrosine was detected in the same blot (data not shown). Upon immunoprecipitation of lysates with an anti-GFP antibody, serine phosphorylation was evident only in Hsp90 co-immunoprecipitated from GFP-p23-expressing cells (Fig. 5*B*, *bottom*). To examine the possibility that Δp23 functions as a phosphatase for Hsp90, we incubated recombinant Δp23 with phosphorylated Hsp90 purified from HeLa cell

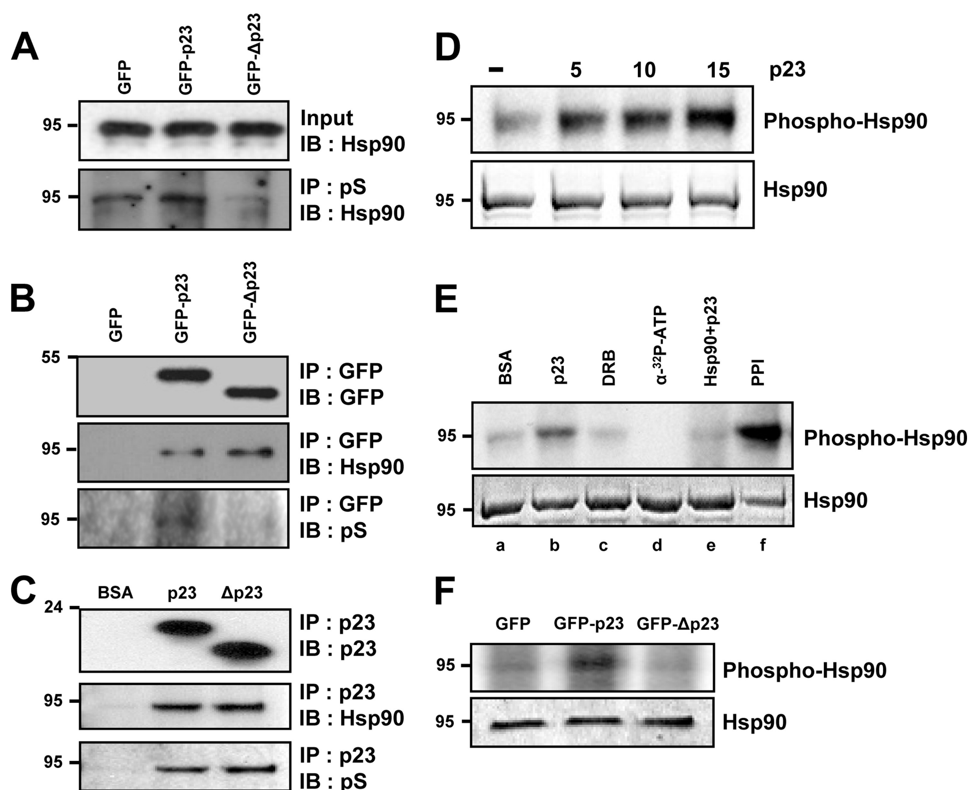

FIGURE 5. **Hsp90 phosphorylation is enhanced by full-length p23, but not by its truncated form.** HeLa cells were transfected with GFP, GFP-p23, or GFP-Δp23. After 24 h, cell lysates were immunoprecipitated with anti-phosphoserine (*pS*) (*A*) or anti-GFP antibodies (*B*), and immunoprecipitates were analyzed by Western blotting. *C*, purified Hsp90 was incubated with BSA, p23, or Δp23. Reactions were immunoprecipitated with an anti-p23 antibody, and immunoprecipitates were analyzed by Western blotting. *D*, rabbit reticulocyte lysates were incubated with the indicated doses of p23 (in μg) in the presence of γ-[32P]ATP, as described under "Experimental Procedures." *E*, rabbit reticulocyte lysates were incubated with 10 μg of BSA (*lane a*), 10 μg of p23 (*lanes b* and *d*), 10 μg of p23 plus 1 mM 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (*DRB*, *lane c*), or 10 μg of p23 plus 1× phosphatase inhibitor mixture 1 (*PP1*, Sigma, *lane f*) in the presence of γ-[32P]ATP or α-[32P]ATP (*lane d*), as described under "Experimental Procedures." Hsp90 (5 μg) was incubated with 10 μg of p23 in the presence of γ-[32P]ATP (*lane e*). *F*, HeLa cells were transfected with the indicated constructs and labeled with [32P]orthophosphate. Reactions and cell lysates were immunoprecipitated with an anti-Hsp90 antibody. Immunoprecipitates were separated by SDS-PAGE and analyzed by autoradiography. The autoradiogram shown is representative of three separate experiments.

lysates. However, the phosphoserine status of Hsp90 bound to Δp23 was unchanged relative to that of Hsp90 bound to full-length p23 (Fig. 5*C*). To investigate whether full-length p23 participates in Hsp90 phosphorylation, we reconstituted the complete chaperone machinery of Hsp90 using a rabbit reticulocyte lysate system. Interestingly, incubation of rabbit reticulocyte lysates with recombinant p23 and γ-[32P]ATP resulted in significant labeling of Hps90 (Fig. 5*D*). No radiolabeled band appeared when α-[32P]ATP was substituted for γ-[32P]ATP in the reaction, and treatment with a serine phosphatase inhibitor increased the intensity of the labeled band in the presence of γ-[32P]ATP, consistent with the identity of the band as phospho-Hsp90 (Fig. 5*E*). Although the molecular mechanisms underlying Hsp90 phosphorylation and dephosphorylation during the chaperone cycle are not clearly understood, it has been suggested that CKII and protein phosphatase 5 (PP5) are involved in the phosphorylation and dephosphorylation of Hsp90 serine residues, respectively (29, 31). To determine whether CKII is involved in p23-induced Hsp90 phosphorylation, we preincubated the reaction mixture with 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole, an inhibitor of CKII. Phosphorylation of Hsp90 by full-length p23 was completely abolished by 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole, indicating that CKII participates in this process (Fig. 5*E*). To confirm that p23 stimulates Hsp90 phosphorylation *in vivo*, we grew HeLa cells in the presence of [32P]orthophosphate and then immunoprecipitated Hsp90 with an anti-Hsp90 antibody. 32P-Labeled Hsp90 was observed in cells transfected with GFP-p23 but not in those transfected with GFP-Δp23 (Fig. 5*F*).

Regulation of Telomerase Activity via Hsp90 Phosphorylation—To clarify the mechanism of p23-mediated Hsp90 phosphorylation, we examined the interaction between Hsp90 and CKII in cells expressing GFP-p23. CKII is comprised of α and β subunits, of which the α subunit directly binds to Hsp90 (34). Co-immunoprecipitation analyses revealed that the interaction between Hsp90 and CKIIα was enhanced in GFP-p23-expressing cells (Fig. 6*A*). Because Hsp90 binds to and enhances CKII kinase activity (34), we conclude that p23 accelerates Hsp90 phosphorylation by promoting its association with CKII.

Next, we tested whether phosphorylation of Hsp90 directly affected telomerase activity. Two Hsp90 serine residues, Ser-231 and Ser-263, have been reported to be phosphorylation targets (29). Constructs encoding phosphorylation-defective (S231A/S263A) and phospho-mimetic (S231E/S263E) Hsp90 mutant proteins were prepared and introduced into HeLa cells. The double mutant S231A/S263A displayed decreased Hsp90 serine phosphorylation, indicating that these residues are authentic targets for phosphorylation (Fig. 6*B*). Importantly, the S231A/S263A mutant did not enhance telomerase activity; in contrast, the S231E/S263E phospho-mimetic mutant enhanced telomerase activity to an extent similar to that of wild-type Hsp90 (Fig. 8*C*).

We next examined the mechanism of Δp23-induced regulation of Hsp90 phosphorylation. We initially assumed that Δp23 did not affect the interaction between Hsp90 and CKIIα but found instead that Δp23 increased Hsp90 binding to CKIIα (Fig. 6*A*). PP5 is a known component of the Hsp90 chaperone complex (35). The yeast ortholog of PP5, Ppt1, has been identified as a serine phosphatase of yeast Hsp90 (31). Accordingly, we tested whether Δp23 affected the interaction between Hsp90 and PP5. As shown in Fig. 7*A*, overexpression of GFP-Δp23 effectively increased the binding of PP5 to

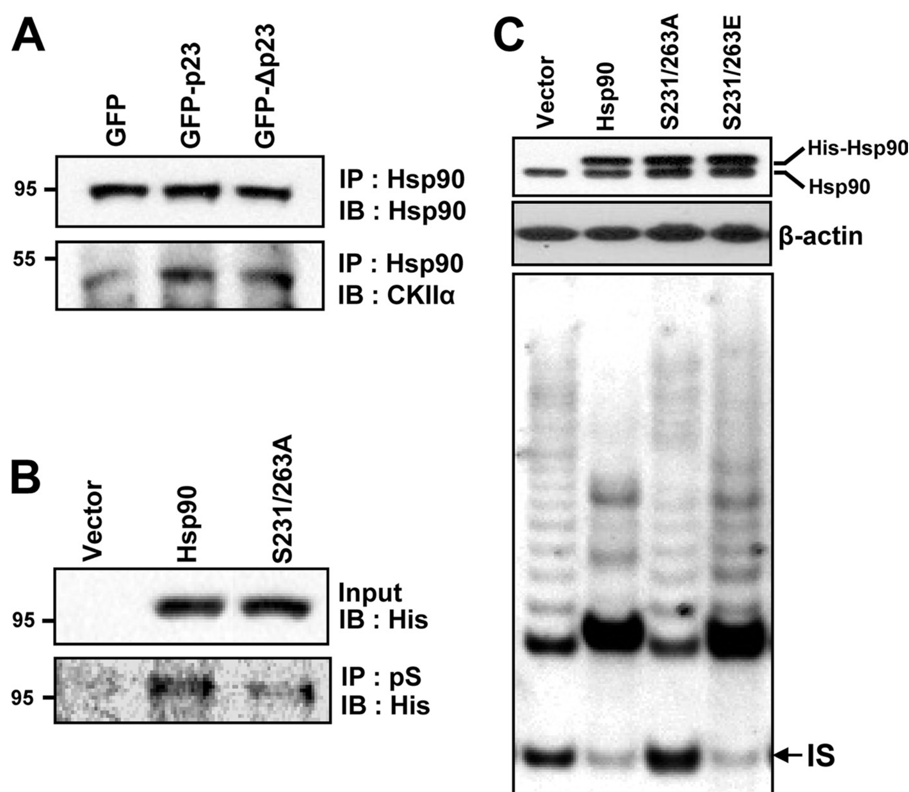

FIGURE 6. **Regulation of telomerase activity via Hsp90 phosphorylation.** *A*, HeLa cells were transfected with GFP, GFP-p23, or GFP-Δp23. After 24 h, cell lysates were immunoprecipitated with an anti-Hsp90 antibody, and immunoprecipitates were analyzed by Western blotting with anti-Hsp90 and anti-CKIIα antibodies. *B*, HeLa cells were transfected with empty vector, Hsp90, or His-tagged Hsp90 mutant (S231A/S263A). After 24 h, lysates were immunoprecipitated with an anti-pS antibody, and immunoprecipitates were analyzed by Western blotting. *C*, HeLa cells were transfected with empty vector, Hsp90, or His-tagged Hsp90 mutants (S231A/S263A or S231E/S263E). After 24 h, cell lysates were assayed for telomerase activity by TRAP analysis.

Hsp90. Likewise, co-immunoprecipitation experiments using purified recombinant proteins confirmed that PP5 bound more tightly to Hsp90 in the presence of Δp23 than in the presence of p23 (Fig. 7*B*).

To determine whether Hsp90 dephosphorylation influences telomerase activity, we assessed the effects of PP5 knockdown using small interfering RNAs. As expected, PP5-small interfering RNA decreased PP5 protein levels in HeLa cells and markedly increased telomerase activity (Fig. 7*C*). Small interfering RNA-mediated depletion of p23 protein led to a decrease in telomerase activity, consistent with the reported role of p23. These results indicate that PP5 is a negative regulator of telomerase. Accordingly, we conclude that Δp23 inhibits telomerase activity by promoting the interaction between PP5 and Hsp90 and further propose that full-length p23 induces serine phosphorylation of Hsp90, which is essential for enhancing telomerase activity. In contrast, Δp23 fails to enhance phosphorylation of Hsp90, resulting in destruction of the maturing hTERT peptide.

Effects of Δp23 on Cell Growth and Death—To address the physiological relevance of p23-mediated regulation of telomerase activity, we generated 293 cell lines stably expressing GFP, GFP-p23, or GFP-Δp23. Isolated clones were assayed for GFP, GFP-p23, or GFP-Δp23 expression by immunoblot and immunofluorescence analyses (Fig. 8*A*, *middle panel*, and data not shown). Telomerase activity and hTERT expression in stably transfected 293 cells were regulated in a manner similar to that in HeLa cells transiently expressing these proteins (Figs. 3*B* and 8*A*). Telomerase is essential for the maintenance of genomic integrity in rapidly growing cells, and inactivation of telomerase through depletion of human telomerase RNA rapidly inhibits the growth of human cancer cells expressing hTERT (36). Therefore, we reasoned that suppression of hTERT by Δp23 could block proper progression of cell growth. Consistent with this idea, we observed that the rate of growth in the GFP-Δp23 cell line was retarded compared with that in control and GFP-p23 cell lines (Fig. 8*B*).

A recent study showed that depletion of hTERT facilitates the induction of apoptotic cell death by genotoxic agents, particularly cisplatin (37). We thus evaluated whether overexpression of Δp23 influenced the chemosensitivity of cells to cisplatin. Analysis of apoptosis using annexin-V-phycoerythrin staining revealed a substantial increase in apoptotic cells in the cisplatin-treated GFP-Δp23 cell line compared with cisplatin-treated control and GFP-p23 cells (Fig. 8*C*). Inhibition of cisplatin-induced apoptosis in the GFP-p23 cell line is consistent with a previous model suggesting

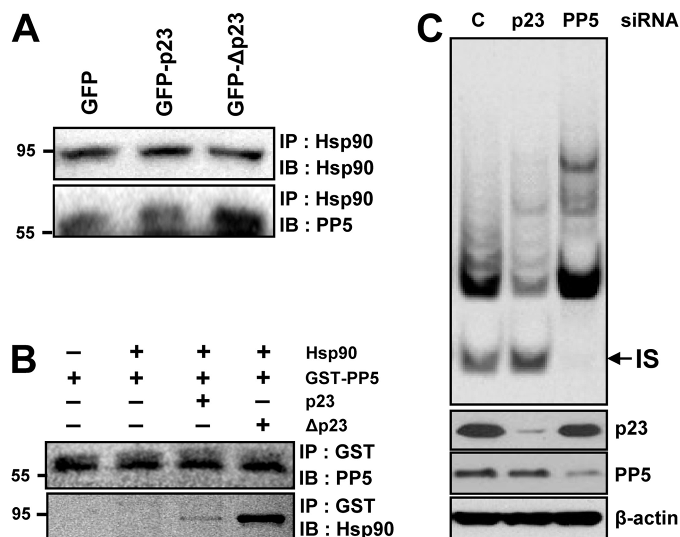

FIGURE 7. **Truncated p23 decreases telomerase activity by enhancing the interaction of Hsp90 with PP5.** *A*, HeLa cells were transfected with GFP, GFP-p23, or GFP-Δp23. After 24 h, lysates were immunoprecipitated with an anti-Hsp90 antibody, and immunoprecipitates were analyzed by Western blotting with anti-Hsp90 and anti-PP5 antibodies. *B*, recombinant GST-tagged PP5 (2 μg, Novus Biologicals, Littleton, CO) was incubated with purified Hsp90 (2 μg) in the presence of 2 μg of p23 or Δp23. Reactions were immunoprecipitated with glutathione-agarose, and immunoprecipitates were analyzed by Western blotting. *C*, small interfering RNAs targeting p23 or PP5 (Ambion, Austin, TX) were introduced into HeLa cells. After 48 h, cell lysates were examined by Western blotting and TRAP analysis.

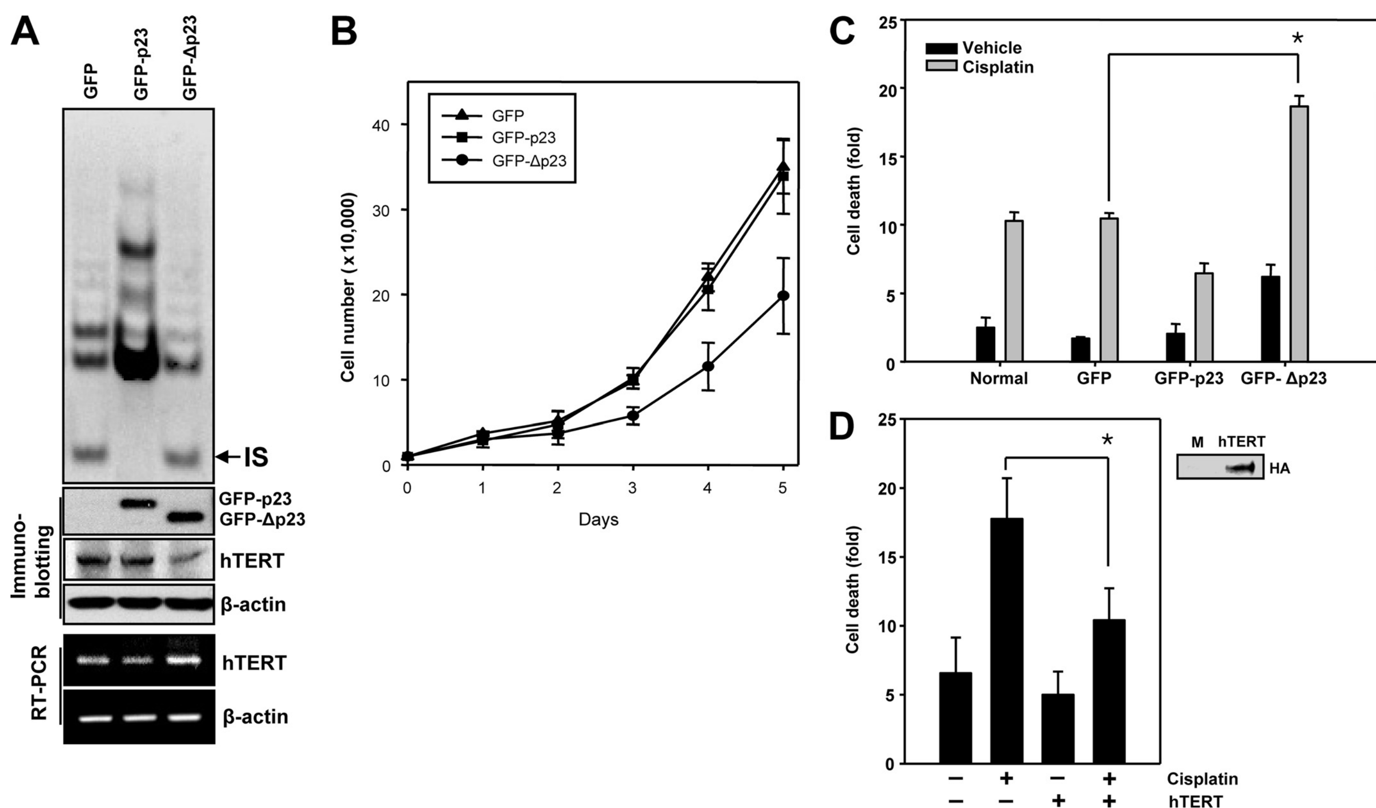

FIGURE 8. **Effects of truncated p23 on cell growth and death.** *A*, 293 cells were stably transfected with GFP, GFP-p23, or GFP-Δp23. hTERT mRNA and protein levels in lysates of stably transfected cells were examined by Western blotting and reverse transcription-PCR (*RT-PCR*), respectively, and telomerase activity was measured using TRAP analysis. *B*, stable 293 cell lines were grown for different periods of time, and cell numbers were determined as described previously (24). *C*, stable 293 cell lines were treated with 50 μM cisplatin for 24 h or left untreated. *D*, the GFP-Δp23 cell line was transfected with hemagglutinin (*HA*)-tagged hTERT or empty vector (*M*) and treated with 50 μM cisplatin for 24 h or left untreated. Cytotoxicity was determined by staining with annexin V-phycoerythrin, as described under "Experimental Procedures." Results are presented as means of at least three independent experiments. The *bars* denote standard deviation (*, $p < 0.01$).

that hTERT is a novel endogenous inhibitor of apoptosis (37). Restoring hTERT expression in the GFP-Δp23 cell line by transfection with a hemagglutinin-tagged *hTERT* gene decreased cisplatin-induced apoptosis, confirming that sensitization of the GFP-Δp23 cell line to cisplatin resulted from suppression of hTERT expression (Fig. 8*D*).

DISCUSSION

Telomerase activity is tightly regulated in cells, and hTERT is a key molecule in this process (2). Although hTERT protein levels are primarily controlled by transcriptional regulation in normal human cells, recent reports suggest the additional involvement of ubiquitination and proteasome-mediated degradation due to Hsp90 malfunction (28). The role of Hsp90 and its co-chaperone p23 in mediating the functional maturation of hTERT is well documented (6), but the precise mechanisms are not fully understood. Here, we propose that p23 regulates Hsp90 phosphorylation, a process that is modulated by caspase-dependent cleavage of p23. By disrupting Hsp90 phosphorylation and function, truncated p23 induces proteasomal degradation of hTERT and down-regulates telomerase activity. The findings presented here provide support for the idea that Hsp90 co-chaperones regulate the post-translational modification of Hsp90 and help to determine the mechanisms underlying the regulation of telomerase activity during cell death.

As part of this mechanism, p23 enhances CKII binding to Hsp90. We found that partial proteolysis of p23 by active caspase did not compromise CKII binding but did promote the binding of PP5 to Hsp90 (Fig. 9). Δp23 apparently binds Hsp90 by displacing pre-bound p23, consistent with our preliminary data showing that Δp23 has a greater affinity for Hsp90 than does full-length p23 (data not shown). It is currently unclear if cleavage of p23 increases PP5 recruitment into the Hsp90 chaperone complex, or if it blocks release of PP5 from the complex. Because PP5 is an essential component of the Hsp90 chaperone complex (35), the latter mechanism is more likely. However, further investigation is necessary to clarify this issue.

Hsp90 is a phosphoprotein that is primarily phosphorylated at serine residues but can be phosphorylated to a lesser extent on tyrosine and threonine residues (29–31). Earlier studies reported that a number of kinases are capable of phosphorylating Hsp90, including CKII, double-stranded DNA-activated kinase, and Akt (29, 38, 39). However, proteins that regulate Hsp90 phosphorylation by these kinases have yet to be identified. In a cell-free reticulocyte lysate system, we found that p23 alone had no effect on Hsp90 phosphorylation status, but was able to increase phosphorylation of Hsp90, an effect that was blocked by a CKII inhibitor (Fig. 5, *C* and *E*). In addition, expression of p23 in intact cells enhanced the interaction between Hsp90 and CKII. Collectively, these data indicate that full-

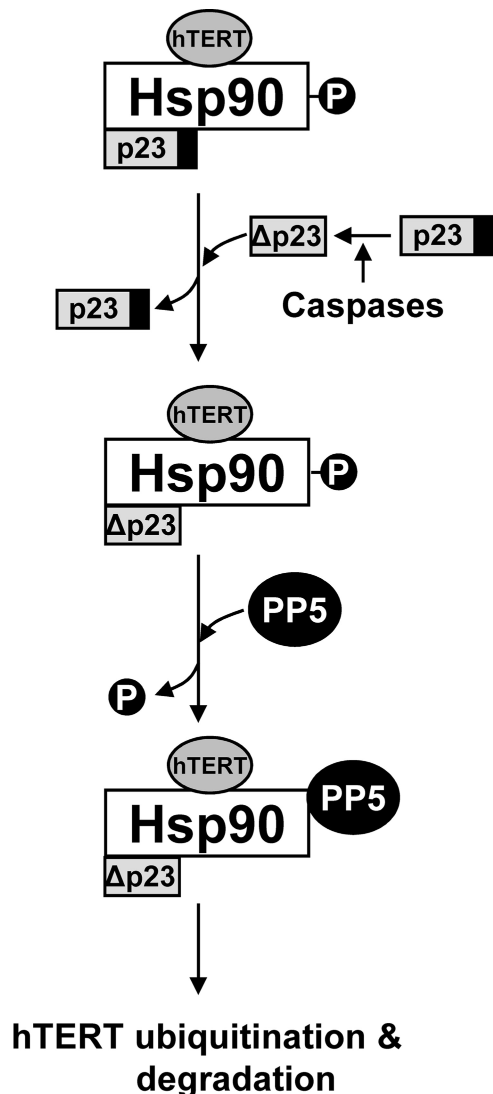

FIGURE 9. **Model for down-regulation of telomerase activity by p23 cleavage.** Interaction of p23 and Hsp90 increases serine phosphorylation at the linker region of Hsp90. Active caspases cleave p23 after Asp-142, generating truncated p23 (Δ*p23*). Δp23 binds to Hsp90 instead of p23 and then promotes PP5-mediated dephosphorylation of Hsp90. hTERT levels decrease due to ubiquitination-dependent degradation.

length p23 stimulates CKII-mediated phosphorylation of Hsp90. Interestingly, overexpression of Δp23 also enhanced binding of CKII to Hsp90. Because CKII is a client protein of Hsp90 (34), it is possible that the interaction between CKII and Hsp90 in the presence of Δp23 is not an enzyme-substrate interaction, but rather a client-chaperone interaction, thus, enhanced binding of CKII to Hsp90 by Δp23 overexpression might not increase Hsp90 phosphorylation.

To our knowledge, CKII is the only kinase capable of phosphorylating Hsp90 at conserved serine residues located in the charged region. Although this region is apparently dispensable in *Escherichia coli* and yeast, it has been suggested that phosphorylation in this region influences Hsp90 chaperone function by modulating the interactions between Hsp90 and client proteins (40–42). Here, we present evidence that phosphorylation of the charged region regulates the interaction between Hsp90 and the client protein, hTERT. Because the charged region of yeast Hsp90 does not contain any conserved serine residues that can be phosphorylated by CKII, we would not expect p23-mediated Hsp90 phosphorylation to be replicated in the yeast system. The length of this charged region is increased from six amino acids in *E. coli* to 67 in human Hsp90, suggesting that this region and its phosphorylation sites have been evolutionary targets for gain of function in higher organisms.

To date, more than a dozen distinct Hsp90 co-chaperones have been identified (43). Some of these co-chaperones facilitate activation of a specific set of substrate proteins. In this context, p23 participates in the maturation of steroid hormone receptors and hTERT. Interestingly, most client proteins are released from Hsp90 after completion of protein folding, although p23 and Hsp90 remain associated with mature hTERT (44). Our results clearly demonstrate that Δp23 induces proteasomal degradation of hTERT protein by blocking Hsp90 phosphorylation. Although it is possible that p23-regulated Hsp90 phosphorylation only affects the stability of hTERT, we cannot exclude the possibility that the levels of other client proteins, such as steroid hormone receptors, are also regulated by p23 cleavage. Further experiments are required to investigate this possibility.

Research from several laboratories confirms that p23 is a substrate of active caspases (20–22). The mechanisms by which caspase-cleaved p23 acts in dying cells are unclear, but two hypotheses have been proposed. One possibility is that the loss of p23 function, independent of its role as an Hsp90 co-chaperone, is the central factor. In this scenario, the resulting increase in Δp23 reduces anti-aggregating activity, and the depletion of p23 sensitizes cells to endoplasmic reticulum stress; thus, the loss of p23 function potentially plays a protective role against endoplasmic reticulum stress, passive chaperone activity, and cytosolic prostaglandin E2 synthetase activity (8, 22, 45). The second possibility involves the loss of p23 co-chaperone functions, dependent on Hsp90 interactions. Several reports demonstrate that hTERT requires the co-chaperone function of p23 as well as Hsp90 for full activity (6, 46); Hsp90 promotes the proper folding of hTERT and subsequent binding to the telomeric primer, whereas p23 is involved in primer dissociation from telomerase. The p23-regulated serine phosphorylation of Hsp90 is essential for telomerase activity, and p23 cleavage leads to down-regulation of telomerase activity, as demonstrated here. In addition, telomerase activity is a prerequisite for cancer cell survival, and hTERT plays a protective role against certain apoptosis-inducing stimuli. Consistent with the role of full-length p23 in this process, we found that overexpression of Δp23 inhibited the growth of transformed cells and increased their sensitivity to anticancer drugs. Thus, reducing telomerase activity by enhancing p23 proteolysis may be an attractive strategy for cancer treatment. Further research is required to generalize the effects of p23 cleavage during cell death.

In view of the protective roles of p23 in stress environments, it is reasonable to assume that the cleaved fragment of p23 in apoptotic cells undergoes rapid degradation. However, our results show that the p23 proteolytic fragment remained stable during cell death. It also displayed a binding activity to Hsp90 similar to that of full-length p23. Thus, the cleaved form of p23 is not merely apoptotic debris that has to be eliminated, but may be a functional molecule that turns off telomerase activity,

and is required for apoptotic progression. In a recent report, Dix and colleagues showed that more than one-third of proteolytic fragments of newly characterized caspase substrate proteins are stable for at least 4 h (47). Additional research is required to determine the general significance of this finding and to identify additional substrates regulated by caspase-mediated proteolysis.

During the chaperone cycle, Hsp90 undergoes continuous phosphorylation and dephosphorylation (32). Evidently, dephosphorylation of Hsp90 is also important for the maintenance of chaperone activity. Another recent report showed that the serine/threonine phosphatase PP5/Ppt1 directly dephosphorylates Hsp90 and modulates the maturation of Hsp90 client proteins (31). Δp23 strengthened the interaction between Hsp90 and PP5, suggesting that the truncated protein deregulates the Hsp90 phosphorylation cycle (Fig. 7, *A* and *B*). Although PP5 specifically dephosphorylates CKII-phosphorylated Hsp90 *in vitro* (31), we found no evidence to suggest that PP5 directly dephosphorylates Hsp90 at CKII-phosphorylated serine residues in intact cells. Further studies are needed to confirm that PP5 bound to Δp23-containing Hsp90 complexes directly dephosphorylates serine residues located in the charged region of Hsp90. PP5 interacts with Hsp90 via the tetratricopeptide repeat domain (35). The tetratricopeptide repeat domain-binding region of Hsp90, located at the C terminus, is the binding site for several co-chaperones. Recent structural data show that the acidic C-terminal tail of p23 becomes well ordered upon binding to Hsp90. The mechanism by which Δp23 enhances the interaction of Hsp90 with PP5 is currently unclear. However, we speculate that the structure of the tetratricopeptide repeat acceptor site of Hsp90 is altered depending on the presence or absence of the p23 C-terminal tail.

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