p53 Suppresses the c-Myb-induced Activation of Heat Shock Transcription Factor 3*

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Expression of heat shock proteins (HSPs) is controlled by heat shock transcription factors (HSF’s). Vertebrates express multiple HSFs whose activities may be regulated by distinct signals. HSF3 is specifically activated in unstressed proliferating cells by direct binding to the c-myb proto-oncogene product (c-Myb), which plays an important role in cellular proliferation. This suggests that the c-Myb-induced HSF3 activation may contribute to the growth-regulated expression of HSPs. Here we report that the p53 tumor suppressor protein directly binds to HSF3 and blocks the interaction between c-Myb and HSF3. In addition, p53 stimulates the degradation of c-Myb through a proteasome-dependent mechanism, which is, at least partly, mediated by induction of Siah in certain types of cells. Induction of p53 by a genotoxic reagent in DT40 cells disrupts the HSF3-c-Myb interaction and down-regulates the expression of certain HSPs. Mutated forms of p53 found in certain tumors did not inhibit c-Myb-induced HSF3 activation. The regulation of HSF3 activity by c-Myb and p53 sheds light on the molecular events that govern HSP expression during cellular proliferation and apoptosis.

Many heat shock proteins (HSPs) remain essential even under nonstressful conditions, a fact that is consistent with their established role in protein folding and translocation (for review see Ref. 1). The transcription of HSP genes is not only induced by various stresses (reviewed in Ref. 1) but is also growth-regulated (2). This is consistent with the view that actively proliferating cells, which express various proteins to high levels, require more HSPs than nonproliferating cells. HSPs also play a role in the regulation of apoptosis, because overexpression of HSPs is known to block apoptosis (3, 4). Therefore, HSP expression might be modulated during apoptosis. However, the mechanism through which HSP expression is linked to cellular growth or apoptosis remains unknown.

In response to stress, heat shock factors (HSFs) bind to heat shock element (HSE) and mediates transcriptional induction of HSP genes (reviewed in Ref. 5). HSF activation is regulated in a number of steps. Upon heat shock, a cryptic form of an inactive HSF1 monomer is converted to a DNA-binding trimer via the hydrophobic repeat (HR-A/B) located in the central region of HSF1 molecule (6, 7). The C-terminal hydrophobic repeat (HR-C) was suggested to function in suppression of trimer formation by interacting with HR-A/B (8, 9). Some HSPs such as Hsp90 and Hsp70 bind to HSF1 and maintain it in the inactive state in the absence of stress (10, 11). Heat shock stress not only regulates the DNA binding activity of HSF1 but also regulates its trans-activating capacity. The activity of the transcriptional activation domain is negatively modulated by other cis-regulatory domains and by phosphorylation (12, 13). In addition to HSF1, vertebrates express multiple HSFs (14–18), suggesting that they are regulated by a diverse array of environmental and developmental cues. HSF1 and HSF3 are activated by heat shock at different temperature (19), but heat shock does not significantly affect the activity of HSF2 (20). The c-myb proto-oncogene product (c-Myb) directly binds to HSF3 via the DNA-binding domain of both proteins and induces activation of HSF3 (21). Although c-Myb is a transcriptional activator that binds to the specific DNA sequence, 5′-AAGNG-3′ (22, 23), it activates HSF3 by a protein-protein interaction. c-Myb is induced at G1/S transition in the cell cycle and plays an important role in the regulation of cellular proliferation and apoptosis (reviewed in Ref. 24). These facts suggest that HSF3 activation mediated by c-Myb may contribute to the growth-regulated expression of HSP genes. Although a lot of evidence indicates that HSF1 is activated by heat stress, disruption of the HSF3 gene also results in a reduction in HSP gene expression in response to heat stress (25). This suggests that HSF3 is also involved in the transcriptional induction of HSP genes following heat stress. However, little is known about how HSF3 regulates heat stress-induced transcription. Thus, HSF3 acts both in Myb- and heat shock-mediated HSP induction.

The products of proto-oncogenes such as c-Myb activate cellular proliferation, whereas the products of tumor suppressor genes have an opposite effect. The p53 protein is the most frequently mutated tumor suppressor to be identified so far in a wide range of human tumors (26, 27). Loss of function of p53 appears to confer selective advantages on cells through deregulated growth and resistance to apoptosis (28). The p53 protein contains the three functional domains responsible for the transcriptional activation, sequence-specific DNA binding, and tetramerization from the N terminus (reviewed in Ref. 29). Most of the p53 mutations found in tumor are localized in the sequence-specific DNA-binding domain in the central portion of...
the molecule (30).

Although p53 is present in small quantities in most cells, several different types of DNA damage produced by γ-irradiation, ultraviolet irradiation, or genotoxic reagents, induces p53 (31). p53 induces cell cycle arrest in essentially all types of cells or triggers apoptosis in some specific cell lineages and under certain cellular context (reviewed in Ref. 29). This was shown to be related, at least partly, to its function as a sequence-specific transcription factor for the induction of a group of target genes (32) including p21WAF1/CIP1 (33), GADD45 (34), and Bax (35). Among these, the p21WAF1/CIP1 gene has received the most attention (33). p21WAF1/CIP1 binds to the cyclin/cyclin-dependent protein kinase complexes and inhibits the function of cyclin-dependent protein kinases during the G1 phase of the cell cycle, leading to DNA-damage-induced G1 arrest. p53 also induces the expression of GADD45, which interacts with the replication and repair factor PCNA, thereby inhibiting the entry of cells into S phase (34, 36). The p53 target gene, bax, encodes a protein that promotes cell death. Siah-1A, which is a murine homologue of Drosophila Sina (seven in absentia) (37), is also induced by p53 and inhibits cell growth (38). Drosophila Sina interacts with a ubiquitin-conjugating enzyme (UBCD1) and assists in the proteasome-dependent degradation of the transcription factor, Tra4 (39, 40). Human Siah also regulates the degradation of tumor suppressor DCC (deleted in colorectal cancer) and transcriptional co-repressor N-CoR via the ubiquitin-proteasome pathway (41, 42). Thus, p53 induces the expression of multiple target genes whose products act in a variety of processes including cell cycle regulation, replication, apoptosis, and protein degradation. p53 not only induces the expression of specific target genes but also directly binds to and acts on several cellular proteins such as TFIIB (43). TFIIB is a component of the RNA polymerase II basal transcription factor and consists of two helicases. p53 binds to these two helicases and modulate their activities (43). Thus, p53 can induce cell cycle arrest and apoptosis through multiple mechanisms. Here we demonstrate that p53 directly binds to HSF3 and inhibits the c-Myb-induced activation of HSF3.

**EXPERIMENTAL PROCEDURES**

**Co-transfection Assays**—Plasmids to express chicken HSF3, mouse c-Myb, and human p53 were constructed using pcDNA3 (Invitrogen) containing the cytomegalovirus promoter. For luciferase assays using 293T cells (see Fig. 1A–E; see also Fig. 3C), a mixture containing 0.5 μg of the HSE-containing luciferase reporter plasmid (44), various amounts of the c-Myb, p53, and HSF3 expression plasmid, or control blank plasmid as indicated in the figures, and 0.5 μg of the internal control plasmid pCMV-β-gal was transfected using the CaPO4 method. Luciferase assays were performed 36–42 h after transfection. Adriamycin treatment (Sigma) (see Fig. 1C) was performed for 24 h before the luciferase assays. Heat shock treatment (42 °C for 1 h) (see Fig. 1E) was performed 36 h after transfection, and the cells were then further cultured at 37 °C for further 5 h, and luciferase assays were performed. To investigate the effect of heat shock on p53 activity, a mixture of 0.5 μg of the luciferase reporter containing tandem repeats of the p53-binding site (45), 0.8 μg of the p53 expression plasmid or control plasmid, and 0.5 μg of the internal control plasmid pCMV-β-gal was transfected into 293T cells using LipofectAmine (Life Technologies, Inc.), and the heat treatment was performed as described above. For luciferase assays using CV-1 cells (see Fig. 4, A, C, and G), a mixture containing 0.5 μg of the HSE-containing luciferase reporter plasmid, various amounts of c-Myb, p53, or control blank expression plasmid as indicated in the figure, and 0.5 μg of the internal control plasmid pCMV-β-gal was transfected using LipofectAmine, and luciferase assays were performed.

**Subcellular Localization of HSF3**—A mixture of 0.5 μg of the HSF3 expression plasmid, 8 μg of the c-Myb expression plasmid DNA or control DNA, and 0.5 μg of the p53 expression plasmid DNA or control DNA was transfected into 293T cells using the CaPO4 method. Immunostaining of HSF3 was performed as described (21).

**In Vitro Binding Assays**—The mutants of p53 and Siah-1A were constructed by the polymerase chain reaction-based method or using appropriate enzyme sites. The vector pSPUTK (Stratagene) was used for in vitro translation of p53, c-Myb, and Siah-1A. GST pull-down assays were performed using approximately 3 μg of GST fusion protein/assay as described previously (46). The following binding buffers were used: Tris buffer (50 mM Hepes, pH 7.5, 125 mM NaCl, 1 mM EDTA, 0.1% Nonidet P-40, and 5 mM dithiothreitol for HSF3-p53 interaction; 20 mM Hepes, pH 7.5, 150 mM NaCl, 0.1% Nonidet P-40, and 1 mM dithiothreitol for interaction between Siah-1A and c-Myb.

**Co-immunoprecipitation**—Rabbit anti-HSF3 antibody was described previously (18). To study the interaction among HSF3, c-Myb, and p53 plasmids, the signal of various target genes or FLAG-HSF3, p53, and c-Myb expression plasmid as indicated in the figures, or the blank control plasmid, and 0.5 μg of the internal control plasmid pCMV-β-gal was transfected into 293T cells using the CaPO4 method. In the plasmids used in these transfections, the chicken cytoplasmic β-actin promoter was linked to the HSF3, c-Myb, or p53 cDNA. 40 h after transfection, cells were lysed in lysis buffer (50 mM Hepes, pH 7.5, 250 mM NaCl, 0.2 mM EDTA, 10 μM NaF, 0.5% Nonidet P-40). The amounts of lysates used for immunoprecipitation was normalized by β-galactosidase activity. After decreasing the NaCl concentration to 175 mM by addition of lysis buffer lacking NaCl, lysates were immunoprecipitated using anti-FLAG M2 (KODAK) or anti-HSF3 antibodies, and the immunocomplex was analyzed on a 10% SDS-polyacrylamide gel (30). Western blotting with anti-c-Myb monoclonal antibody (DO-1) antibody (Santa Cruz). To investigate the interaction between c-Myb and Siah-1A (see Fig. 5B), a mixture of 5 μg of c-Myb expression plasmid, 5 μg of the Siah-1A222 expression plasmid (47), or the control blank DNA, and 0.5 μg of pCMV-β-gal DNA was transfected into 293T cells using the CaPO4 method. Cells were lysed in lysis buffer (50 mM Hepes, pH 7.4, 100 mM NaCl, 0.1% Tween 20, 20% glycerol) by sonication (10 s × 3), and immunoprecipitation was performed using anti-FLAG M2 antibody. For co-immunoprecipitation of endogenous proteins, approximately 106 DT40 cells were lysed in 5 ml of the lysis buffer (50 mM Hepes, pH 7.4, 50 mM NaCl, 0.1% Tween 20, 20% glycerol) by sonication (10 s × 3), and lysates were immunoprecipitated with anti-HSF3 antibody.

**Determination of c-Myb and p53 Protein Levels**—In the experiments using CV-1 cells (see Fig. 4, B, C, E, and F), a mixture of 1 μg of the c-Myb expression plasmid pCDNA3-c-Myb, the control blank DNA, and 0.5 μg of pCMV-β-gal was transfected. 40 h after transfection, cells were lysed in lysis buffer (50 mM Hepes, pH 7.5, 250 mM NaCl, 0.2 mM EDTA, 10 μM NaF, 0.5% Nonidet P-40), and cell lysates, whose amount was normalized using the β-galactosidase activity, were used for Western blotting with the anti-c-Myb monoclonal antibody and ECL detection reagents (Amersham Pharmacia Biotech). Lactacystin (Sigma) treatment was performed for 2 or 4 h at a final concentration of 25 μM before lysis preparation. In the experiments using 293T cells (see Fig. 1B), transfection was performed as described for luciferase assays, lysates were prepared using an aliquot of the cells, and Western blotting was performed as described above. To examine the effect of Siah-1A on the Myb protein level (see Fig. 5A), a mixture of 1 μg of the c-Myb expression plasmid pCDNA3-c-Myb, 1 or 2 μg of the Siah-1A expression plasmid pCDNA3-Siah-1A or the control blank DNA, and 0.5 μg of pCMV-β-gal was transfected. 40 h after transfection, cells were lysed in lysis buffer (50 mM Hepes, pH 7.5, 250 mM NaCl, 0.2 mM EDTA, 10 μM NaF, 0.5% Nonidet P-40), and cell lysates, whose amount was normalized using the β-galactosidase activity, were used for Western blotting with the anti-Myb monoclonal antibody and ECL detection reagents (Amersham Pharmacia Biotech).

To analyze the half-life of c-Myb (see Fig. 4D), CV-1 cells were transfected with a mixture of 3 μg of the c-Myb expression plasmid, 0.6 μg of the p53 expression plasmid or the control DNA, and 0.5 μg of pCMV-β-gal. 24 h after transfection, cells were labeled with [35S]Selenomethionine and [35S]Cysteine for 2 h, and then the radioactivity was chased with an excess of cold amino acids. At various times, lysates were prepared using the lysis buffer described above and immunoprecipitated by anti-c-Myb polyclonal antibody, which was raised against the c-Myb protein containing the N-terminal 200 amino acids. The amount of lysates used for immunoprecipitation was normalized by the [35S]galactosidase activity.

**Determination of p53 Protein Level** (Figs. 1C and 6A), the CV-1 cells transfected for luciferase assays or DT40 cells were treated with the following concentrations of adriamycin for 24 h, and lysates were prepared as described above. Western blotting was performed using anti-p53 antibody PAh240 (Santa Cruz).

**Northern Blotting**—DT40 cells were cultivated to approximately 10 × 106 cells/10-cm dish and treated with adriamycin for 24 or 4.5 h. Total RNA was prepared using TRIzol reagent (Life Technologies, Inc.), subjected to agarose gel electrophoresis, and transferred onto nylon
RESULTS

p53 Inhibits the c-Myb-induced Activation of HSF3—To identify proteins other than c-Myb that modulate HSE-dependent transcription, we examined the effect of various proto-oncogenes and tumor suppressor gene products on HSE-dependent transcription. When the p53 expression plasmid was co-transfected into 293T cells with the HSE-containing luciferase reporter, luciferase activity was significantly repressed to 40% of that of the control (Fig. 1A). This p53-induced repression was relieved by co-expression of c-Myb. Next, we investigated the effect of p53 on HSE-dependent transcription activation mediated by c-Myb (Fig. 1B). In 293T cells, c-Myb activated 57-fold the luciferase expression from the HSE-containing promoter. Addition of increasing amounts of the p53 expression plasmid inhibited this c-Myb-induced luciferase expression in a dose-dependent manner. The c-Myb protein level was not dramatically lowered by p53 expression in the transfected cells (Fig. 1B). Furthermore, we investigated the effect of a genotoxic reagent, which induces p53 protein, on HSF3-mediated transcriptional activation (Fig. 1C). The HSE-containing luciferase reporter was transfected into 293T cells together with the c-Myb expression plasmid, and luciferase activity was examined in the absence or presence of adriamycin. Adriamycin treatment inhibited c-Myb-induced luciferase expression from the HSE-containing promoter in a dose-dependent manner. Overexpression of HSF3 alone activates transcription from the HSE-containing promoter even in the absence of a co-transfected c-Myb expression vector (21). p53 also inhibited this HSF3-dependent transcription (Fig. 1D).

HSF3 is involved not only in c-Myb-dependent HSP induction but also in heat shock-induced transcription (25). To investigate whether p53 affects heat shock-induced transcription, the HSE-containing luciferase reporter was transfected into 293T cells with or without the p53 expression plasmid, and the level of luciferase expression was measured in the presence or absence of heat shock. Heat shock enhanced the luciferase expression 10-fold in the absence of p53, and this degree of induction was not decreased by p53 (Fig. 1E, left panel). Because p53 was not reported to be a temperature-sensitive protein, we confirmed this by the co-transfection assay using the luciferase reporter containing tandem repeats of the p53-binding site. Heat shock treatment did not affect p53-induced luciferase expression from this reporter (Fig. 1E, right panel).

On exposure to heat shock, HSF1 localizes within seconds to discrete nuclear granules (46). This appearance of these HSF1 stress granules correlates with the trimeric DNA-binding state of HSF1 and the temporary induction of heat shock genes. In the presence of c-Myb, approximately 70% of the HSF3 signals were also localized to discrete granules in the nucleus as shown by the intense areas of punctuate staining, whereas in the absence of c-Myb expression the staining was uniformly distributed over the entire nucleus (Fig. 1F) as reported previously (21). Because HSF granules have been found to correlate with HSF trimerization and activation (46), these results suggest that c-Myb affects the subnuclear localization and trimerization of HSF3. We have examined whether p53 inhibits the Myb-induced subnuclear localization of HSF3. Co-expression of p53 with HSF3 and c-Myb blocked the formation of HSF granules, and only about 10% of the HSF3 signals were localized to the discrete granules.

Competition between p53 and c-Myb for Binding to HSF3—To study the mechanism by which p53 blocks c-Myb-induced HSF3 activation, we examined for a direct interaction between p53 and HSF3. In vitro translated 35S-labeled p53 preferentially bound to GST-HSF3 when presented with the three GST fusion proteins containing HSF1, HSF2, or HSF3 (Fig. 2A). We next investigated which domain of HSF3 interacts with p53 by performing binding assays using various forms of the GST-HSF3 fusion protein (Fig. 2B). The results indicate that the DNA-binding domain located in the N-terminal region and the adjacent hydrophobic repeat region, which mediates trimerization, are sufficient and necessary for interaction with p53. Because our previous study indicated that the DNA-binding domain of HSF3 is responsible for interaction with c-Myb, this result raised the possibility that p53 may
To confirm that p53 interacts with HSF3 in vivo, a co-immunoprecipitation study was performed (Fig. 2C). Whole cell lysates were prepared from 293T cells transfected with the two plasmids to express FLAG-linked HSF3 and p53 and incubated with anti-FLAG antibodies or the control IgG. The anti-FLAG antibodies co-precipitated p53, but the control IgG did not. To investigate whether p53 and c-Myb competitively bind to HSF3, the HSF3 and c-Myb expression plasmids were transfected into 293T cells together with increasing amounts of the p53 expression plasmid, and the amount of c-Myb that interacted with HSF3 was analyzed by co-immunoprecipitating with anti-HSF3 antibody (Fig. 2D). In the absence of the p53 expression plasmid, significant amounts of c-Myb and endogenous p53 were precipitated by the anti-HSF3 antibody. The amount of c-Myb that co-precipitated with HSF3 decreased as the amount of the p53 expression plasmid raised, whereas the amount of p53 that co-precipitated with HSF3 increased. Thus p53 competes with c-Myb for binding to HSF3.

**p53 Point Mutants Do Not Inhibit the c-Myb-HSF3 Interaction**

To identify the specific domain of p53 that interacts with HSF3, binding assays using in vitro translated p53 mutants and GST-HSF3 were performed (Fig. 3A). The results indicated that the C-terminal 79-amino acid region of the p53 molecule is responsible for the interaction with HSF3. This region contains the tetramerization domain and the C-terminal region, which is rich in basic residues (47). We observed that in vitro translated HSF3 could not bind to GST-p53, but that in vitro translated p53 could interact with GST-HSF3 (data not shown). This suggests that tetramerization of p53 is required for binding to HSF3, because most of the GST-p53 on the glutathione resin is in a monomeric form. The capacity of p53 mutants to bind to

**Fig. 2.** p53 competes with c-Myb for binding to HSF3. A, binding of p53 to HSF3. Left panel, the GST fusions containing HSF1–3 were purified by glutathione beads, analyzed by SDS-polyacrylamide gel electrophoresis, and revealed by Coomassie Blue staining. Right panel, binding of in vitro translated p53 to GST-HSF1, 2, or 3 was analyzed. B, domain analysis of HSF3. The structures of the HSF3 mutants used are shown, and the results of the binding assays are indicated on the right. Bottom left panel, the GST fusions containing various HSF3 mutants were analyzed by SDS-polyacrylamide gel electrophoresis and visualized by Coomassie staining. Bottom right panel, binding of in vitro translated p53 to various GST-HSF3 was analyzed. C, co-immunoprecipitation of p53 with HSF3. Whole cell lysates were prepared from 293T cells transfected with a mixture of the indicated amount of FLAG-HSF3 and p53 expression plasmids, and the cell extracts were immunoprecipitated by anti-FLAG or control IgG. The immunoprecipitated proteins were analyzed by Western blotting using anti-p53 antibody. D, effect of p53 on co-immunoprecipitation of c-Myb and HSF3. The indicated amount of HSF3 and c-Myb expression plasmids were transfected into 293T cells with or without increasing amount of the p53 expression plasmid. Lysates were used for co-immunoprecipitation with anti-HSF3 antibody. Western blotting was performed with anti-p53 and anti-c-Myb antibodies. Note that endogenous p53 is co-precipitated by anti-HSF3 antibody in the lanes without p53 expression plasmid. a.a., amino acids; WT, wild type; Co-IP, co-immunoprecipitation.
HSF3 and to compete with c-Myb for binding to HSF3 was investigated by co-immunoprecipitation assays (Fig. 3D). The c-Myb and HSF3 expression plasmids were transfected into 293T cells with or without the plasmid expressing wild type or mutant p53, and co-immunoprecipitation was performed using the anti-HSF3 antibody. The five p53 point mutants, A138V, Q143A, R175H, R248W, and R273H, which occur frequently in the tumors of cancer patients, were co-precipitated with HSF3, indicating that these point mutants can form a complex with HSF3. In contrast to wild type p53, however, the anti-HSF3 antibody was able to co-precipitate c-Myb in the presence of these p53 mutants. These results suggest that none of these mutants could block the interaction between c-Myb and HSF3, although they were able to bind to HSF3. In the presence of the p53 mutant lacking the C-terminal 93 amino acids (CT300), anti-HSF3 could not co-precipitate CT300. Thus, the p53 interaction with HSF3 depends on the C-terminal 79-amino acid region of p53, and the interaction between p53 and HSF3 is apparently unaffected by many p53 point mutants commonly found in the tumors of cancer patients. Because the crystal structure of p53 indicates that many point mutations cause structural defects (30), the conformation of p53 protein may be important for its ability to compete with c-Myb (see “Discussion”). The p53 mutants were also used in co-transfection assays to examine their capacity to inhibit the c-Myb-induced activation of HSF3 (Fig. 3C, left panel). CT300 and the four point mutants A138V, Q143A, R248W, and 273H did not efficiently inhibit c-Myb activation of HSF3-dependent transcription. In the control co-transfection experiments (Fig. 3C, right panel), effect of the p53 mutants on the HSE-containing promoter was examined in the absence of c-Myb. All the p53 mutants used did not affect the HSE-linked promoter activity in the absence of c-Myb. Therefore, the co-transfection assay used is not monitoring the HSF3-mediated transcription induced by the denatured form of the p55 mutants.

p53-induced Degradation of c-Myb in CV-1 Cells—When we used CV-1 cells for co-transfection experiments, p53 inhibited again the c-Myb-induced luciferase expression from the HSE-containing promoter like with 293T cells (Fig. 4A). In addition, the C-truncated p53 mutant lacking the HSF3-interacting domain could not inhibit HSE-dependent transcription induced by c-Myb (data not shown). In these cells, however, the c-Myb protein level was dramatically lowered by p53 (Fig. 4B). Treatment of the transfected CV-1 cells with the proteasome inhibitor, lactacystin (48), blocked the p53-induced degradation of c-Myb, suggesting that the p53-mediated degradation of c-Myb occurs via the proteasome system. In the presence of lactacystin, furthermore, p53 did not inhibit the c-Myb-induced luciferase expression from the HSE-containing promoter, indicating that degradation of c-Myb is required for p53-mediated repression of HSF3 in CV-1 cells (Fig. 4C). These results suggest that p53 induces c-Myb degradation in addition to inhibiting the c-Myb-HSF3 interaction in CV-1 cells. After transfection of CV-1 cells with the c-Myb and p53 expression plasmids, cells were pulse-chase labeled with [35S]methionine, and the level of c-Myb proteins was examined at various times after the chase by immunoprecipitation (Fig. 4D). In the absence of p53, the half-life of c-Myb was 120 min, but this level decreased to 35 min in the presence of p53. To determine which domain of c-Myb is critical for p53-dependent degradation, the stability of various c-Myb mutants was examined in the presence and absence of p53 (Fig. 4E). The c-Myb mutant lacking the DNA-binding domain (ΔDBD) was stable even in the presence of p53, whereas all other mutants containing the DNA-binding domain were degraded in the presence of p53. Thus, the DNA-binding domain of c-Myb serves as a target for p53-induced degrada-
lated 35S-labeled c-Myb to the GST-Siah-1A protein was examined. Domain analysis of c-Myb. Binding of various forms in vitro by Western blotting using anti-c-Myb and anti-FLAG antibodies. Anti-FLAG or control normal IgG. The immunocomplex was analyzed.

Siah1A prepared from CV-1 cells transfected with a mixture of FLAG-c-Myb and Siah-1A expression plasmids and immunoprecipitated by anti-FLAG or control normal IgG. The immunocomplex was analyzed by Western blotting using anti-c-Myb and anti-FLAG antibodies. The domain structure of Siah-1A and the mutants used are shown, and the results of binding assays are indicated on the right. The in vitro binding experiments were performed using the 35S-labeled Siah-1A proteins and the GST-c-Myb fusion protein containing the full-length c-Myb. Co-IP: co-immunoprecipitation; WT: wild type; a.a.: amino acids; TA: transcriptional activation.

Siah-1A mediated c-Myb degradation. A, c-Myb degradation induced by Siah-1A. CV-1 cells were transfected with the c-Myb expression plasmid together with the Siah-1A expression plasmid. The level of the c-Myb protein was determined by Western blotting. B, co-immunoprecipitation of c-Myb with Siah-1A. Whole cell lysates were prepared from CV-1 cells transfected with a mixture of FLAG-Siah1A22 and c-Myb expression plasmids and immunoprecipitated by anti-FLAG or control normal IgG. The immunocomplex was analyzed by Western blotting using anti-c-Myb and anti-FLAG antibodies. C, domain analysis of c-Myb. Binding of various forms of in vitro translated c-Myb to the GST-Siah-1A protein was examined. D, domain analysis of Siah-1A. The domain structure of Siah-1A and the mutants used are shown, and the results of binding assays are indicated on the right. The in vitro binding experiments were performed using the 35S-labeled Siah-1A proteins and the GST-c-Myb fusion protein containing the full-length c-Myb. Co-IP: co-immunoprecipitation; WT: wild type; a.a.: amino acids; TA: transcriptional activation.

Siah Mediates c-Myb Degradation.—Among the numerous genes that are induced by p53, Siah is known to mediate protein degradation in a proteasome-dependent manner (41, 42), raising the possibility that Siah might be involved in the p53-induced c-Myb degradation. To investigate this, the Siah-1A expression plasmid was co-transfected with the c-Myb expression plasmid into CV-1 cells, and the c-Myb protein level was examined. Co-expression of Siah-1A with c-Myb significantly decreased the c-Myb protein level (Fig. 5A). Siah was demonstrated to bind directly to and stimulate the degradation of the target proteins DCC and N-CoR. To confirm the association between c-Myb and Siah-1A in vivo, the two plasmids expressing c-Myb and FLAG tag-linked Siah-1A22 were transfected into 293T cells, and co-immunoprecipitation was performed using the anti-FLAG antibody (Fig. 5B). Because it was reported that Siah-1A stimulates the its own degradation, a plasmid expressing the N-terminally truncated Siah-1A (Siah-1AΔ22), which has lower autodegradation activity (38), was used. The anti-FLAG antibody co-precipitated c-Myb, whereas the control normal IgG did not. Using a series of deletion mutants of in vitro translated c-Myb and the GST-Siah-1A resin, we examined which domain of the c-Myb molecule was responsible for the interaction with Siah-1A (Fig. 5C). The c-Myb mutant lacking the DNA-binding domain (3DBD) failed to bind to the GST-Siah-1A resin. This is consistent with our results indicated above, which showed that the DNA-binding domain of c-Myb is a target for p53-induced degradation. In vitro translated Siah-1A also bound to the GST-c-Myb resin (Fig. 5D). Use of the deletion mutants of Siah-1A for the in vitro binding assays indicated that the N-terminal 231-amino acid region of Siah-1A is responsible for its binding to the GST-c-Myb resin.

p53 Induction Leads to Down-regulation of HSPs mRNAs—To further confirm that p53 is really involved in the
regulation of HSPs expression, we examined the level of various HSPs mRNAs in chicken DT40 cells before and after treatment with adriamycin (Fig. 6A). Treatment of DT40 cells with adriamycin for 24 h led to the induction of endogenous p53 protein. The levels of HSP70 and HSP90α mRNAs were decreased in a dose-dependent manner compared with that of control glyceraldehyde-3-phosphate dehydrogenase mRNA after treatment with two different concentration of adriamycin. In contrast, the HSP90β mRNA level was not affected by adriamycin treatment, suggesting that HSP90β transcription is not regulated by HSF3. In fact, it was reported that avian HSP90β mRNA is not inducible by thermal stress (49). Thus, induction of p53 results in a decrease in the level of certain HSPs. To confirm that endogenous c-Myb forms a complex with endogenous HSF3 in DT40 cells, co-immunoprecipitation experiments were performed using the anti-HSF3 antibody (Fig. 6B). The antibody co-precipitated c-Myb with HSF3. We then examined whether adriamycin treatment really disrupted the c-Myb-HSF3 complex. To examine for this, we treated DT40 cells with adriamycin for only 4.5 h to observe the molecular interaction occurring before the onset of the nonspecific effects of the drug. When DT40 cells were treated with adriamycin (1.0 μg/ml) for 4.5 h, the anti-HSF3 antibody did not co-precipitate c-Myb, although the c-Myb protein level was not affected. Thus, induction of p53 blocks complex formation between HSF3 and c-Myb and results in the down-regulation of certain HSPs.

**DISCUSSION**

Our results indicate that p53 inhibits c-Myb-induced activation of HSF3 by two mechanisms (Fig. 7); (i) it competes with c-Myb for binding to HSF3 and (ii) it targets c-Myb for degradation. The mechanism through which p53 blocks the c-Myb-HSF3 interaction appears to be more than a simple competition for c-Myb binding to HSF3. The p53 fragment containing only the C-terminal 79-amino acid region binds to the N-terminal 231-amino acid region of c-Myb directly binds to the N-terminal 231-amino acid region of c-Myb, suggesting that degradation may depend on the cell type. These two cell lines may display differences in their levels of Siah-1 gene transcriptional regulators. Cell type-dependent variations in proteasome-dependent protein degradation were also observed for Mdm2-induced degradation of p53 (55). The c-Myb degradation induced by wild type p53 was not observed for the five point mutations of p53. Because none of these p53 mutants can bind to the target DNA sequence, Siah-1 should not be induced by these p53 mutants. Actively proliferating cells display high expression of numerous proteins and require a higher level of HSP expression for protein folding and translocation. HSPs are critical for cell cycle progression; for instance, Wee1 tyrosine kinase, which regulates the length of the G2 phase, requires an interaction with Hsp90 (56). Overexpression of Hsp70 also blocks the apoptosis induced by tumor necrosis factor/heat shock (3, 4). Thus, HSPs play a role in the progression of the cell cycle and protect the cell against apoptosis. c-Myb also plays a positive role for cell cycle progression and protects against apoptosis. It was reported that c-Myb induces the expression of S phase-specific genes and Bcl-2 and that the induction of these target genes mediate the capacity of c-Myb to induce cell cycle progression and to protect cells against apoptosis (24). In addition to these pathways, however, c-Myb activation of HSF3 could induce HSP expression and thereby contribute to cell cycle progression and the inhibition of apoptosis. In contrast to c-
Myb, the p53 tumor suppressor protein induces cell cycle arrest at cell cycle check points such as the G1/S or G2/M transition and induces apoptosis. A group of target genes such as p21WAF1/CIP1 (33), GADD45 (34), and Bax (35), whose expression is induced by p53, are thought to be responsible for cell cycle arrest and the induction of apoptosis by p53. Our study indicates that p53 also decreases the level of HSPs by blocking the c-Myb-mediated activation of HSF3. The present study suggests that suppression of HSP expression may also be an important pathway for cell cycle arrest and induction of apoptosis by p53.

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p53 Suppresses the c-Myb-induced Activation of Heat Shock Transcription Factor 3
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