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The Physical Association of Multiple Molecular Chaperone Proteins with Mutant p53 Is Altered by Geldanamycin, an hsp90-Binding Agent

LUKE WHITESELL,[1]* PATRICK D. SUTPHIN,[1] ELIZABETH J. PULCINI,[1] JESSE D. MARTINEZ,[2] AND PAUL H. COOK[1]

Department of Pediatrics and Steele Memorial Children's Research Center[1] and Department of Radiation Oncology, Arizona Cancer Center,[2] University of Arizona, Tucson, Arizona 85724

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Wild-type p53 is a short-lived protein which turns over very rapidly via selective proteolysis in the ubiquitin-proteasome pathway. Most *p53* mutations, however, encode for protein products which display markedly increased intracellular levels and are associated with positive tumor-promoting activity. The mechanism by which mutation leads to impairment of ubiquitination and proteasome-mediated degradation is unknown, but it has been noted that many transforming p53 mutants are found in stable physical association with molecular chaperones of the hsp70 class. To explore a possible role for aberrant chaperone interactions in mediating the altered function of mutant p53 and its intracellular accumulation, we examined the chaperone proteins which physically associate with a temperature-sensitive murine p53 mutant. In lysate prepared from A1-5 cells grown under mutant temperature conditions, hsp70 coprecipitated with $p53^{Val135}$ as previously reported by others, but in addition, other well-recognized elements of the cellular chaperone machinery, including hsp90, cyclophilin 40, and p23, were detected. Under temperature conditions favoring wild-type p53 conformation, the coprecipitation of chaperone proteins with p53 was lost in conjunction with the restoration of its transcriptional activating activity. Chaperone interactions similar to those demonstrated in A1-5 cells under mutant conditions were also detected in human breast cancer cells expressing two different hot-spot mutations. To examine the effect of directly disrupting chaperone interactions with mutant p53, we made use of geldanamycin (GA), a selective hsp90-binding agent which has been shown to alter the chaperone associations regulating the function of unliganded steroid receptors. GA treatment of cells altered heteroprotein complex formation with several different mutant p53 species. It increased p53 turnover and resulted in nuclear translocation of the protein in A1-5 cells. GA did not, however, appear to restore wild-type transcriptional activating activity to mutant p53 proteins in either A1-5 cells or human breast cancer cell lines.

The wild-type p53 transcription factor is a nuclear tumor suppressor involved in cell cycle regulation, and loss of its normal function through mutation results in genetic instability and abnormalities in the induction of apoptotic cell death (14). Many *p53* mutations are also associated with positive tumor-promoting activity, and their protein products are found to display markedly increased intracellular levels. Wild-type p53 is a very short lived protein which turns over rapidly via selective proteolysis in the ubiquitin-proteasome pathway (22). We have recently shown, however, that for several common p53 mutants, the normal processing of the protein is impaired, which results in the marked accumulation of dysfunctional molecules with a prolonged intracellular half-life (40). The mechanism by which mutation leads to impairment of ubiquitination and proteasome-mediated degradation is unknown at this time, but it has been noted that many transforming p53 mutants are found in stable physical association with hsc70, a member of the hsp70 class of molecular chaperones (9). Studies of both yeast and vertebrate cells have suggested a role for heat shock proteins in modulating the transit of target proteins through proteolytic processing pathways (6, 32). Consequently, we hypothesized that mutant p53 molecules, presumably due to alterations in conformation, might be retained within the molecular chaperone machinery and protected from ubiquitination and subsequent degradation.

To examine such a possible role for aberrant chaperone interactions in mediating mutant p53's intracellular accumulation, we have examined the chaperone proteins which physically associate with a temperature-sensitive murine p53 mutant ($p53^{Val135}$) in A1-5 fibroblasts which stably overexpress the protein. This protein behaves like other p53 mutants at 39°C, but when cells are maintained at 32°C, most of the protein assumes wild-type p53 activity, including the ability to transactivate gene expression and induce cell cycle arrest (23). Using this system, we were able to examine the pattern of chaperone proteins which coprecipitated with mutant versus wild-type p53 within the same cellular background. Next, using a panel of human breast cancer cell lines, we were able to demonstrate that clinically relevant p53 mutants also physically associate with multiple chaperones. Finally, we examined the effects of geldanamycin (GA), a selective hsp90-binding agent (37, 39) which recent work has shown restores the ubiquitination and proteasome-mediated degradation of mutant p53 in tumor cells (40). We found that drug interaction with hsp90 altered heteroprotein complex formation with several mutant p53 species and increased their turnover. Unlike a temperature shift in A1-5 cells, however, GA treatment did not appear to restore function as a transcription factor to mutant p53 in any of the cell lines tested.

* Corresponding author. Mailing address: Department of Pediatrics, AHSC Room 3336, 1501 N. Campbell Ave., Tucson, AZ 85724. Phone: (520) 626-6527. Fax: (520) 626-4220. E-mail: whitelj@peds.arizona.edu.

MATERIALS AND METHODS

Cells, antibodies, and reagents. The rat embryo fibroblast cell line A1-5 (26) was routinely cultured at 37°C in an atmosphere of 10% CO_2 in air in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum. The breast cancer cell lines MCF-7, SkBr3, MDA-MB-468, and T47D were obtained from the American Type Culture Collection (ATCC, Rockville, Md.) and cultured in RPMI 1640 supplemented with 10% fetal bovine serum. Cells were passaged when 80% confluent, and all experiments were performed on cells within 10 serial passages. Mouse monoclonal anti-53 antibodies (PAb421, PAb242, and DO-1) were obtained from Oncogene Science. Anti-p23 monoclonal antibody JJ3 was a gift from D. Toft (Mayo Clinic, Rochester, Minn.). Monoclonal anti-hop (hsp-organizing protein; previously called p60) antibody F5 and anti-hsp70 antibody BB70 were provided by D. Smith (University of Nebraska, Omaha). Polyclonal rabbit antiserum to cyclophilin 40 (Cyp40) (PA3-022) was purchased from Affinity BioReagents (Golden, Colo.); polyclonal antiserum to human WAF-1 was from Pharmingen (San Diego, Calif.; catalog no. 15431E). Monoclonal antibodies to hsp90 (AC88) and hsp70 (N27F3-4) were purchased from StressGen (Vancouver, British Columbia, Canada). GA was obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute. It was prepared as a 2-mg/ml stock in dimethyl sulfoxide and maintained at −20°C in the dark. All other chemical reagents were purchased from Sigma unless otherwise stated.

Analysis of p53-chaperone protein complexes. Cell monolayers were rinsed twice with cold Tris-buffered saline (pH 7.4) and scraped into ice-cold lysis buffer containing Tris-HCl (pH 7.4, 10 mM), $MgCl_2$ (1 mM), Tween 20 (0.2%, vol/vol), sodium molybdate (10 mM), aprotinin (20 μg/ml), leupeptin (20 μg/ml), and phenylmethylsulfonyl fluoride (1.0 mM). Cells were sonicated for 5 s two times with 1 min of cooling on ice between bursts, followed by centrifugation at 16,000 × *g* for 30 min at 4°C. The protein content of the supernatant fraction was determined with bicinchoninic acid reagent (Pierce), and immunoprecipitation (IP) was routinely performed with 1 to 2 mg of total protein in a final volume of 300 to 400 μl. Incubation with primary antibody was performed at 4°C for 60 min with gentle agitation followed by addition of protein G-Sepharose (15-μl resin pellet; Pharmacia) and further incubation for 60 min. For IP experiments, bead pellets were then washed four times in lysis buffer and extracted into 1× Laemmli sample loading buffer by heating to 95°C for 5 min. For immunodepletion studies, supernatants were collected from bead pellets and subjected to a second round of IP to fully deplete the target chaperone. Supernatants were then collected, and aliquots were added to Laemmli sample buffer and heated as described above. All samples were then fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on either 7.5 or 12.5% polyacrylamide gels, and proteins were transferred to nitrocellulose by electroblotting. Membranes were blocked in 3% nonfat powdered milk and probed with primary antibodies followed by species-appropriate peroxidase-conjugated secondary antibody. Reactivity was detected by using chemiluminescent substrate and exposure to Kodak XAR-5 film as previously described (40). Multiple exposure times were evaluated for each blot to ensure that the band intensities observed were within the dynamic response range of the film.

Metabolic labeling. A1-5 cells were incubated for 1 h at 39°C in methionine-free RPMI 1640 containing 5% dialyzed, heat-inactivated fetal bovine serum. [^{35}S]methionine (11,750 Ci/mmol; NEN) was added to yield 100 μCi/ml in the medium, and incubation was continued for an additional hour. Dishes were rinsed three times with cold phosphate-buffered saline and lysed in nonionic detergent-containing buffer (50 mM Tris-HCl [pH 7.4], 1% Nonidet P-40, 2 mM EDTA, 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 20 μg of leupeptin per ml, 20 μg of aprotinin per ml). p53 IP was performed with PAb421 from equal amounts of trichloroacetic acid-precipitable material (10^8 cpm) in 400 μl (total volume) as described above, and immunoprecipitates were fractionated by SDS-PAGE (7.5% gel). After enhancement (Enlightening; NEN), radiolabeled proteins were visualized by autoradiography.

Immunocytochemistry. The intracellular localization of p53 in A1-5 cells growing on glass coverslips under various experimental conditions was visualized by immunocytochemistry as previously described (23) except that cells were reacted with purified primary antibody (PAb421) at a dilution of 1:100. Detection was achieved by using biotinylated goat anti-mouse and streptavidin-peroxidase reagents supplied in kit form as recommended by the manufacturer (HistoMark; Kirkegaard & Perry Laboratories, Gaithersburg, Md.).

Reporter construct transfection. The p53-responsive reporter constructs PG13/βgal and WAF1/βgal were a generous gift of P. Abarzua (Roche Research Center, Nutley, N.J.). PG13/βgal contains 13 copies of oligonucleotide PG and the polyomavirus promoter, while WAF1/βgal contains a 2.4-kb *Hin*dIII *WAF1* promoter fragment cloned upstream of the β-galactosidase gene (1). A1-5 cells were cotransfected by electroporation with each of these reporter constructs plus a vector encoding G-418 resistance (pRcCMV; Invitrogen) at a 3:1 molar ratio. G-418 was added 48 h after electroporation at a concentration of 500 μg/ml, and selection continued for 14 days, at which time both individual clones and a pool of colonies were isolated and subcultured for subsequent experimentation. Following a temperature shift or drug exposure overnight, β-galactosidase activity was assayed in 0.5% Nonidet P-40-containing cytosolic extracts, using *o*-nitrophenyl-β-D-galactopyranoside as a substrate and measuring absorbance at 450 nm. The protein content of each lysate was also measured by using the bicinchoninic acid reagent, and specific β-galactosidase activity was calculated as optical density units per milligram of cellular protein. All determinations were performed in triplicate.

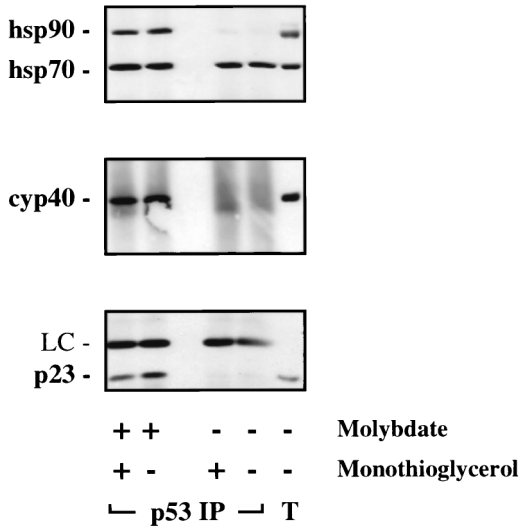

FIG. 1. Mutant p53 is physically associated with multiple molecular chaperones. A1-5 cells were grown at 39°C (mutant conditions), and lysates were prepared in buffer containing sodium molybdate (10 mM) and/or monothioglycerol (10 mM) as indicated. Anti-p53 IP was performed with PAb421, and precipitates were analyzed by immunoblotting for the presence of the indicated chaperone proteins. Total lysate (lane T) was analyzed to verify the presence and migration position of each chaperone. LC, antibody light chain.

RESULTS

Mutant p53 exists in multiprotein chaperone complexes. We made use of A1-5 cells, which stably overexpress a $p53^{Val135}$ mutant that is temperature sensitive for conformation, localization, and function. In this way, the chaperone proteins which physically associate with mutant versus wild-type p53 could be examined within identical cellular backgrounds. We found in this system that under conditions favoring mutant localization and function, p53 coprecipitated with multiple chaperone proteins in addition to hsp70. Demonstration of these novel associations was dependent on the presence of millimolar concentrations of the transition metal molybdate (Fig. 1). hsp70 coprecipitation, however, did not appear molybdate sensitive, which may explain why its stable association with many mutant p53 species has long been appreciated. The finding that hsp90, as well as the large immunophilin Cyp40 and the recently cloned accessory chaperone p23, coprecipitated with mutant p53 in a molybdate-sensitive fashion was intriguing given that unliganded steroid receptors display similar patterns of chaperone protein associations which are also stabilized by molybdate. Unlike steroid receptors, however, reducing agents such as monothioglycerol were found to enhance complex stability under the lysis and precipitation conditions used in this study. The failure to coprecipitate hsp90 or the other chaperones examined in Fig. 1 in buffers which did not contain molybdate was not due to degradation or inadequate lysis, as all chaperones were readily detected in total cytostolic extract prepared in buffer which contained neither molybdate nor monothioglycerol (lane T).

To assess the proportion of p53 in A1-5 cells found in complex with chaperone proteins, we performed immunodepletion studies using anti-p23 antibody or anti-hsp70 antibody. As seen in Fig. 2A, this approach was able to selectively deplete either

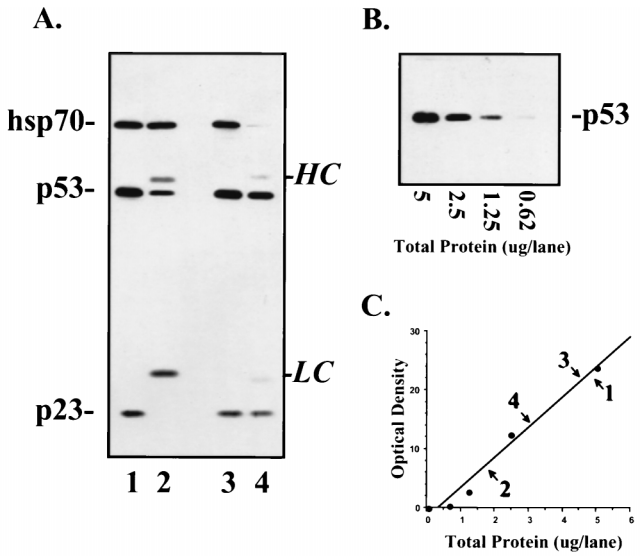

FIG. 2. Most of the mutant p53 in A1-5 cells is complexed with molecular chaperones. Cells were grown at 39°C, and lysates were prepared in molybdate-containing buffer. (A) IPs were performed on replicate aliquots by using control mouse IgG (lanes 1 and 3), anti-p23 antibody (JJ3; lane 2), or anti-hsp70 antibody (BB70; lane 4) in order to deplete the lysate of the relevant chaperone. Aliquots of immunodepleted supernatant were then analyzed for remaining hsp70, p53, and p23 content by Western blotting. HC and LC indicate the positions of residual antibody heavy chain and light chain, respectively, remaining in some of the supernatants after IP. (B) Aliquots of cell lysate containing the indicated amounts of total protein were immunoblotted on the same membrane as the samples depicted in panel A in order to generate a standard curve for the estimation of p53 levels. The position of p53 as detected by PAb421 is indicated. (C) Scanning densitometry (Bio-Rad GS-700 instrument and Molecular Analyst software) was performed to quantitate the p53 signals displayed in panels A and B. The optical densities (arbitrary units) of the bands depicted in panel B are plotted on the *y* axis, while the corresponding amounts of total cellular protein are plotted on the *x* axis. A linear curve fit ($r^2 = 0.98$) for the data points is shown as a solid line. Numbered arrows indicate the optical densities of the p53 bands visible in each of the corresponding lanes displayed in panel A.

the cochaperone p23 (compare p23 signals in lanes 1 and 2) or hsp70 (compare hsp70 levels in lanes 3 and 4) from A1-5 lysates. As expected from the data in Fig. 1, depletion of these chaperones resulted in significant reductions in the p53 contents of the lysates (compare p53 bands in lanes 1 and 2 and in lanes 3 and 4). To quantitate the extent of p53 reduction, a standard curve relating p53 level to band intensity was generated by simultaneously blotting various amounts of A1-5 protein onto the membrane used for the immunodepletion samples. Figure 2B demonstrates visually the expected titration of p53 signal as less protein is loaded, while Fig. 2C depicts optical density measurements of the bands in Fig. 2B plotted against various amounts of cell protein. Using this standard curve as a basis for interpolation, we have indicated the optical densities of the p53 bands seen in Fig. 2A by arrows numbered to correspond to the relevant lanes of Fig. 2A. Based on this analysis, it appears that 70% of p53 existed in a complex or complexes containing p23, while 30% could be coprecipitated with hsp70. Whether hsp70- and p23-containing complexes with p53 represent distinct entities as appears to be the case with the progesterone receptor (36) cannot be determined definitively from the data presented. The finding illustrated by Fig. 2A that immunodepletion of p23 did not result in much decline in hsp70 signal and that depletion of hsp70 did not deplete p23 suggests, however, that distinct complexes do exist. Finally, care must be taken in the interpretation of these results because immunodepletion is by nature performed under nonequilibrium conditions. Some dissociation or alteration of intrinsically dynamic complexes may have occurred during the procedure. As a result, the percentages cited above are best viewed as minimal estimates of mutant p53 involvement in chaperone complexes rather than absolute determinations.

Using hsp90 as a representative of the novel associations demonstrated in Fig. 1 and 2, we next examined the effects of temperature shift and the hsp90-binding agent GA on the composition of p53-chaperone protein complexes. As seen in Fig. 3, a shift to the wild-type temperature for 6 h prior to preparation of cytosol resulted in loss of hsp90 coprecipitation with p53 as well as a substantial reduction in hsp70 (compare lanes 3 and 2). The declines in hsp70 and hsp90 were not due to obvious differences in p53 levels in the total lysate (Fig. 3B, lanes 2 and 3). The relative amount of p53 actually present in the immune complexes analyzed in Fig. 3A could not be readily determined in this experiment because the heavy chain of the immunoglobulin used for IP comigrated with p53 and obscured its detection (data not shown). Unlike the temperature shift, exposure of A1-5 cells maintained at 39°C to GA for 10 min resulted in loss of hsp90 but not hsp70 coprecipitation (compare lanes 3 and 4). Interestingly, GA treatment also induced the association with p53 of another chaperone formerly termed p60 and now known as hop (lane 4). This protein is a well-recognized component of progesterone receptor heteroprotein complexes, where its association with the hormone-binding protein is also stimulated by GA (see below for discussion).

Our findings that manipulations such as temperature shift and exposure of intact cells to GA prior to cytosol preparation resulted in alterations in the composition of the heteroprotein complexes coprecipitating with p53 suggest that these complexes are biologically significant and not formed artifactually during cytosol extraction and IP. Likewise, the detection of the chaperone proteins demonstrated in Fig. 1 and 3 did not result from nonspecific cross-reactivity of the IP reagents used. Spec-

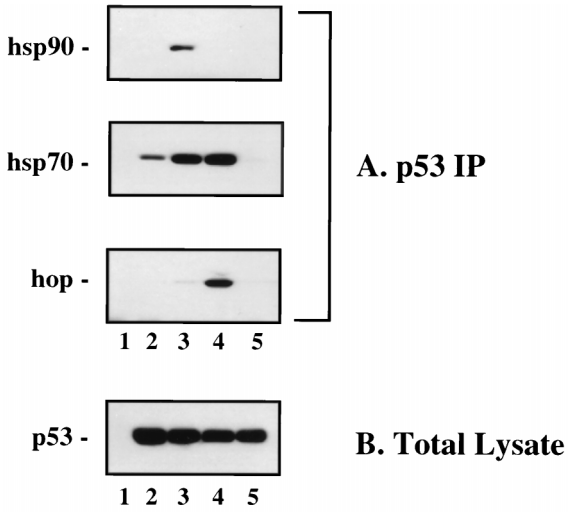

FIG. 3. GA treatment of cells alters the composition of chaperone complexes coprecipitating with p53 in a manner distinct from temperature shift. A1-5 cells were incubated at 32°C (lanes 2) or 39°C (lanes 3 to 5) for 6 h, followed by the addition of 1.8 μM GA (lanes 4) or an equal volume of dimethyl sulfoxide vehicle (lanes 2, 3, and 5) for 10 min prior to lysis in molybdate-containing buffer. As a control, PC-3M carcinoma cells, which do not express p53, were lysed in the same buffer (lane 1). (A) IP with PAb421 (lanes 1 to 4) or irrelevant isotype-matched mouse IgG (lanes 5) was performed, and precipitates were analyzed by immunoblotting for the presence of the indicated chaperones. (B) Total proteins (25 μg) from the lysates analyzed in panel A were analyzed by immunoblotting for the level of p53 present.

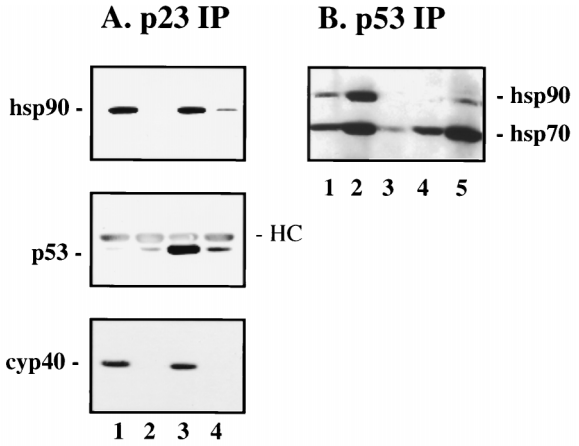

FIG. 4. IP of p53 with chaperone complexes is not epitope dependent. (A) A1-5 cells were incubated at 32°C (lanes 1 and 2) or 39°C (lanes 3 and 4) for 6 h, followed by the addition of 1.8 μM GA (lanes 2 and 4) or control vehicle (lanes 1 and 3) for 10 min prior to lysis as described in the legend to Fig. 3. Anti-p23 IP with monoclonal antibody JJ3 was performed, and precipitates were analyzed by immunoblotting for the presence of the indicated proteins. HC refers to the position of the immunoglobulin heavy chain, which was used for IP and detected by the secondary antibody used in the immunoblotting procedure. (B) A1-5 cells were incubated at 39°C for 6 h followed by the addition of GA (lanes 4 and 5) or control vehicle (lanes 1 to 3) for 10 min and lysis as described for panel A. p53 IP was performed with PAb242 (lanes 1 and 4), PAb421 (lanes 2 and 5), or no primary antibody (lane 3). Precipitates were analyzed by immunoblotting for the presence of hsp70 and hsp90 as indicated.

ificity controls presented in Fig. 3 include IP from cells which do not express p53 (lane 1) and control IP from cytosol derived from A1-5 cells maintained at 39°C with isotype-matched mouse immunoglobulin (Ig) (lane 5). No chaperone protein precipitation was detected under these conditions.

The binding of monoclonal antibody PAb421 near the carboxy terminus of some missense p53 mutants has been reported to alter their function and restore their DNA binding activity (17). To determine whether the novel coprecipitation of chaperones with p53 seen in our system was dependent on PAb421 interaction with the Val135 mutant, we used alternate IP targets and evaluated the pattern of coprecipitating proteins. For the data presented in Fig. 4A, cytosol was prepared from A1-5 cells shifted to wild-type (lanes 1 and 2) or mutant (lanes 3 and 4) temperature conditions for 6 h with (lanes 2 and 4) or without (lanes 1 and 3) the addition of GA 10 min prior to lysis. IP was performed with monoclonal antibody JJ3, which recognizes p23, a component of the mutant p53 heteroprotein complexes demonstrated in Fig. 1. Using this alternate target, we found that p53 coprecipitated efficiently with p23 only in cytosol from cells grown at 39°C (Fig. 4A, lane 3). hsp90 and Cyp40 were seen to coprecipitate with p23 at both 32 and 39°C, and as previously reported, GA treatment of cells prior to lysis disrupted this coprecipitation (18, 36). As an additional means to ensure that precipitation of mutant p53 with chaperone proteins was not epitope specific, we also performed IPs with the anti-p53 monoclonal antibody PAb242, which recognizes a conformation-insensitive epitope at the amino-terminal end of the molecule (Fig. 4B, lanes 1 and 4). Although PAb242 was not as efficient as PAb421 (lanes 2 and 5), it did coprecipitate hsp70 and hsp90. Coprecipitation of hsp90 with p53 by this antibody was disrupted by pretreatment of cells with GA in a fashion similar to that observed in assays using PAb421.

To demonstrate the biologic relevance of the chaperone interactions with p53 shown above, we examined complex formation with several clinically relevant p53 hot-spot mutants in

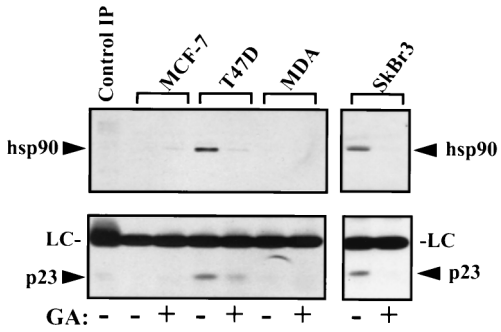

FIG. 5. Certain p53 mutants are stably associated with molecular chaperones in human breast cancer cells. Subconfluent cultures of the cell lines MCF-7 (wild-type p53), T47D (mutant p53, codon 194), MDA-MB-468 (mutant, codon 273), and SkBr3 (mutant, codon 175) were lysed in molybdate-containing buffer with or without prior incubation at 37°C in medium containing GA (1.8 μM) for 15 min as indicated. Anti-p53 IP was performed with antibody DO-1. Control IP consisted of mouse IgG at the same concentration and lysate from non-GA-treated T47D cells. Precipitates were analyzed by immunoblotting for the presence of the indicated chaperones. LC refers to the position of the antibody light chain used for IP and detected by the secondary antibody used in the blotting procedure.

human breast cancer cells. As seen in Fig. 5, the chaperones p23 and hsp90 were found to specifically coprecipitate with the mutant p53 species expressed in the cell lines T47D (mutated codon 194/other allele deleted) and SkBr3 (mutated codon 175/other allele deleted). This coprecipitation was disrupted by brief exposure of cells to GA prior to lysis, as was observed with the Val135 mutant in A1-5 cells. No coprecipitation of chaperones was detected in lysate of MCF-7 cells, which express wild-type p53 alleles, and no coprecipitation was observed in MDA-MB-468 cells, which overexpress p53 from an allele mutated at codon 273. These findings demonstrate that simple overexpression of p53 is not sufficient for detection of the stable complexes that we have observed but rather requires mutation within specific regions of the protein as previously described for hsp70 association with p53 in a panel of human breast tumor specimens (7).

Chaperone complex disruption alters mutant p53 stability and localization. Having demonstrated that chaperone protein complexes involving mutant p53 are disrupted by GA, we examined the consequences of this disruption on p53 function in A1-5 cells. Consistent with our findings for cells carrying other p53 mutants (40), we found that drug treatment of A1-5 cells maintained at 39°C resulted in a marked decline in the total cellular p53 level (Fig. 6A). This decline resulted from enhanced turnover and not decreased synthesis of the protein. In Fig. 6B, cells were pulse-labeled with [^{35}S]methionine for 1 h

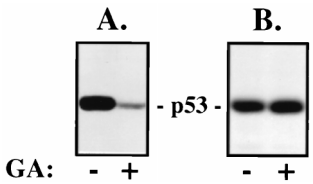

FIG. 6. GA treatment of A1-5 cells decreases p53 levels without altering its rate of synthesis. Cells were cultured overnight at 39°C in the presence or absence of GA (1.8 μM) as indicated. (A) Cells were lysed, and the level of p53 in equal amounts of total protein was evaluated by immunoblotting. (B) Cells were metabolically labeled with [^{35}S]methionine for 1 h, followed by lysis and IP of p53 with PAb421 from equal amounts of trichloroacetic acid-precipitable material. Precipitates were fractionated by SDS-PAGE and visualized by autoradiography.

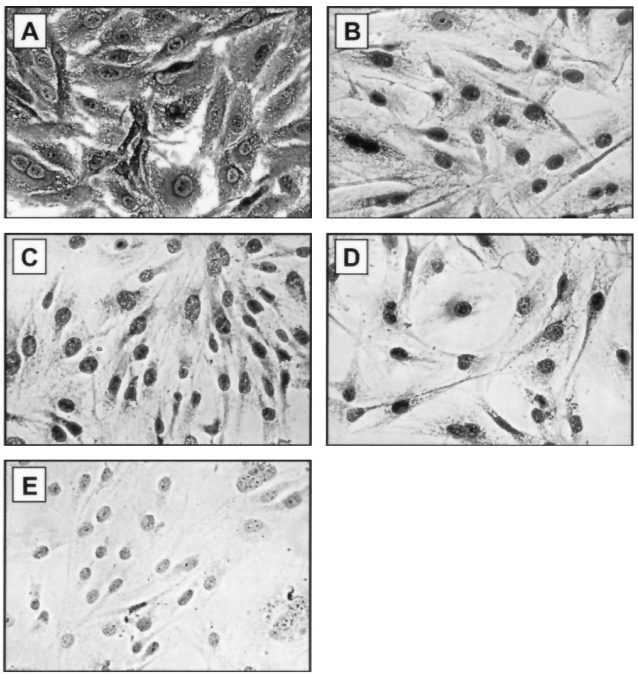

FIG. 7. GA treatment of A1-5 cells induces nuclear localization of mutant p53. Cells growing on coverslips were incubated at 39°C (A, C, and E) or 32°C (B and D) for 6 h in the presence (C and D) or absence (A, B, and E) of GA. Following fixation in cold methanol-acetone, p53 localization was visualized by indirect immunocytochemistry using PAb421 except for panel E, where an irrelevant control antibody was applied. The dark diaminobenzidine signal represents p53 immunoreactivity. All panels were photographed at the same magnification and exposure settings.

after an overnight exposure to GA. Immunoprecipitation of p53 from these cells revealed comparable levels of newly synthesized p53 despite the markedly lower level of total p53 seen in Fig. 6A. Such a finding is consistent with restoration of the normally rapid turnover characteristic of wild-type p53 following disruption of chaperone protein-mutant p53 complex formation by GA.

In addition to destabilizing the mutant protein, GA treatment of cells induced nuclear translocation of the protein in a manner similar to that observed with the temperature shift. Figure 7A demonstrates the clear cytoplasmic immunolocalization of mutant p53 characteristic of A1-5 cells at 39°C. As previously reported (24), a temperature shift to 32°C for 6 h resulted in a dramatic shift to nuclear localization (Fig. 7B). Interestingly, GA treatment of cells maintained at 39°C resulted in a nuclear pattern of p53 localization that was indistinguishable from that observed with a temperature shift (Fig. 7C). Localization associated with concurrent temperature shift plus GA treatment appeared the same as after either manipulation alone (Fig. 7D). The specificity of the immunostaining protocol used was confirmed by the results in Fig. 7E, where isotype-matched control antibody was used to stain A1-5 cells maintained at 39°C and minimal background reactivity was observed.

GA does not restore transcriptional activating activity to mutant p53. To assess the transcriptional activating activity of mutant p53 relocalized to the nucleus by GA treatment, we made use of A1-5 cells stably transfected with reporter constructs under the control of two different p53 response elements. Plasmid PG13/βgal carries the β-galactosidase gene driven by the mouse polyomavirus promoter and a multimer of a genomic p53 binding site from the ribosome gene cluster (1). As seen in Fig. 8A, this plasmid displays very low basal activity at 39°C in A1-5 cells and robust induction following a shift to 32°C. Although GA treatment of cells at 39°C clearly induced nuclear translocation of p53 (Fig. 7), it did not confer significant transactivating activity for the PG13 response element, presumably for reasons to be discussed below.

To be certain that the negative results obtained with PG13/βgal were not restricted to this response element alone, we also examined the effect of drug treatment on the activity of the native *WAF-1* promoter, which drives β-galactosidase expression in the vector WAF1/βgal. Substantial basal activity at 39°C was observed with this plasmid, consistent with a previous report (1), but approximately fourfold-higher induction was observed following temperature shift to wild-type conditions (Fig. 8B). The effect of GA treatment on β-galactosidase activity was more complex in this reporter system, however, than with the PG13/βgal vector. Here drug treatment of cells at 39°C resulted in a small but reproducible increase in β-galactosidase activity, while treatment at 32°C actually resulted in a small decline. Given the complexity of the native *WAF-1* pro-

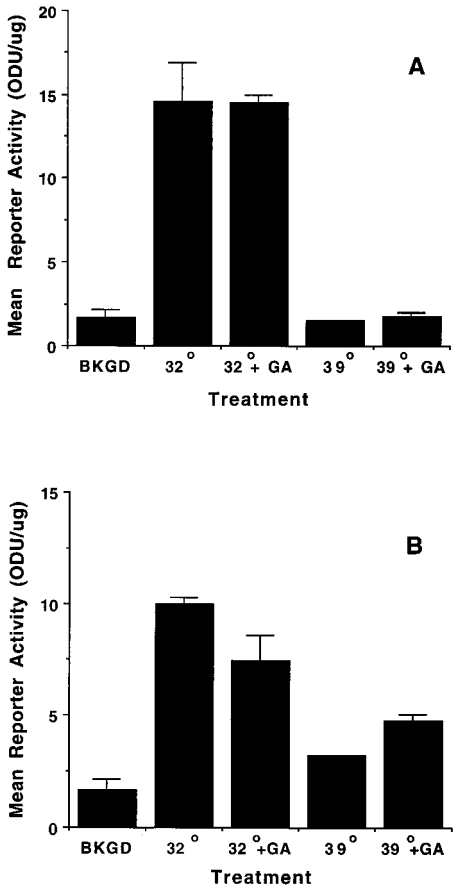

FIG. 8. GA treatment of A1-5 cells does not restore wild-type p53 transcriptional activating activity. Cells were stably transfected with the p53-responsive reporter construct PG13/βgal (A) or WAF1/βgal (B). The level of β-galactosidase activity was quantitated in cell lysates following overnight incubation with or without GA at the indicated temperatures. BKGD refers to the level of β-galactosidase activity measured in nontransfected A1-5 cells. All determinations were performed in triplicate, and mean values are depicted, with the standard deviations of the means indicated by error bars. The results presented are derived from analysis of a clonal isolate derived from transfections with each of the two vectors, but similar results were obtained in assays using pooled colonies. ODU, optical density units.

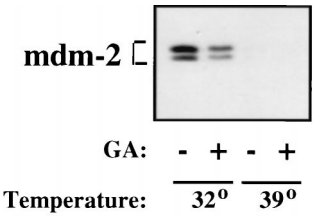

FIG. 9. GA treatment does not induce mdm-2 expression in A1-5 cells. Cells were incubated overnight at the indicated temperatures with or without the addition of GA (1.8 μM). Lysates were prepared, and the induction of mdm-2 was detected by immunoblotting with monoclonal antibody 2A10.

moter, it seems most likely that these conflicting results represent p53-independent effects of GA on activation of this response element in A1-5 cells (27).

As a final approach to demonstrating GA effects on the transactivating activity of mutant p53, we examined cellular levels of the endogenous, p53-regulated gene products mdm-2 in A1-5 cells and WAF-1 in human breast cancer cells. The expression of mdm-2 is tightly regulated by p53, and it appears to serve as a negative regulator of p53 function (28). As seen in the immunoblot of whole-cell lysates presented in Fig. 9, temperature shift from mutant to wild-type conditions increased cellular mdm-2 to readily detectable levels in A1-5 cells as previously reported for other cells carrying a $p53^{Val135}$ mutant (2). Consistent with our findings in assays using the PG13 reporter construct, GA treatment of A1-5 cells did not induce detectable levels of mdm-2 in cells maintained at 39°C. Likewise, as evident in Fig. 10, we did not observe GA-induced increases in the level of WAF-1 protein in human tumor cells expressing two different mutant p53s, one of which we have shown above coprecipitates in a chaperone complex which is disrupted by GA (Fig. 5, T47D). Consistent with its demonstrated target of action (11, 31) and lack of DNA-damaging activity, GA did not increase WAF-1 levels in MCF-7 cells. These cells were used as a control because they express wild-type p53 and were able to respond to doxorubicin-mediated damage by increases in both p53 and WAF-1 levels as expected. GA treatment decreased the level of mutant p53 in T47D cells (and to a lesser extent in MDA-MB-468 cells), as anticipated based on our previous findings (40) and data presented above. Thus, although GA treatment decreased mutant p53 levels (Fig. 6 and 10) and induced its nuclear translocation in A1-5 cells (Fig. 7), GA-mediated disruption of chaperone complex formation did not appear sufficient to confer wild-type transactivating activity to several different mutant species, as monitored by the level of p53-regulated gene products in drug-treated cells.

DISCUSSION

It has long been appreciated that many mutant p53 species display unusually stable physical association with the heat shock protein hsc70 both in tissue culture cells and in human tumor specimens (7). This association has been reported to regulate p53 conformation in vitro (12) and correlates with increased transforming activity in intact cells (13, 16). In the present study, we made use of a temperature-sensitive p53 mutant and the selective hsp90-binding agent GA to establish a role for multiple molecular chaperones, not just hsc70, in modulating the stability, localization, and function of mutant and wild-type p53 within a conserved cellular background. The biologic relevance of these findings was then confirmed by demonstrating a similar pattern of chaperone interactions with the mutant p53 species expressed by some human breast cancer cell lines.

Although the biochemical activity of many molecular chaperones is well established, much less is known about how chaperones act together in large heteromeric complexes to regulate the posttranslational function of diverse kinases, receptors, and transcription factors (29). The best-studied example of chaperone-mediated conformational regulation in eukaryotic cells is that of steroid hormone receptors, which require the interaction of multiple chaperones to acquire or maintain a state competent to bind hormone (30, 35). Through a series of ATP-dependent reactions, an immature hormone receptor complex that contains hsp90, hsp70, and at least the two co-chaperones hip (p48) and hop (p60) is maintained in dynamic equilibrium with a more favored mature complex which is competent to bind hormone (4). This mature complex lacks hsp70, hip, and hop but contains at least two new proteins, p23 and one of the three large immunophilins FKBP52, FKBP54, and Cyp40. Upon hormone binding, the receptor is no longer found in association with chaperone proteins and becomes active as a transcription factor (8). Treatment with GA has been shown to block the transition of steroid receptors from immature to mature complexes, thus preventing hormone binding (36) and resulting in enhanced ubiquitination and proteasome-mediated degradation of the hormone-binding protein (38).

We now report that analogous to steroid receptors, p53 coprecipitated in lysate with multiple molecular chaperones in addition to hsp70 when cells were maintained under conditions favoring mutant p53 conformation and function (Fig. 1). As recently reported by Sepehrnia et al. for A1-5 cells (34) and Selkirk et al. for T47D breast cancer cells (33), we also found hsp90 coprecipitating with mutant p53. In addition, however, we detected coprecipitation of p23 and the large immunophilin Cyp40, components characteristic of more mature steroid receptor complexes. Immunodepletion studies indicated that at least 70% of the p53 in A1-5 cells grown under mutant temperature conditions was associated with a p23-containing chaperone complex (Fig. 2), while a significantly smaller fraction appeared to be associated with hsp70. Such a pattern is consistent with that reported for unliganded progesterone receptors (35). Upon a temperature shift, coprecipitation of all of these chaperones was markedly reduced (Fig. 3), yielding a p53 species capable of acting as a transcription factor (Fig. 8 and 9).

Treatment of A1-5 cells with GA resulted in an effect on p53 similar to that observed with steroid receptors, namely, loss of mature complex components and enhancement of intermedi-

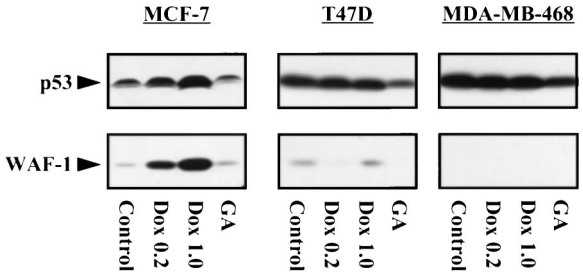

FIG. 10. GA treatment does not induce WAF-1 expression in human breast cancer cells. Subconfluent cultures of the indicated cell lines were treated overnight with control vehicle, doxorubicin (Dox; 0.2 or 1.0 μM), or GA (1.8 μM). Lysates were fractionated by SDS-PAGE, and proteins were transferred to nitrocellulose. The upper half of the membrane was probed with anti-p53 antibody DO-1, while the lower half was probed with antiserum to human WAF-1.

ate components, as indicated by the increased hop signal seen in Fig. 3A, lane 4. Despite these similarities, however, differences also were apparent. Specifically, hsp90 is present in both intermediate and mature progesterone and glucocorticoid receptor complexes (36, 38), while with mutant p53, we found that hsp90 was lost in complexes from GA-treated A1-5 and breast cancer cells even though p53 appeared to be trapped in intermediate, hop-containing complexes (Fig. 3 to 5). In this respect, mutant p53 appeared to behave more like transforming tyrosine kinases such as v-Src. With these targets, association with hsp70 and hsp90 is detectable under control conditions and hsp90 association is disrupted by GA (39). While chaperone interactions appear relatively well conserved, differences do exist in the associations seen with specific targets, the functional significance of which is unclear at this time (29).

As with the glucocorticoid receptor, trapping of p53 in an apparently intermediate complex by GA stimulated its degradation (Fig. 6 and 10) but did not render it active as a transcription factor (Fig. 8 to 10). The concept that association with other proteins can modulate the degradation of p53 is supported by recent studies demonstrating such a role for mdm-2 (15, 19). GA-stimulated degradation of mutant p53 in our system, however, does not appear to be mediated through mdm-2, as no induction of the protein could be detected following GA treatment of A1-5 cells. Our finding that GA failed to restore transcription factor activity to mutant p53 species agrees with a previous study reporting that although GA treatment alters the conformation of mutated p53 as measured by IP with conformation-specific antibody, it only partially restores its ability to bind a consensus DNA sequence (3). It seems most likely that GA's failure to restore p53 function as a transcription factor results from the continued association of p53 with chaperones such as hsp70 and hop in GA-treated cells. These persistent associations may impair oligomerization or posttranslational modifications such as phosphorylation which are required for p53 activity as a transcription factor (21, 24). It is also possible that GA interferes directly with the function of some of the various kinases which have been proposed to be involved in activating p53 such as casein kinase II or raf-1 (reviewed in reference 25).

At this point, we do not know whether the chaperone components that we have identified coprecipitate with p53 as a single complex or several distinct complexes (Fig. 2), but in vitro reconstitution experiments in reticulocyte lysate may be able to address this issue directly in future studies. It is not possible to comment on the stoichiometry of the components observed in our coprecipitation experiments for two reasons. First, complexes are isolated by IP under nonequilibrium conditions, which may allow for their gradual dissociation during the procedure. Second, inherent variation in the affinity of the antibodies used to detect components by Western blotting makes it impossible to directly compare the absolute amounts of each protein detected.

It is interesting to speculate that the extended chaperone interactions we have observed with mutant p53 actually represent a pathologic exaggeration of normal, physiologic interactions of wild-type p53 with the chaperone machinery. Due to its intrinsic conformational lability, the turnover and function of wild-type p53 could be regulated to a significant extent by ongoing posttranslational interactions with components of the chaperone machinery. Such interactions may become detectable by coimmunoprecipitation only when they become pathologically extended as a consequence of mutation of the target. Under normal conditions, their transient nature may serve to modulate the presentation of p53 for degradation by the ubiquitin-proteasome system. Because they involve heat shock proteins, these same interactions could also serve as sensors of cell stress or damage. Altering their levels and availability by insults such as ionizing radiation or alkylating agents could lead to p53 stabilization and provide a mechanism for its rapid activation as a transcription factor in response to cellular damage. Consistent with this proposal, cellular stresses that do not involve DNA damage have been shown to induce p53 activation (20). In addition, salicylate concentrations which inhibit the heat shock response have been shown to inhibit p53 activation in response to UV irradiation and the chemotherapeutic agent adriamycin (5).

In summary, we have shown that several mutant p53 species, but not wild-type p53, are stably associated with a conserved group of molecular chaperones. Mutant p53 molecules, presumably due to specific alterations in conformation, appeared to be retained within this molecular chaperone machinery, leading to their mislocalization and protection from degradation. Alteration of specific chaperone interactions by GA treatment resulted in destabilization of mutant proteins, supporting the view that posttranslational interaction with certain chaperone heteroprotein complexes may stabilize a target while interaction with others may actually stimulate its degradation (10). Taken together, our findings demonstrate that chaperone proteins play an important role in modulating the function of many mutant p53 species and suggest that they could be involved in regulating the activity of wild-type protein in response to cellular stress.

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