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Gambogic Acid-Induced Degradation of Mutant p53 is Mediated by Proteasome and Related to CHIP

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ABSTRACT

As an oncoprotein, mutant p53 is a potential tumor-specific target for cancer therapy. Most mutated forms of the protein are largely accumulated in cancer cells due to their increased stability. In the present study, we demonstrate that mutant p53 protein stability is regulated by gambogic acid (GA). Following GA exposure, protein levels of mutant p53 decreased while the mRNA levels were not affected in MDA-MB-435 cells, which indicate that GA down-regulates mutant p53 at post-transcription level. Co-treatment with GA and cycloheximide, a protein synthesis inhibitor, induced a decrease of half-life of mutant p53 protein. These findings indicated that the reduction of mutant p53 by GA was due to the destabilization and degradation of the protein. Furthermore, inhibition of proteasome activity by MG132 blocked GA-induced down-regulation of mutant p53, causing mutant p53 accumulation in detergent-insoluble cellular fractions. Further studies revealed that mutant p53 was ubiquitinated and it was chaperones related ubiquitin ligase carboxy terminus of Hsp70-interacting protein (CHIP) rather than MDM2 involved in the degradation of mutant p53. In addition, GA prevented Hsp90/mutant p53 complex formation and enhanced interaction of mutant p53 with Hsp70. Depletion of CHIP stabilized mutant p53 in GA treated cells. In conclusion, mutant p53 may be down-regulated by GA through chaperones-assisted ubiquitin/proteasome degradation pathway in cancer cells. J. Cell. Biochem. 112: 509–519, 2011.

KEY WORDS: GAMBOGIC ACID; MUTANT P53; CHAPERONE; CHIP; PROTEASOME

P53 mutation is one of the most frequent alterations in human malignancy [Hainaut and Hollstein, 2000]. The primary outcome of these mutations is the loss of the transcription function of wild-type p53 protein. Although the loss of wild-type p53 function could be sufficient to promote tumor progression, evidence has shown that mutant p53 protein may acquire novel activities as an oncoprotein [Strano et al., 2007]. These activities range from enhanced proliferation in culture, to increased tumorigenicity in vivo [Liu et al., 2000; Bossi et al., 2006], and enhanced resistance to a variety of anticancer drugs commonly used in clinical practice [Blandino et al., 1999; Tsang et al., 2005; Wong et al., 2007]. Moreover, mutant p53 can abolish the function of wild-type p53 by interacting through the oligomer formation, known as a dominant negative function [Milner and Medcalf, 1991].

The majority of mutant p53 in tumor cells display markedly increased intracellular levels, and the mutant proteins have much longer half-lives (more than 24 h) regardless of the presence of environmental stimuli to the cells [Nagata et al., 1999]. Stabilization of mutant p53 is significant considering the process of tumor formation and the therapeutic strategies such as gene therapy against a variety of human malignancies [Roth and Cristiano, 1997]. p53 protein level is mainly regulated at the post-translational level by ubiquitin ligase MDM2, while expression of MDM2 is activated by p53 at the transcription level, forming a negative feedback loop to maintain p53 protein at low levels under normal conditions [Haupt et al., 1997]. The stabilization of mutant p53 is mostly ascribed its inability to activate the expression of MDM2 [Strano et al., 2007]. However, this is thought to be an oversimplification because high levels of MDM2 are found in many tumor cell lines expressing high levels of mutant p53, and even though MDM2 interacts with mutant p53, it still fails to degrade the protein in some cancer cell lines [Buschmann et al., 2000; Peng et al.,

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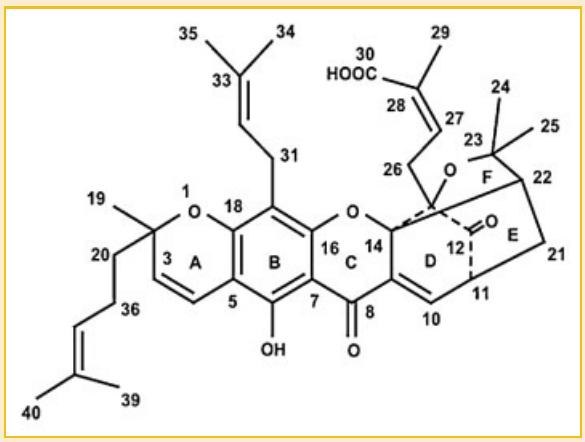

Fig. 1. The molecular structure of GA and its atomic numbering scheme.

2001]. These researches indicate that a failure to activate the expression of MDM2 is not the underlying cause of mutant p53 stability in tumors.

Molecular chaperones including Hsp70 and Hsp90 also play important roles in the stabilization of mutant p53. Mutated p53 proteins are specifically recognized by Hsp90 and Hsp70. These two chaperones can present mutant p53 to chaperone-associated ubiquitin ligase carboxy terminus of Hsp70-interacting protein (CHIP) [Esser et al., 2005; Lukashchuk and Vousden, 2007; Muller et al., 2008]. Through binding to the carboxyl termini of Hsp70 and Hsp90, CHIP mediates the ubiquitylation of chaperone-bound client proteins in conjunction with E2 enzymes and induces client degradation by the proteasome [Jiang et al., 2001; Murata et al., 2001].

Gambogic acid (GA, $C_{38}H_{44}O_8$, Fig. 1) is a xanthone isolated from the dry latex of *Garcinia hanburyi*, which is used as a traditional Chinese medicine. Previous studies have demonstrated that GA exhibits potent in vitro and in vivo activities such as activation of the apoptotic pathway, induction of cell cycle arrest, inhibition of telomerase and topoisomerase II activities, and suppression of metastatic and angiogenesis [Kasibhatla et al., 2005; Lu et al., 2007; Qin et al., 2007; Yang et al., 2007; Yu et al., 2007; Qi et al., 2008; Zhao et al., 2008]. All studies reported to date demonstrate that GA is a potent anticancer agent with diverse molecular targets through different mechanisms. As an antitumor candidate, GA is currently undergoing a phase II clinical trial approved by Chinese Food and Drug Administration. Nevertheless, GA shows its anticancer effect under other mechanisms which need further study. Previous study identified p53/MDM2 as new molecular target that GA modulates to perform its potent anticancer activity. GA inhibits wild-type p53 harboring cancer cell growth both in vitro and in vivo, which due to its enhancement of wild-type p53 at protein level through inhibition of MDM2 expression [Gu et al., 2008; Gu et al., 2009]. It also has been demonstrated that GA down-regulates the cellular level of MDM2 and elevates the expression of p21Waf1/CIP1 independent of wild-type p53 [Rong et al., 2009]. However, p53 mutation occurs in more than half of all human cancers and little is known about the effect of GA on mutant p53. In this study, we focused on investigating the effects of GA on cancer cells harboring mutant p53 and analyzing the mechanisms of mutant p53 regulation by GA.

MATERIALS AND METHODS

MEDICINE, REAGENT AND ANTIBODIES

Gamboge resin (lot# 20000526) of *Garcinia hanburyi* was purchased from Jiangsu Provincial Medicinal Materials Company, China. GA was isolated and purified according to the established methods [Zhang et al., 2004]. The purity of GA used in all experiments was 95% or higher. It was solubilized in arginine dissolved phosphate-buffered saline (PBS, 0.01 M) to 10 mM and stored at −20°C. Arginine dissolved PBS buffer with 0.01% dimethyl sulfoxide (DMSO) was used as a solvent control. Cycloheximide (CHX) was purchased from Beyotime Instiute of biotechnology (Hangzhou, China), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), *N*-benzoyloxycarbonyl (Z)-Leu-Leu-Leu-al complex (MG132) and Chloroquine were obtained from Sigma–Aldrich, Inc. (St. Louis, MO). All the drugs were diluted in corresponding culture medium to desired concentrations before use. The biotinylated anti-goat IgG secondary antibodies were from Santa Cruz Biotech Ltd. (Santa Cruz, CA). The standard avidinbiotin–peroxidase complex (ABC) kit was from VECTASTAIN, Vector Industries, Inc. (Burlingame, CA). Monoclonal anti-p53 antibody (clone DO-1, Pab 240, and Pab 1620) was purchased from Calbiochem (La Jolla, CA,) and monoclonal anti-β-actin antibody was bought from Boster Biological Technology Ltd. (Wuhan, China). Monoclonal anti-green fluorescent protein (GFP), -Hsp70, -CHIP, and rabbit polyclonal anti-p53, -MDM2 (N20) antibodies were obtained from Santa Cruz Biotechnology Inc. Rabbit polyclonal anti-Hsp90, -ubiquitin antibodies were from Bioworld Technology Co. Ltd (Minneapolis, MN).

CELL CULTURE

Human breast cancer cell lines MDA-MB-435 and MDA-MB-231 (contain different p53 mutations for G266E and R280K, respectively) were purchased from Cell Bank of Shanghai Institute of Biochemistry and Cell Biology. The p53 mutation was confirmed by direct sequencing of PCR products in MDA-MB-435 and MDA-MB-231 (Supplementary Fig. 1). H1299 (p53-null) was a gift from Dr. Caicun Zhou (Shanghai Pulmonary Hospital, Shanghai, China). All cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U ml^{-1} benzylpenicillin and 100 μg ml^{-1} streptomycin in a humidified environment with 5% CO_2 at 37°C.

CELL VIABILITY ASSAY

Cells were plated on 96-well flat-bottom culture plates (8,000 cells per well) and then exposed to the various agents. Following 48 h exposure, MTT solution in PBS was added to each well for 4 h. After removal of the medium, dimethyl sulfoxide (DMSO) was added to each well to dissolve the formazan crystals. Then absorbance at 570 nm was determined. The experiment was performed three times in a parallel manner for each concentration of drugs used and the results were presented as mean ± SEM. Triplicate wells were analyzed at each dose. Cell viability inhibitory rate was calculated

as follows: Inhibitory rate $= (1 - A_{treatment}/A_{control}) \times 100\%$. IC_{50} was taken as the concentration that caused 50% inhibition of cell viability and calculated with Graphpad Prism V 5.0 (GraphPad Software, San Diego, CA).

ANIMAL STUDIES

Female athymic BALB/c nude mice (35–40 day old) with body weight of 18–22 g were supplied by Shanghai Institute of Materia Medica, Shanghai Institute for Biological Sciences, Chinese Academy of Sciences. The animals were kept at $22 \pm 2^{\circ}C$ and 55–65% humidity in stainless steel cages under controlled lights (12 h light/day) and were fed with standard laboratory food and water ad libitum. The animal care was followed in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals published by the National Institute of Health, USA. MDA-MB-435 cells were resuspended in icy 0.9% normal saline at a density of $2 \times 10^6/200\,\mu l$ and implanted into the mammary fat pad of BALB/cA nude mice. When a distinct tumor mass (4–5 mm in diameter) was detectable, the animals were randomly divided into control group and GA treated groups (10 mice per group). Test compounds or vehicle was administrated every other day for 9 weeks by intravenous injection. Twenty-four hours after the last drug administration, the animals were sacrificed and tumors were carefully peeled off for Western blot analysis.

IMMUNOCYTOCHEMISTRY

Cells were plated onto glass coverslips in 6-well plates. The following day, cells were treated with GA for 6 h, then washed with PBS and fixed with 4% paraformaldehyde for 30 min at 4°C and followed by blocking with 10% bovine serum albumin (dissolved in PBS) for 1 h. Then cells were incubated with mouse monoclonal antibody p53 (DO-1) at 4°C overnight. After washing, cells were incubated with biotinylated anti-mouse IgG secondary antibodies for 1 h. Then immune complexes were visualized using the ABC kit and nuclei were counterstained with hematoxylin. Cells were mounted by neutral gum and examined by an inverted microscope. In all cases, negative controls were treated in the same way except that the primary antibody was omitted.

REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION ASSAY

Total cellular RNA was extracted using the RNAiso Reagent from Takara (Japan), quantified by UV spectrophotometry with BioPhotometer (Eppendorf, Germany). Purity of the RNA extracted was determined by the ratio of A260/A280. Single-stranded oligo (dT)-primed cDNA was generated using MMLV reverse transcriptase (TaKaRa, Japan). Primers used for reverse transcription-PCR (RT-PCR) analysis of human p53 were 5′-GCGTGAGCGCTTCGAGAT-3′ (sense) and 5′-AGCCTGGGCATCCTTGAG-3′ (anti-sense). The amplification reaction was carried out for 35 cycles, and each cycle consisted of 95°C for 20 s, 55°C for 30 s, and 72°C for 1 min, followed by a final 10-min elongation at 72°C. Comparability of RNA quantities was ensured using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal standard [Yu et al., 2007]. Primers for human GAPDH RNA amplification were 5′-GTCGTACCACAGGCATTGTGATGG-3′ (sense) and 5′-GCAATGCCTGGGTACATGGTGG-3′ (anti-sense).

PLASMID AND TRANSFECTION

The plasmids pcDNA3/p53 (R273H) and pcDNA3/p53 (R175H) were gifts from Dr. Petr Muller (Institute of Molecular and Cell Biology, Proteos, Singapore) and Dr. Jörg Höhfeld (Institute for Cell Biology and Bonner Forum Biomedizin, Bonn, Germany). The p53 siRNA, CHIP siRNA, and control siRNA was purchased from Santa Cruz Biotechnology, Inc. Both transient plasmid and siRNA transfections were performed according to the manuals of LipofectamineTM 2000 regent (Invitrogen).

WESTERN BLOT ANALYSIS

Total proteins were extracted with TNES buffer (50 mM Tris–Cl, pH 7.6, 150 mM NaCl, 1 mM EDTA, 1% (m/v) NP-40, 0.2 mM PMSF, 0.1 mM NaF, 1.0 mM DTT, 0.1 mM Na3VO4, $2\,\mu g\,ml^{-1}$ Pepstatin A, $2\,\mu g\,ml^{-1}$ Leupeptin, $1\,\mu g\,ml^{-1}$ Aprotinin). Cellular lysates were cleared by centrifugation at 12,000*g* for 15 min at 4°C. The supernatants were collected as the NP40-soluble fractions. The pellets (NP40-insoluble fractions) were lysed in TNES buffer containing 2% SDS, and boiled for 15 min. Protein concentration was determined using the BCA assay with Varioskan multimode microplate spectrophotometer (Thermo). Equal amounts of protein samples were electrophoresed on 10% SDS-polyacrylamide gels (SDS–PAGE) and transferred onto nitrocellulose membranes. Membranes were blocked with 10% non-fat milk in PBS and probed overnight with primary antibodies at 4°C followed by washing and incubated for 1 h with IRDyeTM800-conjugated secondary antibodies. Immunoreactive protein bands were detected using an Odyssey Scanning System (LI-COR Biosciences, Lincoln, Nebraska).

IMMUNOPRECIPITATION

For co-immunoprecipitation of p53 complexes, cells were lysed in TNES buffer as described above. Cell lysate (1 ml) containing 1.5 mg total protein was incubated with 1 μg p53 (clone DO-1) antibody and 20 μl protein A/G-conjugated beads (Santa Cruz) overnight. After four washes in TNES buffer, samples were centrifuged at 3,000*g* for 2 min and resuspended in 20 μl SDS-sample buffer (0.5 M Tris–HCl, pH6.8, 20% glycerol, 2% SDS, 5% 2-mercaptoethanol, 4‰ bromophenol blue). For Western blot analysis, 10 μl samples were used.

STATISTICAL ANALYSIS

All data were expressed as means ± SD or means ± SEM as indicated and statistically compared by one-way ANOVA. $P < 0.05$ was taken as statistically significant and $P < 0.01$ was considered as dramatically significant. All the experiments in this study were repeated at least twice.

RESULT

GA DOWN-REGULATES MUTANT P53 AND INCREASES THE SENSITIVITY OF CANCER CELL HARBORING MUTANT P53 TO CHEMOTHERAPEUTIC AGENTS

The apoptosis inducing effect of GA partially depending on up-regulation of p53 is reported previously [Gu et al., 2008]. In our study, we firstly investigate the effects of GA on two human cancer

cell lines harboring mutant p53 (MDA-MB-435 and MDA-MB-231 with different p53 mutations for G266E and R280K, respectively). As shown in Figure 2A, GA inhibited the viability of the cells in a concentration-dependent manner. IC_{50} values for MDA-MB-435 and MDA-MB-231 were 2.57 ± 0.96 and 2.04 ± 0.46, respectively. Being different from wild-type p53, most of mutant p53 is usually detected at high levels in tumor cells, and the mutant proteins have much longer half-lives regardless of the presence of environmental stimuli to the cells [Zambetti and Levine, 1993]. Western blot analysis was performed to examine the effects of GA on mutant p53 expression in MDA-MB-435 and MDA-MB-231, (Fig. 2B). We found that mutant p53 protein level was decreased in the tested cell lines with a concentration-dependent manner after GA exposure. Identical results were essentially obtained in H1299 cells (p53 null) which were transfected with exogenous mutant p53-R273H and mutant p53-R175H (Fig. 2C). Results from immunocytochemical staining confirmed that from Western blot analysis. Compared to control, GA treatment decreased the amount of mutant p53, especially in cell nucleus as shown in Figure 2D.

Researches have demonstrated that over-expression of mutant p53 increases tumorigenicity and tissue invasiveness in mice [Sun et al., 1993; Liu et al., 2000]. Human breast cancer cell line MDA-MB-435 is a highly invasive cell line, which expresses high levels of mutant p53. We have previously reported that GA suppresses invasion and lung metastasis of MDA-MB-435 cells in vivo [Qi et al., 2008]. This implied the regulative effect of GA on mutant p53 in vivo. MDA-MB-435 xenograft model was constructed to demonstrate this hypothesis. Tumor tissues were retrieved after the final administration of GA and analyzed by Western blotting to determine the change in mutant p53 protein level. Administration of 4 and 8 mg kg^{-1} GA produced remarkable inhibition of mutant p53 compared with vehicle control, whereas little change was observed in the adriamycin (ADM) treatment group (Fig. 2E).

A growing number of studies have provided compelling evidence that mutant p53 gains a new function in tumor cells by increasing chemoresistance to a variety of anticancer drugs [Pugacheva et al., 2002; Tsang et al., 2005; Bossi et al., 2006; Wong et al., 2007]. To evaluate whether in our experimental conditions the depletion of mutant p53 expression affects the chemosensitivity of tumor cells, mutant p53 was knocked down by transient transfecting with p53 siRNA in MDA-MB-435 cells. Results of the MTT assay showed that, cells were more sensitive to chemotherapeutic agents such as etoposide, ADM, and 5-fluorouracil (5-FU) after mutant p53 knock-down (Fig. 3A). As GA promoted down-regulation of mutant p53, we proceeded to test whether GA can increase chemotherapeutic agents sensitivity. As shown in Figure 3B, pre-treatment with GA increased cytotoxic effects induced by etoposide, ADM, and 5-FU in MDA-MB-435 cells.

GA DOWN-REGULATES MUTANT P53 AT POST-TRANSCRIPTIONAL LEVEL

Based on the observed decrease in mutant p53 protein level caused by GA, we proceeded to evaluate whether this decrease was regulated at transcription or post-transcription level in GA treated MDA-MB-435 cells. As shown in Figure 4A, there was no significant reduction of p53 mRNA observed in cells treated with GA comparing with that in controls, which suggested that GA down-regulated mutant p53 at post-transcription level.

Since the transcription of mutant p53 is not affected by GA, the reduction of mutant p53 was probably due to protein degradation. To demonstrate this hypothesis, we measured the half-life of mutant p53 by treating the cells with CHX, an inhibitor of protein synthesis. As shown in Figure 4B, mutant p53 is a very stable protein exhibiting a long half-life in MDA-MB-435 cells. When cells were treated with CHX alone, mutant p53 protein decreased to 73.76% of the initial amount after 6 h. However, following co-treatment of GA and CHX, pre-existing mutant p53 protein was markedly decreased, indicating a protein half-life of 3.5 h. This finding is consistent with the hypothesis that GA decreases mutant p53 protein by inducing its degradation.

GA STIMULATES UBIQUITIN/PROTEASOME-MEDIATED DEGRADATION TO MUTANT P53

In order to determine whether the degradation of mutant p53 caused by GA was mediated by the proteasome and/or lysosomal proteases, we tested the abilities of proteasome inhibitor MG132 and lysosome inhibitor chloroquine to block GA-induced degradation of mutant p53. As previous studies reported that inhibition of proteasome function led to the accumulation of ubiquitinated mutant p53 which was insoluble in non-ionic detergent such as NP-40 and redissolved in SDS buffer [Whitesell et al., 1997], we analyzed the level of mutant p53 and ubiquitinated mutant p53 in NP-40-insoluble fraction which was redissolved in SDS. As shown in Figure 5A, inhibition of proteasome function with MG132 resulted in a marked accumulation of p53 in NP-40-insoluble fractions. This NP-40-insoluble material represented aggregated or precipitated p53 species, which cannot be degraded when proteasome activity is inhibited. However, inhibition of lysosomal function with chloroquine had no such effect. Moreover, GA treatment in the presence of MG132 enhanced the redistribution of mutant p53 in MDA-MB-435 cells to insoluble form, resulting in quit small fractions in soluble form. The result of total p53 analysis was the same. This demonstrates that GA selectively stimulates transit of mutant p53 through proteasome-dependent degradation.

Furthermore, we immunoprecipitated (IP) p53 from the NP-40-insoluble fraction of MDA-MB-435 cell lysates to test whether mutant p53 was ubiquitinated prior to its degradation in GA treated cells. The immunoprecipitates were analysed by SDS–PAGE followed by Western blot analysis for p53 (Fig. 5B, left panel) and ubiquitin (Fig. 5B, right panel). Treatment with GA or MG132 resulted in accumulation of a ladder of immunoprecipitable p53 species (Fig. 5B, lane 2, 3), and an increase in higher molecular weight form was evident following treatment with GA and MG132 together (Fig. 5B, lane 4). Meanwhile, there was no significant ubiquitinated p53 in control and GA-treated cells (Fig. 5B, lane 5, 6). However, an increase in ubiquitinated p53 was detected after treatment with MG132 alone (Fig. 5B, lane 7) and the ubiquitinated species were more evident in the cells treated with both GA and MG132 (Fig. 5B, lane 8). These results demonstrate that GA stimulates polyubiquitination of mutant p53.

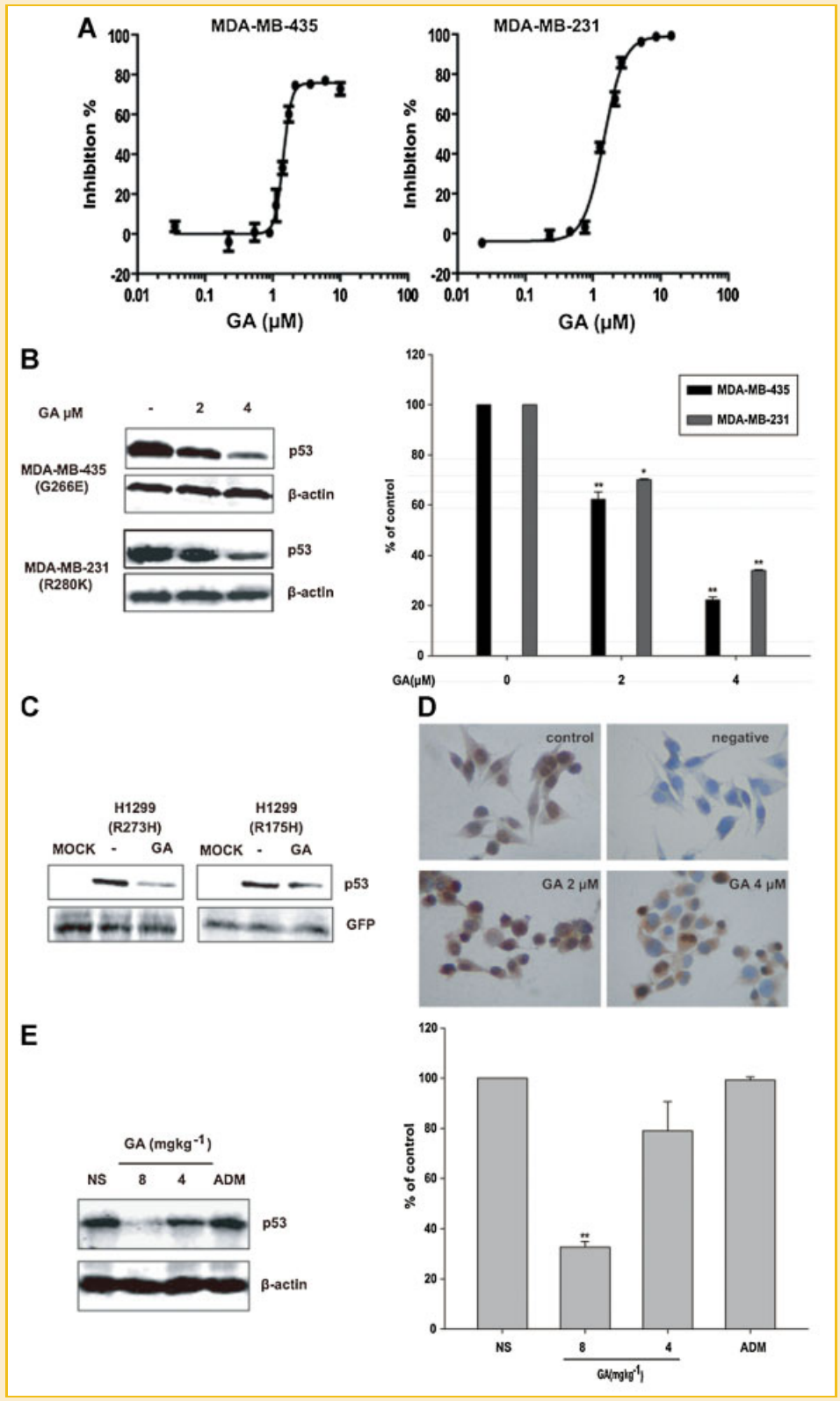

Fig. 2. GA down-regulates mutant p53 in vitro and in vivo. A: GA inhibits the viability of cells carrying mutant p53. MDA-MB-435 and MDA-MB-231 cells were treated with indicated concentrations of GA for 48 h. Cell viability was determined by MTT assay. B: MDA-MB-435 and MDA-MB-231 cells were treated with indicated concentrations of GA for 6 h. The protein level of p53 was detected by clone DO-1 antibody. C: H1299 cells were transfected with empty pcDNA3 or pcDNA3 vectors expressing mutant p53 (R273H and R175H) and treated with 4 μM GA for 6 h. The expression of exogenous mutant p53 protein was observed by Western blot analysis with clone DO-1 antibody. GFP was used as the measurement of transfection efficiency. D: Immunocytochemistry analysis of GA on down-regulation of mutant p53 protein level in MDA-MB-435 cells. Cells were treated with solvent (control), 2 μM and 4 μM GA, respectively. Immune complexes were visualized with ABC reagents (brown), and the nucleuses were counterstained with hematoxylin (blue). No primary antibody was used in negative control. Amplification: 200×. E: GA inhibited the expression of mutant p53 in MDA-MB-435 tumor tissues. NS, normal saline, as vehicle control; ADM, adriamycin.

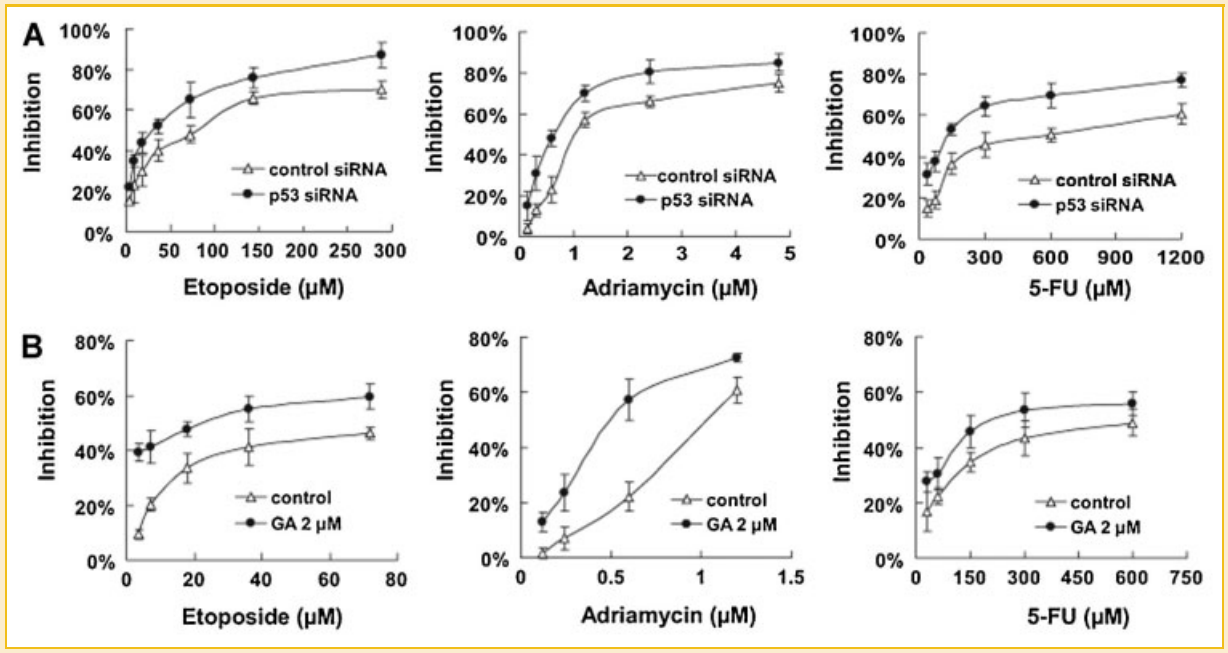

Fig. 3. GA increases chemotherapeutic agents induced cytotoxic effects in MDA-MB-435 cells. A: Knocking down mutant p53 sensitive MDA-MB-435 cells to chemotherapeutic agents. Cells were plated on 96-well plates and transfected with p53 siRNA (0.3 pmol per well) and control siRNA. After 24 h, the cells were treated with etoposide, ADM, and 5-FU at different concentrations for 48 h. Then cell viability was determined. B: MDA-MB-435 cells were pre-treated with 2 μM GA or solvent (indicated as control) for 6 h. Then supernatant was discarded and chemotherapy drugs were added with different concentrations. After 48 h, cell viability was determined. Data were plotted as means ± SEM (n = 3).

CHAPERONES INVOLVES IN THE DEGRADATION OF MUTATED P53 STIMULATED BY GA

Ubiquitin ligase MDM2 is a major factor responsible for the degradation of wild-type p53. Previously studies show that GA down-regulates MDM2 expression and decreases p53-MDM2 interaction in MCF-7 cells, which express wild-type p53. Interestingly, we found that the effect of GA on MDM2 was different in cells harboring mutant p53. As shown in Figure 6, following GA treatment, neither MDM2 protein level nor the p53-MDM2 interaction was affected significantly in MDA-MB-435 cells. Another pathway for mutant p53 degradation, which involves a close cooperation of molecular chaperones Hsp70 and Hsp90 with the ubiquitin/proteasome system has been reported recently [Esser et al., 2005; Muller et al., 2008]. Major importance on this degradation pathway is the chaperone associated ubiquitin ligase CHIP. Results showed that GA up-regulated Hsp70 and down-regulated Hsp90 expression, whereas the protein level of CHIP was not affected significantly (Fig. 6A). Further studies by immunoprecipitation exhibited that GA treatment altered the interaction between mutant p53 and chaperones. Figure 6B shows that GA disrupted the Hsp90-mutant p53 interaction concentration-dependently, and promoted Hsp70/mutant p53 and CHIP/mutant p53 complexes formation. Then protein levels of CHIP were depleted in MDA-MB-435 cells following transfection with CHIP siRNA (Fig. 6). Intriguingly, depletion of the chaperone associated ubiquitin ligase in GA treated cells caused an increase of mutant p53 levels. The findings emphasize the critical role of chaperones in the degradation of mutant p53 caused by GA.

DISCUSSION

Recently, GA has been deeply investigated as a novel anti-neoplastic agent. Previously studies demonstrate that GA mediates apoptosis as a p53 inducer through down-regulation of MDM2 in wild-type p53 expressing cancer cells [Gu et al., 2008]. The p53 tumor suppressor is mutated in 50% of human cancers, and mutant p53 is reported to gain new function in induction of drug resistance in cells. Clinical studies have also revealed higher resistance to chemotherapy of tumors expressing p53 mutants [Cabelguenne et al., 2000]. It may suggest that the mutant p53 itself may serve as a potential target for drug development in overcoming drug resistance in cancer therapy. In this study, we focus our effort on investigating the effect and corresponding mechanisms of GA on mutant p53 regulation. The results obtained here demonstrated that GA strikingly reduced mutant p53 protein level and increased chemotherapeutic agents induced cytotoxicity in cancer cells harboring mutant p53. Subsequent results showed that GA did not influence p53 mRNA synthesis but induced a decrease of half-life of the mutant p53 protein to 3.5 h. These results indicate that the down-regulation of mutant p53 by GA is due to degradation of mutant p53 protein rather than decreasing synthesis.

We further analyzed how GA-induced degradation of mutant p53 was biochemically executed. Since the lysosomal and proteasomal systems are of primary importance on intracellular proteolysis [Barrett, 1992; Magnani, 2000], we focused on them, and found that the proteasome inhibitor MG132 protected mutant p53 from GA-induced degradation, while lysosome inhibitor chloroquine was

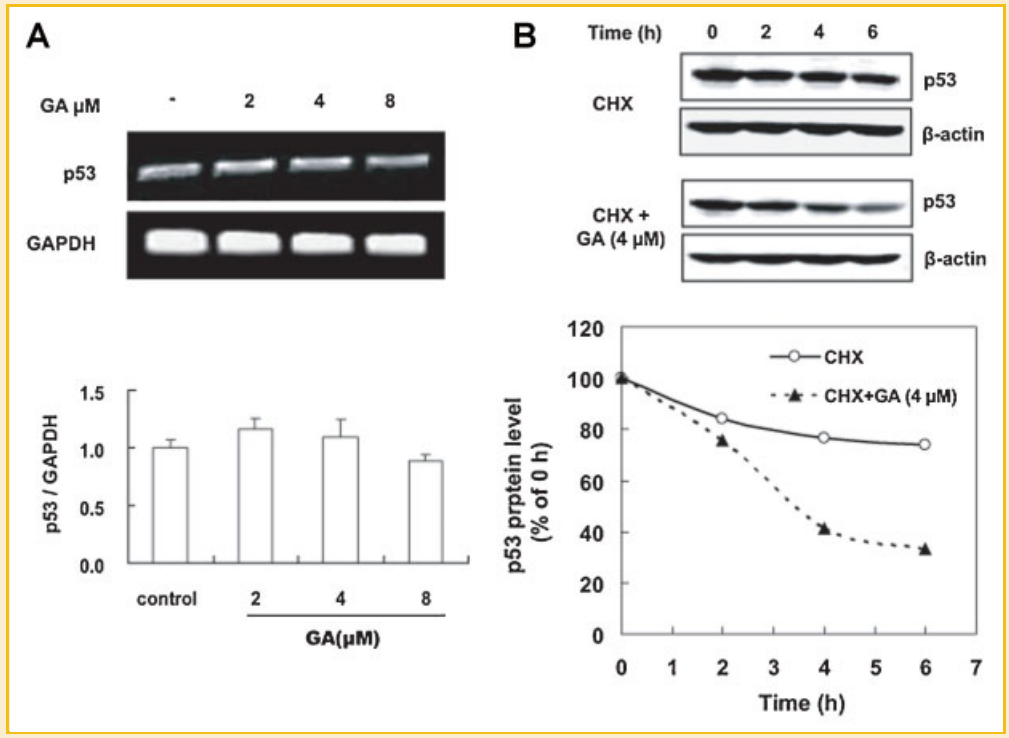

Fig. 4. GA down-regulates mutant p53 at post-transcriptional level. A: Effect of GA on p53 mRNA expression. MDA-MB-435 cells were treated with indicated concentrations of GA for 6 h followed by extraction of total RNA. cDNA fragments of p53 and GAPDH were amplified by RT-PCR, using the primers indicated in material. Data was obtained from three independent experiments and presented as the means ± SEM. B: GA destabilizes mutant p53. MDA-MB-435 cells were pre-incubated with 4 μM GA or solvent control for 1 h. Then, cells were treated with 20 μg ml^{-1} cycloheximide (CHX, an inhibitor of protein synthesis) or a combination of CHX and GA, and harvested at different time points. Western blot analysis was performed to detect the protein level of mutant p53 (with clone DO-1 antibody) in whole cell lysates. Following scanning densitometry of mutant p53 band intensities, the amount of p53 protein relative to β-actin was plotted against the time course of culture. Values from cells not treated with CHX were set at 100%, and other values were expressed as a percentage of these.

unable to inhibit mutant p53 degradation. It is noteworthy that mutant p53 protected from GA-induced degradation by proteasome inhibitor could be found in higher molecular weight form in NP-40-insoluble fractions than in NP-40-soluble ones. This is probably because inhibition of proteasome action led to accumulation of ubiquitinated p53, which was insoluble in non-ionic detergent, presumably due to protein denaturation and aggregation [Whitesell et al., 1997]. Furthermore, ubiquitinated mutant p53 from p53 immunoprecipitates could be visualized after co-treatment with GA and MG132, which demonstrates that GA promotes efficient ubiquitination of mutant p53.

p53 is mainly regulated by phosphorylation, acetylation, sumoylation, and ubiquitination at post-translational level [Jayaraman and Prives, 1999; Vogelstein et al., 2000; Melchior and Hengst, 2002]. Central to the regulation of p53 is the ubiquitin ligase MDM2 [Freedman and Levine, 1999; Freedman et al., 1999]. Although previously data establish that GA up-regulates p53 protein level through down-regulation of MDM2 in cells harboring wild-type p53, it may be different in mutant p53 expressing cells. Results of this study indicated that both MDM2 protein level and the interaction between mutant p53 and MDM2 were not affected significantly by GA in MDA-MB-435 cells. Although MDM2 drive the degradation of both mutant and wild-type p53, increasing number of studies suggest that the ability of MDM2 to functioning as a ubiquitin ligase is less important in the degradation of mutant p53 [Lukashchuk and Vousden, 2007; Muller et al., 2008].

Other ubiquitin ligases which can promote the degradation of p53 independently on MDM2 have recently been identified, including CHIP, Pirh2, Cop1, Topors, Synoviolin, and Carps [Leng et al., 2003; Dornan et al., 2004; Rajendra et al., 2004; Esser et al., 2005; Tang et al., 2006; Yamasaki et al., 2007]. It has been identified that mutant p53 associate with Hsp70 and Hsp90, and be presented to CHIP-induced degradation [Finlay et al., 1988; Whitesell et al., 1998; Esser et al., 2005]. Further studies show that Hsp90 forms a stable complex with p53 mutants that adopt native conformation recognizing by monoclonal antibody PAb1620 [Muller et al., 2008]. Inhibition of Hsp90 chaperone activity by benzoquinone ansamycin antibiotic such as geldanamycin lead to an elevation in the fraction of denatured p53 mutants recognizing by monoclonal antibody PAb240. The denatured p53 mutants preferentially interact with Hsp70 and CHIP, leading to CHIP-mediated ubiquitination and proteasomal degradation [Muller et al., 2008]. In our study, MDA-MB-435 cells contain both native (PAb1620-positive) and denatured conformation of p53 (PAb240-positive) (Supplementary Fig. 1). Following the cells treated with GA, the p53-Hsp90 binding decreased, while the combination between Hsp70-CHIP complex and p53 increased. We questioned whether the destabilization of mutant p53 caused by GA was chaperone assisted. As ubiquitin

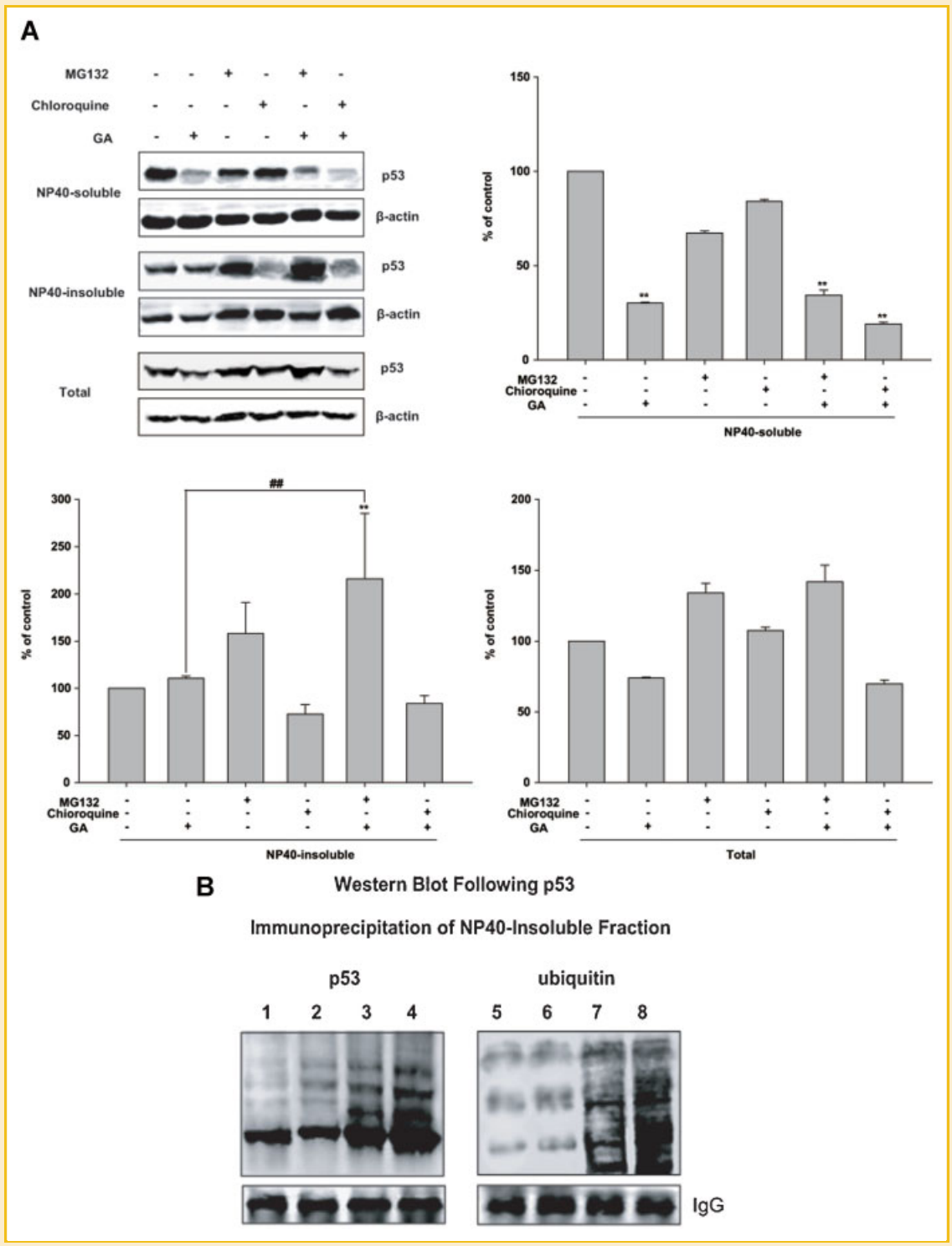

Fig. 5. GA stimulates ubiquitin/proteasome-mediated degradation to mutant p53 in MDA-MB-435 cells. A: Stimulation of the proteasomal processing of mutant p53 by GA. MDA-MB-435 cells were treated with 4 μM GA, 10 μM MG132 (proteasome inhibitor), and 100 μM chloroquine (lysosome inhibitor) as indicated. Inhibitors were added 1 h prior to GA treatment (6 h). After rinsing in PBS, cells were lysed in TNES buffer or SDS-sample buffer. The soluble and insoluble fractions in cells lysed with TNES buffer were separated. Total NP-40-soluble proteins (80 μg per lane) were fractionated by 10% SDS–PAGE and blotted for p53. Insoluble precipitates were resolubilized in boiling TNES buffer containing 2% SDS and total SDS-soluble (NP-40-insoluble) proteins (80 μg per lane) were analyzed. The cells lysed with SDS-sample buffer were boiled for 15 min and 20 μl sample were blotted for total p53. B: Effect of GA on mutant p53 ubiquitination. MDA-MB-435 cells were treated with control vehicle (lanes 1 and 5), GA 4 μM (lanes 2 and 6), MG132 10 μM (lanes 3 and 7) or GA + MG132 (lanes 4 and 8). MG132 was added 1 h prior to GA treatment (6 h). Total SDS-soluble (NP-40-insoluble) proteins were extracted as described in the legend of Figure 4. Mutant p53 was IP with p53 (clone DO-1) monoclonal antibody from 1,000 μg of SDS-solubilized cellular proteins. Immunoprecipitates were fractionated and blotted for p53 with rabbit polyclonal antibody (left panel, lanes 1–4) or rabbit polyclonal anti-ubiquitin antiserum (right panel, lanes 5–8).

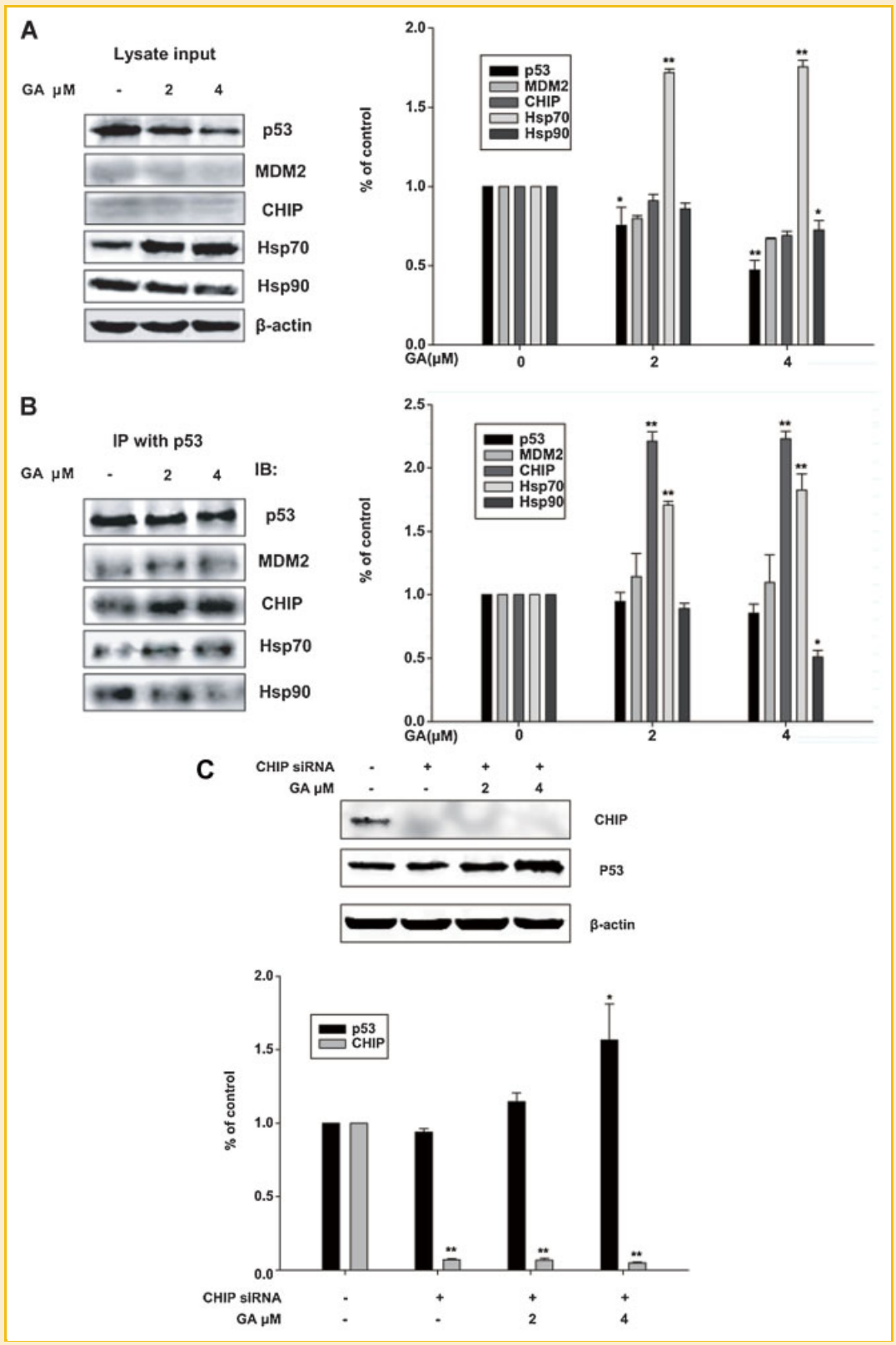

Fig. 6. Chaperones rather than MDM2 involve in the degradation of mutant p53 induced by GA. MDA-MB-435 cells were treated with GA (2, 4 μM) for 6 h. A: mutant p53, MDM2, CHIP, Hsp70, and Hsp90 protein levels were detected in cell lysates. p53 was blot with clone DO-1. B: Cell lysates were IP with agarose-conjugated p53 (clone DO-1) monoclonal antibody and immunoblotted with rabbit polyclonal anti-p53, -MDM2, -Hsp90, and monoclonal anti-CHIP, -Hsp70 antibodies to detect coprecipitated p53 mutants. C: CHIP ablation by siRNA stabilizes mutant p53. MDA-MB-435 cells were transfected with CHIP siRNA (10 nmol per well). Levels of CHIP and mutant p53 were analyzed with Western blot analysis. Actin served as loading control.

ligase, CHIP plays an important role in the degradation of chaperone-bound client proteins. Depletion of endogenous CHIP stabilized mutant p53 in GA treated MDA-MB-435 cells. These data demonstrated the critical role of chaperones in the degradation of mutant p53 caused by GA.

Taken together, our data reveals that GA accelerates the degradation of mutant p53 by chaperones-assisted ubiquitin/proteasome pathway. The mechanism of GA regulated mutant p53-chaperone interaction and the influence of GA on chaperone function need further investigation. As mutant p53 may serve as a potential target for drug development in overcoming drug resistance in cancer therapy, our findings highlight the important potential of GA to be used both alone and in combination with other chemotherapeutic drugs for future cancer therapy.

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