CHIP Chaperones Wild Type p53 Tumor Suppressor Protein*

Veenu Tripathi, Amjad Ali, Rajiv Bhat, and Uttam Pati

From the School of Biotechnology, Jawaharlal Nehru University, New Delhi 110067, India

Wild type p53 exists in a constant state of equilibrium between wild type and mutant conformation and undergoes conformational changes at elevated temperature. We have demonstrated that the co-chaperone CHIP (carboxyl terminus of Hsp70-interacting protein), which suppressed aggregation of several misfolded substrates and induced the proteasomal degradation of both wild type and mutant p53, physically interacts with the amino terminus of WT53 and prevented it from irreversible thermal inactivation. CHIP preferentially binds to the p53 mutant phenotype and restored the DNA binding activity of heat-denatured p53 in an ATP-independent manner. In cells under elevated temperatures that contained a higher level of p53 mutant phenotype, CHIP restored the native-like conformation of p53 in the presence of geldanamycin, whereas CHIP-small interfering RNA considerably increased the mutant form. Further, under elevated temperatures, the levels of CHIP and p53 were higher in nucleus, and chromatin immunoprecipitation shows the presence of p53 and CHIP together upon the DNA binding site in the p21 and p53 promoters. We propose that CHIP might be a direct chaperone of wild type p53 that helps p53 in maintaining wild type conformation under physiological condition as well as help resurrect p53 mutant phenotype into a folded native state under stress condition.

CHIP (carboxyl terminus of Hsp70-interacting protein) is a dimeric 35-kDa ubiquitin ligase comprising three functional domains: a tetratricopeptide repeat (TPR) at the amino terminus, a U-box domain at the COOH terminus, and a highly charged region separating the two. CHIP-mediated events were Hsp70-dependent (20). Although the full range of cellular substrates of CHIP remains to be explored, misfolded CFTR (17, 21), tau (22, 23), and polyglutamine aggregation (24) are suppressed by CHIP-assisted quality control.

In this study, we have analyzed in detail the interaction of p53 with CHIP and have discovered that the co-chaperone CHIP regulates p53 conformation and activity under physiological and elevated temperatures both in vitro and in vivo independent of Hsp90 function. CHIP prevents p53 from irreversible thermal inactivation and restores the DNA binding activity of heat-denatured p53 in an ATP-independent manner. In cells under elevated temperatures, CHIP helps resurrect the p53 mutant conformation into a folded native state. CHIP was shown to co-associate with WT p53 upon DBS in chromatin. We have proposed that CHIP might be a direct chaperone of WT p53 both under physiological and elevated temperatures.

*This work was supported in part by a grant from University Grant Commission (University Potential of Excellence), India (to U. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 To whom correspondence should be addressed: School of Biotechnology, Jawaharlal Nehru University, New Delhi 110067, India. Tel.: 91-11-26704081; Fax: 91-11-26717580; E-mail: uttam@mail.jnu.ac.in.

2 The abbreviations used are: WT, wild type; TPR, tetratricopeptide repeat; PBS, phosphate-buffered saline; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; siRNA, small interfering RNA; DBS, DNA binding site(s).
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EXPERIMENTAL PROCEDURES

Expression Vectors, Recombinant Proteins, and Antibodies—HA-p53 was generated by subcloning p53 cDNA into pNHA1 vector. All His-tagged p53 constructs were cloned in pET 32a vector. All His and GST (pGEX-CHIP, pGEX-MDM2) recombinant proteins were expressed in Escherichia coli strain BL21 (DE3) (Stratagene) and subsequently purified using Ni\(^{2+}\)-nitrilotriacetic acid (Qiagen) and glutathione-Sepharose (Sigma) column, respectively. The purification procedure for GST-CHIP and GST-MDM2 is described elsewhere (25, 26). p53\(^{1–393}\), p53\(^{126–293}\), and p53\(^{121–285}\) were in pET32(a) vector with His tag. For the expression of recombinant p53 proteins, culture was induced with 0.2 mM isopropyl 1-thio-D-galactopyranoside when \(A\) reaches 0.8 at 600 nm, for 3 h at 25 °C. Cells were harvested, lysed by sonication, and purified by Ni\(^{2+}\) chelation chromatography. TopoI51d-CHIP\(^{1–126}\) having His-tagged TPR-deleted CHIP, was expressed in BL21 (DE3) after induction with 0.5 mM isopropyl 1-thio-\(\beta\)-D-galactopyranoside and purified by Ni\(^{2+}\) chelation chromatography. Antibodies anti-p53 (pAb 1801), anti-p53 (pAb 421), anti-p53 (pAb 1620), anti-p53 (pAb 240) (all from Oncogene), anti-CHIP (Calbiochem), anti-actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-HA (Babco), anti-Hsp90 (Santa Cruz Biotechnology), and anti-Hsp70 (Santa Cruz Biotechnology) were used.

ELISA-based Assay—An ELISA-based protein-protein interaction assay was utilized for CHIP-p53 and MDM2-p53 interaction studies. 96-Well Maxisorp ELISA plates (Nunc) were coated with 50 μl of a 10 μg/ml CHIP protein in PBS at 4 °C overnight. The wells were rinsed with PBS at 4 °C three times. Blocking was done with 2% BSA (Sigma) in PBS at 4 °C for 4 h. Following the blocking step, the wells were washed three times with PBS containing 0.01% (v/v) Tween 20 (Sigma). Native or denatured p53 protein (0.5 μg) was added into 50 μl of PBS, 0.05% (v/v) Tween 20, 0.2% (w/v) BSA and put in the CHIP-coated wells. After an incubation period of 90 min at 4 °C, the ELISA plates were then washed with PBS containing 0.01% Tween 20 three times. The p53 protein was detected by adding 0.2 μg of mouse monoclonal antibody pAb 1620 or pAb 240 in 50 μl of PBS, 0.05% (v/v) Tween 20, 0.2% (w/v) BSA, and then anti-mouse secondary antibody to the wells. Finally, 100 μl of alkaline phosphate substrate with 50 mM Na\(_2\)CO\(_3\), 1 mM MgCl\(_2\), and 1 mg/ml PNPP was added, and the enzymatic reaction was allowed to take place for 10–30 min at room temperature. The reaction was terminated by adding 50 μl of 0.1 M EDTA, pH 8.0. The optical density at 405 nm was determined using a Microplate reader.

Cell Culture, Immunoprecipitation, and Immunoblotting—KB cells were purchased from NCCS (Pune, India) and maintained in Dulbecco’s modified Eagle’s medium (Biological Industries) with 10% fetal calf serum. For immunoprecipitation, 70% confluent KB cells (both untreated and treated with proteasome inhibitor MG-132 as well as with geldanamycin (1.0 μM for 4 hours)) were washed with PBS, harvested, lysed in radioimmune precipitation buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1.0% Triton X-100, 0.1% SDS, and 1.0% sodium deoxycholate). The whole cell lysate was precleared, and 1.0 μg of anti-CHIP polyclonal antibodies was used for immunoprecipitation. After a 2-h incubation with antibodies, 10 μl of protein A-agarose was added to the lysate and further incubated at 4 °C for 4 h. Washing was done twice with Nonidet P-40 buffer (20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10% glycerol, 1.0% Nonidet P-40, and 1 mM EDTA) and once with radioimmune precipitation buffer. Immunocomplexes were released by the addition of SDS loading dye, boiled for 5 min, centrifuged, and loaded to 10% SDS-PAGE. Transfer of proteins to nitrocellulose membrane was done and immunoblotted with target antibodies. The whole cell lysate was excluded from Hsp70 and Hsp90 by passing it through columns containing anti-Hsp70 and anti-Hsp90 antibodies and then used for immunoprecipitation. KB cells were transfected with HA-tagged expression vector pNHA1-p53, and cells were grown further for 30–36 h, followed by immunoprecipitation with anti-HA antibodies. To assess the effect of CHIP on the conformation of p53 under heat shock conditions, KB cells were transfected with increasing amounts (1.0 and 2.0 μg) of pcDNA3-CHIP, followed by immunoprecipitation as described above.

GST Pull-down Assay—2.0 μg of purified GST, GST-CHIP, and GST-MDM2 recombinant proteins were conjugated to glutathione-Sepharose beads and incubated with 2.0 μg of wild type and various truncated recombinant p53s for 3 h at 4 °C in PBST buffer (PBS with 0.1% Tween 20). After incubation, beads were washed five or six times with PTX (PBS with 0.01% Triton X-100) (1 ml each), and the complex was eluted from the beads with 10 mM reduced glutathione in 50 mM Tris-HCl (pH 8.0). SDS loading dye was added to the eluted proteins, boiled for 5 min, and subjected to SDS-PAGE analysis followed by immunoblotting with anti-His antibodies (Oncogene).

Electrophoretic Mobility Shift Assay—The DNA binding activity of p53 was monitored by EMSA. A 42-mer oligonucleotide containing a p53 binding site (5'-GGTACCAATTCCTCGAGGAAACATGTCCCCAAACATGTGGCTGAGATCTTTT-3') was used as a probe. The reaction was performed in 5 μl of buffer containing 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 10% glycerol, 0.5% Nonidet P-40, and 1 μg of purified recombinant p53 protein. The reaction mixture was incubated at 37 °C for 30 min, followed by gel retardation assay performed as described previously (25, 26).

The purified GST and GST-CHIP and GST-MDM2 recombinant proteins were conjugated to glutathione-Sepharose beads and incubated with 2 μg of wild type and various truncated recombinant p53s for 3 h at 4 °C in PBST buffer (PBS with 0.1% Tween 20). After incubation, beads were washed five or six times with PTX (PBS with 0.01% Triton X-100) (1 ml each), and the complex was eluted from the beads with 10 mM reduced glutathione in 50 mM Tris-HCl (pH 8.0). SDS loading dye was added to the eluted proteins, boiled for 5 min, and subjected to SDS-PAGE analysis followed by immunoblotting with anti-His antibodies (Oncogene).

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3') was derived from the p21 promoter and synthesized from Microsynth. Sense and anti-sense oligonucleotides were end labeled by T4 polynucleotide kinase using [γ-32P]ATP, annealed, and purified by G-50 column. 5× EMSA buffer, 100 ng of poly(dI-dC), 100 ng of recombinant p53 protein, and 3.0 ng of radiolabeled probe DNA were added to make the reaction mixture, and water was added to make up the volume. The reaction mixture was incubated for 30 min at 4 °C. For the supershift assay, antibodies were added to the reaction mixture 0.5 h prior to the addition of radiolabeled nucleotide, the mixture was loaded to 4% native PAGE containing 0.5× TBE buffer, electrophoresed for 3 h at 175 V. Gel was then dried and exposed for autoradiography. To see the effect of CHIP on the DBS binding of p53, recombinant CHIP (up to 1.5 μg) was added to the reaction mixture prior to the addition of radiolabeled oligonucleotide. Further, in order to see the effect of CHIP on DNA binding activity of denatured p53, 100 ng of human recombinant p53 was diluted in 10 μl of EMSA buffer, and samples were supplemented with or without 500 ng of CHIP (native or denatured at 37, 45, and 50 °C) as well as in the presence and absence of 5 mM ATP. Such samples were incubated at 37 °C for 60 min. The activation step was followed by the addition of EMSA reaction mixture containing 1× EMSA buffer, poly(dI-dC), and radiolabeled nucleotide.

Heat Shock Treatment—KB cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FCS at 37 °C in a humidified chamber containing 5% CO2. For heat shock experiments, cells were grown at 42 °C for 70 min in a CO2 incubator and then taken back to 37 °C for the indicated period of time.

siRNA Transfections—siRNA templates specific for CHIP were synthesized from Sigma (sense, 5′-AGGCCCTTGGCCGAAGACGATCTGCGG-3′; antisense, 5′-CGGCGTGCCACGGCCGAGCCGG-3′) (as per Ambion’s instruction manual), annealed, and cloned into psiSilencer linear. For endogenous CHIP knockdown studies, KB cells were plated at a density of 1 × 106 on a 35-mm dish 18 h before transfection, and 0.5 and 1.0 μg of CHIP siRNA and control siRNA (scrambled) were transfected into KB cells at 37 and 42 °C, followed by Western blotting with anti-CHIP antibodies. Whole cell lysate of CHIP siRNA-transfected KB cells was taken for immunoprecipitation with conformation-specific antibodies.

Aggregation Assay—p53 (1.0 μM) was incubated at 37, 45, and 50 °C with or without CHIP (2.0 μM). Thermal aggregation kinetics were monitored by measuring light scattering in a Cary Eclipse-fluorescence spectrophotometer in a Peltier-controlled thermostatted quartz cuvette. The measurements were done at excitation and emission wavelengths of 340 nm with excitation and emission bandwidths of 2.5 nm each. The above aggregation kinetics experiment was also repeated at 37 and 45 °C with CHIP53-126—130. Thermal denaturation of CHIP was carried out by monitoring the absorbance at 280 nm in a Cary100 Bio UV spectrophotometer from Varian.

Chromatin Immunoprecipitation Assay—The chromatin immunoprecipitation assay was performed as described previously (27). KB cells were cross-linked with 1.0% formaldehyde at 37 °C for 10 min, and cross-linking was stopped by the addition of 125 mM glycine. Cells were then rinsed twice with ice-cold PBS and centrifuged for 5 min. The pellet was then resuspended in 0.4 ml of lysis buffer (1.0% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1, and 1.0% protease inhibitor mixture and sonicated five times for 5 s each at an interval of 2 min at submaximal input, followed by centrifugation for 10 min. Supernatant was collected and diluted in dilution buffer (1.0% Triton X-100, 2 mM EDTA, 150 mM NaCl, and 20 mM Tris-HCl, pH 8.1), followed by immunoclearing with 2 μg of sheared salmon sperm DNA, 20 μl of preimmune serum, and protein A-agarose (20 μl of a 50% slurry in 10 mM Tris-HCl, pH 8.1, and 1 mM EDTA) for 2 h at 4 °C. Immunoprecipitation was performed overnight with anti-p53 and anti-CHIP antibodies. After immunoprecipitation, 20 μl of protein A-agarose and 2 μg of salmon sperm DNA were added, and then incubation was continued for another hour. Agarose beads were washed sequentially for 10 min each in TSEI (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, and 150 mM HCl), TSEII (0.1% SDS, 1.0% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, and 500 mM HCl), and buffer III (0.25 M LiCl, 1.0% Nonidet P-40, 1.0% deoxycholate, 1.0 mM EDTA, and 10 mM Tris-HCl, pH 8.1). Beads were then washed three times with TE buffer and extracted three times with 1.0% SDS, 0.1 M NaHCO3. Cross-linking was reversed by incubation of eluted samples for 6 h at 65 °C. The DNA was extracted with phenol-chloroform, precipitated with ethanol. The same amounts of eluted samples were taken for PCR. An equal amount of chromatin solution was precipitated with nonspecific antibody as a negative control. Input DNA was diluted in 50 μl of water, 1 μl of sample was taken for PCRs, and the same amounts of all PCR products were loaded on 10% native PAGE followed by silver staining. Sequences of the primers used in PCRs are as follows: p21-5’ site, sense (5′-GGGGTACAGAAGAAGCCTGTCC-3′) and antisense (5′-GGGAAGATCTAATTACTGACATCCAGT-3′); p19-3′ site, sense (5′-GGGGTACCCCGACGGACGT-3′) and antisense (5′-GGGAAGATCTAATTACTGACATCCAGT-3′); DBS-I, sense (5′-GGGGTACCCCTTGGGACGGACTGT-3′) and antisense (5′-GGGAAGATCTAATTACTGACATCCAGT-3′); DBS-II, sense (5′-GGGGTACCCCGGATTTTCCTT-3′) and antisense (5′-GGGAAGATCTAATTACTGACATCCAGT-3′); DBS-III, sense (5′-CTTTTGAATCGAAGACGTTCGAGTTGATCC-3′) and antisense (5′-GGGAAGATCTAATTACTGACATCCAGT-3′).

RESULTS

CHIP Binds to the Amino Terminus of p53—Although the significance of molecular interactions between p53 and heat shock proteins is unclear, both an ELISA-based assay and co-immunoprecipitation were utilized in detecting their interactions (6). Since the TPR domain of co-chaperone CHIP mediates the interaction of CHIP with Hsp90 and Hsp70 (17) in targeting p53 as substrate for its proteosomal degradation (18), we opted to determine in detail if there is a direct physical interaction between CHIP and p53 and whether this interaction is independent of Hsp90. We utilized both an ELISA-based assay and GST pull-down assay in binding experiments with highly purified recombinant proteins. The various constructs containing cDNAs encoding WT p53, core domain, p53Δ126—393, and p53Δ1—285 (Fig. 1A) were expressed in pET32a vector, and the His-tagged recombinant proteins were highly purified to homogeneity (>98%) (Fig. 1B). In order to confirm that purified
fractions were not contaminated with bacterial Hsp70 and Hsp90, the proteins were run on SDS-PAGE and were immuno-blotted with anti-Hsp70 and anti-Hsp90 antibodies. The results showed no bands, thus suggesting that the recombinant proteins were free of bacterial Hsp70 and Hsp90 (Fig. 1C). These highly purified proteins were further sequentially passed through immobilized Hsp70 and Hsp90 antibodies columns prior to loading onto the GST-CHIP column. In the ELISA, the wells were either coated with MDM2 or CHIP, followed by the addition of WT p53 or its truncated products. The retained p53 or its truncated products were probed with anti-p53 antibody pAb 421 (Fig. 1E). The control plate that was coated with BSA failed to retain p53, whereas both MDM2 (positive control) and CHIP-coated plates showed an increased binding (Fig. 1D) after the addition of p53 in a dose-dependent manner. We then determined the location of the CHIP binding site in p53. To analyze the domain interaction in detail, we bound different p53 fragments to the MDM2 and CHIP-columns. The amino terminus of p53 (p53Δ1–285) showed strong binding for both MDM2 and CHIP (Fig. 1E), whereas the COOH terminus and the core domain (not shown) failed to bind to CHIP. Since CHIP was shown to bind to p53 in the same region that binds to MDM2, we asked whether CHIP could compete with MDM2 binding to p53. In the first experiment, p53 was plated on ELISA plate followed by the addition of MDM2. After washing, an excess of CHIP was added, followed by incubation with anti-MDM2 antibodies. Alternatively, p53 was plated, followed by the addition of CHIP. After washing, excess MDM2 was added, followed by the addition of anti-CHIP antibodies. The results showed that CHIP did compete with MDM2 in binding to p53 (Fig. 1F). In a parallel approach, recombinant His-tagged WT p53 and its truncated fragments were passed through GST-MDM2 and GST-CHIP columns. After washing thoroughly, half of the eluted fragments were run on SDS-PAGE and Coomassie-stained (Fig. 2A), and the other half of the eluents were further transferred onto nitrocellulose for immunoblotting with anti-His antibodies (Fig. 2B). Both the stained and the immunoblotted gels showed that WT p53 and p53Δ1–285 were bound to CHIP (Fig. 2, A and B, lanes 4 and 5), whereas the core and COOH terminus domains failed to bind CHIP, thus confirming that the amino-terminal domain interacted with CHIP. As a positive control, GST-MDM2 was shown to bind to WT p53 and p53Δ1–285 (Fig. 2, A and B, lane 3).

**Preferential Binding of p53 Mutant Phenotype to CHIP**—In order to determine which conformation phenotype of p53 binds to CHIP, we developed a sandwich ELISA assay by utilizing conformation-specific antibodies pAb 240 (specific for denatured p53) (28) and pAb 1620 (specific for wild type p53) (29) both at room and elevated temperatures. The ratio of wild type to mutant p53 in recombinant preparations at room temperature varied between
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CHIP Prevents p53 from Irreversible Thermal Inactivation—Since both the structured and the unstructured regions of p53 are required for the proper functioning of p53 (2, 30), a multitude of regulators could potentially bind to p53. Hsp90 was shown to bind to both wild type and mutant p53 to stabilize their conformations, suppressing aggregation and catalyzing disaggregation of p53 at elevated temperatures (5, 6). Our results showed that CHIP binds to the flexible amino terminus domain of CHIP and preferentially to the p53 mutant conformation. In order to understand the significance of this interaction, we then explored whether CHIP could prevent aggregation of thermally denatured p53 both at physiological and at elevated temperatures. The recombinant CHIP, p53, and p53 \textsuperscript{A126–393} were shown to be of high purity both by SDS-PAGE and immunoblot (Fig. 4A). Wild type p53 was first incubated at 37 °C, and the thermal aggregation kinetics was monitored by measuring light scattering in a fluorescence spectrophotometer. Structural changes occur at temperature above 25 °C, and decreased DNA binding activity correlates with the loss of secondary structure (not shown). The wild type p53 aggregated and reached a plateau after 40 min at 37 °C. Upon the addition of a 2-fold molar excess of CHIP, p53 aggregation was completely prevented (Fig. 4C). A similar pattern was also observed at 45 °C, although a plateau was reached after 10 min, and the addition of CHIP in a 2-fold molar excess again suppressed p53 aggregation. However, at 50 °C, CHIP did aggregate faster and could not suppress the aggregation of p53; A steep rise in scattering was observed with CHIP (Fig. 4C). This could be due to the fact that CHIP starts denaturing at 50 °C, as is evident from the thermal denaturation curve for CHIP (Fig. 4B). In order to check whether the Hsp70/90-interacting TPR domain of CHIP is responsible for preventing irreversible thermal aggregation of p53, we then utilized recombinant CHIP\textsuperscript{A1–126} (CHIP depleted of the TPR domain) for the aggregation assay. CHIP\textsuperscript{A1–126} was able to prevent p53 aggregation both at 37 and 45 °C (Fig. 4D).

Restoration of DBS Binding of Heat-denatured p53 by CHIP—The observed prevention of aggregation of p53 by CHIP led us to analyze whether CHIP could restore DBS binding of heat-denatured p53. EMSA was performed between radiolabeled p21 5′-DBS and WT p53, and highly purified recombinant CHIP was added in a dose-dependent manner. To avoid the effect of non-sequence-specific COOH terminus binding to the DNA, all p53 binding assays were done in the presence of short 50-bp DNA competitor DNA fragments to the DNA binding reaction as described (5). The addition of antibody pAb 421 to the complex resulted in supershift (Fig. 5A, lane 3), thus authenticating the p53 DBS binding. The addition of CHIP to the reaction mixture increased the DNA binding potential of p53, although it did not cause supershift.
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A

Comassie Staining

Pull-down

104K
80K
60K
40K
20K

M
GST
GST-Mdm2
GST-CHIP
GST-CHIP

p53
p53

393
126-393

1
2
3
4
5
6

B

Western Blot

Pull-down

80K
60K
40K
20K

M
GST-Mdm2
GST-CHIP
GST-CHIP

p53
p53

393
126-393

WB: anti-His

1
2
3
4
5
6

CHIP Binds to both Exogenous and Endogenous p53 in Cells in the Absence of Hsp90—It was suggested that CHIP acts as a molecular switch between proteasomal and lysosomal degradation pathways and was recently shown to induce the proteasomal degradation of both mutant and wild type p53, although no direct interaction was reported (18). CHIP was earlier shown to physically associate with an Hsp70-dependent ternary complex in activating HSF1 (31), with Smad1 (32), and with α-synuclein and synphilin-1 (33) by co-immunoprecipitation analysis. In order to examine whether CHIP and p53 could associate in cells, we co-immunoprecipitated CHIP and p53 both endogenously and in transfected cell lines under physiological conditions. Oral epithelial KB whole cell lysates, both treated and untreated with proteasome inhibitor MG132, were first immunoprecipitated with anti-CHIP antibodies, followed by immunoblotting with anti-p53 antibody pAb 1801 (Fig. 6A, lanes 2 and 3). The endogenous CHIP was shown to interact with endogenous p53. In order to confirm that this interaction was independent of Hsp90, geldanamycin was utilized as an Hsp90 inhibitor during the immunoprecipitation assay (Fig. 6A, lane 4). Further, prior to immunoprecipitation analysis, the whole cell extract was sequentially passed through anti-human Hsp90 and Hsp70 antibody columns. The whole cell extract and the beads contained Hsp70, since it was detected by anti-Hsp70 antibodies (Fig. 6C, lanes 1 and 2) and Hsp90 (Fig. 6C, lanes 4 and 5). Immunoprecipitation with preimmune serum was utilized as a negative control (Fig. 6A, lane 6). In a parallel manner, HA-tagged p53 cDNA was transiently transfected into KB cells, and the cell lysates were immunoprecipitated with anti-HA antibodies followed by immunoblot analysis with either anti-HA or anti-CHIP antibodies. The result showed that exogenously expressed HA-tagged p53 also interacted with CHIP (Fig. 6B, lane 2) and that the cellular interaction between CHIP and p53 was independent of Hsp90 and Hsp70 (Fig. 6D, lane 3).

CHIP Restores the Folded Native Conformation of p53 in Cells under Elevated Temperatures—WT p53 undergoes transient conformational transformation under heat stress similar to that of misfolding caused by structural mutations (7, 8). As CHIP prevented p53 aggregation in vitro and restored DBS binding of denatured p53, we opted to explore the role of CHIP upon p53 conformation in KB cells under stress conditions. It was observed earlier that an overexpression of CHIP in fibroblasts increased the refolding of proteins after thermal denaturation (20). In order to probe whether CHIP could restore p53 native conformation in KB cells that are subjected to heat stress both at 42 and 45 °C, KB cell lysates were, independently, immunoprecipitated, with p53 antibodies pAb 421 (both wild type- and of the p53-DNA complex (Fig. 5A, lane 4) (in the presence of CHIP antibodies; data not shown), suggesting a probable transient association in vitro prior to DBS binding. Adding an increasing amount of CHIP into the p53-DNA complex (200, 400, 800, and 1500 ng) (Fig. 5B, lanes 3, 4, 5, and 6) resulted in an enhancement of DBS binding by p53 by 1.8-, 2.2-, 3.8-, and 4.6-fold, respectively. It was further necessary to analyze whether CHIP could restore the ability of denatured p53 in binding to DBS in vitro. A 1-h incubation of the WT p53 at 37 °C completely abolished the DBS binding activity (Fig. 5, C (lane 3) and D (lane 4)). The addition of CHIP either prior to or after the denaturation step at 37 °C restored the DBS binding significantly (Fig. 5C, lanes 5 and 6). Interestingly, unlike Hsp90 (5), the function of CHIP in enhancing p53 DBS binding was shown to be ATP-independent. Since CHIP was stable up to 53 °C, we explored the role of heat-denatured CHIP (at 37, 45, and 50 °C) upon p53 DBS binding. CHIP that was preheated at 37 and 45 °C restored the DBS binding of heat-denatured p53, whereas preheated CHIP at 50 °C was unable to restore p53 activity (Fig. 5D). The independent chaperoning of p53 by CHIP in an ATP-independent manner thus suggests that an alternate pathway of conformational modulation might exist that is independent of Hsp90.

FIGURE 2. GST pull-down assay showing interaction of CHIP with p53. A, 1.0 μg of GST or GST-CHIP and GST-MDM2 fusion proteins were, separately, incubated with 1.0 μg of His-p53-(1–393), His-p53 126–393, and His-p53 31–285 for 4 h at 4 °C. M denotes molecular weight marker. The agarose was extensively washed, and the complexes were eluted with 10 mM reduced glutathione in 50 mM Tris-HCl (pH 8.0). A, eluents were subjected to SDS-PAGE by Coomassie Blue staining or Western blot analysis. p53 (lanes 2 and 5) were taken as positive and negative controls. B, the eluents were transferred onto nitrocellulose and immunoblotted with anti-His antibodies (lanes 4 and 5). GST-MDM2 (lane 3) and GST (lane 2) were taken as positive and negative controls.
mutant-specific), pAb 1620 (wild type-specific), and pAb 240 (mutant-specific). The control cell lysate at physiological temperature showed higher level of native p53 (pAb 1620) (Fig. 7A, lane 2) in comparison with the mutant form (pAb 240) (Fig. 7A, lane 3), whereas the lysate of the cells at 42 °C showed a rise in the mutant form (Fig. 7A, lanes 5 and 11). However, in cells that

**FIGURE 3. Interaction of CHIP with wild type and mutant phenotype p53 by ELISA, sandwich ELISA, and immunoprecipitation.** A, onto the plates coated with p53 conformational antibodies, p53 was added, followed by CHIP. The CHIP that was bound to p53 was detected by anti-CHIP antibodies. B, alternatively, onto CHIP-coated plates, p53 was added, followed by the addition of p53 conformational antibody pAb 1620 or pAb 240. CHIP was shown to preferentially bind to p53 mutant. C, WT p53 and heat-denatured p53 (37 and 42 °C) were added to plates coated with CHIP. The WT and mutant that were bound to CHIP were detected by adding pAb 1620 or pAb 240 mutant P53. D, alternatively, CHIP was preferentially shown to bind to the p53 mutant phenotype by sandwich ELISA. E, co-immunoprecipitation between WT p53 or denatured p53 and CHIP in an equimolar ratio, followed by immunoprecipitation with p53 conformation-specific antibodies pAb 240, pAb 1620, and pAb 421 and then analysis by SDS-PAGE. The immunoblotted material was run on SDS-PAGE. The mutated form was preferentially immunoblotted with CHIP. RT, room temperature.
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**FIGURE 4. CHIP prevents irreversible thermal aggregation of p53.**

A, Coomassie staining of purified recombinant p53, CHIP, and CHIP^{1–126} protein as well as Western blot analysis of the same. M denotes molecular mass markers. B, denaturation curve was taken with recombinant CHIP (50 μg/ml in phosphate-buffered saline) protein. C, the kinetics of p53 aggregation were monitored at 37, 45, and at 50 °C by measuring the light scattering of the samples at 340 nm by incubating WT p53 (1.0 μM) with or without CHIP (2.0 μM). D, the kinetics of p53 aggregation at 37 and 45 °C with or without CHIP deleted of its TPR domain (CHIP^{Δ1–126}).
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were transfected with CHIP cDNA at an increasing concentration, the restoration of native p53 level (Fig. 7A, lanes 6, 8, 12, and 14) as well as a decline in the mutant level (Fig. 7B, lanes 7, 9, 13, and 15) was observed, thus confirming that CHIP, in vivo, restored WT p53 conformation under elevated temperature. In order to exclude the possibility that Hsp90 might be involved in modulating p53 conformation, the transfection experiments were further carried out in a similar manner as above in the presence of geldanamycin, an Hsp90 inhibitor. No difference in the protein band was observed between geldanamycin-treated and untreated cells, suggesting that the conformational modulation of WT53 by CHIP might be independent of Hsp90. At 37 °C, a lower amount of the mutant form was observed (Fig. 7B, lane 3), and at 42 and 45 °C, the mutant form was increased (Fig. 7B, lanes 5 and 11). The addition of CHIP in the presence of geldanamycin resulted in a lowering of the mutant form (Fig. 7, lanes 7 & 9). Further, we transfected a mutant CHIPK30A, a TPR domain mutant unable to interact with Hsp70/Hsc70 or Hsp90 (42), into KB cells and subjected the cells to heat stress, followed by immunoprecipitation with pAb 1620 and pAb 240. Exogenous expression of CHIPK30A in KB cells under elevated temperatures resulted in an increase in the wild type form (Fig. 7C, lanes 6 and 10, shown with arrows) and decrease in the mutant form (Fig. 7C, lanes 7 and 11), thus strongly suggesting that the chaperoning of p53 by CHIP was independent of Hsp70 and Hsp90. We further knocked out CHIP by siRNA in KB cells and subjected the transfected cells to heat stress and measured the levels of both the wild type and mutant p53 phenotype by immunoprecipitation with p53 conformational antibodies. In a standardization experiment, no CHIP was detected by Western blot when 1.0 µg of CHIP siRNA was used (Fig. 7D), whereas scrambled RNA and cells showed positive bands. Actin was used as an internal control. In KB cells, both at 42 and 45 °C, the levels of p53 wild type (pAb 1620) and mutant conformation (pAb 240) were found to be in approximately the same ratio (Fig. 7D, lanes 2, 3, 6, and 7). At a 1 µM concentration of CHIP siRNA, there was invariably an increase in the level of conformational mutant both at 42 and 45 °C (Fig. 7D, lanes 5 and 9). It is thus assumed that in cells there was a direct interaction between CHIP and p53, and CHIP helped in resurrecting the mutant phenotype into the wild type form under elevated temperatures.

Synchronization of CHIP and p53 Levels in the Nucleus under Elevated Temperatures and Their Cooperative Binding to DBS in Chromatin—Both p53 (37) and CHIP (31) were earlier shown to rapidly translocate into the nucleus during heat probe alone. C, EMSA was performed with recombinant p53 (100 ng), as shown in lane 2. Lane 3 shows p53 (100 ng) denatured at 37 °C in the absence of CHIP; lane 4 contains a mixture of p53 (100 ng) and CHIP (500 ng) at 37 °C; lane 5 shows p53 that was denatured first at 37 °C for 60 min, and CHIP was then added for EMSA; lane 6 shows gel shift of denatured p53 (100 ng) in the presence of CHIP (500 ng) and 5 mM ATP. D, EMSA was performed with recombinant p53 (100 ng) as shown in lane 2; lane 3 shows p53 in the presence of pAb 421; lane 4 shows p3 (100 ng) denatured at 37 °C in the absence of CHIP; lane 5 contains denatured p53 (100 ng) and 500 ng of CHIP (heated at 37 °C for 30 min); lane 6 shows EMSA mixture having denatured p53 (100 ng) and CHIP (500 ng heated at 45 °C for 30 min); lane 7 contains denatured p53 (100 ng) and CHIP (500 ng, heated at 50 °C for 30 min).

FIGURE 5. CHIP restores p21 DBS binding activity of denatured p53. A, 5 ng of 32P-labeled probe containing 5′ p21 DBS was incubated with 100 ng of recombinant p53 (lane 1), p53 + CHIP (lane 4), and p53 + pAb 421 (lane 3). Anti-p53 antibodies were added to the reaction mixture to ensure the specific DNA-p53 interaction. B, gel shift assay of p53 (100 ng) in the absence (lane 2) or in the presence of an increasing amount of CHIP (200 ng (lane 3), 400 ng (lane 4), 800 ng (lane 5), and 1500 ng (lane 6). Lane 1 contains radiolabeled probe alone.
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FIGURE 6. CHIP interacts with p53 in vivo. A, KB cell lysate of both untreated (lane 2) and treated cells (lane 3) with proteasome inhibitor MG132 and geldanamycin (lane 4) (1.0 μM, for 4 h) were incubated with 1.0 μg of anti-CHIP antibodies at 4 °C overnight, and immunocomplexes were captured with protein A-agarose and subjected to Western blotting (WB) for p53 (pAb 1801) and CHIP. Immunoprecipitation (IP) of equal amounts of cell lysates with serum was taken as a negative control. B, KB cells were transfected with HA-tagged expression vector pNHA1 containing p53 cDNA, and cell lysates of both untreated and proteasome inhibitor-treated MG132 were immunoprecipitated with 1 μg of anti-HA antibodies and immunoblotted with anti-CHIP and anti-p53 (pAb 1801) antibodies. C, the whole cell lysates of KB cells were first excluded for Hsp70 and Hsp90 proteins with anti-Hsp70 and anti-Hsp90 antibody columns. The gel shows Western blot analysis with anti-Hsp70 and anti-Hsp90 antibodies showing whole cell lysate (lanes 1 and 4), beads containing Hsp70 (lane 2) and supernatant (lane 3), and beads containing Hsp90 proteins (lane 5) and supernatant (lane 6). D, KB cell lysate purified over anti-Hsp70 and anti-Hsp 90 antibody columns was incubated with 1.0 μg of anti-CHIP antibodies at 4 °C overnight, and immunocomplexes were captured with protein A-agarose and subjected to Western blotting for p53 (pAb 1801) and CHIP. Immunoprecipitation of equal amounts of cell lysates with serum was taken as a negative control.

The chaperone function of CHIP in resurrecting p53 mutant under elevated temperature led us to examine whether their co-association is linked to their co-nuclear translocation. The analysis of whole KB cell lysates under heat shock conditions at 42 °C showed a sudden rise in their level in a synchronized manner (Fig. 8A, lane 2), and over a recovery period of 4 h, their levels were further raised (Fig. 8A, lanes 3–5). Under heat shock conditions at 42 °C, a sharp increase in both p53 and CHIP levels in the nucleus (Fig. 8B, lane 7) and a lowering of p53 and CHIP levels in the cytoplasm was observed (Fig. 8B, lane 2). Over a recovery period of 4 h, both p53 and CHIP were found to relocate into the cytoplasm (Fig. 8B, lanes 3–5). However, the total intracellular level of both the proteins remains much higher than the untreated cells. We then performed the chromatin immunoprecipitation assay in order to determine whether CHIP co-associated with p53 upon p53 DBS in chromatin by utilizing both anti-p53 and anti-CHIP antibodies. We chose both p21 and p53 promoters for this purpose, p21 has two class I DBS in its promoter, whereas two p53 DBS were earlier identified in the p53 promoter (DBSI, class II, −157 to −156, and DBSII, class I, −216 to −235) (Fig. 8C). The class I DBS has a “C” at position 4 of the pentamer PuPuPuC (A/T), and its binding is enhanced by pAb 421 (41). We have further identified a third DBSIII (class II) at positions −388 to −397.3 Primers flanking all of the DBS in the p21 and p53 promoters were utilized for PCR to determine any simultaneous but differential occupancy by p53 and CHIP. Interestingly, the two DBS in p21 promoter and all three DBS in the p53 promoter could be amplified, thus confirming that both p53 and CHIP assembled together upon p53 DBS (Fig. 8D). It was surprising to notice that in the p21 promoter, the 3’-DBS (class I) and the DBS III (class II) in the p53 promoter were both preferentially occupied by both CHIP and p53 (Fig. 8D, arrow). Also, a double chromatin immunoprecipitation assay has thus confirmed the presence of both CHIP and p53 upon p21 and p53 DBS in their promoters (Fig. 8E), which is assumed that the co-occupancy of both CHIP and p53 upon DBS might be discriminatory under different cellular conditions.

DISCUSSION

Wild type p53 regulates cellular response to stress, abnormal cell proliferation, and DNA damage. Under stress condition p53 is activated, stabilized, and imported into the nucleus, where it promotes transcription of several genes whose products induce cell cycle arrest, DNA repair, and apoptosis. Wild type p53 shows intrinsic conformational lability (4, 25) and might undergo dynamic associations with molecular chaperones at a post-translational stage, and the conformation of the p53 is essential for its biological activity. A little is known about the functional consequence of interaction of WT 53 with molecular chaperones. It was shown that conformational mutants but not wild type p53 form stable complexes with molecular chaperones (26, 27), and wild type p53 might undergo highly transient interactions with molecular chaperones (28). Under heat shock conditions WT p53 undergoes conformational changes and acquires mutant phenotype conformation that undergoes irreversible loss of DNA binding activity. Our results demonstrate that CHIP selectively binds to p53 amino terminus and regulates its conformation in an alternate pathway that is independent of Hsp90. CHIP was shown

3 U. Pati, unpublished results.
earlier to assist protein folding in association with Hsp70 (20). Under physiological and elevated temperatures, CHIP preferentially binds to the mutant conformation, and the binding was progressive when the wild type conformation was being switched into a mutant one. Hsp90 was shown earlier to bind to a folded native-like conformation \textit{in vitro} with micromolar affinity and stabilize p53 at physiological temperature (6). Our data showed that CHIP, \textit{in vitro}, binds to both wild type and mutant p53, although it showed preferential binding to mutant p53 at elevated temperatures. There are two alternate possibilities. CHIP might bind to wild type form in order to prevent its transition into the mutant form; under stress conditions, where the transition to the mutant form has already occurred, CHIP could bind to the mutant form in order to resurrect the mutant conformation. While this manuscript was in review, Rosser et al. (40) reported that CHIP possesses an intrinsic chaperone activity that enables it to selectively recognize and bind to nonnative proteins, and this chaperone function of CHIP is enhanced by heat stress. In a similar manner, MDM2 binding to the amino terminus could induce a pronounced accumulation of mutant conformation prior to WT p53 degradation, and this conformational change was opposed by Hsp90 (14).

The TPR domain mediates the interaction of CHIP with Hsp90 and Hsp70 during the regulation of signaling pathways and during protein quality control (17). Since CHIP$^{Δ26}$ (without TPR domain), \textit{in vitro}, was able to prevent WT p53 from irreversible thermal aggregation at elevated temperatures, it could be suggested that this event could be independent of Hsp90 and Hsp70 function. Besides, the recombinant proteins utilized for the aggregation studies were thoroughly depleted of any bacterial Hsp90 and Hsp70 contamination. This was further supported by the fact that, in cells under stress, CHIPK30A, a TPR domain mutant unable to interact with Hsp70/Hsp90, was able to restore the wild type form and reduced the mutant form. It was further observed in EMSA that heat-denatured p53, which irreversibly lost p21 DBS binding, was restored of its activity in the presence of CHIP (and Hsp90 inhibitor geldanamycin) in an ATP-independent manner. On the contrary, Hsp90 was shown to positively modulate p53 DNA binding after

**FIGURE 7.** CHIP restores the native conformation of p53 under elevated temperatures. A, CHIP restores the native conformation of p53. KB cells were transfected with the indicated amount of CHIP expression vector and grown at 37 °C in a CO$_2$ incubator. After incubation, cells were heat-shocked at 42 and 45 °C, and lysates were immunoprecipitated (IP) with pAb 1620 and pAb 240 at 37, 42, and 45 °C and immunoblotted (WB) with pAb 421. The levels of wild type and mutant at 37 °C (lanes 2 and 3), at 42 °C (lanes 4 and 5), and at 45 °C (lanes 12–15) were shown. The arrows show restoration of native p53 level (lanes 8 and 14). B, shown is the repetition experiment as above in the presence of geldanamycin (1.0 μM for 4 h). C, CHIP mutant, CHIPK30A, when transfected in to KB cells showed an increase in the wild type form (lanes 6 and 10, with arrows) and decrease in the mutant form (lanes 7 and 11). D, 0.5 and 1.0 μg of CHIP and scrambled siRNA were transfected into the KB cell at 37 and 42 °C, followed by Western blotting with anti-CHIP antibodies. Actin was taken as loading control. E, 1.0 μg of CHIP siRNA was transfected, and whole cell lysate was immunoprecipitated with pAb 1620 and pAb 240 antibodies at 42 and 45 °C. An increase in the level of mutant form in the presence of siRNA (lanes 5 and 9, with arrows showing comparison) was shown.
incubation at physiological temperature in an ATP-dependent manner. CHIP chaperone function remained intact when it was preheated at 45 °C, although the chaperone activity was lost at 50 °C. It is thus assumed that heat-denatured p53 that existed in a mutant form was transiently associated with CHIP prior to its conversion into wild type conformation.

FIGURE 8. Co-migration of CHIP and p53 into the nucleus under elevated temperatures. A, the levels of CHIP and p53 increase under heat shock. KB cells were heat-shocked at 42 °C for 90 min, and whole cell lysates were immunoblotted with anti-p53 and anti-CHIP antibodies under normal, no shock (NS) and heat stress conditions and during recovery conditions. The right-hand image shows the densitometric plot of the above Western blot (WB) analysis with the Bio-Rad gel documentation system. B, subcellular distribution of endogenous CHIP and p53 under heat stress. Nuclear and cytoplasmic fractions of KB cells at 37 and 42 °C and during recovery over a period of 4 h were immunoblotted with anti-p53 and anti-CHIP antibodies. Actin was taken as control. Images below show the densitometric plot of the above Western blot analysis with the Bio-Rad gel documentation system. C, the location of two p21 DBS and three p53 DBS in the p21 and p53 promoters. The class I and class II DBS sequences are shown below. D, chromatin immunoprecipitation assay showing the presence of both CHIP and p53 upon all of the class I and class II DBS in p21 and p53 promoters. The arrows show the preferential amplifications of p33 DBS III and p21 3'-DBS. E, double chromatin immunoprecipitation assay showing the presence of p53 and CHIP together upon DBS in p21 and p53 promoters.
In KB cells, CHIP physically associated with p53, and this association was not influenced in the presence of geldanamycin. Under physiological conditions, p53 was present in wild type conformation in a higher amount than the mutant form, whereas under elevated temperatures (42 and 45 °C), there was an increase in the mutant form. The addition of CHIP exogenously, at 42 and 45 °C, restored the wild type form, and the same result was obtained in the presence of geldanamycin. As it was expected, CHIP-siRNA considerably increased the amount of mutant form, thus suggesting the role of CHIP as a chaperone in helping resurrect p53 mutant phenotype into wild type form.

The levels of both p53 and CHIP were higher in the nucleus under elevated temperatures, whereas during recovery, their levels were higher in the cytoplasm. The simultaneous co-migration of CHIP and p53 in to the nucleus under elevated temperatures might be linked to the observed chaperone function of CHIP in the cytoplasm. An increase in the p53 level was reported after heat treatment of A-172 glioma cells, and this increase was attributed to an induction at the transcription level (34). A similar observation of a rise in p53 level was made in MCF-7 cells (7), and it was thus suggested that this rise in p53 level could have been due to reduced p53 degradation by the proteasome. This is further supported by a new observation that MDM2 binding could drive wild type p53 into a mutant conformation, and proteasome-dependent degradation was blocked during recovery from stress treatment and in cells co-expressing p53 and MDM2 (14). Under heat stress, the level of p53 mutant phenotype increases in the cytoplasm, and at the same time it is required that p53 should enter into the nucleus in the wild type DNA-binding form for transcriptional activation of genes that might also include both p21 and p53. An increase in CHIP level, under elevated temperature, could then probably be to maintain p53 in wild type form for its transport into the nucleus. Our finding thus corroborates a recent observation that mRNA level of CHIP is significantly increased in the cells exposed to oxidative, endoplasmic reticulum, and proteasomal stress, and it could be an adaptive response of the cells to deal with the excess burden of misfolded protein (39). Interestingly, the chromatin immunoprecipitation shows the presence of p53 and CHIP together upon DBS in the p21 and p53 promoters. Since CHIP does not bind to DNA alone, its presence upon the DBS of p21 and p53 promoter in the chromatin could obviously be due to its association with p53. The direct physical association of CHIP with p53 right from the cytoplasm to the nucleus reasonably justifies the CHIP function of consistently monitoring and restoring native p53 conformation for maximal DBS binding under physiological and heat stress conditions.

In conclusion, the function of CHIP as a direct chaperone of wild type p53 both in vitro and in vivo might be due to an alternate pathway that is independent of Hsp90 and MDM2 function. We propose that the independent chaperoning of p53 by CHIP might provide the cell with means of responding to heat stress, thus maintaining cell homeostasis and protection against cellular transformation. Further work is needed in order to understand the biological relevance of alternate chaperoning of p53 by CHIP in p53-mediated gene transcription.

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Acknowledgments—We thank C. Patterson, H. Band, T-P. Yao, M. Oren, S. Misra, and L. Neckers for the vectors pcDNA3-CHIP, pGEX-CHIP, pGEX-MDM2, and 2.4-kg promoter, Topo1151d-CHIP, (127-303), and CHIPK30A, respectively; D. Mitra for anti-Hsp90 antibodies; and A. Sharma for help in the Western blot experiment.
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