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Hsp70 and Hsp90 oppositely regulate TGF-β signaling through CHIP/Stub1

Yu Shang [a,b,1], Xialian Xu [b,1], Xiaolin Duan [c], Junwei Guo [b], Yinyin Wang [b], Fangli Ren [b], Dacheng He [a], Zhijie Chang [b,*]

[a] The Key Laboratory for Cell Proliferation and Regulation Biology of Ministry of Education, College of Life Sciences, Beijing Normal University, Beijing 100875, China
[b] State Key Laboratory of Biomembrane and Membrane Biotechnology, School of Medicine, Tsinghua University, Beijing 100084, China
[c] The Second People's Hospital of Zhuhai, Guangdong 519000, China

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ABSTRACT

Transforming growth factor-β (TGF-β) signaling plays an important role in regulation of a wide variety of cellular processes. Canonical TGF-β signaling is mediated by Smads which were further regulated by several factors. We previously reported that E3 ubiquitin ligase CHIP (carboxyl terminus of Hsc70-interacting protein, also named Stub1) controlled the sensitivity of TGF-β signaling by modulating the basal level of Smad3 through ubiquitin-mediated degradation. Here, we present evidence that Hsp70 and Hsp90 regulate the complex formation of Smad3/CHIP. Furthermore, we observed that over-expressed Hsp70 or inhibition of Hsp90 by geldanamycin (GA) leads to facilitated CHIP-induced ubiquitination and degradation of Smad3, which finally enhances TGF-β signaling. In contrast, over-expressed Hsp90 antagonizes CHIP mediated Smad3 ubiquitination and degradation and desensitizes cells in response to TGF-β signaling. Taken together, our data reveal an opposite role of Hsp70 and Hsp90 in regulating TGF-β signaling by implicating CHIP-mediated Smad3 ubiquitination and degradation. This study provides a new insight into understanding the regulation of the TGF-β signaling by chaperones.

1. Introduction

Transforming growth factor-β (TGF-β) is a secreted cytokine superfamily that exerts broad biological activities including early embryo development, organ morphogenesis and the basis homeostasis maintenance of the living body [1]. Canonical TGF-β signaling is mainly transduced by Smad2/3 and Smad4, regulated by Smad6/7. When TGF-β signaling is initiated by TGF-β, the type II (RII) and type I (RI) receptors form a complex to recruit Smad2/3 and Smad4 for dimerization, which further translocate into the nucleus and promotes targeted gene expression [2]. Aberrant TGF-β responses lead to various human diseases are frequent in human diseases, such as cancers, fibrosis, and autoimmune and cardiovascular ailments [3]. To avoid an excess of TGF-β family signaling in cells, the duration and intensity of the TGF-β family signal appear to be subject to elaborate regulation.

Recently, Smad proteins were reported to be regulated by the ubiquitination proteasomal pathway. Several E3 ligases including Smurf1, Smurf2, WWP1, RNF111, Skp2 and ROC1-SCF^{Fbw1a} have been implicated in Smad degradation (see review [4]). We also reported that CHIP/Stub1 as a Smad interacting partner functions on the regulation of Smad protein stability through the ubiquitination proteasomal pathway [5–7].

CHIP/Stub1 was originally identified as a novel tetratricopeptide repeat (TPR)-containing protein, which interacts with Hsc70 [8]. CHIP has been reported to induce degradation of a number of proteins such as ErbB2 [9], p53 [10], CIP2A [11] and SRC-3 [12] to regulate cell proliferation, metastasis and tumor progression. We have demonstrated that CHIP mediated ubiquitination and degradation of Smads [5–7], Runx1/2 [13,14] and NF-κB [15] *in vitro*. Recently, we observed that CHIP expression is related to colon cancer, seemingly targeting eIF5A [16]. Genetic deficiency of CHIP resulted in super-sensitive phenotype for stress [17]. We recently found that CHIP deficiency mouse suffered from bone loss [18].

Here we provide evidence that Hsp70 and Hsp90 regulate the activity of CHIP to degrade Smad3 protein. Interestingly, we observed that Hsp70 promotes, but Hsp90 inhibits, CHIP-mediated Smad3 degradation.

* Corresponding author. Fax: +86 10 62773624.
E-mail address: zhijiec@tsinghua.edu.cn (Z. Chang).
[1] These authors equally contributed to the work.

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2. Materials and methods

2.1. Cell culture and transfection

HEK293T (human kidney cells), COS7 (Monkey kidney cells) and Mv1Lu (Mink lung) cells were maintained in Dulbecco's modified Eagle medium (DMEM, GIBCO) supplemented with 10% heat inactivated fetal bovine serum (FBS, Gibico) at 37 °C in the presence of 5% CO_2. Stable cell lines were selected by 1 mg/ml G418 (Sigma–Aldrich) after transfection of the related plasmids, and the obtained clones were maintained in media containing 400 μg/ml G418. Cells were transfected with various expression plasmids by using the Lipofectinamine 2000 reagent (Invitrogen) according to the manufacturer's instructions.

2.2. Antibodies and reagents

Anti-HA (A-7), anti-Myc (9E10), and anti-GFP (FL), anti-Hsp70 (W27), anti-Hsp90 (Hsp90α/β), anti-Smad3 (38-Q) were purchased from Santa Cruz. Anti-β-Actin (AC-15) and anti-Flag (M2) were purchased from Sigma. Anti-Smad2/3 antibody was obtained from BD. TGF-β1 and geneticin (G418) were purchased from R&D and CLONTECH, respectively. MG132 and Geldanamycin (GA) were obtained from Calbiochem.

2.3. Plasmids

Plasmid of F-Hsp70 was kindly provided by Dr. Zhimin Yin. HA-Hsp70, HA-Hsp90α and HA-Hsp90β were constructed by PCR amplification and sub-cloned into pEF-neo (from Dr. Xinyuan Fu) vector. Myc-CHIP and Myc-CHIP(K30A) were constructed in our lab [14]. Flag-Smad3, TGF-β RI(TD), and $(CAGA)_{12}$-Luc constructs were kindly provided by Dr. Xiaofan Wang. pEGFP-N1 was purchased from Clontech. The HA-ubiquitin plasmid was a gift from Dr. Ying Zhang.

2.4. Immunoprecipitation and Western blot

Whole-cell lysates were incubated with 2 μg of the indicated antibodies and 30 μl of protein G-plus beads (Santa Cruz) for 8 h at 4 °C. Precipitated proteins were eluted with 2× SDS–PAGE loading buffer and analyzed by Western blot. Western blot was performed as previously described [6].

2.5. Surface plasmon resonance analysis

The full-length cDNA of hSmad3 in pET15b (Novagen) and hHsp70 and hHsp90β in pET28a (Novagen) were expressed in *Escherichia coli* BL21 and the recombinant 6× His fusion protein was purified as previously described [7]. Interaction of Smad3 with Hsp70 or Hsp90β was analyzed by surface plasmon resonance (SPR) using Biacore 3000 (GE Healthcare) at 298 K. Recombinant Hsp70 or Hsp90β was immobilized onto a sensor GLC-chip using the standard amine-coupling method (GE Healthcare). 1 μg/ml or 10 μg/ml concentrations of Smad3 proteins were injected in the running buffer (HEPES 10 mM, NaCl 150 mM, EDTA 3 mM, polysorbate 0.005%) over the chip at a flow rate of 30 μl/min. The complex was allowed to associate for 60 s and dissociate for 60 s. Regeneration was accomplished by passing binding buffer over the chip surface until dissociation completed. Data was analyzed with Biacore 3000 evaluation software by fitting to a 1:1 Langmuir binding fitting model.

2.6. Pulse–chase analysis

COS7 cells were used for the experiment by co-transfection of indicated plasmids. Cells were starved with methionine-free medium without addition of FBS for 1 h and then metabolically labeled with 50 μCi/ml [^{35}S] methionine (PerkinElmer Life Sciences) for 15 min, and chased for indicated points. The cells were lysed and subjected to anti-Flag immunoprecipitation for Smad3 and separated by SDS–PAGE. The gels were dried and ^{35}S-labeled F-Smad3 was visualized with PhosphorImager.

2.7. In vivo ubiquitination assay

The ubiquitination assay was performed as described [13]. In brief, the HEK293T cells transfecting the indicated plasmids were lysed by buffer A and then sonicated. After incubating with 50 μl of equilibrated (50%) Ni–NTA agarose for 3 h at room temperature, beads were washed. Precipitated proteins were eluted with 2× SDS–PAGE loading buffer (containing 250 mM imidazole) and subjected to SDS–PAGE followed by Western blot.

2.8. Luciferase assay

Luciferase assay was performed according to a previous study [6]. In brief, to activate the TGF-β signaling, cells were transfected with 100 ng of TGF-β RI(TD) or treated with TGF-β1 for 18 h before harvest. Luciferase activities were determined by Top Count (Packard). The data were normalized with an internal control. The experiments were done in triplicate, and the standard error and mean were calculated.

3. Results

3.1. Hsp70 and Hsp90 are important for CHIP in negatively regulating TGF-β signaling

Lysine at position 30 in the TPR domain was reported to be the unique site critical for CHIP to bind Hsp90 and Hsc/Hsp70 [9]. To evaluate the ability of chaperones on CHIP activity, we used CHIP (K30A), a mutant lost the ability to interact with chaperones. An IP experiment confirmed that Myc-CHIP(K30A) lost interaction with either Hsp70 or Hsp90 (Fig. 1A). As expected, Myc-CHIP(-K30A) failed to interact with Flag-Smad3 (Fig. 1B). These results suggest that chaperones affected CHIP binding ability to Smad3.

Consistently, over-expression of Myc-CHIP(K30A) failed to mediate Smad3 degradation while the wild type CHIP led to F-Smad3 degradation (Fig. 1C). A pulse–chase experiment demonstrated that CHIP(K30A) seemingly increased the half-life of Smad3 while wild type CHIP shortened the half-life to 3.5 h (Fig. 1D and E). These results suggest that chaperones are involved in the regulation of CHIP activity in mediating Smad3 degradation.

Based on the fact that CHIP(K30A) failed to degrade Smad3, we next examine the inhibitory effect on TGF-β signaling using $(CAGA)_{12}$-Luc, a synthetic TGF-β responsive reporter responding to Smad3 activation. A luciferase reporter experiment demonstrated that over-expression of wide type CHIP potently suppressed activation of the reporter, whereas the mutant CHIP(K30A) had no inhibitory effect on the reporter (Fig. 1F). This result indicates that the interaction of CHIP with chaperones is important for the efficient inhibition of TGF-β signaling by CHIP.

3.2. Hsp70 and Hsp90 regulate the complex formation of Smad3 and CHIP

To investigate the binding feature of CHIP and Smad3 to chaperones, we first examined the interaction of Hsp70 and Hsp90 with Smad3. An SPR experiment demonstrated that both Hsp70 (Fig. 2A)

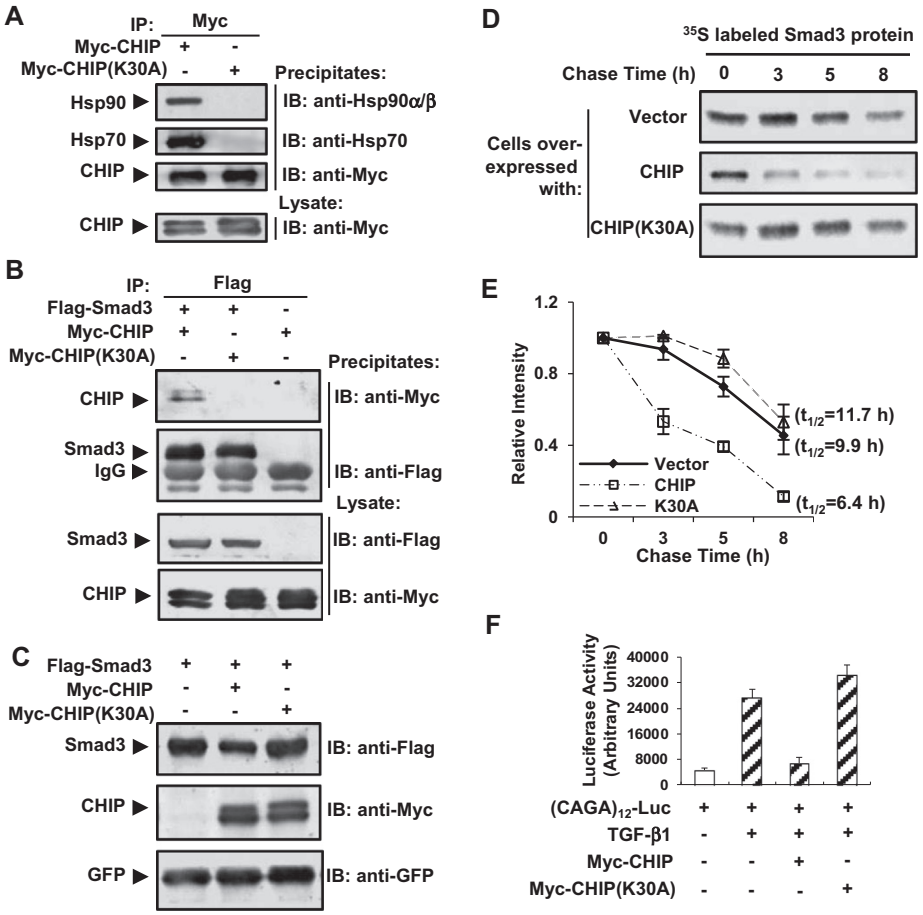

Fig. 1. The activity of CHIP in negative regulation of TGF-β signaling is dependent on the interaction of chaperone. (A) CHIP(K30A) lost the interaction with Hsp90/Hsp70. Immunoprecipitation experiments were performed using an anti-Myc antibody. (B) CHIP(K30A) lost the interaction with Smad3. An anti-Flag antibody were used during the immunoprecipitation experiment. (C) CHIP(K30A) failed to decrease the Smad3 protein level. The steady-state protein level of Smad3 was determined by immunoblotting. GFP was used as a control. (D and E) CHIP(K30A) lost the ability in mediating the turnover of Smad3 protein. A pulse-chase assay (D) was performed using 35S-methionine. Quantitative presentation of the results repeated by three times is shown and the half-life (t1/2) was calculated. (F) CHIP(K30A) fails to inhibit the Smad3 transcriptional activity in Mv1Lu cells. Luciferase assays were performed independently by three times. The luciferase activity was expressed as arbitrary unit normalized to an internal control. Data were presented as means ± SD.$^{**}p < 0.01$.

and Hsp90 (Fig. 2B) associated with Smad3 *in vitro*. The interaction of Hsp70 and Hsp90 with CHIP and Smad3 prompted us to examine whether these proteins form a complex. An IP experiment indicated that an antibody against Hsp70 precipitated down both Flag-Smad3 and Myc-CHIP simultaneously (Fig. 2C). This result indicates that Hsp70, Smad3 and CHIP formed a complex.

To further reveal the presence of Hsp70 and Hsp90 in the complex of CHIP/Smad3, we precipitated down the Myc-CHIP/Flag-Smad3 complex and examined the protein levels of Hsp70 and Hsp90. The results showed that strong Hsp70 but less Hsp90 was presented in the Myc-CHIP/Flag-Smad3 complex (Fig. 2D, first two lanes). Interestingly, when the activity of Hsp90 was inhibited by GA, a specific inhibitor for Hsp90, the presence of Hsp90 in the Myc-CHIP/Flag-Smad3 complex was further reduced while the presence of Hsp70 remained stably strong in the complex (Fig. 2D, last two lanes).

To further reveal the role of chaperones on the complex of CHIP-Smad3, we precipitated down the Myc-CHIP/Flag-Smad3 complex in the presence of Hsp70 or Hsp90. The results showed that the Smad3/CHIP complex was increased when Hsp70 was over-expressed, whereas, the complex was decreased when Hsp90 was over-expressed (Fig. 2E). These results suggest that Hsp70 promotes but Hsp90 inhibits the complex formation of Smad3 and CHIP.

3.3. Inhibition of Hsp90 enhances CHIP-mediated Smad3 protein degradation and ubiquitination

Since Hsp70 and Hsp90 both associated with CHIP and Smad3, we questioned whether Hsp70 and Hsp90 regulate Smad3 stability. To this end, we firstly used GA to inhibit the activity of Hsp90 and measured the Smad3 protein levels in the presence of CHIP. A Western blot result showed that over-expression of CHIP resulted in decreased Smad3 and addition of GA further promoted the degradation of Smad3 by CHIP (Fig. 3A). To reveal the mechanism of the Hsp90 on CHIP activity, we examined the ubiquitination of Smad3 by CHIP. A Western blot experiment showed that inhibition of Hsp90 by GA dramatically increased the ubiquitination of Smad3 by CHIP (Fig. 3B). These results suggest that Smad3 could be easily ubiquitinated and degraded by CHIP when Hsp90 activity was inhibited.

3.4. Hsp70 facilitates, while Hsp90 inhibits, CHIP-mediated Smad3 ubiquitination and degradation

To demonstrate the role of Hsp70 and Hsp90 on the regulation of Smad3 by CHIP, we over-expressed Hsp70 and Hsp90 in mammalian cells. A Western blot showed that the protein level of Smad3 was significantly decreased when Hsp70 was co-expressed

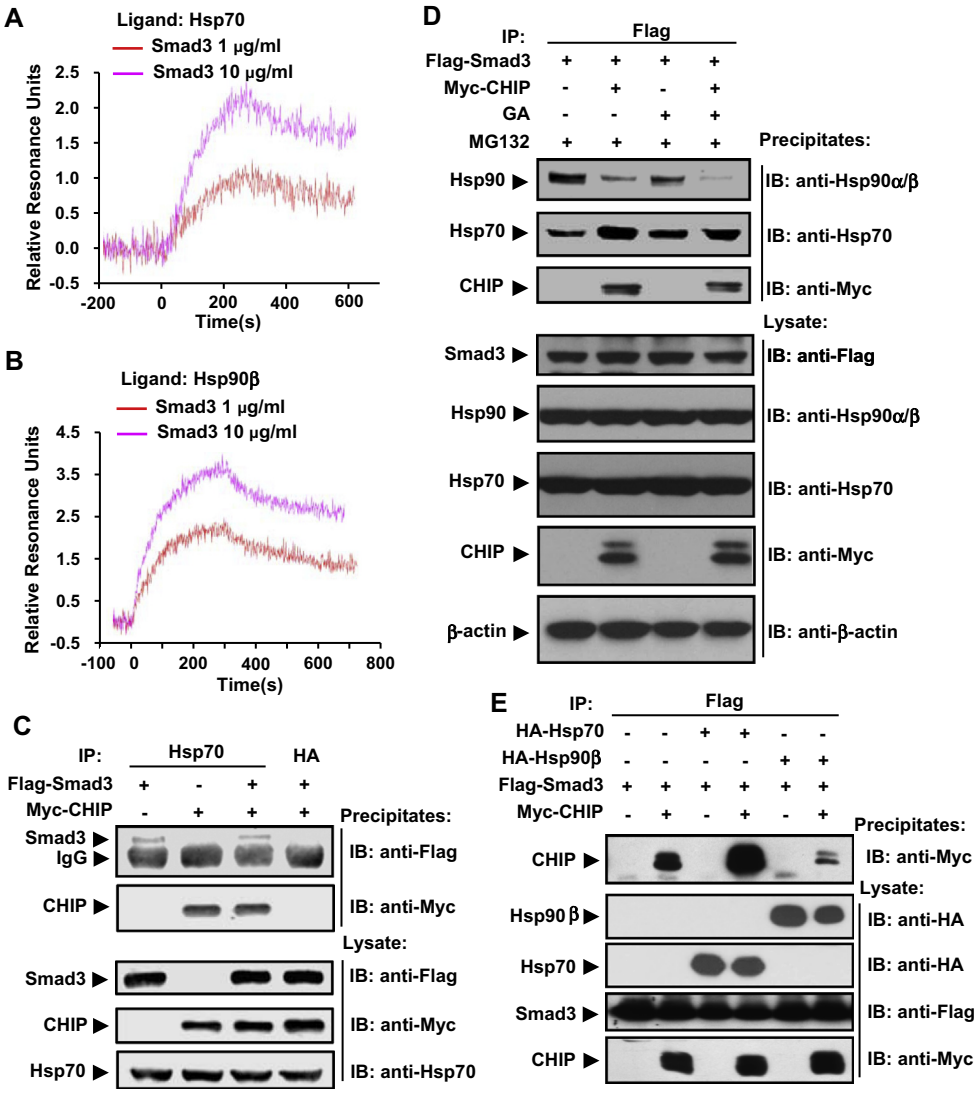

Fig. 2. Hsp70 and Hsp90 regulate the complex formation of CHIP/Smad3. (A and B) Hsp70 or Hsp90 binds to Smad3 protein *in vitro*. SPR analyses were performed to demonstrate the binding features of Smad3 with Hsp70 (A) and Hsp90 (B). (C) Hsp70 forms a complex with both CHIP and Smad3. Co-immunoprecipitated Smad3 and CHIP were examined by immunoblotting with anti-Flag or anti-Myc antibody, respectively. (D) Abundant Hsp70 but weak Hsp90 was present in the CHIP/Smad3 complex. Co-immunoprecipitation assay was performed to harvest the CHIP/Smad3 complex. Endogenous Hsp70 or Hsp90 in the complex was examined by immunoblotting. The cells were treated by MG132 for 4 h with 25 μM to avoid the degradation of Smad3 by CHIP during the experiment. (E) Hsp70 promotes, but Hsp90 inhibits, Smad3/CHIP complex formation. Co-immunoprecipitation assay was performed to demonstrate the CHIP/Smad3 complex.

with CHIP, while co-expressed Hsp90β with CHIP resulted in a marked increase in Smad3 protein level (Fig. 3C). These results suggested that Hsp70 and Hsp90 functions oppositely on the CHIP activity in regulation of Smad3 stability.

Furthermore, we examined the protein level of endogenous Smad3 in Mv1Lu cells. Consistently, over-expression of Hsp70 with CHIP further decreased the level of endogenous Smad3 (Fig. 3D, lanes 1–3). On the other hand, over-expression of Hsp90β (but not Hsp90α) recovered the Smad3 level degraded by CHIP (Fig. 3D, last lane). These results suggest that Hsp70 accelerates, while Hsp90 inhibits, Smad3 degradation mediated by CHIP.

The opposite role of Hsp70 and Hsp90 on Smad3 was further demonstrated by ubiquitinated Smad3 level. A Western blot analysis revealed that over-expression of Flag-Hsp70 increased, but HA-Hsp90β decreased, the ubiquitinated Smad3 protein in the presence of Myc-CHIP (Fig. 3E). The effect of Hsp70 and Hsp90 on the ubiquitinated Smad3 protein was in a dose dependent manner (Fig. 3E). These results suggested that Hsp70 facilitates CHIP mediated ubiquitination of Smad3 and Hsp90 inhibits the process.

3.5. Hsp70 facilitates while Hsp90 antagonizes CHIP-down-regulated TGF-β signaling

To test the role of Hsp70 and Hsp90 on TGF-β signaling, we assessed the Smad3-dependent transcriptional activities. A luciferase reporter experiment demonstrated that TGF-β1 induced the reporter activity by 180-fold but over-expression of CHIP inhibited the reporter activity in Mv1Lu cells (Fig. 3F, columns 1 and 2). Interestingly, over-expression of Hsp70 further decreased the reporter activity and over-expression of Hsp90β (not Hsp90α) recovered the reporter activity (Fig. 3F, last three groups of columns). Similar results were obtained under the over-expression of TGF-β RI(TD) (data not shown). These results suggest that Hsp70 facilitates CHIP mediated inhibition of TGF-β signaling while Hsp90 desensitize cells in response to TGF-β signaling.

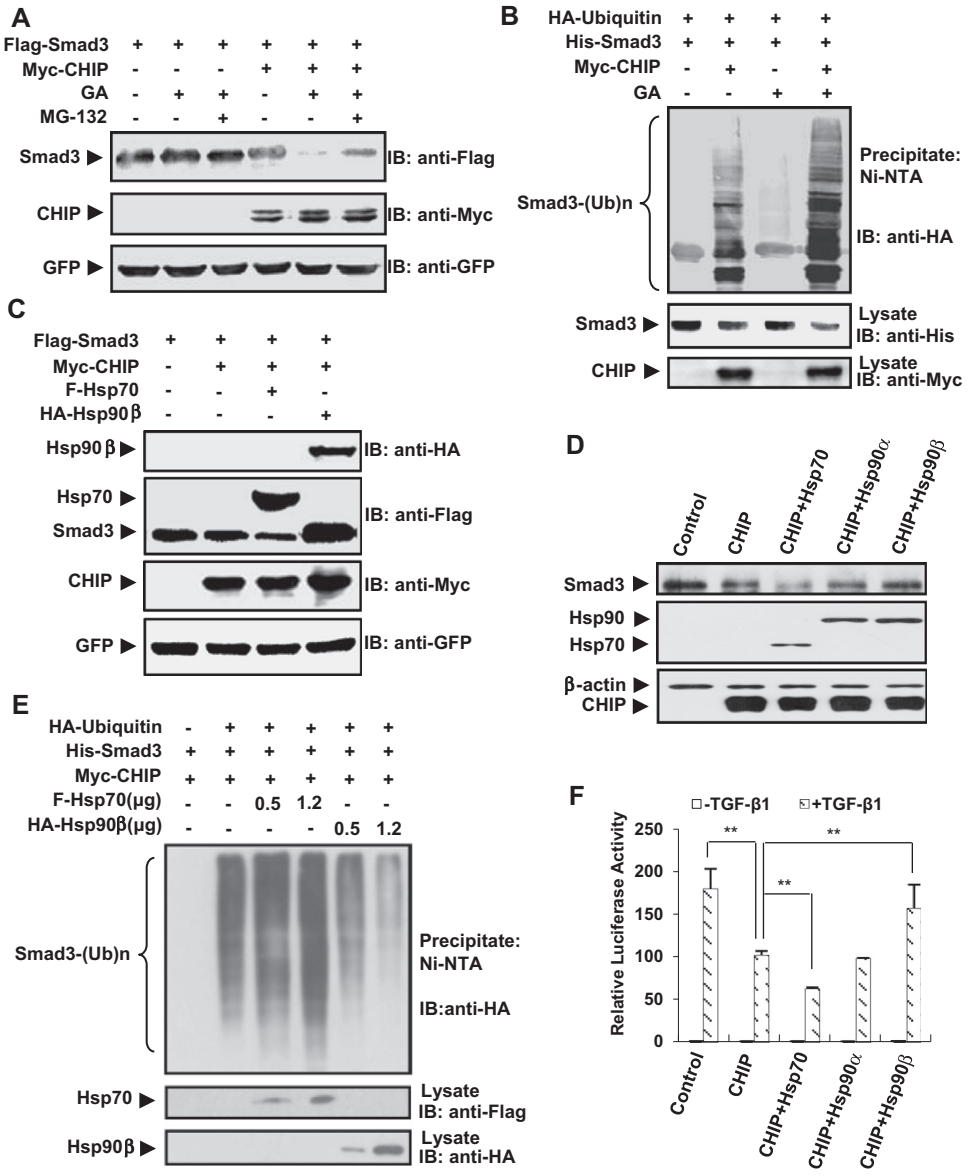

Fig. 3. Hsp70 promotes, but Hsp90 inhibits, the activity of CHIP on Smad3 degradation, ubiquitination and signaling. (A) Inhibition of Hsp90 activity by GA enhances Smad3 protein degradation mediated by CHIP. F-Smad3 protein level was measured by immunoblotting. (B) CHIP promotes Smad3 ubiquitination in the presence of GA. (C and D) Hsp70 enhances, while Hsp90 inhibits, CHIP-mediated Smad3 degradation. The protein level of F-Smad3 (C) in COS7 cells or endogenous Smad3 (D) in Mv1Lu cells were examined by immunoblotting. (E) Hsp70 accelerates, but Hsp90 blocks, CHIP-mediated Smad3 ubiquitination. HA-ubiquitin was co-expressed with Smad3 and CHIP in the presence of Hsp70 or Hsp90. (F) Hsp70 inhibits TGF-β in a synergy with CHIP and Hsp90 rescued the signaling. Luciferase assays were performed using $(CAGA)_{12}$-Luc reporter in Mv1Lu cells in the absence or presence of TGF-β1. Values were normalized using an internal control and presented as means ± SD from three independent repeats. $^{**}p < 0.01$.

4. Discussion

Smad proteins, key mediators in TGF-β signaling, have previously been shown to be degraded through ubiquitin–proteasome pathway [19]. We previously demonstrated that CHIP regulated TGF-β signaling by controlling the basal protein level of Smad3 [6]. In this study, we showed that chaperone protein Hsp70 and Hsp90 are involved in the interaction of Smad3 with CHIP. We provided evidence that Hsp70 facilitated the activity of CHIP to ubiquitinate and degrade Smad3 but Hsp90 just had an opposite effect. Since more Hsp70 and less Hsp90 are present in the complex of CHIP/Smad3 when Hsp90 activity was inhibited, we envision that Hsp70-CHIP-Smad3 complex is in favor of ubiquitination and degradation.

The role of Hsp70/Hsp90 has been investigated in TGF-β signal pathway. Inhibition of Hsp90 was reported to increase TGF-β RI and TGF-β RII ubiquitination and degradation, dependent on the Smurf2, another ubiquitin E3 ligase for Smads [20]. Also Hsp90 inhibition was found to abrogate Smad3 activity [21] and HSP72 inhibits TGF-β-induced EMT by modulating protein level of phosphorylated Smad2/3 and its nuclear translocation [22,23]. Our study revealed an opposite role of Hsp70 and Hsp90 in regulation of CHIP activity for Smad3 ubiquitination and degradation. Our results echo the observations of the role of Hsp70 and Hsp90 in regulation of TGF-β signaling and functions.

As CHIP is identified as a C-terminal Hsc70 interaction protein [8], most of CHIP targets are chaperone-dependently regulated [24]. In one of our previous studies, we found that CHIP-regulated

Runx1 protein stability was independent of Hsp70/90 [13]. Recently, chaperones were reported to sequentially regulate biogenesis and degradation of NCC (NaCl cotransporter) by CHIP [25]. Others reported that inhibition of Hsp90 or over-expressed Hsp70 promoted nNOS or Nox5 degradation mediated by CHIP [24,26]. In our study, we demonstrated that Hsp70 and Hsp90 influence Smad3 turnover in an opposing manner through altering the association of Smad3 with CHIP. The data presented added a member to the list of CHIP-regulated substrates that Hsp70 and Hsp90 function in an opposing way.

Disclosure

The author discloses no conflicts of interest.

Acknowledgments

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