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Citation	Biochemical and Biophysical Research Communications, 300(4), 847-852 https://doi.org/10.1016/S0006-291X(02)02941-8
Issue Date	2003-01-24
Doc URL	http://hdl.handle.net/2115/28121
Type	article (author version)
File Information	BBRC300-4.pdf



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Title: Involvement of Heat-shock protein 90 in the interleukin-6-mediated signaling pathway through STAT3

Authors: Noriko Sato *§, Tetsuya Yamamoto*‡ §, Yuichi Sekine *, Taro Yumioka*, Akira Junicho¶, Hideki Fuse¶ and Tadashi Matsuda *

Affiliation: *Department of Immunology, Graduate School of Pharmaceutical Sciences, Hokkaido University, Kita-Ku Kita 12 Nishi 6, Sapporo 060-0812, Japan

‡ Toyama Chemical Company, 4-1-2 Shimookui , Toyama, 930-8508, Japan

¶Department of Urology, Faculty of Medicine, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930-0194, Japan

Footnotes: §These authors contributed equally to this work. This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture in Japan, the Sagawa Foundation for Promotion of Cancer Research, the Osaka Foundation for Promotion of Clinical Immunology, the Akiyama Foundation, the Suhara Memorial Foundation and Mochida Memorial Foundation for Medical and Pharmaceutical Research.

Address for manuscript correspondence: Dr. Tadashi Matsuda, Department of Immunology, Graduate School of Pharmaceutical Sciences, Hokkaido University, Kita-Ku Kita 12 Nishi 6,

Sapporo 060-0812, Japan TEL: 81-11-706-3243, FAX: 81-11-706-4990, E-mail:

tmatsuda@pharm.hokudai.ac.jp

Running title: Interaction between STAT3 and Hsp90

ABSTRACT

Interleukin-6 (IL-6) is a multifunctional cytokine playing roles in the immune system, hematopoiesis and acute phase reactions. IL-6 also regulates the growth of in various types of human malignant tumors. Here we demonstrate that IL-6-induced gene expression was suppressed by a specific Heat-shock protein 90 (Hsp90) inhibitor, geldanamycin (GA) in human hepatoma Hep3B cells. GA also suppressed the IL-6-induced activation of signal transducer and activator of transcription 3 (STAT3) in a human embryonic kidney carcinoma 293T cells. This inhibitory effect of GA on STAT3 activation was reversed by overexpression of Hsp90. Furthermore, Hsp90 directly bound to STAT3 via its N-terminal region, which interacted with GA. We provide evidence that the action of GA on IL-6 functions was due to the inhibition of direct physical interactions between STAT3 and Hsp90, which represents a novel role of Hsp90 in the IL-6 signaling pathways.

Key words: IL-6, Heat-shock protein 90 (Hsp90), signal transducer and activator of transcription (STAT3), geldanamycin (GA)

INTRODUCTION

Interleukin-6 (IL-6) is a pleiotropic cytokine that regulates immune and inflammatory responses (1, 2), and also implicated in IL-6-related diseases (2). The IL-6 receptor is a heterodimeric complex, consisting of an IL-6 specific ligand-binding subunit, α chain, and a signal-transducing subunit, gp130. Structural analysis has revealed that both subunits belong to the cytokine receptor superfamily, and gp130 is shared by the receptors for ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), oncostatin M (OSM), and cardiotropin 1 (CT-1) (3, 4). The binding of IL-6 to α chain leads to the formation of receptor complexes, followed by tyrosine phosphorylation and activation of Janus protein tyrosine kinases (Jaks) and various cellular proteins, including gp130 itself. The activated Jaks, in turn, phosphorylate and activate latent signal transducer and activator of transcription (STAT) family of transcription factors (5, 6).

One member of the STAT family of proteins is STAT3, which is mainly activated by IL-6 family of cytokines, epidermal growth factor, and leptin (3, 4). Like other members of the STAT family, STAT3 is tyrosine-phosphorylated by Jaks, upon which it dimerizes, and translocates into the nucleus to activate target genes (5, 6). It has been shown that the activated STAT3 can mediate cellular transformation (7, 8) and constitutively active STAT3 is found in various types of human malignant tumors (9). Furthermore, STAT3 has been recently shown to act as an oncoprotein (7).

Molecular chaperones are a group of proteins whose major roles appear to be the prevention of target protein aggregation and the promotion of their correct folding and assembly (10). Chaperones such as Heat-shock protein (Hsp70) and Hsp40 likely interact with a wide variety of proteins, and thus their involvement in the folding processes is

believed to be of a general and universal nature. Another major molecular chaperone, Hsp90 has been shown to interact mainly with proteins involved in transcription regulation and signal transduction pathways, such as steroid hormone receptors, protein kinases and transcription factors (11-18). Apparently Hsp90 stabilizes these proteins and keeps them in a conformation amenable to activation under appropriate conditions. Previous studies presented the observation that Hsp90 was overexpressed in a wide variety of cancer cells and in virally transformed cells (19). It was also demonstrated that overexpression of Hsps in breast cancer cells resulted in the resistance to some forms of chemotherapy (20). To date, Hsp90 inhibitors, such as geldanamycin, by interacting specifically with a single molecular target, cause the destabilization and eventual degradation of Hsp90 binding proteins, and they have shown promising antitumor activity in preclinical model systems (13, 21, 22).

In this study, we examined the involvement of Hsp90 in the IL-6-mediated signaling pathway. The data demonstrated that Hsp90 may function as a stabilizer of an IL-6-mediated signal transducing molecule, STAT3 by directly interacting with STAT3. Further understanding of the interactions between STAT3 and Hsp90 is therefore important as this new information may provide new therapeutic approaches for the IL-6- or STAT3-mediated diseases.

MATERIALS AND METHODS

Reagents and antibodies

Human recombinant IL-6 was a kind gift from Ajinomoto (Tokyo, Japan). Human recombinant soluble IL-6 receptor α (sIL-6R α) was a kind gift from Dr. Hirano (Osaka Univ., Osaka, Japan). Geldanamycin (GA) was purchased from Calbiochem (San Diego, CA). Expression vectors, Jak1, FLAG-tagged Hsp90 mutants (16), FLAG-tagged STAT3 and STAT3-LUC (23) were kindly provided by Dr. J. N. Ihle (St. Jude CRH., Memphis, TN) and Dr. T. Hirano (Osaka Univ., Osaka, Japan), respectively. Myc-tagged STAT3 mutants were described previously (24). Anti-Myc antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-FLAG M2 antibody was purchased from Upstate Biotechnology (Lake Placid, NY).

Cell culture, transfections, luciferase assays, and cell growth assays

The human hepatoma cell line Hep3B was maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS)(25). Before stimulation, the cells were cultured for 12 h in DMEM containing 1% FCS, followed by treatment with IL-6 and/or GA. Human embryonic kidney carcinoma cell line, 293T was maintained in DMEM containing 10% FCS, transfected by the standard calcium precipitation protocol and followed by treatment with IL-6 and soluble IL-6 receptor α (100ng/ml) in the presence or absence of GA. Luciferase assay was performed as described (26). The cells were harvested and lysed in 100 μ l of PicaGene Reporter Lysis Buffer (Toyo Ink, Tokyo, Japan) and assayed for luciferase and β -galactosidase activities according to the manufacturer's instructions.

Luciferase activities were normalized to the β -galactosidase activities. Three or more independent experiments were carried out.

Northern blot analysis

Hep3B cells were maintained as described above. After serum starvation, cells (1×10^7) were treated with IL-6 (100 ng/ml) and/or geldanamycin (1 μ M) for 3 or 6 h. Total RNAs were prepared using Iso-Gen (Nippon Gene) and used in Northern analysis according to established procedures. A nylon membrane (Hybond N+, Amersham Pharmacia Biotech) and radiolabelled cDNA probes, as indicated, were used.

Immunoprecipitation and immunoblotting

The immunoprecipitation and Western blotting were performed as described previously (26). 293T cells were harvested and lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, containing 1% NP-40, 1 μ M phenylmethylsulfonyl fluoride and 10 μ g/ml each of aprotinin, pepstatin and leupeptin). The immunoprecipitates from cell lysates were resolved on SDS-PAGE and transferred to Immobilon filter (Millipore, Bedford, MA). The filters were then immunoblotted with each antibody. Immunoreactive proteins were visualized using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech).

RESULTS AND DISCUSSION

The Hsp90-specific inhibitor, geldanamycin suppresses IL-6-induced gene expression in hepatoma cells

To examine whether an Hsp90 inhibitor, geldanamycin (GA) has any effects on IL-6-induced transcriptional activation of cellular genes, we carried out Northern blot analysis on RNA samples prepared from human hepatoma Hep3B cells which had been stimulated with IL-6 and/or GA. As a cellular target for IL-6, we analyzed the expression of C/EBP δ which is a regulator of acute phase response genes in hepatocytes and is upregulated by IL-6 treatment through STAT3(25). As shown in Fig. 1, C/EBP δ expression was induced at 3 h after treatment with IL-6 alone in Hep3B cells. This IL-6-induced C/EBP δ expression was markedly suppressed by the addition of GA, whereas GA alone did not affect C/EBP δ expression. These data show that GA suppresses IL-6-induced STAT3-mediated gene transcription of C/EBP δ in Hep3B cells.

Cross-talk between STAT3 and Hsp90 in 293T cells

To further delineate the effect of GA on IL-6 signaling pathway, we carry out the transient transfection assay in a human embryonic kidney carcinoma cell line, 293T. The IL-6-mediated transcriptional responses through STAT3 were measured by using STAT3-LUC, in which the α 2-macroglobulin promoter (23) drives expression of the luciferase (LUC) reporter gene. In these experiments, 293T cells were stimulated with IL-6

in the presence of soluble IL-6 receptor α (sIL-6R α), which was shown to potentiate the biological activities of IL-6 (27). As shown in Figure 2A, STAT3-LUC activity stimulated with IL-6 plus sIL-6R α was suppressed by GA in a dose-dependent fashion.

Several studies demonstrated that GA has an effect on several Hsp90 client proteins such as steroid hormone receptors, protein kinases and transcription factors (11-18). To avoid these effects on inhibition of STAT3 activation by GA, we used a constitutively active form of STAT3, STAT3-C (7). 293T cells were transfected with STAT3-LUC and expression vectors for STAT3-C and the cells were either left untreated or treated with the increasing amounts of GA. As shown in Fig. 2B, STAT3-LUC activation by STAT3-C was inhibited when cells were simultaneously treated with the increasing amounts of GA.

To further assess the specificity of GA function on STAT3 activation, we overexpressed Hsp90 together with STAT3-LUC in 293T cells and cells were treated with GA before stimulation. Inhibitory effect of GA on STAT3 activation was reversed, when cells were transfected with the increasing amounts of Hsp90 (Fig. 2C). These results indicate that the inhibitory effect of GA on STAT3 activation in 293T cells is mediated by Hsp90 and the presence of a cross-talk between Hsp90 and STAT3 in 293T cells.

STAT3 and Hsp-90 physically interact in 293T cells

One of the mechanisms that is consistent with the data described above is that there are direct physical interactions between Hsp90 and STAT3. We tested this possibility by co-immunoprecipitation experiments. To activate STAT3 instead of IL-6 stimulation, we coexpressed Jak1 together with STAT3 as described previously (25). Expression vectors

encoding Hsp90 and/or Myc-tagged STAT3 in the presence or absence of Jak1 were transiently transfected into 293T cells. Cells were lysed, and subjected to immunoprecipitation with an anti-FLAG antibody. Immunoprecipitates were then used in Western blot analysis with an-Myc antibody. As shown in Fig. 3A, STAT3 physically associated with Hsp90 even in the absence of Jak1. Interestingly, STAT3-Hsp90 interactions were substantially increased in the presence of Jak1, suggesting that activated STAT3 may interact well with Hsp90. We next examined whether GA had any effect on STAT3-Hsp90 interactions or not. Expression vectors encoding FLAG-tagged Hsp90 and Myc-tagged STAT3 in the presence of Jak1 were transiently transfected into 293T cells. Before harvest, cells were treated with DMSO or GA (0.1 μ M or 1 μ M) for 6 h and then lysed, subjected to immunoprecipitation with an anti-FLAG antibody. Immunoprecipitates were then used in Western blot analysis with an-Myc antibody. As shown in Fig 3B, STAT3-Hsp90 interactions were affected by GA in a dose-dependent fashion. These data indicate that STAT3 and Hsp90 physically interact in vivo and GA disrupts their interactions.

To delineate the domains in the Hsp90 that mediate the protein-protein interactions between STAT3 and Hsp90, co-immunoprecipitation experiments were performed with a series of mutant Hsp90 proteins. Expression vectors encoding a series of FLAG-tagged Hsp90 mutants and Myc-tagged STAT3 were transiently transfected into 293T cells along with Jak1. As shown in Fig 3C, when only N-terminal domain, which was known as GA-binding site (21), was deleted, Hsp90 did not interact with STAT3. These data suggest that just N-terminal region is required for Hsp90 to interact with STAT3. We next determined the domains of STAT3 that mediate interactions with Hsp90, using deletion mutants of STAT3(24). Expression vectors encoding FLAG-tagged Hsp90 and/or a series of Myc-tagged STAT3 mutants were transiently transfected into 293T cells. Cells were lysed,

and subjected to immunoprecipitation with an anti-FLAG antibody. Immunoprecipitates were then used in Western blot analysis with an anti-Myc antibody. As shown in Figure 3D, the DNA binding domain/STAT3(320-493) interacted with Hsp90. The N-terminal domain/STAT3(1-137), the coiled-coil domain/STAT3(138-319) or the C-terminal region/STAT3(494-750) did not interact with Hsp90. These results indicate that the N-terminal region of Hsp90 interacts with STAT3 via the DNA binding domain of STAT3.

Conclusions

In the present study, we have shown that a specific Hsp90 inhibitor, GA suppressed IL-6-induced gene expression through STAT3 in hepatoma cells. Inhibitory effects of GA on STAT3 activation in 293T cells were reversed by overexpression of Hsp90. Furthermore, Hsp90 directly interacted with STAT3 via its N-terminal region, which interacted with GA.

IL-6 is a pleiotropic cytokine with a wide range of biological activities such as support of hematopoiesis, regulation of acute phase reactions, and generation of immune responses (1). Uncontrolled hyperproduction of IL-6 causes plasmacytosis, hyper-gamma-globulinemia, thrombocytosis, mesangial cell proliferation of the kidney as well as inflammatory symptoms which are frequently observed in autoimmune diseases (2). IL-6 is also involvement in several tumor development and progression (2, 9). An IL-6 signal transducing molecule, STAT3 is tyrosine-phosphorylated by Jaks, upon which it dimerizes, and translocates into the nucleus to activate IL-6-induced genes (5, 6). It has been shown that v-Src- or Q205L $G\alpha_0$ -mediated cell transformation required STAT3 activation (7, 8). Moreover, STAT3 acts as a sole transforming agent (7). Therefore, interference with IL-6 signal transduction through STAT3 may be useful for autoimmune diseases and anti-cancer therapy.

Hsp90 is an abundant molecule and acts as a molecular chaperone that is involved in the folding of various signaling molecules, such as the tyrosine kinases v-Src and Lck, and serine/threonine kinases Raf1 Akt, Bcr-Abl and Pim-1(11-17). Several transcription factors, such as p53 are also Hsp90 client proteins (18, 21). The levels of both IL-6 and Hsp90 have been reported to be elevated in patients with active systemic lupus erythematosus (SLE) (28). It has been also demonstrated that Hsp90 protein accumulates to increased levels in both hepatoma cells and peripheral blood mononuclear cells treated with IL-6, suggesting that IL-6 is able to activate the Hsp90 gene promoter directly (29). Indeed, the promoter of Hsp90 gene is shown to be stimulated by both IL-6-activated transcription factors, nuclear factor IL-6 (NF-IL6) and STAT3(30). These findings suggest that IL-6 positively regulates Hsp90 expression via STAT3 and Hsp90 stabilizes STAT3 proteins. Further work would be required to assess the possible significance of this cross-talk, and the feedback loop that may be established, in the pathophysiology of disease states.

In this study, we demonstrated that Hsp90 is an important regulator of IL-6 functions via STAT3 and thereby may have critical roles in the progression of diseases, such as cancer or autoimmune diseases. More detailed understanding of the cross-talk between Hsp90 and STAT3 is therefore important as this new information may provide new therapeutic approaches for these and other pathological conditions

ACKNOWLEDGEMENTS

We thank Dr. T. Hirano, Dr. J. N. Ihle for their kind gifts of reagents. We also thank Dr. J. Akiyama for encouraging our work.

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FIGURE LEGENDS**Fig. 1. GA inhibits IL-6-induced gene expression.**

Effect of C/EBP δ expression by IL-6 and/or GA in Hep3B cells. Northern blot analysis of 20 μ g of total RNA from Hep3B cells treated with IL-6 (100 ng/ml) and/or GA (1 μ M) for 3 or 6 h. The fold induction of C/EBP δ expression was shown as the densitometric intensity. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA is included as a loading control (lower panel).

Fig. 2. Cross-talk between STAT3 and Hsp90 in 293T cells.

(A) 293T cells were transfected with 1 μ g of reporter constructs STAT3-LUC. 48 h after transfection, cells were stimulated for an additional 12h with or without IL-6 (100ng/ml) and sIL-6R α (100ng/ml) and/or GA as indicated, and LUC activities were determined. The results are presented as the fold induction of luciferase activity from triplicate experiments, and the error bars represent the standard deviations.

(B) 293T cells were transfected with STAT3-LUC (1 μ g) and/or STAT3-C expression construct (1 μ g) or the empty vector (1 μ g). 48 h after transfection, cells were stimulated for 12h with or without GA as indicated. Cells were harvested and relative luciferase activities were measured. The results are presented as fold induction of luciferase activity from triplicate experiments, and the error bars represent the standard deviations.

(C) 293T cells were transfected with STAT3-LUC (1 μ g) together with the increasing amounts of Hsp90 expression vector. 48 h after transfection, cells were stimulated for 12h with or without GA as indicated. Cells were harvested and relative luciferase activities were

measured. The results are presented as fold induction of luciferase activity from triplicate experiments, and the error bars represent the standard deviations.

Fig. 3. STAT3 and Hsp90 physically interact in 293T cells.

(A) 293T cells (1×10^7) were transfected with Myc-tagged STAT3 (7.5 μg) together with FLAG-tagged Hsp90 (10 μg) in the presence or absence of Jak1 (1 μg). 48 h after transfection, cells were lysed and immunoprecipitated with an anti-FLAG antibody, and immunoblotted with an anti-Myc antibody (upper panel) or an anti-FLAG antibody (lower panel).

(B) 293T cells (1×10^7) were transfected with Myc-tagged STAT3 (7.5 μg) together with or without FLAG-tagged Hsp90 (10 μg) in the presence of Jak1 (1 μg). 48 h after transfection, cells were treated with DMSO or GA for 6 h and lysed, immunoprecipitated with an anti-FLAG antibody, and immunoblotted with an anti-Myc antibody (upper panel) or an anti-FLAG antibody (lower panel).

(C) Domain structure of Hsp90 and mutant fragments are schematically shown.

Mapping the STAT3 interaction domain of Hsp90. 293T cells (1×10^7) were transfected with FLAG-tagged Hsp90(1–733) or Hsp90(218–733), Hsp90(405–733), Hsp90(578–733) (10 μg) and Myc-tagged STAT3 (7.5 μg), together with Jak1 (1 μg). 48 h after transfection, cells were lysed and immunoprecipitated with an anti-Myc antibody, and immunoblotted with anti-FLAG antibody (upper panel) or anti-Myc antibody (middle panel). Total cell lysates (20 μg) were blotted with anti-FLAG antibody (lower panel). The asterisks indicate the migration position of the Hsp90 deletion mutants.

(D) Domain structure of STAT3 and mutant fragments are schematically shown. Mapping the Hsp90 interaction domain of STAT3. 293T cells (1×10^7) were transfected with STAT3

(1–137) or STAT3 (138–319), STAT3 (320–493), STAT3 (494–750) (10 μ g) and FLAG-tagged Hsp90 (7.5 μ g). 48 h after transfection, cells were lysed and immunoprecipitated with an anti-FLAG antibody, and immunoblotted with anti-Myc antibody (upper panel) or anti-FLAG antibody (middle panel). Total cell lysates (20 μ g) were blotted with anti-Myc antibody (lower panel). The asterisks indicate the migration position of the STAT3 deletion mutants.

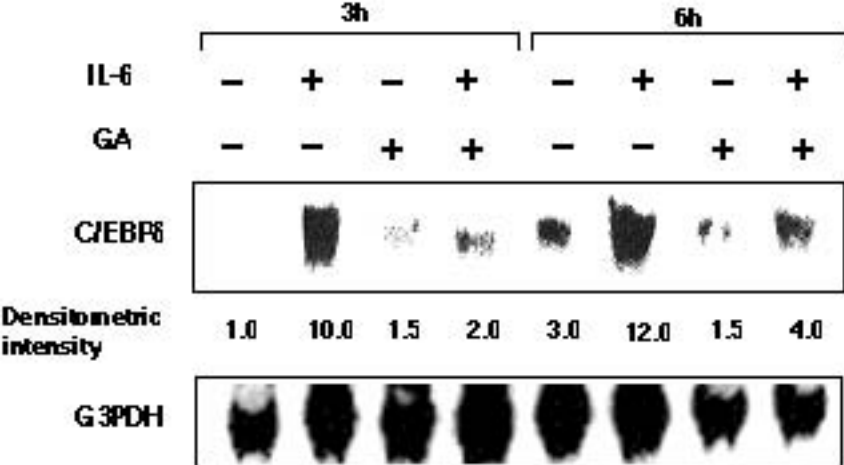
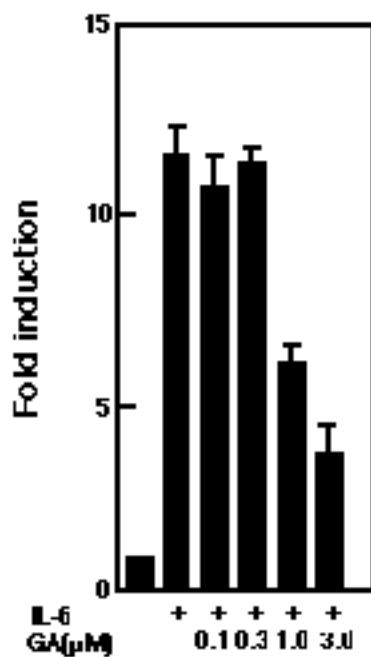
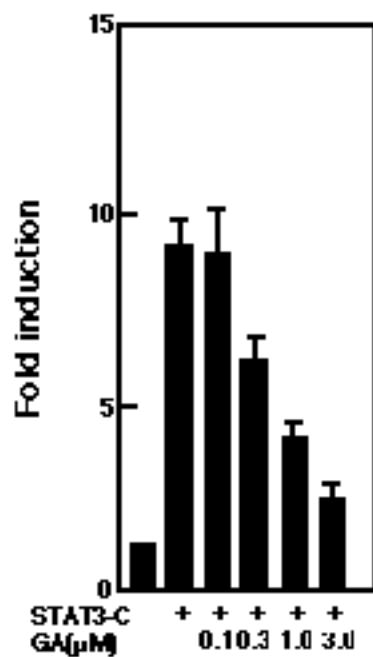
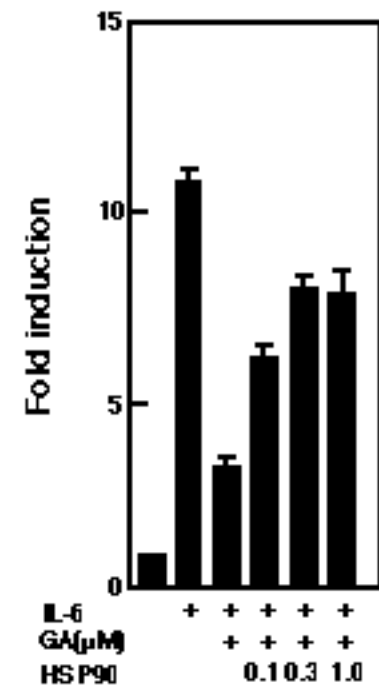


Fig. 1

A**B****C****Fig. 2**

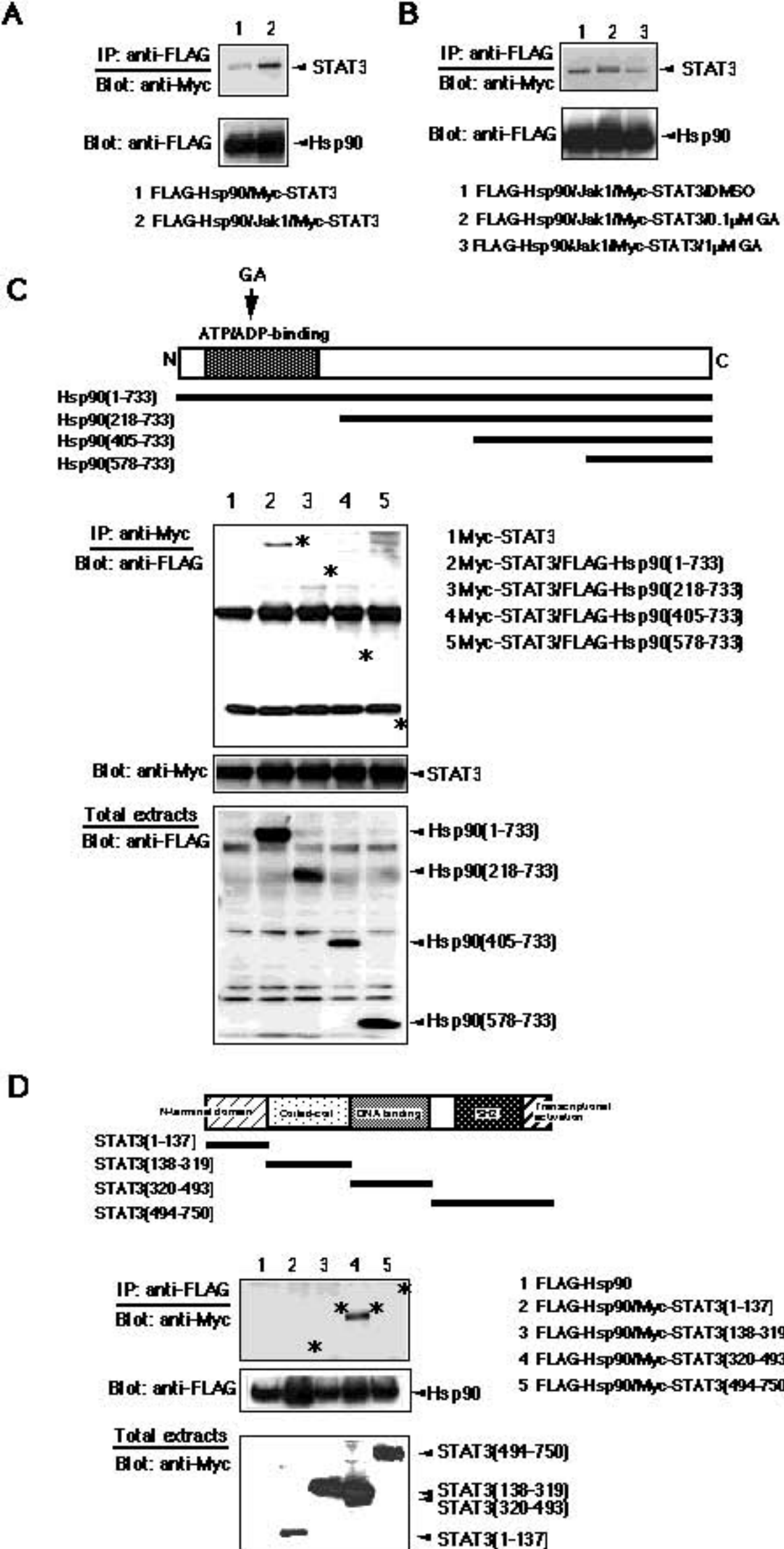


Fig. 3