Title: **Molecular interactions between STAT3 and protein inhibitor of activated STAT3, and androgen receptor**

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Running title: Interactions between STAT3, PIAS3 and AR

ABSTRACT

STAT3 mainly acts as a signal transducer of IL-6 family cytokines and transcriptionally activates specific target genes. The recently discovered protein Protein inhibitor of activated STAT3 (PIAS3) binds directly to STAT3 and blocks transcriptional activation. In our previous report, we demonstrated that PIAS3 directly interacted with androgen receptor (AR) and affected AR-mediated gene activation. Furthermore, we also showed that AR associated with STAT3 and enhanced its activity. Here we examined molecular interactions between STAT3, PIAS3 and AR to underline the mechanism how they regulate each other. AR activation overcame the inhibitory effect on STAT3-mediated transcription by PIAS3. Co-immunoprecipitation experiments revealed that an active form of AR relieved STAT3 from STAT3-PIAS3 complex formation. These results indicate that AR and PIAS3 regulate the STAT3-mediated transcriptional activity by their physical protein-protein competition on STAT3.

Key words: STAT3, protein inhibitor of activated STAT3 (PIAS3), androgen receptor (AR), transcription

INTRODUCTION

One member of the STAT family of proteins is STAT3, which is mainly activated by IL-6 family of cytokines including Leukemia inhibitory factor (LIF) and Leptin (1, 2). Like other members of the STAT family, STAT3 is tyrosine-phosphorylated by Jak kinases, then forms a dimer and translocates into the nucleus to activate target genes (3, 4). It has been shown that the activated STAT3 can mediate cellular transformation (5, 6, 7) and is found in numerous cancers, including prostate (7). Furthermore, STAT3 has been recently shown to act as an oncoprotein (5).

Protein inhibitor of activated STAT3 (PIAS3) was originally identified as a specific inhibitor of signal transducer and activator of transcription 3 (STAT3) (8, 9). PIAS3 binds to STAT3 and inhibits its DNA-binding activity and thereby interferes with STAT3-mediated gene activation. Recently, PIAS3 has been also shown to function as a transcriptional cofactor for AR (10, 11).

Androgen receptor (AR) mediates the effects of androgens and plays a central role in prostate cancer progression (12). In the beginning stages of the disease, the growth of prostate cancer is dependent on androgens. This is the basis for androgen ablation therapy which results in the involution of the tumor (13). However, in most cases, it progresses to an androgen-independent phenotype at which time there is no curative therapy available (14). AR activates transcription through interaction with androgen response elements (AREs) that are in the vicinity of the androgen responsive genes, such as prostate specific antigen (PSA) and probasin (PB) (12). Recent studies have documented that IL-6 activates AR-mediated gene expression through STAT3 (15, 16, 17).

In our previous studies, we demonstrated that the cross-talk between IL-6 and AR signaling occurs by direct physical and functional interactions between STAT3 and AR in prostate cancer cells (17). We also showed that PIAS3 directly interacts with AR and modify the transcriptional activity of AR (11).

In this study, we report that AR activation overcomes the inhibitory effect on STAT3-mediated transcription by PIAS3 and an active form of AR relieves STAT3 from STAT3-PIAS3 complex formation by their physical protein-protein competition.

MATERIALS AND METHODS

Reagents and antibodies.

Human recombinant LIF was purchased from INTERGEN (Purchase, NY). Expression vectors, HA-tagged STAT3 or Jak1, 2xARE-LUC (18), AR(1-714) (18), STAT3-LUC (19) STAT3-C(5) and FLAG-tagged PIAS3 (8), were kindly provided by Dr. J. N. Ihle (St. Jude SRH, Memphis, TN), Dr. F. Saatcioglu (University of Oslo, Norway), Dr. T. Hirano (Osaka Univ., Osaka, Japan), Dr. J. F. Bromberg (Rockefeller Univ., New York, NY) and Dr. K. Shuai (UCLA), respectively. Myc-tagged STAT3 mutants were described previously (20). Anti-AR and anti-HA antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz). Anti-FLAG M2 antibody was purchased from Sigma (St Louis, MO).

Cell culture, transfections, and luciferase assays.

Human embryonic kidney carcinoma cell line, 293T cells were transfected in DMEM containing 1% FCS by the standard calcium precipitation protocol. The cells were harvested 48 h after transfection and lysed in 200 μl 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.

Immunoprecipitation and immunoblotting.

Immunoprecipitation and Western blotting were performed as described previously (21). Briefly, the transfected 293T cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 0.3 M

NaCl, containing 1% NP-40, 1mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride and 10 μg/ml each of aprotinin, pepstatin and leupeptin). Cell lysates were immunoprecipitated with each antibody as indicated. The immunoprecipitates from cell lysates were resolved on 5-20% SDS-PAGE and transferred to Immobilon membrane (Millipore, Bedford, MA). The membranes were then probed with each antibody as indicated. Immunoreactive proteins were visualized using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech).

RESULTS AND DISCUSSION

Transcriptional cross-talk between STAT3 and PIAS3, AR

We previously demonstrated that STAT3 functionally interacts with AR in prostate cancer cells (17) and a STAT3 inhibitor, PIAS3 directly interacts with AR and modify the transcriptional activity of AR (11). However, molecular interactions between STAT3, PIAS3 and AR were still unknown. To delineate the mechanism how they regulate each other, we first access the transcriptional cross-talk between STAT3, PIAS and AR. To examine whether AR has any effect on the transcriptional inhibitory activity of PIAS3 on STAT3, we carried out transient transfection assays. STAT3-LUC, in which the α2-macroglobulin promoter drives expression of the LUC gene (19) was transfected into 293T cells in the presence of increasing amounts of PIAS3, cells were stimulated with LIF, and LUC activities were determined. As shown in Fig.1A, LIF-induced STAT3 activation was inhibited by the expression of PIAS3 in a dose-dependent manner, the result being in accordance with that previously reported (8). When we expressed the increasing amounts of an active form of AR, AR (1-714) (18) in the presence of PIAS3 in 293T cells and stimulated with LIF (Fig. 1A), the inhibitory effect of PIAS3 on STAT3-mediated transcriptional activation was reversed by the expression of an active form of AR. To avoid the activation of other signaling pathways by LIF stimulation, we also used a constitutively active form of STAT3, STAT3-C (5). 293T cells were transfected with STAT3-LUC together with expression vectors for STAT3-C and/or the increasing amounts of PIAS3, and the LUC activities were measured. PIAS3 expression suppressed STAT-LUC activity induced by STAT3-C (Fig, 1B). Furthermore,

when we co-expressed the increasing amounts of AR(1-714) together with STAT3-C and PIAS3, the similar results as described above were obtained as shown in Fig. 1B.

Conversely, to assess whether STAT3 activation has an effect on the cross-talk between PIAS3 and AR, we examined the effect of STAT3 activation on the PIAS3-modified AR activation in 293T cells. AR activity was monitored by 2xARE-LUC reporter in which two copies of an androgen response element (ARE) drive expression of the LUC gene (18). As shown in Fig.1C, PIAS3 enhanced AR-induced 2xARE-LUC activation in a dose-dependent manner in 293T cells. The enhanced AR activation by PIAS3 was slightly suppressed by the expression of the increasing amounts of STAT3-C (5).

These results indicate that AR can reverse the inhibitory effect of PIAS3 on STAT3-mediated transcriptional activation, but STAT3 has a few effect on the transcriptional cross-talk between PIAS3 and AR.

Mapping of PIAS3 or AR interacting domain on STAT3

In our previous reports, we demonstrated that either STAT3 or PIAS3 directly interacts with AR and an intact N-terminus is required for AR to interact with either STAT3 or PIAS3 (11, 17). These results also suggested that AR interact with either STAT3 or PIAS3 in the similar region. We then examined the interacting domains on STAT3 with AR or PIAS3. To delineate the domains in the STAT3 that mediate the protein-protein interaction with AR or PIAS3, co-immunoprecipitation experiments were performed with a series of mutant STAT3 proteins. Expression vectors encoding an active form of AR(1-714) or FLAG-tagged PIAS3 and a series of Myc-tagged STAT3 mutants were transiently transfected into 293T cells. Cells were lysed, and subjected to immunoprecipitation with an AR or an anti-FLAG

antibody. Immunoprecipitates were then used in Western blot analysis with an anti-Myc antibody. As shown in Fig. 2B, both the DNA binding domain/STAT3(320-493) and the C-terminal region/STAT3(494-750) of STAT3 interacted with AR(1-714). The N-terminal domain/STAT3(1-137) or the coiled-coil domain/STAT3(138-319) did not interact with AR(1-714). Interestingly, PIAS3 also interacted with the similar regions of STAT3 (Fig. 2C).

These results indicate that either AR or PIAS3 interacts with STAT3 via a large C-terminal region of STAT3 including the DNA binding domain.

Protein-protein interactions between STAT3 and PIAS3, AR

To further delineate the molecular mechanisms how AR can overcome the STAT3-mediated transcriptional activation by PIAS3 block, we performed co-immunoprecipitation experiments by introducing these three expression vectors into 293T cells. We also introduced Jak1 expression vector together with them to activate STAT3. Expression vectors encoding FLAG-tagged PIAS3 and HA-tagged STAT3 together with Jak1 were transiently transfected into 293T cells. Cells were lysed, and subjected to immunoprecipitation with an anti-HA antibody. Immunoprecipitates were then used in Western blot analysis with an anti-FLAG antibody. As shown in Fig. 3, STAT3 interacted with PIAS3 strongly. Interestingly, when we expressed both HA-tagged STAT3 and FLAG-tagged PIAS3, Jak1 together with the increasing amounts of an active form of AR (1-714), the STAT3-bond PIAS1 proteins remarkably decreased in a dose-dependent manner.

These results strongly suggested AR can relieve STAT3-mediated transcriptional transactivation from PIAS3 block by their physical protein-protein competition.

Conclusions

In this report, we have shown that AR activation overcomes the inhibitory effect on STAT3-mediated transcription by PIAS3. Either AR or PIAS3 interacted with STAT3 in the similar regions of STAT3 molecule. An active form of AR relieved STAT3 from STAT3-PIAS3 complex formation by their physical protein-protein competition.

Recently, a similar observation has been reported (22). A zinc finger protein, Gfi-1, which is to act as a dominant oncogene and cooperates in the process of lymphomagenesis with Myc and Pim-1(23), was identified as a potential binding partner for PIAS3 using a yeast two-hybrid screening (22). It was also shown that Gfi-1 interacts with PIAS3 and affects its function, as it is able to relieve the inhibitory effect of PIAS3 on STAT3 activity (22), suggesting that the oncogenic potential of Gfi-1 may be at least in part mediated by an enhancement of STAT3-triggered events. Thus, Gfi-1 in the lymphoma development might be an analogue with AR in the progression of prostate cancer.

Prostate cancer frequently progresses from an initial androgen dependence to androgen independence, rendering the only effective androgen ablation therapy useless (13, 14). The mechanism underlying the androgen-independent progression is unknown. Recent studies have shown that STAT3 may play a role in the progression of prostate cancer (24, 25). The levels of activated STAT3 are associated with the progression of androgen-independent prostate cancer (24). Activation of STAT3 in androgen-sensitive LNCaP prostate cancer cells results in enhancement of tumor growth in both intact and castrated male nude mice and

enhances AR-mediated transcription (26).

In this study, we present experimental evidence that supports the hypothesis of a biochemical and functional interaction between the STAT3, PIAS3 and AR. Understanding the more details of the interactions between STAT3, PIAS3 and AR would be important since this may provide new mechanisms that can be the basis for new drug development for prostate cancer.

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REFERENCES

1. Akira, S., Nishio, Y., Inoue, M., Wang, X. J., Wei, S., Matsusaka, T., Yoshida, K., Sudo, M., Naruto, M., and Kishimoto, T. (1994) Molecular cloning of APRF, a novel IFN-stimulated gene factor 3 p91-related transcription factor involved in the gp130-mediated signaling pathway. *Cell* **77**, 63-71

2. Kishimoto, T., Taga, T., and Akira, S. (1994) Cytokine signal transduction. *Cell* **76**, 253-262

3. Ihle, J. N. (1995) Cytokine receptor signalling. *Nature* **377**, 591-594

4. Darnell, J. E., Kerr, I. M., and Stark, G. R. (1994) Jak-Stat pathway and transcriptional activation in response to IFNs and other extracellular signaling proteins. *Science* **264**, 1415-1421

5. Bromberg, J. F., and Darnell, J. E., Jr. (2000) The role of STATs in transcriptional control and their impact on cellular function. *Oncogene* **19**, 2468-2473

6. Ram, P. T., Horvath, C. M., and Iyengar, R.(2000) Stat3-mediated transformation of NIH-3T3 cells by the constitutively active Q205L Gαo protein. *Science* **287**, 142-144

7. Jove, R. (2000). STAT signaling. *Oncogene* **19**, 2466-2467

8.Chung, C. D., Liao, J., Liu, B., Rao, X., Jay, P., Berta, P., and Shuai, K. (1997). Specific inhibition of STAT3 signal transduction by PIAS3. *Science* **278**, 1803-1805

9. Shuai,K. (2000) Modulation of STAT signaling by STAT-interacting proteins. *Oncogene***19**, 2638-2644

10. Gross, M., Liu, B., Tan, J., French, F. S., Carey, M., and Shuai, K. (2001) Distinct effects of PIAS proteins on androgen-mediated gene activation in prostate cancer cells. *Oncogene* **20**, 3880-2887

11. Junicho, A, T. Matsuda, T. Yamamoto, H. Kishi , K. Korkmaz, F. Saatcioglu, H. Fuse and A. Muraguchi. (2000) Protein inhibitor of activated STAT3 regulates androgen receptor signaling in prostate carcinoma cells. *Biochem. Biophys. Res. Commun.* **278**, 9-13

12. Roy A. K., Lavrovsky, Y., Song, C. S., Chen, S., Jung, M. H., Velu N. K., Bi, B. Y., Chatterjee, B. (1999) Regulation of androgen action. *Vitam Horm,* **55**, 309-352

13. Huggins C., and Hodges C. V. (1941) Studies on prostatic cancer; effect of castration of estrogen and of androgen injection on serum phosphatases in metastatic carcinoma of the prostate. *Cancer Res.* **1**, 293-297

14. Crawford, E. D., Rosenblum, M., Ziada, A. M., and Lange, P. H. (1999) Hormone refractory prostate cancer. *Urology* **54**, 1-7

15. Hobisch, A., Eder, I. E., Putz, T., Horninger, W., Bartsch, G., Klocker, H., and Culig, Z. (1998) Interleukin-6 regulates prostate-specific protein expression in prostate carcinoma cells by activation of the androgen receptor. *Cancer Res.* **58**, 4640-4645

16. Chen, T., Wang, L. H., and Farrar, W. L. (2000) Interleukin 6 activates androgen receptor-mediated gene expression through a signal transducer and activator of transcription 3-dependent pathway in LNCaP prostate cancer cells. *Cancer Res.* **60**, 2132-2135

17. Matsuda, T., Junicho, A, Yamamoto, T., Kishi, H., Korkmaz, K., Saatcioglu, F., Fuse, H., and Muraguchi, A. (2001) Cross-talk between signal transducer and activator of transcription 3 and androgen receptor signaling in prostate carcinoma cells. *Biochem. Biophys. Res. Commun.* **283,** 179-187

18. Frϕnsdal, K., Engedal, N., Slagsvold, T., and Saatcioglu, F. (1998). CREB binding protein is a coactivator for the androgen receptor and mediates cross-talk with AP-1. *J. Biol. Chem.* **273**, 31853-31859

19. Nakajima, K., Yamanaka, Y., Nakae, K., Kojima, H., Ichiba, M., Kiuchi, N., Kitaoka, T., Fukada, T., Hibi, M., and Hirano, T. (1996). A central role for Stat3 in IL-6-induced regulation of growth and differentiation in M1 leukemia cells. *EMBO J.* **15**, 3651-3658

20. Yamamoto, T., Sekine, Y., Kashima, K., Kubota, A., Sato, N., Aoki, N., and Matsuda, T. (2002) The nuclear isoform of protein tyrosine phosphatase TC-PTP regulates

Interleukin-6-mediated signaling pathway through STAT3 dephosphorylation. *Biochem. Biophys. Res. Commun.* **297**, 811-817

21. Matsuda, T., Yamamoto, T., Kishi, H., Yoshimura, A, and Muraguchi, A.(2000). SOCS-1 can suppress CD3ζ- and Syk-mediated NF-AT activation in a non-lymphoid cell line. *FEBS Lett.* **472**, 235-240

22. Rodel, B., Tavassoli, K., Karsunky, H., Schmidt, T., Bachmann, M., Schaper, F., Heinrich, P., Shuai, K., Elsasser, H. P., and Moroy, T. (2000) The zinc finger protein Gfi-1 can enhance STAT3 signaling by interacting with the STAT3 inhibitor PIAS3. *EMBO J.* **19,** 5845-5855.

23. Schmidt, T., Karsunky, H., Gau, E., Zevnik, B., Elsasser, H. P. and Moroy, T. (1998) Zinc finger protein Gfi-1 has low oncogenic potential but cooperates strongly with pim and myc genes in T-cell lymphomagenesis. *Oncogene*, **17**, 2661-2667.

24. Dhir, R., Ni, Z., Lou, W., DeMiguel, F., Grandis, J. R., and Gao, A. C.(2002) Stat3 activation in prostatic carcinomas. *Prostate* **51**,241-246.

25.Mora, L. B., Buettner, R , Seigne, J., Diaz, J., Ahmad, N., Garcia, R., Bowman, T., Falcone, R., Fairclough, R., Cantor, A., Muro-Cacho, C., Livingston, S., Karras, J., Pow-Sang, J., and Jove, R. (2002) Constitutive activation of Stat3 in human prostate tumors and cell lines: direct inhibition of Stat3 signaling induces apoptosis of prostate cancer cells. *Cancer Res.* **62**, 6659-6666.

26. DeMiguel, F., Lee, S. O., Lou, W., Xiao, X., Pflug, B. R., Nelson, J. B., and Gao, A. C. (2002) Stat3 enhances the growth of LNCaP human prostate cancer cells in intact and castrated male nude mice. *Prostate* 52, 123-129

FIGURE LEGENDS

Fig 1. **Transcriptional cross-talk between STAT3 and PIAS3, AR.**

(A) 293T cells in 6-well plate were transfected with STAT3-LUC (1.0μg) together with PIAS3 expression construct (0.3 or 1.0μg), and the increasing amounts of AR(1-714) (0.1-1.0μg), 48 h after transfection, cells were stimulated for 12h with LIF (100ng/ml), 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

(B) 293T cells in 6-well plate were transfected with STAT3-LUC (1.0μg) together with STAT3-C (1.0μg), PIAS3 expression construct (0.3 or 1.0μg), and the increasing amounts of AR(1-714) (0.1-1.0μg), 48 h after transfection, 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 in 6-well plate were transfected with or 2xARE-LUC (1.0μg) together with AR(1-714) expression construct (0.1-1.0μg), and the increasing amounts of PIAS3 (0.1-1.0μg) and/or and the increasing amounts of AR(1-714) (0.1-1.0μg), 48 h after transfection, 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. 2. **Mapping of PIAS3 or AR interacting domain on STAT3.**

(A) Domain structure of STAT3 and mutant fragments are schematically shown.

(B) Mapping the AR(1-714) interaction domain of STAT3. 293T cells ($1x10^7$) were transfected with STAT3 (1–137) or STAT3 (138–319), STAT3 (320–493), STAT3 (494–750) (10 μg) and AR(1-714) (7.5μg). 48 h after transfection, cells were lysed and immunoprecipitated with an anti-AR antibody, and immunoblotted with anti-Myc antibody (upper panel) or anti-AR antibody (middle panel). Total cell lysates (20μg) were blotted with anti-Myc antibody (lower panel).

(C) Mapping the PIAS3 interaction domain of STAT3. 293T cells ($1x10^7$) were transfected with STAT3 (1–137) or STAT3 (138–319), STAT3 (320–493), STAT3 (494–750) (10 μg) and FLAG-tagged PIAS3 (7.5μg). 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 (20μg) were blotted with anti-Myc antibody (lower panel). The asterisks indicate the migration position of the STAT3 deletion mutants.

Fig. 3. **Protein-protein interactions between STAT3 and PIAS3, AR**.

293T cells ($1x10^7$) were transfected with HA-tagged STAT3 (7.5μg) together with FLAG-tagged PIAS3 (7.5μg) and/or the increasing amounts of AR(1-714) (1.0 or 5.0 μg) in the presence of Jak1(1.0μg). 48 h after transfection, cells were lysed and immunoprecipitated with an anti-HA antibody, and immunoblotted with anti-FLAG antibody (upper panel), anti-AR antibody (middle panel) or anti-HA antibody (lower panel). Total cell lysates (20μg) were blotted with either anti-Myc antibody or anti-AR antibody.

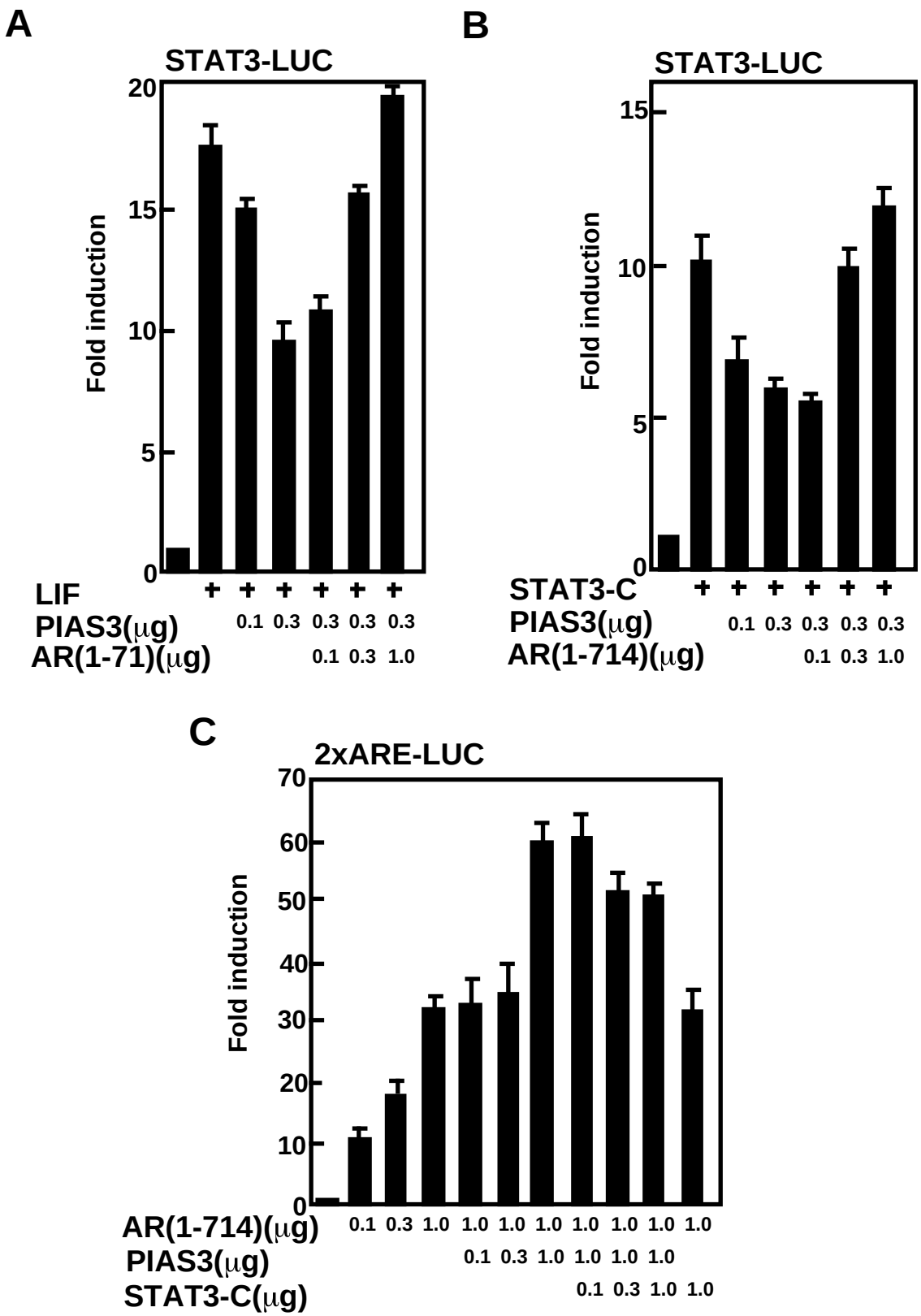


Fig. 1 Yamamoto, T. et al.

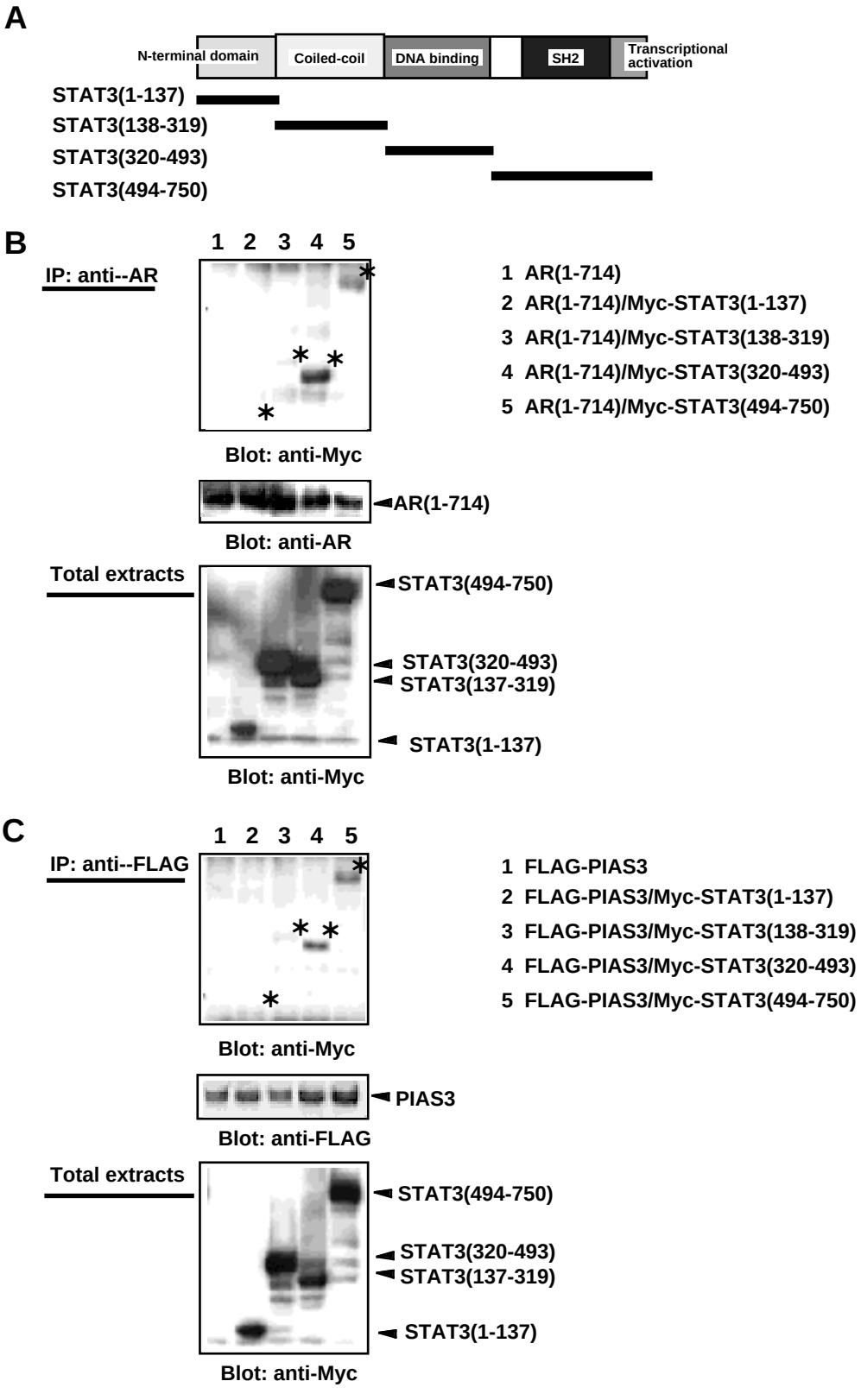
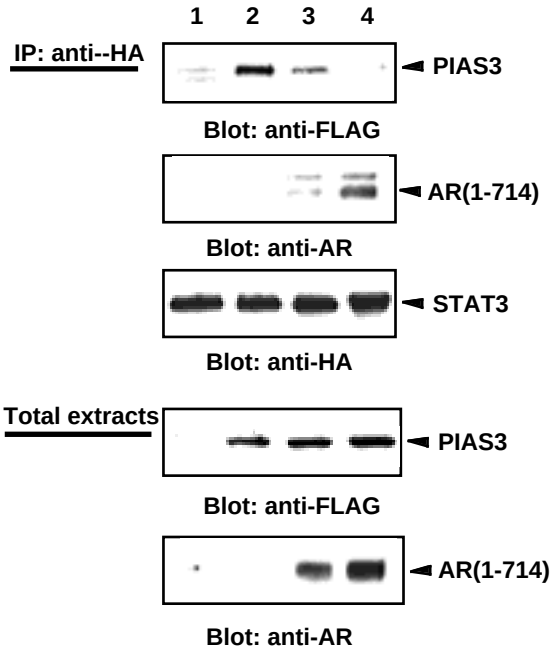


Fig. 2 Yamamoto, T. et al.



1 HA-STAT3/Jak1

2 HA-STAT3/Jak1/FLAG-PIAS3

3 HA-STAT3/Jak1/FLAG-PIAS3/AR(1-714) 1μg

4 HA-STAT3/Jak1/FLAG-PIAS3/AR(1-714) 5μg

Fig. 3 Yamamoto, T. et al.