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Protein Inhibitor of Activated STAT3 Regulates Androgen Receptor Signaling in Prostate Carcinoma Cells

Akira Junicho,*[,1] Tadashi Matsuda,†[,1,2] Tetsuya Yamamoto,† Hiroyuki Kishi,† Kemal Korkmaz,‡ Fahri Saatcioglu,‡ Hideki Fuse,* and Atsushi Muraguchi†

†*Department of Immunology and* **Department of Urology, Faculty of Medicine, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930-0194, Japan; and* ‡*Biotechnology Centre of Oslo, University of Oslo, Gaustadalleen 21, 0349 Oslo, Norway*

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Protein inhibitor of activated STAT3 (PIAS3) is a specific inhibitor of signal transducer and activator of transcription 3 (STAT3). PIAS3 binds to STAT3 and inhibits its DNA-binding activity, and thereby STAT3-mediated gene activation. PIAS1, another member of the PIAS family, was recently shown to interact with the androgen receptor (AR), a nuclear hormone receptor that has an important role in both physiological and pathological processes, and acts as a cofactor for AR. Here we demonstrate that PIAS3 is expressed in prostate cancer cells and its expression is induced in response to dihydrotestosterone (DHT) treatment. Ectopic overexpression of PIAS3 suppressed AR-mediated gene activation induced by DHT-stimulation in LNCaP cells. We provide evidence that these activities were due to direct physical interactions between PIAS3 and AR. These results indicate that PIAS3 acts as a coregulator of AR signaling pathway in prostate cancer cells. © 2000 Academic Press

Key Words: **protein inhibitor of activated STAT3 (PIAS3); androgen receptor (AR); cofactor.**

Protein inhibitor of activated STAT3 (PIAS3) was originally identified as a specific inhibitor of signal transducer and activator of transcription 3 (STAT3). STAT3 is mainly activated by IL-6 family of cytokines, epidermal growth factor, and leptin (1–4). Like other members of the STAT family, STAT3 is tyrosine-phosphorylated by Jak kinases, upon which it forms a dimer and translocates into the nucleus to activate target genes (2, 3). PIAS3 binds to STAT3 and inhibits its DNA-binding activity and thereby interferes with STAT3-mediated gene activation. Although STAT1 is also phosphorylated and activated by IL-6, PIAS3 does not bind to or alter the function of STAT1 (5, 6). PIAS1, another member of PIAS family, was originally identified as an inhibitor of STAT1 (7). PIAS1 was recently identified as an androgen receptor (AR) interacting protein isolated from a HeLa cell cDNA library by the yeast two hybrid assay (8); PIAS functions as a transcriptional coactivator for AR and the glucocorticoid receptor (GR), but serves as a corepressor for the progesterone receptor (PR) (8).

Other coregulators of AR, a member of the nuclear receptor superfamily, along with GR and PR, have been identified (9–12). Recently, a testis-specific AR coregulator, AR interacting protein 3 (ARIP3), was identified that interacted with the DNA binding domain of AR and modulated AR-dependent transcription. Interestingly, ARIP3 is highly homologous to PIAS3 and Gu/RH-II binding protein (GBP) (12), raising a possibility that this new family of proteins may widely be involved in the regulation of AR function.

In this study, we report that PIAS3 expression is induced by androgens in prostate cancer cells. Furthermore, we provide an evidence that PIAS3 acts as a coregulator of AR in prostate cancer cells.

MATERIALS AND METHODS

Reagents and antibodies. Dihydrotestosterone (DHT) was purchased from Wako Chemicals (Osaka, Japan). Human recombinant LIF was purchased from INTERGEN (Purchase, NY). STAT3-LUC (14), −285PB-LUC (15), and FLAG-tagged PIAS3 (5) were kindly provided by Dr. T. Hirano (Osaka Univ., Osaka, Japan), Dr. J. Palvimo (University of Helsinki, Finland), and Dr. Ke Shuai (UCLA), respectively. C-terminal deletion mutants of AR have previously been described (16). Anti-PIAS3 and anti-AR antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-FLAG M2 antiserum was purchased from Upstate Biotechnology (Lake Placid, NY).

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[1] These authors contributed equally to this work.

[2] To whom correspondence should be addressed. Fax: 81-76-434-5019. E-mail: tmatsuda@ms.toyama-mpu.ac.jp.

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Cell culture, transfections, and luciferase assays. Human prostate carcinoma cell line LNCaP was maintained as described (17). Before stimulation, the cells were maintained for 24 h in RPMI 1640 containing 2% TCM (ICN) followed by treatment with DHT. LNCaP cells (2–2.5 $\times$ 10^5 in a 6-cm dish) were transfected by using LipoTAXI (Stratagene) following the manufacturer's instructions. 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. Since 293T cell line is a very useful cell lines for transfections of various plasmid DNAs with extremely high transfection efficiency, we used 293T cells for a detection of PIAS3 protein (as a size marker of PIAS3) in Fig. 1A, for testing whether PIAS3 inhibits LIF-induced STAT3 activation in Fig. 2A, and finally for verifying the physical interaction of AR and STAT3 in Fig. 3.

Northern analysis. LNCaP cells were maintained as described above. After serum starvation, LNCaP cells (1×10^7) were treated with DHT (10^{-8} M) for 3 or 6 h. Total RNAs were prepared by using Iso-Gen (Nippon Gene) and used in Northern analysis according to the established procedures. A nylon membrane (GeneScreen, NEN Life Science Products) and ^{32}P-labelled cDNA probes, as indicated, were used.

Immunoprecipitation and immunoblotting. Immunoprecipitation and Western blotting were performed as described previously (18). Briefly, the transfected 293T cells were lysed in lysis buffer (50 mM Tris–HCl, pH 7.4, 0.3 M NaCl, containing 1% Triton X-100, 10% glycerol, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of aprotinin, pepstatin, and leupeptin). Cell lysates were immunoprecipitated with either anti-AR antibody (Santa Cruz) or anti-FLAG M2 antibody (Upstate Biotechnology). 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

PIAS3 Is Expressed and Induced in Response to Androgens in a Prostate Carcinoma Cell Line

Since one of AR cofactors ARIP3 was found to be highly similar to PIAS3 (13), we assessed the possibility that PIAS3 can also serve as a cofactor for AR. To that end, we first examined if PIAS3 is expressed in an AR-positive prostate cancer cell line, LNCaP. Western analysis on whole cell extracts of LNCaP with a PIAS3-specific antibody showed that PIAS3 was expressed in LNCaP cells at a low level and its expression is induced approximately twofold in response to dihydrotestosterone (DHT) treatment (Fig. 1A). The PIAS3 band in LNCaP was similar in migration compared with PIAS3 that was ectopically expressed in a human embryonic kidney carcinoma cell line, 293T.

In order to clarify if the DHT-induced increase in PIAS3 expression is mediated on the mRNA level in LNCaP cells, total RNA was prepared from LNCaP cells that had been incubated with or without DHT and

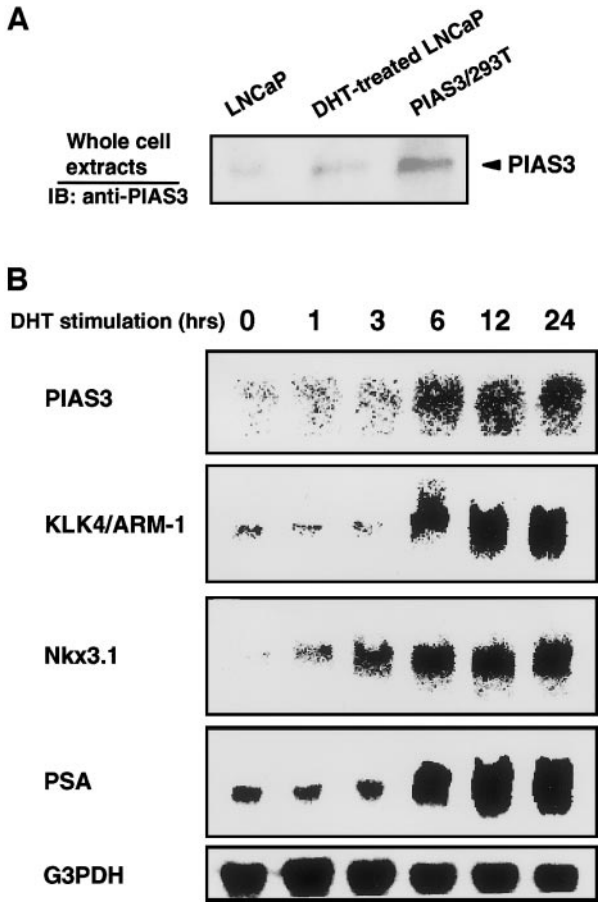

FIG. 1. Induction of PIAS3mRNA by DHT in LNCaP cells. Total cell lysates (20 μg) of LNCaP left untreated and treated with DHT(10^{-8} M) for 12 h were blotted with anti-PIAS3. Total lysates (20 μg) from PIAS3-transfected 293T cells were also blotted with anti-PIAS3. (A) Induction of PIAS (upper panel) and Nkx3.1, KLK4/ARM-1 and PSA (middle panel) mRNAs by DHT in LNCaP cells. Northern blot analysis of 20 μg of total RNA from LNCaP cells treated with DHT (10^{-8} M) for the indicated time. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA is included as a loading control (lower panel).

Northern analysis was performed. As shown in Fig. 1B, the level of PIAS3 mRNA expression increased 1.5-fold at 3 h, fivefold at 6 h, and did not alter until at least 24 h. The similar change of mRNA expression level after DHT-stimulation was observed in the previously characterized AR-responsive genes, such as tissue kallikrein 4 (KLK4/ARM-1) (19, 20), prostate-specific homeobox gene NKX3.1 (21), and prostate specific antigen (PSA) (17). These results indicate that PIAS3 is an androgen-inducible gene in a prostate cancer cell line, LNCaP.

PIAS3 Inhibits AR-Mediated Transcription

Recently, studies demonstrated that PIAS1, another member of PIAS family, directly binds to AR and enhanced its transcriptional activity (8). To examine

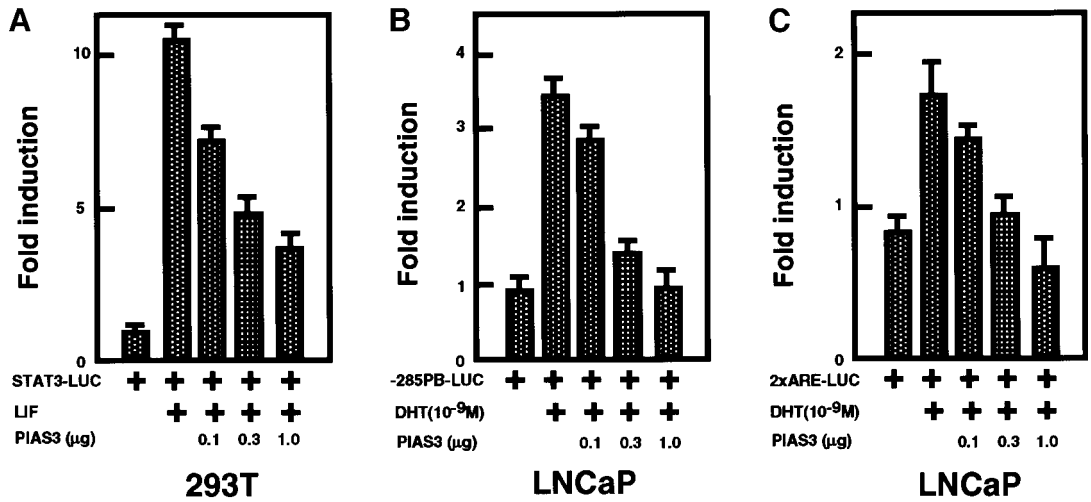

FIG. 2. PIAS3 regulates AR-dependent transcriptional activation by DHT in LNCaP cells. LNCaP cells in 6-cm dishes were transfected with or without −285PB-LUC (1 μg) reporter (1 μg) (A) or 2 × ARE-LUC (1 μg) reporter (1 μg) (B) together with either PIAS3 expression construct or the pCMV empty vector, and then stimulated with DHT as indicated. Forty-eight hours after transfection, cells were stimulated for an additional 12 h. 293T cells in 6-cm dishes were transfected with STAT3-LUC reporter (1 μg) (A) together with PIAS3 expression construct (1 μg) or the empty pCMV (1 μg), and then stimulated with LIF as indicated. Forty-eight hours after transfection, cells were harvested and relative luciferase activity was measured. The results are presented as the fold induction of luciferase activity from triplicate experiments, and the error bars represent the standard deviations.

whether PIAS3 has any effect on the transcriptional activity of AR, we carried out transient transfection assays.

First, we examined whether PIAS3 can inhibit Leukemia Inhibitory factor (LIF)-induced STAT3 activation in 293T cells. STAT3-LUC, in which the α2-macroglobulin promoter drives expression of the LUC gene (14) 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. 2A, 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 (5).

AR activity was monitored by −285PB-LUC in which a deletion derivative of the probasin promoter sequences drive expression of the LUC gene (15). In LNCaP cells, −285PB-LUC activity increased in a dose-dependent manner upon stimulation of DHT as expected. When we overexpressed PIAS3 in LNCaP cells, DHT-induced −285PB-LUC activation was inhibited in a dose-dependent manner (Fig. 2B).

To confirm this finding, the same experiment was performed with 2×ARE-LUC reporter in which two copies of an androgen response element (ARE) drive expression of the LUC gene (16). As shown in Fig. 2C, in accordance with the data obtained with −285PB-LUC, PIAS3 also inhibited DHT-induced 2×ARE-LUC activation in a dose-dependent manner. These results indicate that PIAS3 acts as negative regulator of AR in a prostate carcinoma cell line.

STAT3 and AR Physically Interact in Vivo

It was reported that PIAS3 inhibits STAT3 activation by a direct protein–protein interaction (5). To test a possibility that PIAS3 inhibits AR-induced gene activation in a similar protein–protein interaction fashion, we performed co-immunoprecipitation experiments. First, expression vectors encoding wild-type AR and FLAG-tagged PIAS3 were transiently transfected into 293T cells. Then the transfected cells were incubated with DHT and their cell lysate were immunoprecipitated with either an anti-AR monoclonal antibody or anti-FLAG antibody, followed by immunoblot analysis using the antibodies as indicated. As shown in Fig. 3A, AR and PIAS3 were found to be in a complex regardless of which one was immunoprecipitated first.

To delineate the domains in the AR that mediate the protein–protein interactions between PIAS3 and AR, coimmunoprecipitation experiments were performed with a series of mutant AR proteins. It was previously shown that the deletion mutants AR(1-566), AR(1-503), and AR(1-333) did not significantly stimulate −285PB-LUC, because they lack a functional DNA-binding domain, whereas wild-type AR and AR(1-714) are efficient activators of −285PB-LUC (16). As shown in Fig. 3B, when most of the ligand-binding domain or the DNA-binding domain was deleted, AR still interacted with PIAS3.

However, AR(1-333), which encodes only part of the N-terminal domain, did not interact with PIAS3. These data suggest that an intact N-terminus is required for AR to interact with PIAS3.

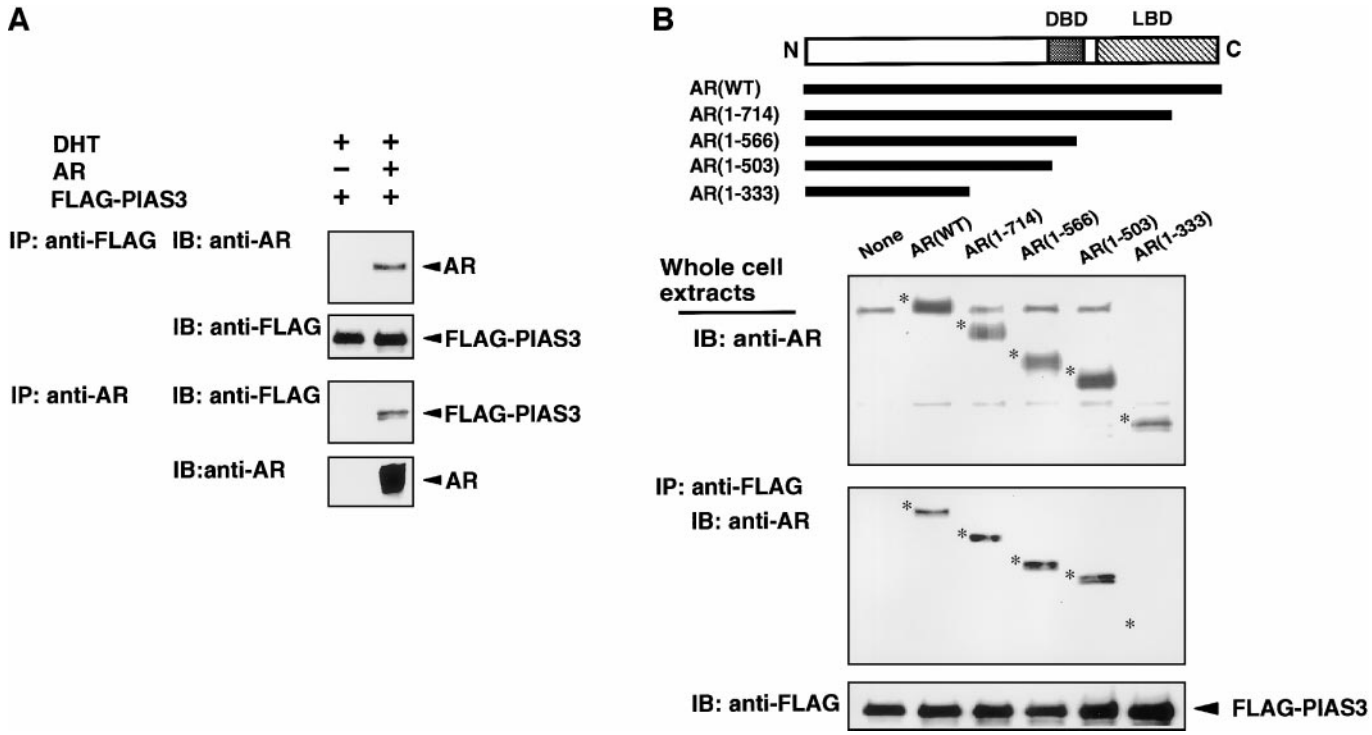

FIG. 3. PIAS3 and AR form a complex. (A) 293T cells were transfected with AR (10 μg) and FLAG-tagged PIAS3 (5 μg). Forty-eight hours after transfection, cells were treated with DHT (10^{-8} M) for 12 h. Cell lysates were then immunoprecipitated and immunoblotted with anti-FLAG M2 (upper panel) or anti-AR (lower panel). (B) Mapping the PIAS3 interaction domain of AR. The domain structure of AR and the location of mutant fragments are schematically shown. The DNA-binding domain (DBD) and the ligand-binding domain (LBD) are indicated. 293T cells were transfected with a series of AR mutants (10 μg) together with wild-type FLAG-PIAS3 (5 μg) by calcium precipitation method. Forty-eight hours after transfection, cells were treated with DHT (10^{-8} M) for 12 h. Cell lysates were then immunoprecipitated and immunoblotted with anti-FLAG M2. Total cell lysates (20 μg) (upper panel) and the immunoprecipites with anti-FLAG M2 were blotted with anti-AR (middle panel). The blot was stripped and reprobed with anti-FLAG M2 (lower panel). The asterisks indicate the migration position of the wild-type AR or of the deletion mutants.

PIAS1 is shown to be a transcriptional coactivator for AR and GR, but it was also reported to be a corepressor for PR shown by cotransfection assays using monkey kidney CV1 cells. Whether PIAS1 can act as a cofactor for these receptors in cells that express these receptors, such as prostate cancer cell line LNCaP for AR and HeLa cells for GR, was not determined.

Regarding the involvement of STAT3 in this system, our present data show that the ectopic expression of PIAS3 suppressed the DHT-induced gene activation by AR in LNCaP, and that there is a physical interaction of PIAS3 and AR when these two molecules were expressed in 293T. In these systems, STAT3 activation signal was not delivered from either JAKs or LIF. Thus, the involvement of activated STAT3 in the present study is not probable. However, this does not rule out a possibility that endogenous activated STAT3 in these cell lines is involved in inhibition of DHT-induced AR gene activation by PIAS3 as well as the interaction of AR and PIAS3. To prove this possibility, coimmunoprecipitation of STAT3 and AR using anti-STAT3 and anti-AR in STAT3- and AR-expressed 293T cells has been done, but we have no definite data showing that STAT3 and AR form a complex in this lysis buffer conditions, in which PIAS3 and AR form a complex (data not shown).

In this report, we demonstrated that PIAS3 binds to AR directly and inhibits its transcriptional activity in a transient transfection assay. Furthermore, PIAS3 mRNA accumulation was induced by androgen treatment in a prostate carcinoma cell line. Taken together, these results demonstrate that PIAS3 is an androgen-dependent gene and acts a negative regulator of AR signaling in a prostate cancer cell line. This is expected to form an auto-regulatory feedback loop where androgenic induction of PIAS3 would inhibit AR function. The possible physiological significance of this mechanism in androgen action, e.g., in prostate cancer cells, remains to be studied. Understanding the details of the interactions between 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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