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Journal of Cellular Biochemistry 112:818–828 (2011)

Journal of Cellular Biochemistry

Associations of Saposin C, Src, and Androgen Receptor Upregulate the Expression and Function of Androgen Receptor in Human Prostate Cancer Cells

Yan Ding,[1,2] Xiaoling Wang,[1,3] Aihui Xu,[1] Xia Xu,[1] Keli Tian,[1] Charles Y.F. Young,[4] and Huiqing Yuan[1*]

[1] *Department of Biochemistry and Molecular Biology, School of Medicine, Shandong University, Jinan 250012, China*
[2] *School of Life Science and Technology, Xi'an Jiaotong University, Xi'an 710049, China*
[3] *The Second Hospital of Shandong University, Jinan 250033, China*
[4] *Department of Urology, Mayo Clinical College of Medicine, Mayo Clinic, Rochester, Minnesota 55905*

ABSTRACT

We previously demonstrated that ectopic expression of neurotrophic peptide (NP) derived from saposin C promotes androgen receptor (AR) expression and transactivation in human prostate cancer cells. This prompted us to investigate how NP or saposin C can function in cells. We constructed plasmids expressing saposin C or a chimeric peptide of a viral TAT transduction domain and saposin C (TAT-saposin C) with His-tag. Intracellular localization of saposin C and NP was predominantly shown in transfected cells, while TAT-saposin C was detected around membrane and in cytosol by immunofluorescence staining. Furthermore, induction of the AR expression and activation of the AR transcriptional function were observed in cells transfected with saposin C or TAT-saposin C, compared to control cells transfected with an empty plasmid. The effects of saposin C and TAT-saposin C on AR activity were examined in the presence of inhibitors of GPCR, MAPK1/2, and PI3K/Akt. Interestingly, we found that these inhibitors only affect AR activities in cells with TAT-saposin C expression but not with saposin C expression. Immunostaining images showed that co-localization of saposin C, Src, and the AR occurred in transfected cells. Physical interactions of saposin C/NP, Src, and the AR were then demonstrated by co-immunoprecipitation assays. Blockage of Src activity by specific inhibitor led to a decrease in the saposin C-mediated enhancement of AR transactivity, suggesting that intracellular expression of saposin C caused stimulation of AR expression and activity by associations with Src in LNCaP cells. This effect may not be mediated by GPCR. J. Cell. Biochem. 112: 818–828, 2011.

KEY WORDS: SAPOSIN C; ANDROGEN RECEPTOR; SRC; PROSTATE CARCINOMA CELLS

Compelling evidence implicates that androgen-androgen receptor (AR) signaling plays a vital role in the initiation and progression of prostate cancer (PCa), because AR remains active in both androgen-dependent and androgen depletion-independent PCa [Roy-Burman et al., 2005]. Continued activation of the AR signaling in a castrate hormone environment could result from overexpression of AR, mutations in the AR gene, excessive production of AR coactivators, alterations in AR nongenomic signaling, and ligand-independent activation of the AR [Heinlein and Chang, 2002; Culig, 2004; Debes and Tindall, 2004; Chmelar et al., 2006; Kung and Evans, 2009]. It has been reported that growth factors such as insulin-like growth factor-I (IGF-I), epidermal growth factor (EGF) [Craft et al., 1999; Gregory et al., 2004], interleukin-6 (IL-6) [Ueda et al., 2002] activate the AR in the absence of ligand. Moreover, recent studies have shown that neurotrophic molecules including gastrin-releasing peptide ligand-independently upregulate AR expression and activity [Kung and Evans, 2009]. Therefore, it is not surprising that growth factors, cytokines,

Abbreviations: AR, androgen receptor; ARE, androgen responsive element; ERK, extracellular signal-regulated kinase; GPCR, G-protein coupled receptor; hK2, human glandular kallikrein; Mib, mibolerone; PSA, prostate-specific antigen.

Grant sponsor: The Scientific Technology Research Grant of the Ministry of Education of China; Grant number: 106101; Grant sponsor: Shandong Scientific Reward Funding Program; Grant number: 2006BS03006; Grant sponsor: Foundation for the Young Researcher Program of The Health Department of Shandong Province; Grant number: 2006J39.

*Correspondence to: Huiqing Yuan, Department of Biochemistry and Molecular Biology, School of Medicine, Shandong University, 44 Wenhua Xi Road, Jinan 250012, China. E-mail: lyuanhq@sdu.edu.cn

Received 16 February 2010; Accepted 17 November 2010 • DOI 10.1002/jcb.22977 • © 2010 Wiley-Liss, Inc.
Published online 6 December 2010 in Wiley Online Library (wileyonlinelibrary.com).

neurotrophic factors and other trophic agents are implicated in the activation of AR in addition to androgens in androgen ablation-resistant PCa. Although it is believed that aberrant activation of AR appears to be essential for outgrowth and survival of hormone-refractory PCa, it still requires a further step towards understanding of how AR is inappropriately activated and involved in progression of PCa.

Saposin C is one of the sphingolipid activator proteins involved in lysosomal hydrolysis of sphingolipids. Recently, saposin C and prosaptides are demonstrated to stimulate PCa cells growth, migration, and invasion via the activation of MAPK and PI3/AKT signal pathways [Koochekpour et al., 2004, 2005]. It has also been demonstrated that saposin C and neurotrophic domain of saposin C (NP) were able to upregulate AR expression and transactivation in a ligand-independent manner [Ding et al., 2007; Koochekpour et al., 2007]. Our study also found that ectopic expression of NP with or without TAT, a transmembrane signal peptide derived from HIV-1, was able to up-regulate AR transactivity in cells. The present work is aimed to understand if saposin C-induced stimulation of AR function in cells is mediated by a membrane receptor, since saposin C was reported to exert their biological effects by binding to a G-protein coupled receptor (GPCR) [Campana et al., 1998; Koochekpour et al., 2007]. Our results revealed that protein–protein interactions of saposin C, Src, and AR were involved in saposin C-mediated effect on AR activity.

MATERIALS AND METHODS

CELL CULTURE AND REAGENTS

Human prostate cancer cell lines, LNCaP (The American Type Culture Collection) and PC3 (The Cell Bank of Chinese Academy of Sciences, Shanghai) were routinely grown in RPMI 1640 medium supplemented with 10% fetal bovine serum. The cells were then maintained in serum-free medium for 24 h to deplete endogenous hormones before experiments. Pertussis toxin, an inhibitor of GPCR was from Calbiochem. The extracellular signal-regulated kinase 1/2 (ERK1/2) inhibitor PD98059 and phosphatidylinositol 3-kinase (PI3K)/Akt-inhibitor LY294002 were purchased from Promega, Src kinase inhibitor PP2 was obtained from Enzo life sciences and dissolved in dimethyl sulfoxide (DMSO). Mibolerone (Mib) were obtained from New England Nuclear and dissolved in ethanol. Control cells received same volume of DMSO and/or ethanol for the same time.

PLASMID CONSTRUCTIONS

The saposin C expression plasmid was constructed with a full length saposin C cDNA with His tag using the standard PCR cloning technique. The primer pairs used for saposin C were synthesized containing *Kpn*I and *Apa*I enzyme restriction sites at 5′ and 3′ ends, respectively. 5′-TTCGGTACCCGGATGTCTGATGTTTACTGTGAG (forward) and 5′-GGGCCCTTTCTGGGCACCAGGCCAGAGCAGAGGTGCAG (reverse). The PCR product was inserted into pcDNA3.1/myc-his B(−) and designated as pcDNA-Saposin C/His (pcDNA-Sa). Similarly, the primers containing of a Tat protein transduction domain sequence in front of the Saposin C sequence were made for TAT-Saposin C, forward primer 1, 5′-GCTTGGTACCATGTACGGCAGGAAGAAGCGGCGGCAGCGCAGGC, forward primer 2, 5′-GCTTGGTACCATGTACGGCAGGAAGAAGCGGCGG CAGCGCAGGC, and reverse primer, 5′-GGGCCCTTTCTGGGCACCAGGCCAGAGCAGAGGTGCAG. The PCR product was inserted into pcDNA3.1/myc-His B(−) to generate the pcDNA-TAT-Saposin C/His construct (pcDNA-TAT-Sa). Based on the functional domain sequence of Saposin C(NP), we synthesized two complementary oligonucleotides containing *Kpn*I and *Apa*I enzyme restriction sites at 5′ and 3′ ends, respectively. 5′-CATGACCGCTCTGATCGACAACAACGCCACCGAAGAAATCCTGTACGAGGGCC (forward) and 5′-CTCGTACAGGATTTCTTCGGTGGCGTTGTTGTCGATCAGAGCGGTCATGGTAC (reverse). After annealing, the DNA fragment was inserted into pcDNA3.1/myc-His B(−) to construct vector pcDNA3.1-NP/His (pcDNA-NP). The authenticity of the above plasmids was confirmed by direct sequencing.

RT-PCR ANALYSIS

After transfection of pcDNA-Sa (4.0 μg/well), or pcDNA-TAT-Sa (4.0 μg/well) into LNCaP cells in six-well plates, the cells were maintained in the serum free medium for 24 h. Total RNA were extracted by using Trizol, and reverse-transcribed with RevertAid™ First Strand cDNA Synthesis kit (Fermentas, Inc.). The primers were used for analysis of expressions of saposin C, TAT-saposin C: 5′-TAATACGACTCACTATAGGG and 5′-TAGAAGGCACAGTCGAGG. The expected lengths of PCR products are: pcDNA-TAT-Sa (470 bp), pcDNA-Sa (440 bp) and pcDNA3.1/his (240 bp). The experiments were repeated at least three times independently.

QUANTITATIVE PCR

Changes in the expressions of AR following transfection of saposin C and TAT-saposin C in LNCaP cells were quantified using real time RT-PCR on the Takara Thermal Cycle Dice TP800 Detection System (Takara Biotechnology, Japan). AR transcript was detected from MMLV reverse transcriptase-amplified cDNA as described above, with one aliquot designated to receive no enzyme. Quantitative PCR (TaqMan PCR) was performed as previously described [Yuan et al., 2008]. PCR assay was performed on each group in triplicate. Values were indicated as percentage of untreated control and set 100%.

TRANSIENT TRANSFECTION ASSAY

Cells were cultured in 24-well plates under the conditions described above. For transfection of DNA into LNCaP cells, pGL3 basic vector with a 2 kb AR promoter (0.8 μg/well), 6 kb PSA promoter (0.8 μg/well), or pGL3-SV40 with three copies of ARE of hk2 gene (hK2-3ARE, 0.8 μg/well), and an expression vector pcDNA-TAT-Sa (0.3 μg/well), pcDNA-Sa (0.3 μg/well), or pcDNA-NP (0.3 μg/well) were cotransfected by using Lipofectamine™2000. For transfection into PC3 cells, a human AR expression vector pSG5-AR (hAR, 0.2 μg/well) was included for cotransfection together with the plasmids as indicated. In all transfections, cells were also transfected with the empty vectors pcDNA3.1, pcDNA3.1/His, pGL3 basic, and pGL3-SV40 as controls. The pRL-TK vector (0.1 μg/well, Renilla luciferase, Promega) was cotransfected and served as an internal control for normalization. After 24 h transfection, cells were either treated with Mib (1 nM) or remained untreated for an additional 24 h. In some experiments, cells were exposed to ERK1/2 inhibitor

PD98059 (50 μM) for 1 h, PI3K/Akt inhibitor LY294002 (50 μM) for 3 h, GPCR inhibitor pertussis toxin (200 ng/ml) for 4 h, and Src kinase inhibitor PP2 (10 μM) for 1 h before transfections. Luciferase activity was detected using a dual-luciferase assay kit (Dual-Luciferase Reporter Assay System, Promega). At least three independent transfection experiments were performed.

WESTERN BLOT ANALYSIS

Cells were grown in 75-ml culture flask under the same treatment described above. After 24 h transfection, whole cell lysates were obtained as described previously [Yuan et al., 2005; Ding et al., 2007]. Protease inhibitors (0.5 mM phenylmethanesulfonyl fluoride (PMSF), 50 μg/ml aprotinin, 1 mM sodium orthovanadate, 10 mM sodium fluoride and 10 mM β-glycerolphosphate) were freshly added. Proteins (50 μg) were run with a SDS polyacrylamide gel and electrotransferred onto nitrocellulose membrane (Bio-Rad). The blots were probed with anti-AR (BD Biosciences), anti-phosphor-ERK 1/2 (Thr202/Tyr204, Cell Signaling Technology), anti-ERK1/2 and anti-β-tublin (Santa Cruz Biotechnology). Immunoblot assay was performed with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG secondary antibody (Santa Cruz Biotechnology), and visualized by enhanced chemiluminescence substrate (Pierce Biotechnology).

CO-IMMUNOPRECIPITATION

Cells were seeded in 100 mm culture dishes and transfected with pcDNA-Sa, pcDNA-TAT-Sa, pcDNA-NP/His, or pcDNA3.1/His, whole cell lysates were prepared and precleared with anti-mouse IgG and protein A-agarose beads (Santa Cruz Biotechnology). Proteins (500 μg) were incubated in binding buffer (20 mM HEPES, pH 7.9, 20% glycerol, 150 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 50 mg/ml aprotinin, 1 mM sodium orthovanadate, 10 mM sodium fluoride, and 10 mM β-glycerolphosphate) with 2 μg of anti-Src antibody (Cell Signaling Technology), or normal IgG as the nonspecific control (Santa Cruz Biotechnology) at 4°C overnight. The protein A-agarose beads were added and incubated for additional 2 h at 4°C. The beads were washed six times with buffer containing 50 mM Tris–HCl, pH 7.5, 0.5% IGEPAL CA-630, 150 mM NaCl, 0.2 mM EDTA, 0.5 mM PMSF, and resuspended in SDS sample buffer. Immunocomplexes were analyzed by Western blotting with the corresponding anti-AR, anti-Src, and anti-His antibodies. The signals were developed using ECL.

IMMUNOFLUORESCENCE STAINING

Cells were cultured on coverslips in 24-well plates and treated as described above. The coverslips were fixed with 4% paraformaldehyde in PBS solution for 30 min. The cells on coverslips were either permeabilized with 0.3% Triton-X-100 in PBS for 15 min or remained nonpermeabilized, rinsed with PBS, and blocked with PBS buffer containing 5% bovine serum albumin for 1 h. After washing with PBS, the cells were incubated with anti-AR antibody (1:50), anti-His antibody (1:300), or anti-Src antibody (1:100) at 4°C overnight, and followed by incubation with rhodamine-labeled anti-rabbit IgG and FITC-labeled anti-mouse IgG (1:100, Zhongshan Golden Bridge Biotechnology, Beijing, China) for 1 h at room temperature respectively. Standard fluorescence was captured with a Nikon E-600 Photomicroscope (Toyko, Japan). Plug-ins from ImageJ software was used to estimate correlation coefficients.

STATISTICAL ANALYSIS

The data are presented as the means ± SD of several independent experiments. The statistical significance of difference between control and treated groups was determined by paired *t*-test. $P < 0.05$ was considered statistically significant.

RESULTS

DETECTION OF THE EXPRESSION OF SAPOSIN C IN TRANSFECTED CELLS

Initially, we demonstrated the expression of saposin C and TAT-saposin C mRNA after transfections on LNCaP cells by RT-PCR analysis. As shown in Figure 1A, the bands corresponding to TAT-saposin C and saposin C were shown to be 470 and 440 bp respectively, as compared to a 240 bp band from the empty vector-transfected cells. Moreover, increases in protein expression levels in cells by transfections were detected by immunobloting using an anti-His antibody (Fig. 1B). The results revealed that TAT-saposin C and saposin C were able to express continuously at the three measured time points (6, 12, and 24 h). No bands were shown in the negative control (Fig. 1B). To visualize expression of TAT-saposin C and saposin C proteins, we also performed immunofluorescence staining using an anti-His antibody in transfected cells. TAT-saposin C/His, saposin C/His or NP/His were confined mainly in transfected cells, and the control pcDNA3.1/His vector also showed mainly cytoplasm staining in the presence of permeabilized agent (Fig. 1C). The difference in overall intensity of His staining was not significant in cells transfected either with TAT-saposin C, saposin C, or NP. To evaluate the ability of TAT-mediated protein transduction in cell membrane, the transfected cells were remained nonpermeabilized to avoid antibodies entering into cells. As shown in Figure 1D, the TAT-saposin C/His, but not saposin C/His or NP/His, was exclusively stained in the cellular membranes. These observations indicate that TAT-saposin C and saposin C were able to express in cells, and only TAT was able to cause saposin C crossing cellular membranes.

EXPRESSION OF SAPOSIN C INDUCES AR EXPRESSION

Previous studies have shown that NP fused with a TAT sequence could regulate the AR activity in prostate cancer cells [Ding et al., 2007]. Of interest is that, NP lacking of the TAT signal peptide was able to give the results similar to NP with TAT sequence. We extended our studies to examine whether saposin C could affect the expression and function of the AR in serum-free medium. The AR mRNA expression level was assessed by real time PCR, and showed that an increase in the AR expression was noticeable in cells transfected with saposin C and TAT-saposin C (Fig. 2A) compared to the control vehicle. Furthermore, the AR promoter activity was examined to characterize the induction effect of saposin C on AR at transcript level. As shown in Figure 2B, Mib treatment, as a positive control, significantly increased the AR promoter reporter expression. Exogenous expression of saposin C resulted in an increased AR promoter reporter as TAT-saposin C did, but this stimulatory effect was less than that of Mib treatment. Changes in AR protein level

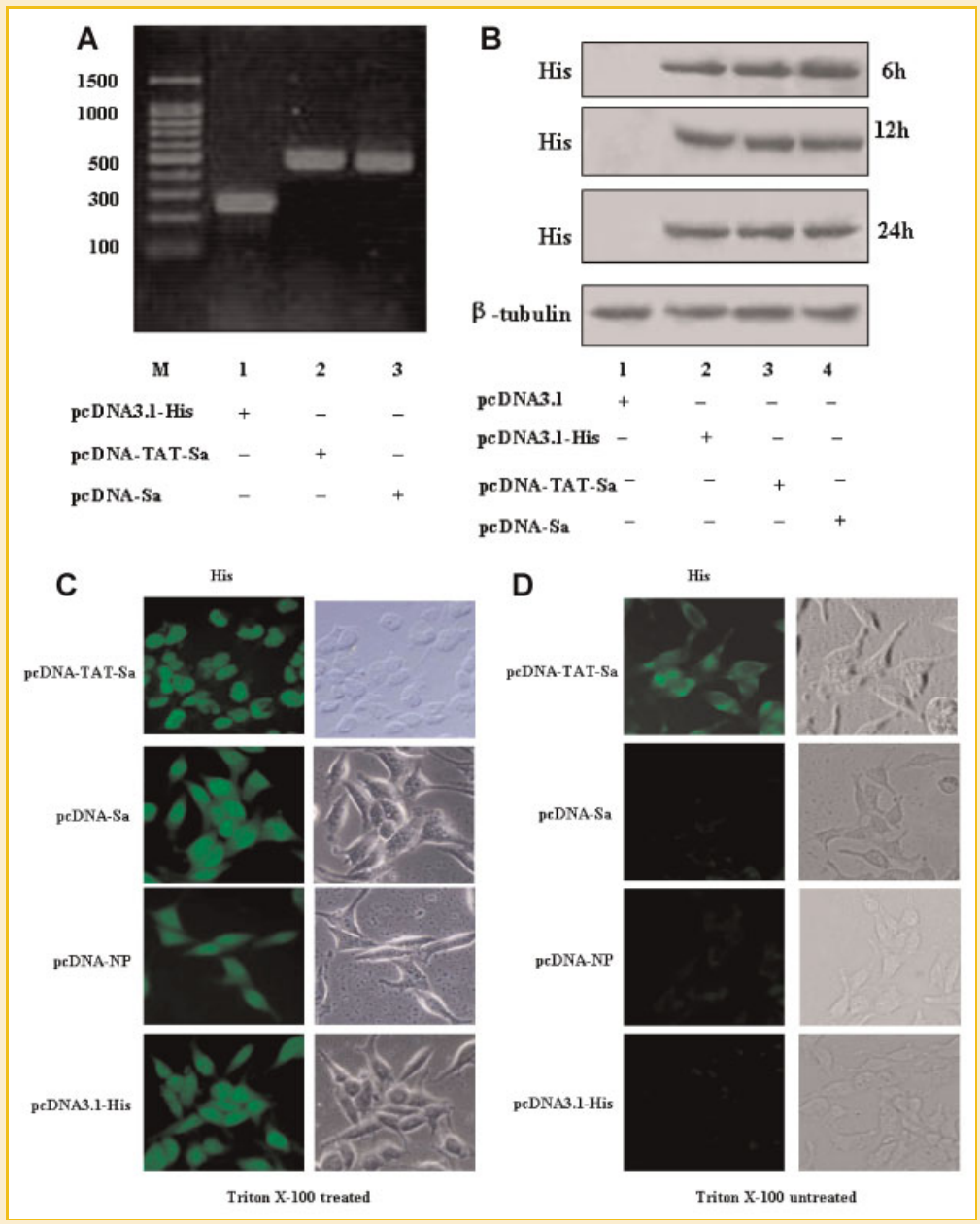


Fig. 1. Detection of ectopic expressions of Saposin C and TAT-saposin C in LNCaP cells. A,B: Analysis of mRNA and protein expression levels of Saposin C in LNCaP cells transfected with pcDNA-Sa or pcDNA-TAT-Sa plasmid by RT-PCR and Western blot. The empty vectors of pcDNA3.1 and pcDNA3.1-His were included as controls. The expression of β-tublin was served as an internal control to monitor sample loading. C,D: Localization of pcDNA-Sa, pcDNA-TAT-Sa and pcDNA-NP-His expression in LNCaP cells by immunofluorescence. After transfection, the cells were incubated with primary mouse His-Tag monoclonal antibody in PBS containing of Triton X-100 (C), or free of Triton X-100 (D). The empty pcDNA3.1-His vector was included as a control.

were also detected in the transfected cells. The results in Figure 2C showed that the expression of AR protein was induced in response to saposin C and TAT-saposin C. These observations strongly indicate that saposin C and TAT-saposin C mediated the induction of AR mRNA and protein expression in an androgen-independent manner.

EXPRESSION OF SAPOSIN C ENHANCES AR TRANSCRIPTIONAL ACTIVITY

Activated AR translocates to the nucleus where it interacts with ARE to transactivate its target genes such as prostate-specific antigen (PSA) and human glandular kallikrein (hk2). To determine whether AR transcriptional function is also affected by saposin C, we next analyzed the PSA promoter activity in response to saposin C under androgen-deprived conditions. As shown in Figure 3A, in addition to TAT-saposin C, saposin C-mediated induction of reporter gene activity was observed by cotransfection of LNCaP cells with a PSA promoter-luciferase reporter construct compared with the control vector. Similarly, Mib caused an induction of reporter gene expression, although at a relatively higher level. The regulatory effect by saposin C was also demonstrable in the AR-negative PC3

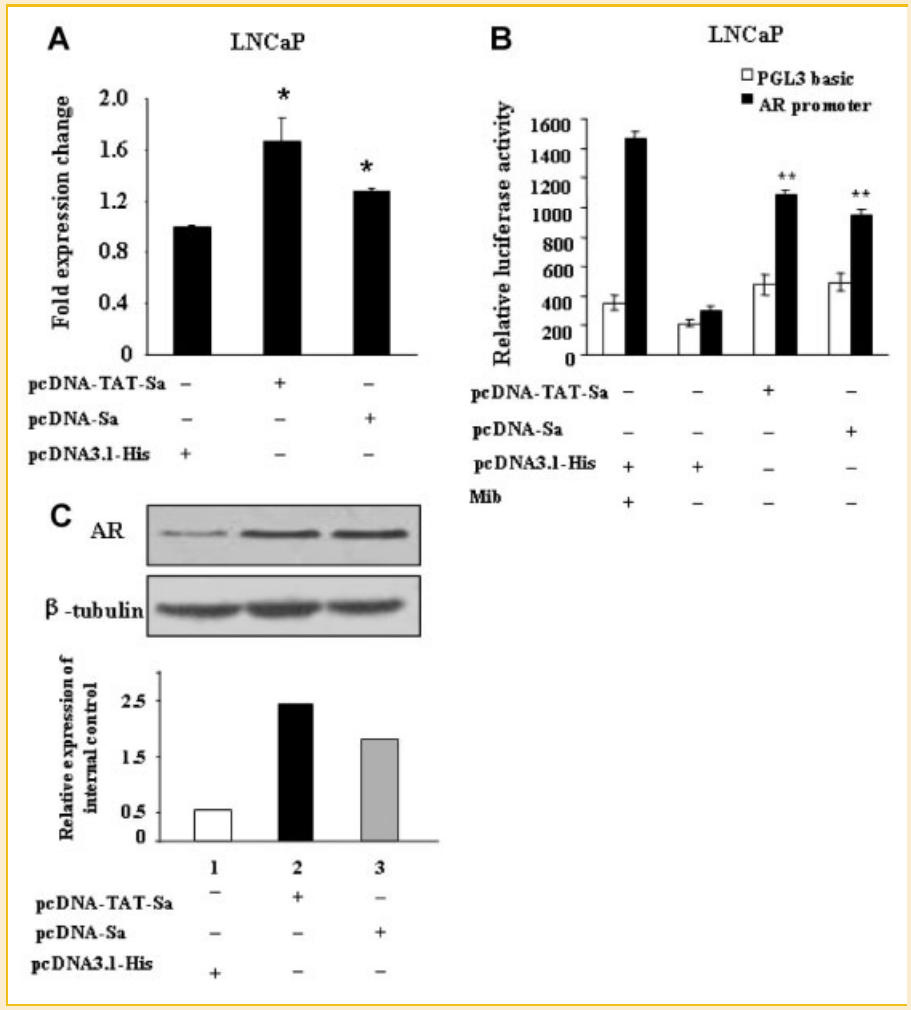


Fig. 2. Induction of AR expression by Saposin C in LNCaP cells. A: Transfection of expression vector pcDNA–Sa, pcDNA–TAT–Sa, or pcDNA3.1–His (a control) into LNCaP cells for 24 h, changes in AR mRNA levels were determined by real time PCR. B: LNCaP cells were cotransfected with an AR promoter plasmid and pcDNA–Sa, pcDNA–TAT–Sa, or pcDNA3.1–His control vector, and reporter activities were measured by Dual–luciferase assay. The normalized relative luciferase activity (means ± SD) of at least three independent experiments were shown. $^{*}P < 0.05$ and $^{**}P < 0.01$, compared with the control. C: LNCaP cells were transfected with constructs as indicated for 24 h, AR protein expression levels in whole cell lysates were analyzed by Western blot assays.

cells, wherein a similar increase pattern was noted upon forced expression of either saposin C or TAT-saposin C with a human AR expression vector (Fig. 3B). To further analyze whether saposin C modulates AR interaction with its response elements, we examined the effect of saposin C on AR binding activity to the AREs using a reporter construct containing of three copies of ARE elements on the hk2 promoter. As shown in Figure 3C, saposin C-stimulated reporter activity was detected as TAT-saposin C did in LNCaP cells. No significant difference was observed between saposin C and TAT-saposin C under androgen-deprived conditions. Additionally, the luciferase activity of hk2-3ARE reporter was investigated in PC3 cells as well. As shown in Figure 3D, both TAT-saposin C and saposin C upregulated AR transcriptional function and subsequently led to an increase of the hk2-3ARE reporter activity. Therefore, forced expression of saposin C caused enhancement on the AR transcriptional activity in addition to the increase of AR expression.

EXPRESSION OF SAPOSIN C UPREGULATES AR ACTIVITY WITH BLOCKING OF THE GPCR

Saposin C is proposed to exert its biological function by interacting with a GPCR [Campana et al., 1998]. However, forced expression of NP (the functional peptide of saposin C) lacking of signal peptide could stimulate AR activity as well [Ding et al., 2007]. It prompted us to investigate the potential role of GPCR in saposin C-mediated stimulation on AR in cells. The results in Figure 4A indicated that a noticeable increase in the PSA promoter reporter activity was shown in cells transfected with either TAT-saposin C or saposin C in the absence of pertussis toxin. Pretreatment of cells with pertussis toxin followed by transfection of TAT-saposin C led to a decrease in the production of PSA promoter reporter compared with that of untreated one, suggesting that androgen-like effect induced by TAT-saposin C is mediated via a GPCR. However, saposin C-stimulated induction of the PSA promoter reporter expression

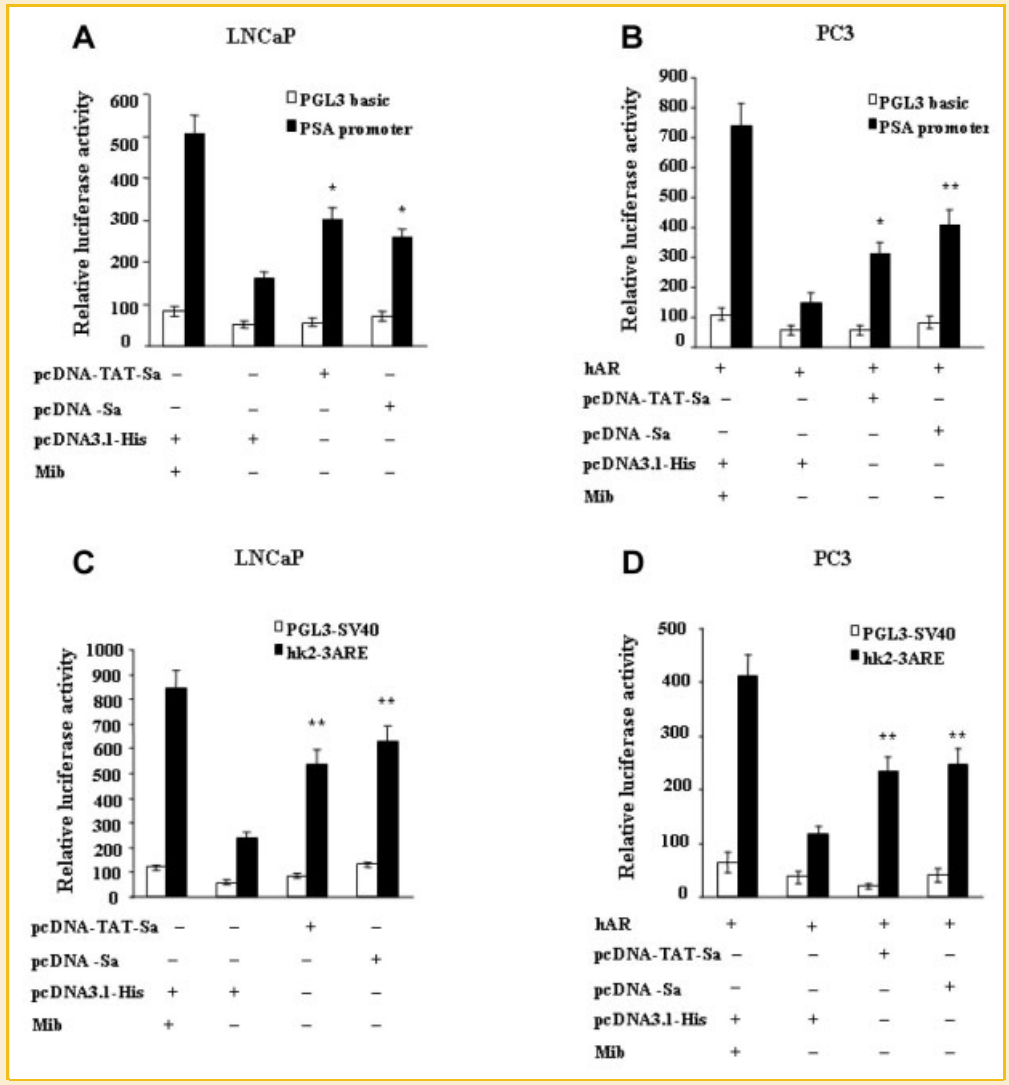


Fig. 3. Enhancement of AR transcriptional activity by Saposin C in prostate cancer cells. A,C: LNCaP cells were cotransfected with a pGL3-PSA6kb promoter-luciferase reporter, or pGL3-hk2-3ARE reporter and pcDNA-Sa, or pcDNA-TAT-Sa plasmid. B,D: PC3 cells were cotransfected a human AR expression plasmid together with the PSA promoter reporter, or hk2-3ARE reporter and pcDNA-Sa or pcDNA-TAT-Sa plasmid. Changes in luciferase activity were detected and normalized as relative luciferase activities (means ± SD) of at least three independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$, compared with the control.

remained unaffected in cells pretreated with pertussis toxin, raising a possibility that saposin C would be functional in cells even when GPCR was blocked. Saposin C-mediated enhancement in the PSA promoter reporter activity was further confirmed in PC3 cells exposed to pertussis toxin as shown in Figure 4B, consistent with the result obtained in LNCaP cells (Fig. 4A). In addition, we used hk2-3ARE luciferase reporter plasmid to test whether DNA sequence dependent AR activity could be affected by saposin C in cells pretreated with pertussis toxin. As expected, the result in Figure 4C revealed that the TAT-saposin C-induced increase in the luciferase activity of hk2-3ARE was evident in the absence of androgen. This induction by TAT-saposin C was impaired with pertussis toxin treatment, while production of reporter gene was remained unaffected in cells transfected with saposin C in the presence of pertussis toxin. Similar results were obtained in PC3 cells exposed to pertussis toxin before transfection (Fig. 4D). Therefore, pertussis toxin treatment impaired TAT-saposin C-stimulated AR transcriptional activity, but had little inhibitory effect on saposin C-mediated augmentation in AR function.

INVOLVEMENT OF THE PI3K/AKT AND MAPK SIGNAL PATHWAYS IN SAPOSIN C-MEDIATED AR TRANSACTIVATION

There is extensive evidence to support the concept that several cell signaling pathways including p42/p44 MAPK and PI3K/Akt have been implicated in the regulation of AR activity and lead to androgen independence. It has been demonstrated that saposin C can activate MAPK, PI3K/Akt, and GPCR signaling pathways in PCa cells [Lee et al., 2004b; Koochekpour et al., 2005]. We analyzed saposin C-mediated effects on ERK phosphorylation in the absence or presence of ERK1/2, and/or PI3K/Akt inhibitors. The results in Figure 5A showed that, in addition to TAT-saposin C, saposin C increased the phosphorylation of ERK1/2, which was

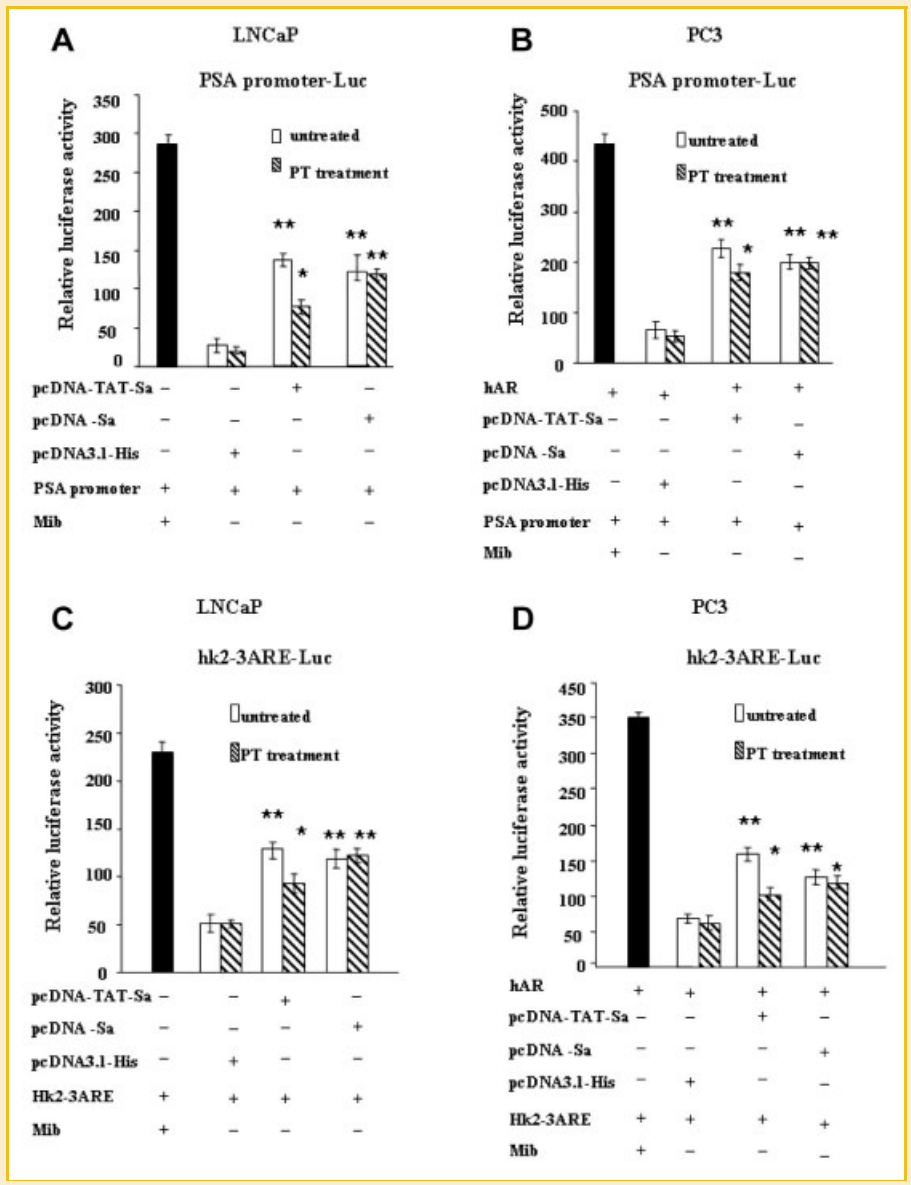


Fig. 4. GPCR-independent upregulation of AR activity by expressed saposin C in prostate cancer cells. Pretreatment of cells with pertussis toxin (PT) (200 ng/ml, 4 h), A,B: LNCaP and PC3 cells were cotransfected with PSA promoter-Luc, and desired expression vector (pcDNA-TAT-Sa, pcDNA-Sa), respectively. For transfection on PC3 cells, an additional human expression plasmid pSG5-AR (hAR) was also cotransfected with desired vectors as indicated. C,D: LNCaP and PC3 cells were cotransfected with hK2-3ARE-Luc and desired expression vectors. After transfection of 24 h, cells exposed to Mib were served to monitor experimental conditions. Cell lysates were prepared following of transfection and luciferase activity assays were performed accordingly. $^{*}P < 0.05$ and $^{**}P < 0.01$, differences between control and any other experimental group of interest.

substantially impaired by pretreating cells with the specific ERK inhibitor PD98059. However, changes in the total ERK1/2 proteins were not observed by either TAT-saposin C or saposin C treatment. No significant difference was observed between TAT-saposin C- and saposin C-mediated effects on the phosphorylation of ERK under treatment conditions. Similarly, the phosphorylation of ERK was further examined in cells pretreated with PI_3K/Akt inhibitor LY294002. Both TAT-saposin C and saposin C significantly induced the levels of phospho-ERK1/2, but only TAT-saposin C induced phosphor-ERK1/2 was inhibited dramatically by LY294002 as illustrated in Figure 5B. Total ERK1/2 expressions remained unchanged, consistent with the result shown in Figure 5A. Moreover, in response to the combination of the above PI3K/Akt and MAPK inhibitors, the phosphor-ERK1/2 levels were abrogated completely as shown in Figure 5C, while this suppression could be restored partially by transfection of saposin C or TAT-saposin C expression plasmid. We next elucidated whether activation of MAPK by saposin C is involved in regulation of AR activity by co-transfection of saposin C expression vector and the PSA promoter reporter in the presence or absence of the inhibitors. As shown in Figure 5D, TAT-saposin C-stimulated induction of the PSA promoter reporter activity decreased slightly in response to PD98059 and LY294002 inhibitors, while saposin C-mediated enhancement on the PSA promoter activity remained almost

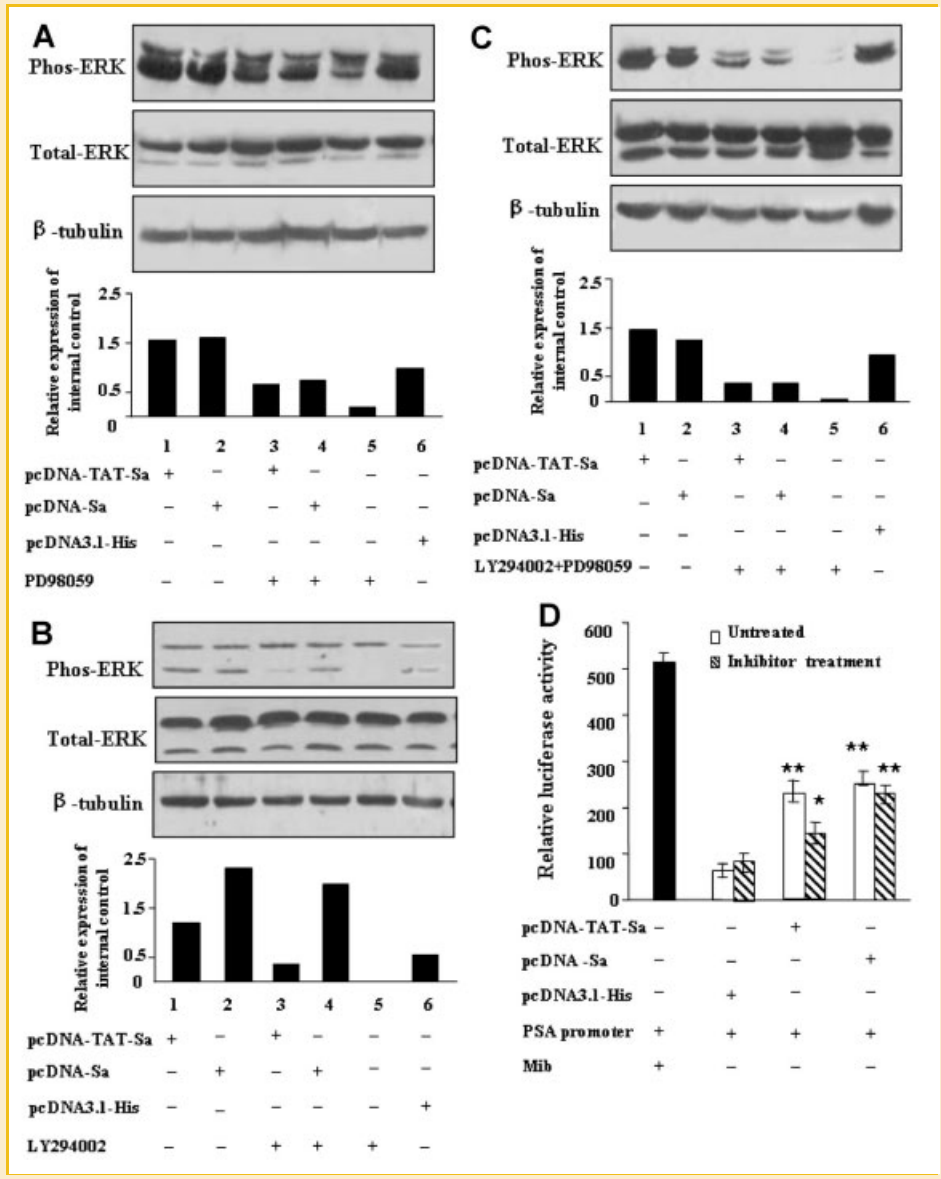
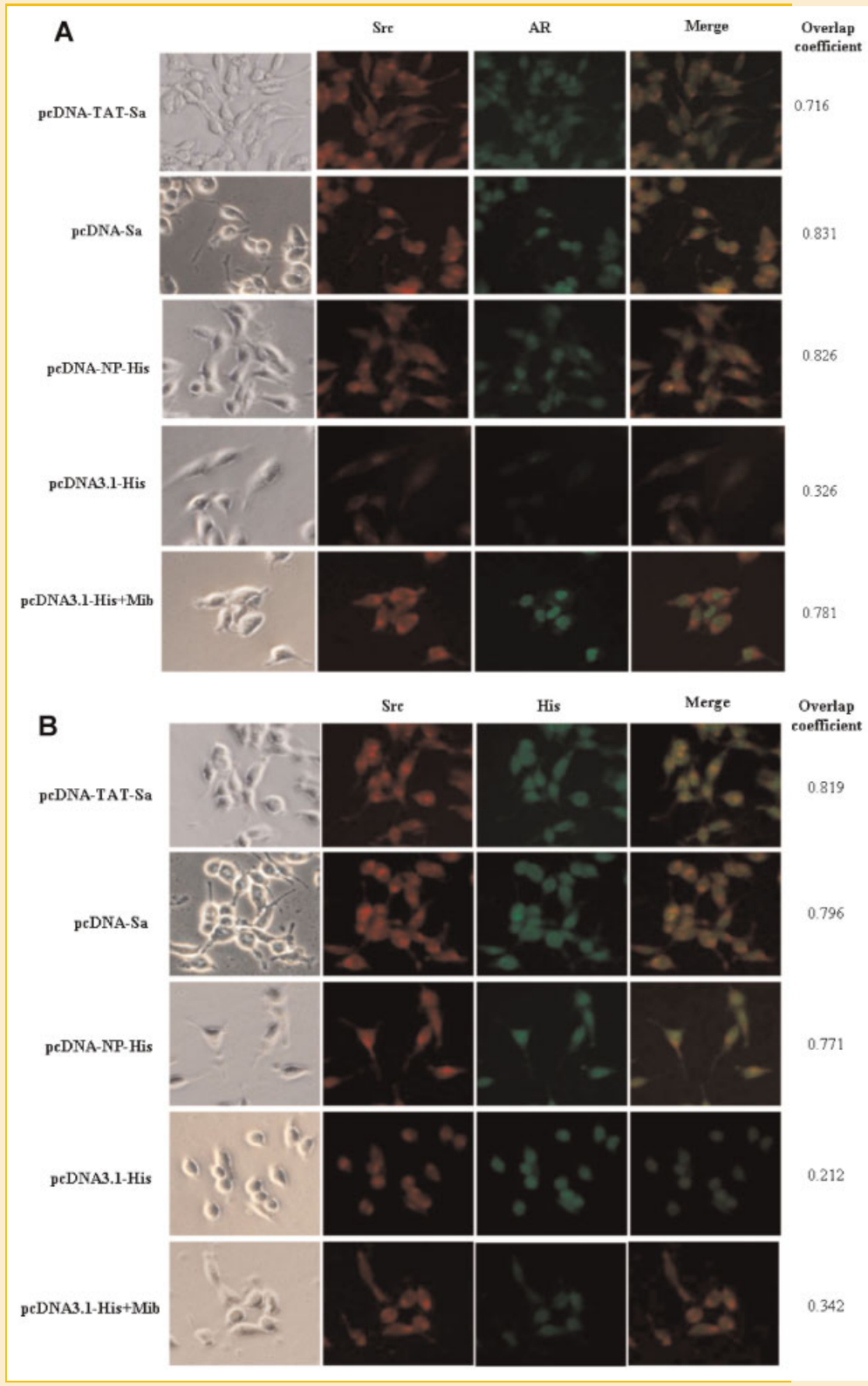


Fig. 5. PI3K/Akt and MAPK signal pathways were involved in saposin C-mediated induction of AR activity. LNCaP cells were cultured in serum-deprived medium for 24 h, and then fresh culture media was changed in the presence of inhibitor PD98059 (A, 1 h), LY294002 (B, 3 h), combination of LY294002 and PD98059 (C), or remained untreated. After pretreatment with inhibitors above, cells were transfected with pcDNA-TAT-Sa, pcDNA-Sa, or empty vector pcDNA3.1-His under androgen-deprived conditions. Changes in expression of phospho-ERK and total ERK were analyzed by immunoblotting. D: LNCaP cells were cotransfected with pGL3-PSA6kb promoter-luciferase reporter and pcDNA-Sa, pcDNA-TAT-Sa, or pcDNA3.1-His control vector, respectively following of pretreatment with inhibitors (combination of LY294002 and PD98059) described above. Cells treated with Mib after transfection were used as a positive control. The normalized relative luciferase activity (means ± SD) of three independent experiments were shown. $^{*}P < 0.05$, compared to the group transfected with pcDNA3.1-His expression construct and PSA 6 kb promoter-luciferase reporter in the presence of inhibitors. $^{**}P < 0.01$, compared to the group transfected with control pcDNA3.1-His expression plasmid and PSA 6kb promoter-luciferase reporter, and cells remained untreated, or in the presence of inhibitors.

Fig. 6. Stimulation of saposin C on the AR activity via Src. LNCaP cells were cultured in androgen-deprived medium for 24 h, and then transfected with pcDNA-TAT-Sa, pcDNA-Sa, pcDNA-NP-His, or pcDNA3.1-His for additional 24 h, respectively. One group of cells treated with Mib was used as a positive control. The cells were permeabilized and subjected to immunofluorescence staining. A: Immunofluorescence staining of Src and AR using anti-Src and anti-AR antibodies. Correlation coefficients were estimated by ImageJ. B: Staining of Src, Saposin C, and NP using anti-Src, anti-His monoclonal antibodies. Correlation coefficients were also analyzed by ImageJ. C: Proteins in whole cell extracts were prepared from LNCaP cells transfected with pcDNA-TAT-Sa, pcDNA-Sa, pcDNA-NP-His, or pcDNA3.1-His under conditions described above. Immunoprecipitation experiment was performed with anti-Src antibody. The co-precipitated AR, saposin C/His, and NP/His proteins were detected using either anti-AR antibody or anti-His antibody. Input represents 10% of the whole cell extracts used in the above experiments. D: Effect of Src kinase inhibitor PP2 on saposin C-, TAT-saposin C-mediated induction of PSA promoter in LNCaP cells. Cells were treated with PP2 inhibitor (10 μM) for 1 h prior to co-transfection of saposin C or TAT-saposin C with PSA promoter into LNCaP cells. After 24 h transfection, cells were either treated with Mib (1 nM) or remained untreated for an additional 24 h. Cell lysates were used for dual-luciferase activity assay. $^{*}P < 0.05$, compared to the group co-transfected with saposin C or TAT-saposin C expression construct and PSA 6 kb promoter-luciferase reporter in the absence of PP2. $^{**}P < 0.01$, compared to the group transfected with control pcDNA3.1-His expression plasmid and PSA 6 kb promoter-luciferase reporter, and cells remained untreated. $^{***}P < 0.001$, compared to the group transfected with control pcDNA3.1-His expression plasmid and PSA 6 kb promoter-luciferase reporter, and cells remained untreated with Mib.


A
Src
AR
Merge
Overlap coefficient
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pcDNA-Sa
0.831
pcDNA-NP-His
0.826
pcDNA3.1-His
0.326
pcDNA3.1-His+Mib
0.781
B
Src
His
Merge
Overlap coefficient
pcDNA-TAT-Sa
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pcDNA-Sa
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0.771
pcDNA3.1-His
0.212
pcDNA3.1-His+Mib
0.342

Fig. 6.

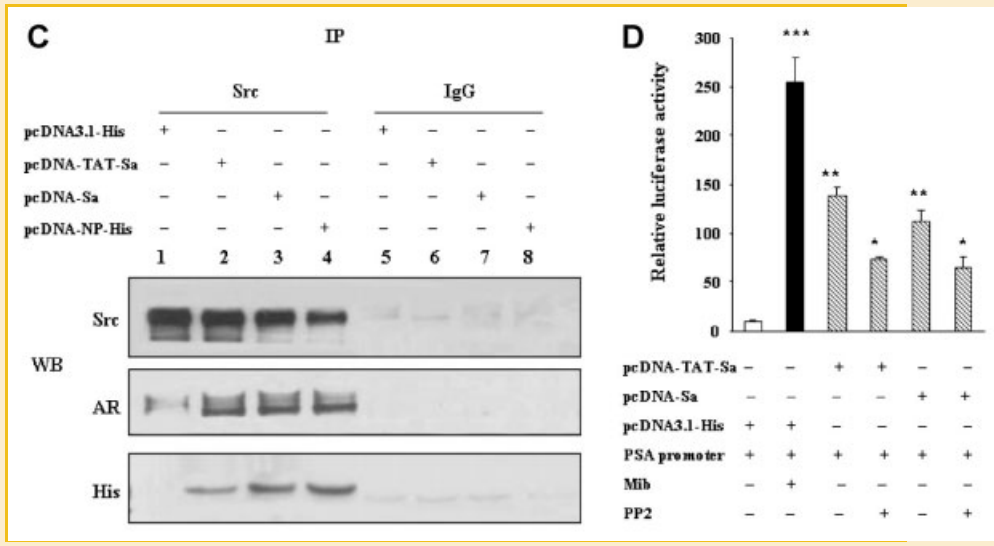

Fig. 6. (*Continued*)

unchanged. Together, these observations demonstrate that expressions of saposin C and TAT-saposin C could activate MAPK pathway, but MAPK activation might not be directly involved in saposin C mediated-regulation of AR activity.

ASSOCIATION OF SRC AND SAPOSIN C LEADS TO ACTIVATION OF THE AR

Since Src is the apical molecule mediating the cross-talk in AR and ERK1/2 signaling [Desai et al., 2006; Kraus et al., 2006]. An effort was directed toward studying of the role of Src in saposin C-mediated effect on AR. Immunofluorescence staining resulting in Figure 6A showed that co-localization of Src and AR were detected in response to the expression of saposin C or TAT-saposin C. The NP also showed the ability to induce the co-localization of Src and AR under androgen-deprived conditions (Fig. 6A). Furthermore, the images presented in Figure 6B showed a diffused signal for both Src and saposin C/NP proteins. Similar results were also obtained with expression of TAT-saposin C, whereas no overlapped fluorescence was seen in the control vehicle, suggesting that Src may contribute to the saposin C-mediated stimulation on AR. Next, we examined whether there are direct physical interactions among Src, AR, and saposin C using coimmunoprecipitation assays. LNCaP cells transfected with saposin C, TAT-saposin C, or NP and grown in serum-free medium. As shown in Figure 6C, endogenous AR bands were detected in anti-Src-precipitated complexes from cells transfected with saposin C, TAT-saposin C, or NP, respectively, whereas a very weak signal was detected in control vector transfected cells. Of importance, TAT-saposin C, saposin C, or NP protein was coimmunoprecipitated with the endogenous Src from transfected cells as shown in Figure 6C. In contrast, no detectable saposin C protein could be precipitated in cells transfected with the control vector. Using PP2, a potent and selective Src tyrosine kinase inhibitor, we further verified the role of Src protein in saposin C-mediated stimulation of AR activity. As shown in Figure 6D, TAT-saposin C or saposin C increased the PSA 6 kb promoter luciferase activity as expected. However, either TAT-saposin C- or saposin C-mediated enhancement on the PSA promoter activity was markedly suppressed in cells pretreated with PP2 inhibitor following co-transfection of TAT-saposin C, or saposin C and the PSA promoter reporter plasmids. The data suggested that Src protein may bridge saposin C and subsequently lead to activation of AR in androgen-independent manner.

DISCUSSION

Neuroendocrine cells are gaining attentions because the observations demonstrated that increased neuroendocrine cells and neuroendocrine cell-derived soluble factors are associated with the development of hormone-refractory prostate cancer [Amorino and Parsons, 2004; Desai et al., 2006]. Many of neurokines released from neuroendocrine cells such as gastrin-releasing peptide, IL6, and IL8 are implicated to activate AR and induce androgen-independent growth [Hobisch et al., 1998; Lee et al., 2001, 2004a]. Prosaposin and NP have been reported to act as androgen-agonists [Ding et al., 2007; Koochekpour et al., 2007]. This study revealed that the ectopically expressed saposin C could regulate AR activity and promote PCa cell growth. The conclusions are based on the following evidence: (a) the expression of saposin C effectively increased endogenous AR expression, and enhanced AR transcriptional activity as indicated in the promoter assays; (b) consistent with published results [Lee et al., 2004b; Koochekpour et al., 2005], in which expressed saposin C functioned to activate MAPK and PI3K/Akt signaling pathways in prostate cancer cells. One novel observation was that the GPCR seemed not to play a significant role in saposin C-mediated activation of AR. Of importance, this activation involves Src-tyrosine kinase pathway.

Post-translational modifications of AR such as phosphorylation [Ikonen et al., 1994], acetylation [Fu et al., 2002], sumoylation [Poukka et al., 2000], and protease cleavage [Devlin and Mudryj, 2009], which affect AR activity, have been well-documented by many studies. Regarding of growth factors and neuropeptides, they employ a phosphorylation cascade, including AKT and MAPK pathways [Papatsoris et al., 2007]. For example, gastrin-releasing peptide can act as a ligand for GPCR, activates Src/FAK tyrosine kinase complex, and promotes AR transcription activity [Lee et al., 2001]. It has been suggested that Src kinase plays an important role in neuropeptide-induced AR activation [Guo et al., 2006; Kraus et al., 2006; Kung and Evans, 2009]. It is reasonable to evaluate the role of Src kinase and the activated ERK and AKT signaling pathways in saposin C-mediated activation of AR in cells. We did observe that expressed saposin C interacted with Src protein, and induced formation of saposin C/Src/AR complex leading to activation of AR. Additionally, the effect of saposin C and androgen on AR activation was synergistic consistent with the result of NP [Ding et al., 2007], although saposin C-mediated AR signaling was less than that of Mib treatment presumably due to the stabilization of AR by the ligand (data not shown). Furthermore, the functional domain of saposin C may be important for interactions of saposin C and Src, because the Src protein was immunoprecipitated in cells transfected with NP as well.

In summary, the novel finding of this study is the demonstration that activation of AR by saposin C requires interactions of saposin C, Src, and AR, and finally results in increased expression of AR-regulated genes and survival of prostate cancer cells in androgen-independent manner.

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