A novel steroid receptor co-activator protein (SRAP) as an alternative form of steroid receptor RNA-activator gene: expression in prostate cancer cells and enhancement of androgen receptor activity

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We have cloned a cDNA coding for a novel steroid receptor co-activator protein termed SRAP from a rat prostate library. Although the nucleotide sequence of the SRAP has 78.2% identity to that of the human steroid receptor RNA activator (SRA), a novel RNA molecule which was reported to act as an RNA transcript without being translated into protein [Lanz, McKenna, Onate, Albrecht, Wong, Tsai, Tsai and O'Malley (1999) *Cell* 97, 17–27], the cDNA of SRAP is capable of generating a functional protein. Glutathione S-transferase pull-down assays showed that SRAP associates with the partial androgen receptor (AR) protein composed of a DNA-binding domain and an activation function 2. Luciferase assays demonstrated that SRAP enhances the transactivation activity of the AR, the glucocorticoid receptor and the peroxisome proliferator-activated receptor γ_1 in a ligand-dependent manner. Using a green fluorescent protein (GFP) fusion-protein construct, we demonstrated *in vivo* translation of the GFP-SRAP fusion protein

in HeLa cells co-transfected with pSG5AR and reporter gene in the presence of 5 α -dihydrotestosterone (DHT). Co-transfection of the GFP-SRAP fusion protein expression plasmid enhanced the transactivation activity of AR whereas incorporation of mutations in SRAP of the fusion protein resulted in loss of enhancement of the transactivation activity. Northern blot analysis and reverse transcriptase PCR assays showed that SRAP and SRA are expressed in rat and human prostate cancer cell lines respectively. In HeLa cells and the human prostate cancer cells line DU-145, co-transfected with SRAP, the DHT-dependent transactivation activities of AR were not completely inhibited by the anti-androgen flutamide, but the transactivation activities still remained high even in the presence of 5 μ M flutamide, suggesting that SRAP may play an important role in enhancing AR activity in prostate cancer.

Key words: anti-androgen, hormone-refractory prostate cancer.

INTRODUCTION

One of the most striking characteristics of prostate cancer is its androgen dependence. Even patients with advanced stage D₂ prostate cancer can be treated successfully with androgen ablation therapy for a while. However, their cancers eventually become androgen- or hormone-independent within several years, resulting in their being refractory to any treatment with a correspondingly poor prognosis.

To elucidate the mechanism by which prostate cancer is initially androgen-dependent and later becomes hormone-refractory, much attention has been paid to androgen receptor (AR)-mediated transcription of the relevant genes. The proposed mechanisms of the hormone-refractory progression of prostate cancer in relation to AR-mediated transactivation include mutation [1,2], amplification [3] and ligand-independent activation [4–6] of AR. On the other hand, recent studies have identified the co-activators of nuclear receptors such as transcriptional mediator/intermediary factor (TIF)-1 [7], CREB-binding protein (CBP)/p300 (where CREB is c-AMP response element-binding protein) [8,9], steroid receptor coactivator (SRC)-1/TIF2 [10,11], nuclear receptor co-activator ('ACTR') [12] and receptor interacting protein ('RIP') 140 [13]. Several AR co-activators, AR-

associated protein (ARA) 70 [14], ARA55 [15], ARA54 [16], ARA160 [17] and ARA267- α [18], have also been extensively isolated and characterized. Despite demonstrating the complexity of nuclear receptor-mediated transcription, alteration of the expression of these molecules has not been observed during the hormone-independent progression of prostate cancer.

The discovery of the oestrogen receptor β (ER β) [19] prompted us to search for a new nuclear receptor activated by androgens. Our attempt to isolate a new nuclear receptor was unsuccessful, for all the nuclear receptors obtained from a rat prostate library were AR and peroxisome proliferator-activated receptor γ_1 (PPAR γ_{1a}) variants [20] in our experiments. However, we have unexpectedly cloned from a rat prostate library a novel steroid receptor co-activator protein (SRAP), and investigated its possible role in anti-androgen-refractory progression of prostate cancer.

MATERIALS AND METHODS

Materials

Male Sprague–Dawley rats were purchased from Clea Japan (Tokyo, Japan); ZAP express cDNA synthesis kit and pCMVscript vector were from Stratagene (La Jolla, CA, U.S.A.);

Abbreviations used: AIB1, a steroid receptor co-activator amplified in ovarian and breast cancer 1; AF, activation function; AR, androgen receptor; ARA, AR-associated protein; CBP, CREB-binding protein; DBD, DNA-binding domain; DHT, 5 α -dihydrotestosterone; ER, oestrogen receptor; FHL2, four and a half LIM domains 2; GFP, green fluorescent protein; GR, glucocorticoid receptor; GST, glutathione S-transferase; MMTV, mouse mammary tumour virus; ORF, open reading frame; PPAR, peroxisome proliferator-activated receptor; RT-PCR, reverse transcriptase PCR; SRA, steroid receptor RNA activator; SRAP, steroid receptor co-activator protein; SRC, steroid receptor coactivator; TIF, transcriptional mediator/intermediary factor.

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[α - 32 P]dATP (800 Ci/mmol), [α - 32 P]dCTP (3000 Ci/mmol) and [35 S]methionine (1000 Ci/mmol) were from Amersham Biosciences; Dual-luciferase reporter assay system, TNT-coupled reticulocyte lysate system and pRL-TK vector were from Promega (Madison, WI, U.S.A.); CheckMate mammalian two-hybrid system was from Clontech (Palo Alto, CA, U.S.A.); SuperFect transfection reagent was from Qiagen (Chatsworth, CA, U.S.A.); an expression vector for glutathione S-transferase (GST) fusion proteins, pGEX-5X-1, was from Amersham Biosciences; and glutathione-agarose beads were from Sigma-Aldrich (St. Louis, MO, U.S.A.).

Mouse mammary tumour virus (MMTV)-Luc reporter plasmid [21] and glucocorticoid receptor (GR) expression vector were generous gifts from N. V. Organon (Oss, The Netherlands). A reporter vector pMAMneo-luc was kindly provided by Dr Barbara M. Sanborn, University of Texas Medical School (Houston, TX, U.S.A.). A human AR expression plasmid pSG5AR, and a rat AR in pGEM3Z, were generous gifts from Dr Chawnshang Chen, University of Rochester (Rochester, NY, U.S.A.) and Dr Shutsung Liao, University of Chicago (Chicago, IL, U.S.A.), respectively. The pAOXPPRE-Luc reporter vector [22] was kindly given by Dr Takashi Osumi, Himeji Institute of Technology (Himeji, Japan). pCDNA/PPAR γ 1 was a kind gift from Dr Tetsu Kamitani, University of Texas, M. D. Anderson Cancer Center (Houston, TX, U.S.A.). Dunning rat prostatic cancer cell sublines, G, AT2 and MAT-Lu, were provided by Nippon Kayaku Corp. (Tokyo, Japan) with the permission of Dr John T. Isaacs, The Johns Hopkins School of Medicine (Baltimore, MD, U.S.A.). An anti-androgen, flutamide, was a generous gift from Nippon Kayaku Corp.

Cloning and sequencing of the SRAP cDNA

A rat prostate cDNA library in the λ ZAPII vector was prepared as described previously [23]. The library contained 5 500 000 independent clones with insert sizes ranging from 0.4 to 5.5 kb. A *Hind*III-digested rat AR fragment containing a DNA-binding domain (DBD) and an activation function 2 (AF2) was radio-labelled with [α - 32 P]dCTP by the random primer labelling method, and hybridization was carried out as described previously [23]. The filters were washed twice at 65 °C for 30 min in 3 $\times$ SSC (where 1 $\times$ SSC = 0.15 M NaCl/0.015 M sodium citrate) and 0.5% (w/v) SDS. The sequence reactions were performed using BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and analysed with an automated sequencer model 377 (Applied Biosystems).

Cell culture, transient transfection and luciferase assay

Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Rat prostate cancer cell lines, Dunning cells, were grown in RPMI 1640 with 0.5 mg of fetuin (Sigma), 1% ITS+TM premix (a culture supplement containing insulin, transferrin, selenium, linoleic acid, and bovine serum albumin; BD Biosciences, Bedford, MA, U.S.A.) and 10⁻⁸ M 5 α -dihydrotestosterone (DHT) for G-cells, and in RPMI 1640 with 10% fetal calf serum and 250 mM dexamethasone for AT-2 and MAT-Lu cells. LNCaP cells were maintained in RPMI 1640 with 10% fetal calf serum. Cells plated on to six-well culture plates were transfected using SuperFect transfection reagent (Qiagen) with 3 μ g of DNA mixture according to the manufacturer's instructions and further grown for 24 h in Dulbecco's modified Eagle's medium with 10% fetal bovine serum dialysed (10 000 molecular-mass cut-off; Sigma). Then ligands were added and cells were further cultured for another

24 h. The luciferase assay was performed using Dual-luciferase reporter assay system (Promega) according to the manufacturer's instructions. The results of the luciferase assay were normalized using the activity of the *Renilla* expression vector pRL-TK. The mammalian expression plasmid of SRAP, pCMV-SRAP, was constructed by subcloning a *Bam*HI-*Xho*I fragment containing the open reading frame (ORF) into pCMVscript at the same cloning sites.

GST pull-down assay

The SRAP cDNA was digested with *Nco*I and *Msp*I, yielding its ORF fragment, which was blunt-ended and inserted into the *Sma*I site of the pGEX-5X-1 expression vector (Amersham Biosciences) in the correct reading frame, obtaining the GST-SRAP fusion protein expression plasmid pGEX-5X-1-SRAP. The GST-SRAP fusion protein was expressed in *Escherichia coli* strain BL21, and purified using glutathione-agarose beads. To express the AF1 of AR and the AF2 with DBD using the TNT-coupled reticulocyte system, the *Eco*RI-*Hind*III fragment and the *Hind*III fragment of AR were respectively subcloned into the corresponding sites of the pGEM3Z vector. The purified GST-SRAP fusion protein bound to agarose beads was resuspended in 100 μ l of interaction buffer [20 mM Hepes, pH 7.9, 150 mM KCl, 5 mM MgCl₂, 0.5 mM EDTA, 0.5 mM dithiothreitol, 0.1% (v/v) Nonidet P-40, 0.1% BSA, 1 mM PMSF and 10% glycerol] [18] and mixed with 7 μ l of 35 S-labelled *in vitro*-expressed AF1, DBD + AF2 and full-length protein of AR with 2 μ M DHT, and incubated for 4 h at 4 °C with a gentle shaking. After five washes with 900 μ l of NETN buffer [20 mM Tris/HCl, pH 8.0, 100 mM NaCl, 6 mM MgCl₂, 1 mM EDTA, 0.5% (v/v) Nonidet P-40, 8% glycerol and protease inhibitors; completeTM (Roche)], the bound proteins were separated by SDS/PAGE (10–20% gel) followed by autoradiography.

Mammalian two-hybrid assay

We used the CheckMate mammalian two-hybrid system (Promega). The expression plasmid of GST-SRAP fusion protein was digested with *Bam*HI and *Not*I, and the SRAP insert was isolated. It was subcloned into pBIND (GAL4) vector at the above cloning sites. The *Hind*III fragment of AR (DBD + AF2) was blunt-ended with T4 DNA polymerase, and subcloned into pACT (VP16) vector at the *Eco*RV site. The amounts of plasmid DNA transfected are given in the figure legends. The Dual-luciferase reporter assay system was employed to determine relative luciferase activities.

Construction of green fluorescent protein (GFP)-SRAP fusion protein expression plasmid and incorporation of mutations in SRAP of the fusion protein

The GST-SRAP fusion protein expression plasmid pGEX-5X-1-SRAP was digested with *Eco*RI and *Sal*I, and the *Eco*RI-*Sal*I fragment containing the ORF of SRAP was ligated in frame at the same cloning sites of the pEGFP-C2 vector (Clontech), constructing a GFP-SRAP expression vector pEGFP-SRAP. Incorporation of mutations into SRAP was performed as follows. For M-1, a frameshift with early termination, pEGFP-SRAP was cut with *Eco*RI, which is in a multiple cloning site of pEGFP-C2 and is located between the GFP and SRAP cDNA, and was blunt-ended followed by ligation of the ends to recircularize. For M-2, a stop codon 5'-adjacent to the initiation codon of SRAP, pEGFP-SRAP was cut with *Hind*III, blunt-ended and recircularized. For M-3, a 5'-deletion mutant of

B

SRAP:	1'	MGSCPASGVDPPSSPVIESETLIEDVLRPLE
SRA isoform 1:	61"	PRVPASETSPGPPPMGPPPPSSKAPRSPPVGSGBPAGSVEPTSFP-VESEAVMEDVLRPLE
SRAP:	32'	QALEDCRGHTKKQVCDDISRRLALLHEQWDGGKLSVPVKRMALLVQELLHHQWDTADDI
SRA isoform 1:	120"	QALEDCRGHTRKQVCDDISRRLALLQEQWAGGKLSIPVKRMALLVQELSSHRWDAADDI
SRAP:	92'	HRSLMVDHVTEVSQWMVGVKRLIAEKRSLSSEE-NKEEKSTVAPE-NQTIPGFQPPSS
SRA isoform 1:	180"	HRSLMVDHVTEVSOWMVGVKRLIAEKRSLSFEEAANEKSAATAEKNHTIPGFOOAS

(A) The clone consists of 893 nucleotides, and has an initiation codon at nucleotide number 227 with a putative Kozak sequence (underlined). The polyadenylation signal is boxed. **(B)** There have been several SRA sequences, such as SRA and SRA isoforms 1, 2 and 3, submitted to GenBank (accession numbers AF092038, AF293024, AF293025 and AF293026). The nucleotide sequence of SRA and SRA isoform 1 are identical with SRA having a foreshortened 5'-sequence. SRA isoforms 1, 2 and 3 were reported to have been successfully translated *in vitro*, and the deduced amino acid sequences of these three SRA isoforms are almost identical with 99.6% identity between isoform 1 and 2, and 99.2% between isoform 1 and 3. The figure shows the alignment of the deduced amino acid sequence of SRAP and SRA isoform 1. They have 79.7% amino acid sequence identity, with SRAP having a foreshortened N-terminal amino acid sequence compared with SRA isoform 1.

M-4, a 5'-deletion mutant of SRAP without frameshift, pEGFP-SRAP was cut with *Bg*III and *Eco*RV, and the linear fore-shortened plasmid was recircularized after blunt-end ligation.

Northern blot analysis and reverse transcriptase PCR (RT-PCR)

Northern blot analysis was performed as described previously [23]. The primers used for detecting the human isoform by RT-PCR were 5'-AGGAACGCGGCTGGAACGA-3' as the sense primer and 5'-AGTCTGGGAACCGAGGAT-3' as the anti-sense primer [24].

RESULTS

In an attempt to isolate a new nuclear receptor sharing similarity with AR, 1700000 independent plaques from a rat prostate library were screened with the DBD+AF2 fragment of AR cDNA as a probe, with very low stringency being used to wash filters. Of the cDNA clones obtained, 20 were clones for AR and one was for PPAR γ_{1a} . Among them, there was a new clone which was ligated to the 3'-fragment of AR in tandem. The fusion of the new clone to the partial AR cDNA was not in frame, and the new clone was ligated to the reverse-complementary cDNA of the partial AR, suggesting that ligation of the two independent clones happened during library construction. The new clone contained 893 nucleotides, and had an initiation codon at nucleotide 227 with a putative Kozak sequence (Figure 1A). Although it has 78.2% nucleotide sequence identity with the reported human steroid receptor RNA activator (SRA), a novel RNA molecule which was reported to co-activate steroid receptors as an RNA transcript [25], our cDNA codes for a functional protein of 146 amino acids with a calculated molecular mass of 16344 Da. Results of a BLAST search using our rat SRAP cDNA sequence showed that there were no other human homologues than the reported human SRA [25], and its isoforms (SRA isoforms 1, 2 and 3) submitted by another group, and the nucleotide sequences of these human SRAs were almost identical. Interestingly, being inconsistent with the notion that the SRA acts as an RNA transcript [25], SRA isoforms 1, 2 and 3 have been submitted to the database with the comment that they have been successfully translated *in vitro* in rabbit reticulocyte lysate although their functional analyses have not yet been reported. Figure 1(B) shows the alignment of the predicted amino acid sequence of SRA isoform 1 and that of rat SRAP. They have 79.7% amino acid sequence identity, with SRAP having a foreshortened N-terminal amino acid sequence.

Since the cDNA coding for the 16 kDa new protein shares nucleotide sequence similarity to SRA, we investigated whether it has effects on nuclear receptor-mediated transactivation. We subcloned the new cDNA into a mammalian expression plasmid pCMVscript, and carried out reporter assays using several nuclear receptor expression plasmids such as pSG5AR for AR, pKCREGR for GR and pCDNA/PPAR γ_1 for PPAR γ_1 , and luciferase reporters containing cognate hormone response elements. As shown in Figure 2(A), co-transfection of our cDNA cloned into pCMVscript increased the transactivation activity of AR in the presence of 50 nM DHT in a dose-dependent manner. The GR- and PPAR γ_1 -mediated transactivations were also enhanced by SRAP in a ligand-dependent manner (Figures 2B and 2C). Thus the new protein was named SRAP.

To address the issue of whether the SRAP enhances the steroid receptor-mediated transactivation as a protein, we performed GST pull-down assays. Prior to expressing the recombinant GST-SRAP fusion protein, *in vitro* transcription/translation reactions were carried out using the TNT T3-coupled reticulocyte system with the SRAP gene in pCMVscript as a template, and the SRAP was successfully translated showing a band of the predicted size range (results not shown). Then we expressed the GST-SRAP fusion protein in *E. coli* strain BL21, and purified it using glutathione-agarose beads to an electrophoretically homo-

Figure 2 SRAP enhances AR, GR and PPAR γ_1 transactivation activities in mammalian cells

(A) HeLa cells were transfected with a DNA mixture containing pMMTV-Luc reporter (1 μg), pSG5-AR (960 ng), pRL-TK (40 ng) and increasing amounts of pCMV-SRAP, and further grown for 24 h. Then 50 nM of DHT was added, cells were incubated for another 24 h and luciferase activities were measured. (B) PC-3 cells were transfected with pMMTV-Luc reporter (1 μg), pRL-TK (40 ng), pKCRE-GR (960 ng) and pCMV-SRAP (1 μg), and GR transactivation activity with or without 50 nM dexamethasone was measured. (C) HeLa cells were transfected with a DNA mixture containing pAOX-PPRE-Luc (3 μg), pRL-TK (40 ng), pCDNA/PPAR γ_1 (60 ng) and pCMV-SRAP (1 μg), and the transactivation activity of PPAR γ_1 with or without 1 μM 15d-PGJ₂ was measured. The values represent the means ± S.E.M. from four (A, B) and five (C) independent transfections.

Figure 3 Direct binding of SRAP protein with DBD + AF2 domain of AR *in vitro* (A) and mammalian two-hybrid assays showing interaction of SRAP protein with the DBD + AF2 domain of AR *in vivo* (B)

(A) The GST-SRAP fusion protein was expressed in *E. coli* strain BL21, and purified using glutathione-agarose beads to an electrophoretically homogeneous state. *In vitro*-translated AF1, DBD + AF2 and full-length protein of AR radiolabelled with [³⁵S]methionine were incubated in batch with purified GST or GST-SRAP bound to glutathione-agarose beads in the presence of 2 μ M DHT. After five washes, the proteins retained by GST-SRAP immobilized on glutathione-agarose beads were analysed by SDS/PAGE (10–20% gel) followed by autoradiography. The input lanes represent 10% of total [³⁵S]-labelled protein used in each interaction. (B) HeLa cells were co-transfected with 1 μ g of pG5luc reporter, 0.5 μ g of either GAL4-SRAP or GAL4 plasmid and 1.5 μ g of VP16-AR[DBD + AF2] or VP16 plasmid. The relative luciferase activities were measured using the Dual-luciferase reporter assay system using *Renilla* luciferase activity as an internal control. The VP16 fusion protein of the DBD + AF2 domain of AR associates with the GAL4 fusion protein of SRAP16, enhancing the luciferase activity from pG5luc reporter plasmid in a DHT-dependent manner. Values represent the means $\pm$ S.E.M. from three independent transfections.

geneous state. Figure 3(A) shows a GST pull-down assay using the purified fusion protein. The results indicate that, in the presence of DHT, [³⁵S]-labelled full-length AR and [³⁵S]-labelled

Figure 4 Functionality of mutant SRAP fused to GFP (GFP-SRAP; A), its protein expression in HeLa cells co-transfected with pSG5AR and pMMTV-Luc in the presence of DHT (B) and the simultaneous nuclear stain with Hoechst 33342 (C)

(A) HeLa cells were co-transfected with pMMTV-Luc reporter (1 μ g), pSG5AR (960 ng), pRL-TK (40 ng) and pEGFP or pEGFP-SRAP (1 μ g), and further grown for 24 h. Then, DHT was added, and relative luciferase activities were measured after incubation for another 24 h. Values represent the means $\pm$ S.E.M. from three independent transfections. (B) In the cells co-transfected with pEGFP-SRAP, pSG5AR and pMMTV-Luc in the presence of 50 nM DHT, *in vivo* translation of the fusion protein was shown as an accumulation of strong fluorescence. (C) The nuclei were simultaneously stained with Hoechst 33342 (2 μ g/ml).

DBD + AF2 of AR proteins were specifically retained by the GST-SRAP fusion protein immobilized on glutathione-agarose beads (Figure 3A, lanes 6 and 9), whereas no significant association was observed with GST alone. Thus SRAP is able to interact with the DBD + AF2 portion of AR *in vitro* as a protein.

Figure 5 SRAP enhances the transactivation activity of AR as a protein

(A) Scheme of mutant constructs. For mutant constructs M-1 and M-2, mutations are incorporated in the junction between GFP and SRAP, i.e. multiple cloning sites (MCS) of pEGFP vector, so that the frameshift with early termination (M-1) and a stop codon adjacent to the initiation codon of SRAP (M-2) occur without modifying any of the SRAP cDNA sequence. M-3 and M-4 are 5'-deletion mutants of SRAP fused to GFP with a frameshift for M-3 and in frame for M-4. (B) HeLa cells were co-transfected with pEGFP, pEGFP-SRAP or mutant construct (1 μ g) together with pMMTV-Luc reporter (1 μ g), pSG5AR (960 ng) and pRL-TK (40 ng), and further grown for 24 h. Then, DHT was added, and relative luciferase activities were measured after incubation for another 24 h. Values represent means $\pm$ S.E.M. from three independent experiments.

To demonstrate that SRAP interacts with DBD+AF2 of AR *in vivo*, mammalian two-hybrid assays were performed in HeLa cells using the GAL4-DBD fusion of SRAP (pBIND-SRAP) and the VP16 fusion of AR [DBD+AF2] (pACT-AR [DBD+AF2]). An interaction of these two hybrid proteins formed a functionally transactive protein complex that activated transfected GAL4 responsive reporter, pG5Luc. As shown in Figure 3(B), the fusion of SRAP to GAL4 enhanced the transactivation activity compared with GAL4 alone. Co-transfection of VP16-AR [DBD+AF2] plasmid enhanced the GAL4-SRAP-mediated transcription of luciferase from pG5Luc reporter plasmid in a ligand-dependent manner, indicating that the SRAP protein interacts with the DBD+AF2 domain of AR *in vivo*.

We further investigated the functional protein expression and localization of SRAP in HeLa cells by using a GFP fusion protein construct. The ORF of SRAP cDNA was ligated in frame to the 3'-end of the GFP gene in pEGFP-C2 vector, constructing a pEGFP-SRAP expression vector. As shown in Figure 4(A), co-transfection of this pEGFP-SRAP enhanced transactivation activity of AR in a ligand-dependent manner

Figure 6 Northern blot analysis of SRAP in Dunning rat prostate cancer cell lines (A) and detection of SRA in human prostate cancer cell lines (B)

(A) A Nytran filter containing 1 μ g of poly(A)⁺ RNA from each of the samples indicated below, was hybridized with a ³²P-labelled SRAP cDNA. Lane 1, MAT-Lu; lane 2, AT2; lane 3, G cells. (B) RT-PCR was carried out with 1 μ g of total RNA from each of the samples indicated below, using a pair of primers as described in the Materials and methods section. For SRA, each PCR consisted of 25 cycles, and for the glyceraldehyde-3-phosphate dehydrogenase (G3PDH) internal control it was 30 cycles. Lane 4, PC-3; lane 5, DU-145; lane 6, LNCaP.

confirming the functionality of the fusion protein. Figure 4(B) shows *in vivo* translation of the GFP-SRAP fusion protein as an accumulation of strong fluorescence in HeLa cells co-transfected with pSG5AR and reporter gene in the presence of DHT, while weak and ubiquitous expression of the control GFP was observed (results not shown). Figure 4(C) shows a simultaneous nuclear stain with Hoechst 33342, and it seems to be difficult to conclude the particular localization, i.e. nuclear localization, of the fluorescence of GFP-SRAP seen in Figure 4(B).

To determine whether SRAP enhances the transactivation activity of AR as a protein rather than an RNA, we made several mutants of GFP-SRAP fusion protein. For mutants M-1 and M-2, the mutations were incorporated in the multiple cloning sites located between the GFP and SRAP cDNA so that a frameshift with an early termination (M-1) and an insertion of a stop codon adjacent to the initiation codon of SRAP (M-2) occur without modifying any of the SRAP cDNA sequence. M-3 and M-4 are 5'-deletion mutants of SRAP fused to GFP with a frameshift for M-3 and in frame for M-4 (Figure 5A). As shown in Figure 5(B), co-transfection of GFP-SRAP enhanced AR-mediated transcription of luciferase in a ligand-dependent manner whereas co-transfection of M-1, M-2, M-3 or M-4 did not enhance AR-mediated transcription of luciferase.

We then examined mRNA expression of SRAP in Dunning rat prostate cancer cell lines and that of SRA [25] in human prostate cancer cell lines. Northern blot analysis showed that mRNA of SRAP is expressed in all the Dunning cell lines (Figure 6A). As shown in Figure 6(B), SRA is expressed in all the human prostate cancer cell lines that we examined, PC-3, DU-145 and LNCaP. The expression levels of SRA seem to be higher in androgen-independent PC-3 cells than in androgen-independent DU-145 and androgen-dependent LNCaP cells. The above results indicate that these co-activators exist and possibly play an important role in nuclear-receptor-mediated transactivation in prostate cancer.

To investigate the possible role of SRAP in anti-androgen-refractory activation of AR in prostate cancer, we performed a reporter assay using AR, MMTV-Luc and SRAP expression plasmids with or without the anti-androgen flutamide. As shown in Figure 7(A), in the HeLa cells co-transfected with SRAP, the

Figure 7 Effect of SRAP on transactivation activity of AR in the presence of flutamide in HeLa (A) and DU-145 (B) cells

(A) HeLa cells were co-transfected with pMMTV-luc reporter (1 μ g), pSG5AR (960 ng) and pRL-TK (40 ng), with or without pCMV-SRAP (1 μ g), and 24 h after transfection the ligand with or without flutamide was added. (B) DU-145 cells were co-transfected with the same plasmids above, and 24 h after transfection the ligand with or without flutamide was added. The relative luciferase activities were measured using the Dual-luciferase reporter assay system. Values represent the means $\pm$ S.E.M. ($n = 5$). Note that the AR activities in both cells co-transfected with pCMV-SRAP were relatively high even in the presence of 5 μ M flutamide.

DHT-dependent transactivation activity of AR was not completely inhibited by adding 5 μ M flutamide. This partially inhibited activity of AR in the SRAP-overexpressing cells was even higher than uninhibited activity of AR in the cells not

transfected with SRAP. Then we investigated the effect of SRAP on AR activity in the presence of flutamide in human prostate cancer cells termed DU-145. In the DU-145 cells co-transfected with SRAP, the DHT-dependent transactivation activity of AR still remained high even in the presence of the 1000–10000-fold molar excess of the anti-androgen (Figure 7B).

DISCUSSION

In this study, we have cloned from a rat prostate library a cDNA coding for a novel nuclear receptor co-activator termed SRAP, which enhances transactivation activities of AR, GR and PPAR γ , and we have shown that this protein molecule associates with AR *in vitro* and *in vivo*. Incorporation of mutations in SRAP resulted in loss of its activity as a nuclear receptor co-activator, suggesting that the active form of this molecule is a protein. We have also demonstrated *in vivo* expression of this novel cofactor protein using a GFP-SRAP fusion protein construct.

On the other hand, the nucleotide sequence of SRAP has similarity with that of an RNA molecule termed SRA, which was reported to co-activate steroid receptors as an RNA transcript based on the experimental results that protein expression of SRA was unsuccessful and that transfected SRA functioned in the presence of the *de novo* protein synthesis inhibitor cycloheximide [25]. It was shown that, even in the presence of 50 μ M cycloheximide, transfected SRA enhanced endogenous GR activity in HeLa cells whereas transfection of SRC-1 and CBP genes did not enhance the activity in the presence of the protein synthesis inhibitor, concluding that SRA acts as an RNA molecule rather than a protein [25]. However, SRC-1 and CBP are large protein molecules with histone acetyltransferase activity, and it is possible that they are much more unlikely to be translated and folded to be functional proteins than such a small protein as SRAP in the presence of 50 μ M cycloheximide. Thus it may be inappropriate to use SRC-1 and CBP as controls in the experiment reported. Subsequently, Watanabe et al. [26] showed that SRA associated directly with p72, an RNA-binding DEAD-box protein which acts as human ER α co-activator, using an RNA gel-shift assay. However, they also reported that the RNA-binding specificity of p72 was very low, mentioning that SRA binding to p72 competed even with its antisense RNA and other RNAs, and that p72 bound to all the RNAs tested [26].

There are several SRA sequences in GenBank. Some of them, named SRA isoforms 1, 2 and 3, are found in the database with the comment that they have been successfully translated *in vitro* in rabbit reticulocyte lysates although their functional analyses have not yet been published, suggesting the possibility that the SRA gene can be translated and may act as a protein as presented here. Although the gene of SRAP appears to be the rat homologue of human SRA, we posit in this paper a new interpretation and the evidence that SRAP acts as a protein rather than an RNA transcript.

It seems that the activation of AR plays a central role in progression of even hormone-refractory prostate cancer. The rationale is the observation that the serum levels of prostate-specific antigen, of which expression is mediated by activated AR binding to an androgen-responsive element upstream of the gene, continue to be a most reliable marker of the disease activity from the hormonally sensitive period to the androgen-independent advanced stage of the illness [27]. Many studies have been performed to elucidate the molecular mechanisms underlying hormone-refractory progression of prostate cancer, focusing on the alterations of AR such as mutation of AR with gain of

function [1,2], AR gene amplification [3] and ligand-independent activation of AR [4–6]. Recently, there have been several reports describing the expression of nuclear receptor cofactors in prostate and breast cancer cell lines in an attempt to associate these molecules with tumour progression. Nessler-Menardi et al. [28] examined, using RT-PCR, the expression of AR co-regulatory protein including a steroid receptor co-activator amplified in ovarian and breast cancer 1 (AIB1), ARA54, ARA70, CBP, cyclin D₁, Her2/neu, Bcl-2-associated gene product-1 ('BAG-1/M/L'), SRC-1, silencing mediator of retinoic acid and thyroid hormone receptors ('SMRT'), TIF2, ARA55, and 'four and a half LIM domains 2' (FHL2) in human prostate cancer cell lines and prostate stroma cells. All of them, with the exceptions of ARA55 and FHL2, were expressed in all the cells, but no significant differences in expression among the cells were observed [28].

On the other hand, Leygue et al. [29] reported that SRA expression in breast cancer was significantly higher in ER+/PR- and ER-/PR+ subgroups than ER+/PR+ and ER-/PR- ones [29]. Thereafter, Murphy et al. [24] reported, using *in situ* hybridization and RT-PCR, that SRA and AIB1 mRNA levels were increased in human breast tumours compared with adjacent normal tissues emphasizing the involvement of these ER regulators in breast tumorigenesis [24]. The above information suggests the possible involvement of SRAP, as an alternative form of SRA, in tumorigenesis and progression of prostate cancer as well. In fact, we have shown that the expression levels of SRAP in androgen-independent MAT-Lu and AT-2 cells seemed to be higher compared with androgen-dependent G cells using Dunning prostate cancer cells, and we have also demonstrated that DHT-dependent transactivation activity of AR in SRAP-overexpressing DU-145 cells remained high even in the presence of an anti-androgen, flutamide.

In summary, we posit a new interpretation and the evidence that a novel steroid receptor co-activator SRAP, as an alternative form of SRA, functions as a protein rather than an RNA transcript. We have also shown the expression of SRAP or SRA in several prostate cancer cell lines and have demonstrated the enhancement of transactivation activity of AR by this novel steroid receptor co-activator using human prostate cancer cells, DU-145. Further study is necessary to elucidate the possible involvement of SRAP in the progression of prostate cancer. Presently, we are making anti-SRAP antibodies to investigate, using Western blot analysis and immunohistochemical methods, the expression of SRAP in clinical specimens of prostate cancer.

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