Expression of Androgen Receptor Coregulators in Prostate Cancer

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ABSTRACT

Purpose: **The androgen receptor (AR)-mediated signaling pathway seems to be essentially involved in the development and progression of prostate cancer. *In vitro* studies have shown that altered expression of AR coregulators may significantly modify transcriptional activity of AR, suggesting that these coregulators could also contribute to the progression of prostate cancer. Here, our goal was to assess alterations in the expression of the AR coregulators in prostate cancer *in vivo*.**

Experimental Design: **The expression of 16 AR coactivators and corepressors (*SRC1*, β-*catenin*, *TIF2*, *PIAS1*, *PIASx*, *ARIP4*, *BRCA1*, *AIB1*, *AIB3*, *CBP*, *STAT1*, *NCoR1*, *AES*, *cyclin D1*, *p300*, and *ARA24*) was measured in prostate cancer cell lines, xenografts, and clinical prostate tumor specimens by using real-time quantitative reverse transcription-PCR. In addition, gene copy number of *SRC1* was analyzed by fluorescence *in situ* hybridization.**

Results: **Both AR-positive and AR-negative cell lines and xenografts expressed the coregulators. Most of the coregulators studied were expressed at equal levels in benign prostatic hyperplasia and untreated and hormone-refractory carcinomas. However, the expression of *PIAS1* and *SRC1* was significantly ($P = 0.048$ and 0.017, respectively) lower in hormone-refractory prostate tumors than in untreated prostate tumors. No overexpression of the coregulators was found in the clinical material. Paradoxically, the *SRC1* gene was found to be amplified and highly expressed in a LuCaP 70 prostate cancer xenograft.**

Conclusions: **These findings suggest that the decreased expression of *PIAS1* and *SRC1* could be involved in the progression of prostate cancer. In addition, gene amplification of *SRC1* in one of the xenografts implies that, in some tumors, genetic alteration of *SRC1* may provide a growth advantage.**

INTRODUCTION

The critical role of androgens in the development of prostate cancer is indicated, for example, by the fact that prostate cancer does not develop in men castrated early in their life (1). In addition, more that 50 years ago, Huggins and Hodges (2) showed that hormonal therapy is an effective treatment for prostate cancer. Subsequently, androgen withdrawal has become the standard and is practically the only effective treatment for advanced prostate cancer. Although most prostate carcinomas are originally androgen dependent, they eventually become hormone refractory during treatment (3). The mechanisms underlying the transition from androgen dependence to androgen independence are incompletely understood.

Androgen action in target tissues, such as the prostate gland, is mediated by nuclear androgen receptor (AR), which functions as a transcription factor. Several findings have already indicated that AR is involved in the development and progression of prostate cancer. First, it has been suggested that certain *AR* polymorphisms are associated with the risk of prostate cancer (4, 5). Second, somatic mutations of the *AR* gene have been found, especially in tumors treated with antiandrogens such as flutamide and bicalutamide (6–8). Third, about one-third of the hormone-refractory prostate carcinomas contain amplification of the *AR* gene, leading to transcriptional up-regulation of the gene (9, 10). In addition, hormone-refractory tumors express more *AR* than the untreated tumors, even without the gene amplification (10). Fourth, other growth factor signaling pathways may activate AR, especially in the presence of only low levels of androgens (11, 12). Finally, recent cDNA microarray studies (13, 14) have demonstrated that many of the androgen-regulated genes become up-regulated at the progression of the disease during androgen withdrawal.

Activation of AR by androgens is a complex process. The apo-AR stays associated with chaperone proteins in the cytoplasm. Ligand binding leads to conformational changes in the receptor and its translocation into the nucleus, where it binds to an androgen response element located in the regulatory regions of target genes (15). In addition to the AR itself, transcriptional regulation involves a large number of activating and repressing proteins (16). *In vitro* studies have indicated that altered expression of some of these coregulators may modify transcriptional activity of AR (17–23). For example, overexpression of a p160 family member, TIF2, in a cotransfection assay enhanced AR transcriptional activity in the presence of several steroids, including adrenal androgens, within physiological concentrations (23). In addition, coregulators may modulate the effects of antiandrogens. For instance, it has been reported that hydroxy-

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flutamide paradoxically increases AR activity when CBP is overexpressed (24). Therefore, changes in the expression of the coregulators might be involved in the development and progression of prostate cancer (25). However, only a few studies on the expression of the coregulators in prostate cancer have been published (22, 23, 26–28).

To evaluate the significance of alterations in the expression of AR coregulators in prostate cancer, we have measured the expression of 16 different putative AR coactivators and corepressors by using quantitative reverse transcription-PCR (RT-PCR). Clinical tumor samples, prostate cancer cell lines, and xenografts were analyzed. The expression data were also combined with the data on the chromosomal alterations of the cell lines and xenografts, obtained previously by comparative genomic hybridization, to identify putative amplifications of the coregulator genes. Finally, the gene copy number of *SRC1* was studied by using fluorescence *in situ* hybridization (FISH).

MATERIALS AND METHODS

Samples. The prostate cancer cell lines (LNCaP, PC-3, DU145, 22Rv1, and NCI-H660) were obtained from the American Type Culture Collection (Manassas, VA) and cultured under the recommended conditions. Freshly frozen samples of 10 prostate cancer xenografts (LuCaP 23.1, 23.8, 23.12, 35, 41, 49, 58, 69, 70, and 73) were made available for the analyses by one of the coauthors (R. L. V.). All xenografts, except LuCaP 49 and LuCaP 58, have been established from hormone-refractory human prostate carcinomas and propagated in intact male mice. Freshly frozen prostate tumor specimens representing benign prostatic hyperplasia (BPH; $n = 9$) and androgen-dependent ($n = 30$) and hormone-refractory ($n = 12$) carcinomas were obtained from Tampere University Hospital (Tampere, Finland). The specimens were histologically examined for the presence of tumor cells using H&E-stained slides. Only samples containing >60% cancerous or hyperplastic epithelial cells were selected for the analyses. The BPH samples were obtained from prostatectomy specimens from cancer patients. However, care was taken to ensure that these samples did not contain any cancerous cells. Samples of hormone-refractory carcinomas were obtained from transurethral resections of prostate of patients experiencing urethral obstruction despite ongoing hormonal therapy. The time from the beginning of hormonal therapy to progression (transurethral resection of prostate) varied from 15 to 60 months. In addition, a tissue microarray containing 47 locally recurrent (transurethral resection of prostate samples), hormone-refractory, formalin-fixed, paraffin-embedded prostate carcinomas from the Tampere University Hospital was constructed.

RT-PCR. Total RNA from the cell lines was isolated using Trizol reagent (Life Technologies, Inc., Gaithersburg, MD) according to the manufacturer's instructions. For RNA extraction from tumor material, tissue was first scratched from frozen tumor blocks using a precooled, sterile scalpel. Subsequently, tissue powder was added directly into Qiagen RNeasy MiniKit tissue lysis buffer (Qiagen, Valencia, CA), and total RNA was isolated according to the manufacturer's instructions. The first-strand cDNA was synthesized with Superscript II reverse transcriptase and oligo(dT)$_{12–18}$ primer according to the manufacturer's protocol (Life Technologies, Inc.). To prepare standard curves, total RNA from LNCaP was used. After first-strand cDNA synthesis, serial dilutions were made corresponding to about 100, 20, 4, 0.8, 0.16, and 0.032 ng of total RNA. Primer sequences used for PCR amplification of each gene are given in Table 1. The primers were designed to correspond to different exons to avoid amplification of genomic DNA. We also checked carefully to ensure that the primers could not detect pseudogenes or retropseudogenes. The PCR reactions were performed in LightCycler apparatus (29) using an LC Fast Start DNA SYBR Green I Kit (Roche Diagnostics, Mannheim, Germany). Thermocycling for each reaction was done in a final volume of 20 μl containing 2 μl of cDNA sample (or standard), 4 mM $MgCl_2$, 0.5 μM each primer, and 1× ready-to-use SYBR Green I reaction mix including Taq DNA polymerase, reaction buffer, and deoxynucleotide triphosphate mix. The cycling conditions were designed according to the manufacturer's guidelines. The annealing temperatures are given in Table 1. The elongation time was calculated by dividing the size of the amplicon (Table 1) by 25. The fluorescence emitted by SYBR Green I was measured in every cycle at the end of the elongation step or, to avoid any fluorescence from nonspecific products, at higher temperatures (for *PIASx*, *CBP*, *cyclin D1*, *p300*, and *ARA24*). After arithmetic background adjustment, the fit point method was used to determine the crossing-point value as described previously (10). Fig. 1 illustrates an example of a standard curve for *PIAS1*. For normalization of the expression levels, the expression of *TATA box-binding protein* (*TBP*) was measured as described previously (10). *TBP* was chosen as the reference gene because there are no known retropseudogenes for it, and the expression of *TBP* is lower than that of many commonly used, abundantly expressed reference genes. We have also shown previously that the use of β-*actin* would give results similar to those obtained with *TBP* (10). The relative expression level was obtained by dividing the values for the gene of interest with the *TBP* value and then multiplying by 100. For xenograft and cell line samples, the *TBP*-normalized results were also normalized against the median expression value detected in those samples. In addition to the melting curve information obtained from the LightCycler, the PCR products were run in 1.5% agarose gel electrophoresis to ensure that a right-sized product (but not other products) was amplified in the reaction. Genomic DNA was also used as a control to ensure that no amplification could result from a residual DNA remaining in the RNA preparation. The intra-assay coefficient of variation for each gene was determined by repeating analyses of two samples 10 times. The coefficient of variation values were 5.7% for *SRC1*, 6.8% for *TIF2*, 6.9% for *BRCA1*, 17.3% for *NCoR1*, 11% for *AES*, 13.2% for *STAT1*, 20% for *cyclin D1*, 10.6% for *AIB1*, 12.6% for *PIAS1*, 4% for β-*catenin*, 19% for *PIASx*, 26% for *ARIP4*, 11.5% for *ARA24*, 8.7% for *AIB3*, 13% for *CBP*, and 28% for *p300*.

FISH. For FISH analysis, human genomic bacterial artificial chromosome clones for *SRC1* (GenBank accession number AC013459) and for *pHyde* (GenBank accession number AC016673) were obtained from ResGen Invitrogen Corp. (Huntsville, AL). Probes for *SRC1* and *pHyde* were labeled by nick translation with digoxigenin-dUTP (Roche Molecular Biochemicals, Mannheim, Germany) and FITC-dUTP (New England Nuclear, Boston, MA), respectively. The dual-color hybrid-

Table 1 The primer sequences used in the real-time RT-PCR

Gene	Primer sequences (5′–3′)	Annealing temperature (°C)	Temperature at the fluorescence measurement	Size of the amplicon (bp)
SRC1	ATGGTGAGCAGAGGCATGACA AAACGGTGATGCTCATGTTG	60	72	349
TIF2	TAATGCACAGATGCTGGCC TCTGTGTATGTGCCATTCGG	67	72	314
PIAS1	CCACATGACACCCATGCCTT CCAAAGATGGATGCCGGGTC	67	72	333
PIASx[a]	TCTTCTGACGAAGAGGAAGACC TCAGAAGATGTTCCAAGCTTCA	59	81	275
ARIP4	ATAGCAAGTTCCTACAGGGC CAGATTCACACCCAAGCATC	61	72	437
BRCA1	TTCAGGGGGCTAGAAATCTG CTACACTGTCCAACACCCACTCTC	62	72	247
β-*Catenin*	AATACCATTCCATTGTTTGTGCAG AGCTCAACTGAAAGCCGTTT	62	72	254
AIB3	TCCAGAACTTCTACCCAGCA ATCAAGTCGCAGTCCTGCTT	61	72	344
AIB1	CGTCCTCCATATAACCGAGC TCATAGGTTCCATTCTGCCG	61	72	255
CBP	CAGAGCGGATCATCCATGACTA GCTTCTCCATGGTGGCATAC	59	83	347
STAT1	TATAGAGCATGAAATCAAGAGCC GGGCATTCTGGGTAAGTTCA	55	72	227
NCoR1	GGCCCTCTTCAGTCTCCTCT GGCAGGTTTTTGACCTGCTA	61	72	364
AES	CGCGATTGACATGATGTTTC CTCTCAATGGCTCCCAAGAC	61	72	339
Cyclin D1	CCCTCGGTGGGTCCTACTTCAA TGGCATTTTGGAGAGGAAGT	55	81	390
p300	CCTGAGTAGGGGCAACAAGA GTGTCTCCACATGGTGCTTG	58	85	353
ARA24	CCACCAGAAGTTGTCATGGAC ACAAGGGATGAGTTCACTTGC	57	82	475

[a] Primers amplify a region that is common to the *PIASx*α and *PIASx*β isoform.

ization was performed essentially as described previously (10, 30). Freshly frozen tissue sections (5 μm) were fixed with a series of 50%, 75%, and 100% methanol-acetic acid (3:1; Carnoy's fixative). The sections from tissue microarray block were pretreated as described previously (30). Subsequently, the slides were denatured and dehydrated in an increasing series of ethanol. Hybridization was done overnight at 37°C. After stringent washes, the slides were counterstained with an antifade solution (Vectashied; Vector Laboratories, Burlingame, CA) containing 4′,6-diamidino-2-phenylindole. The FISH signals were scored from nonoverlapped epithelial cells using an Olympus BX50 epifluorescence microscope (Tokyo, Japan).

Statistical Analyses. The associations of tumor type, clinical stage, and histological grade with expression levels were calculated with nonparametric Kruskal-Wallis and Mann-Whitney *U* tests. Spearman rank test was used to study the correlation of expressions of the AR and coregulators. Grubb's test was used to detect the outlier values in box and whisker plot illustrations.

RESULTS

Expression of Coregulators in Clinical Prostate Tumors. Fig. 2 shows the expression levels of each gene in BPH and untreated primary as well as locally recurrent hormone-refractory prostate carcinomas. The expression levels of the genes varied considerably between individual samples. However, on average, most of the genes were equally expressed in the three tumor groups. Two exceptions were *PIAS1* and *SRC1*, whose expressions were, on average, 2-fold lower in hormone-refractory prostate carcinomas than in untreated prostate carcinomas ($P = 0.048$ and 0.017, respectively). However, no differences in the levels of expression were found between primary tumors and BPH. In the hormone-refractory tumors, the expression of *SRC1* varied considerably. Although the median expression of *SRC1* was lower in hormone-refractory tumors than in BPH or untreated tumors, the highest individual relative expression value was found in a hormone-refractory tumor.

The association between coregulator expression and histological grade and clinical stage of the primary untreated tumors was next analyzed. The decreased expressions of *p300* and *AIB3* were associated with high histological grade ($P = 0.034$ and 0.033, respectively). On the other hand, increased expression of *ARIP4* was associated ($P = 0.006$) with advanced stage of disease (T_{3-4}, or N+ or M+ against $T_{1-2}N_0M_0$). For other coregulators, there were no significant associations. Finally, we determined whether expression of any coregulators correlated with the previously published expression data of *AR* (12). No significant correlations were found.

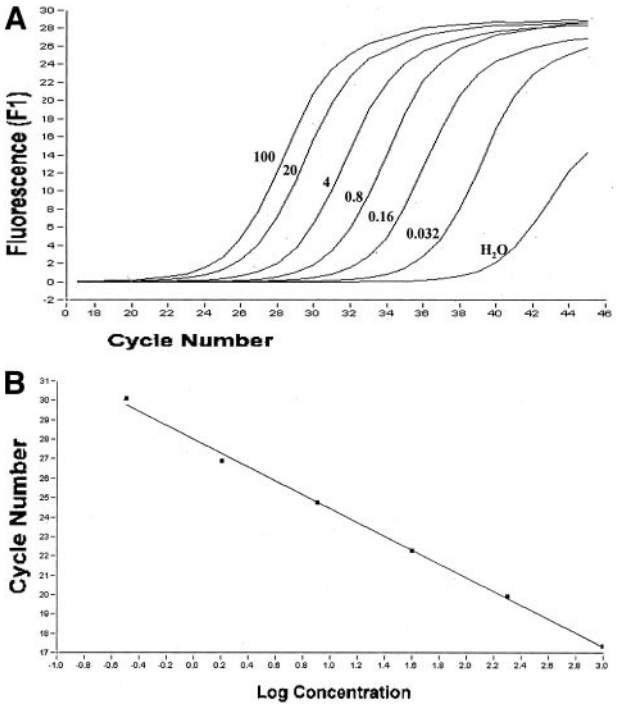

Fig. 1 Standard curve of the PIAS1 mRNA measurement by real-time reverse transcription-PCR. *A*, amplification of serially diluted standards. Cycle numbers are plotted against fluorescent signal measured at the end of the elongation step. Signals are also detected in negative sample due to primer-dimer formation. *B*, standard curve plotting the cycle number at the crossing-point value of each standard presented in *A*.

Expression of Coregulators in Prostate Cancer Cell Lines and Xenografts. Fig. 3 depicts the relative expression levels of the coregulators in the prostate cancer cell lines and xenografts. Xenografts and cell lines were divided into two groups: androgen-independent ones (PC-3, DU145, NCI-H660, 22Rv1, and LuCaP 49); and androgen-sensitive ones (the rest of the cases). Of the xenografts, LuCaP 49 and 58 are the only ones established from tumor samples obtained from untreated patients. LuCaP 49 is a small cell carcinoma with neuroendocrine features and is therefore considered as truly androgen independent (31). The rest of the xenografts originated from patients treated with hormonal therapy. However, after serial passage in intact male athymic mice, the growth of the these xenografts was shown to be inhibited by castration, indicating that they had reacquired an androgen-sensitive phenotype. Of the cell lines examined, only LNCaP is considered to be androgen sensitive. 22Rv1 is androgen independent, expressing mutated *AR* (32), whereas PC-3, DU145, and NCI-H660 do not even express AR. The expression of all AR coregulators was also detected in AR-negative cell lines.

Copy Number Analysis of SRC1. The cell lines and xenografts used in the study have previously been analyzed for DNA sequence copy number changes by comparative genomic hybridization by us (33, 34). To study whether the high-level expression of some coregulators in individual cell lines or xenografts was due to amplification of the corresponding gene, we compared the comparative genomic hybridization profiles and the expression levels. We found that xenograft LuCaP 70 contained a high-level gain at chromosome 2p21-pter (Fig. 4*A*), where the *SRC1* gene is located, and expressed more *SRC1* than the other cell lines or xenografts (Fig. 3). FISH analysis confirmed that *SRC1* is indeed amplified in LuCaP 70 (Fig. 4*B*). Subsequently, we analyzed the other xenografts as well as three hormone-refractory and six untreated prostate tumors, selected based on the high-level expression of *SRC1*, for gene amplification. None of the samples, except LuCaP 70, demonstrated amplification of the gene. Finally, a tissue microarray containing 47 hormone-refractory prostate carcinomas was analyzed by FISH. Six (13%) tumors showed increased copy number (3–4 copies) of SRC1, but none showed amplification of the gene.

DISCUSSION

The transcriptional activity of AR and the other nuclear receptor superfamily members is modulated by different coregulatory proteins. Coactivator proteins enhance AR transactivation in a ligand-dependent manner, whereas the corepressor proteins suppress AR activity either in the absence of androgens or in the presence of antiandrogens (35). AR-mediated transcriptional regulation requires several protein complexes (36) that may interact sequentially, in combination or in a parallel fashion. Whereas recruitment of chromatin remodeling proteins for disruption of histone-DNA interaction by acetyl transferases, such as CBP/p300 (37), is essential to make the target sequences accessible for the ligand-bound receptor, other proteins are needed to bridge the receptor complex to basal transcription machinery (16). Although the critical role of AR-mediated signaling in the development and progression of prostate cancer has become increasingly understood, the role of altered expression of AR coregulators has remained elusive. Thus, the expression of selected AR coactivators and corepressors in prostate cancer was studied in this work.

A real-time RT-PCR approach was chosen to measure the expression of the different coregulatory genes for three reasons. First, good antibodies for immunostaining are available only for a few coregulators. Second, the RT-PCR strategy allows an analysis from small tumor specimens. Third, real-time RT-PCR is a reliable method for quantitation of mRNA abundance (10, 29). The intra-assay coefficient of variation values varied for different genes from 4% to 28%. Thus, at least severalfold expression differences should have been reliably detected, even in the case of the poorest assay (the highest coefficient of variation) performance. The small sample number ($n = 41$) of clinical tumors limits the statistical power of the analyses. However, with the same material, we have previously demonstrated statistically highly significant overexpression of *AR* in the hormone-refractory carcinomas (10), indicating that at least common overexpression of any of the genes analyzed should have been detected in this material.

The most intensively investigated coactivator group is the p160/SRC protein family. It comprises three distinct but structurally and functionally related members, SRC1 (NCoA1), TIF2 (SRC2/GRIP1/NCoA2), and AIB1 [SRC3/RAC3/ACTR (16)]. These proteins interact with steroid receptors and enhance their transcriptional activation in a ligand-dependent manner. In addition, these proteins possess histone acetyltransferase activity,

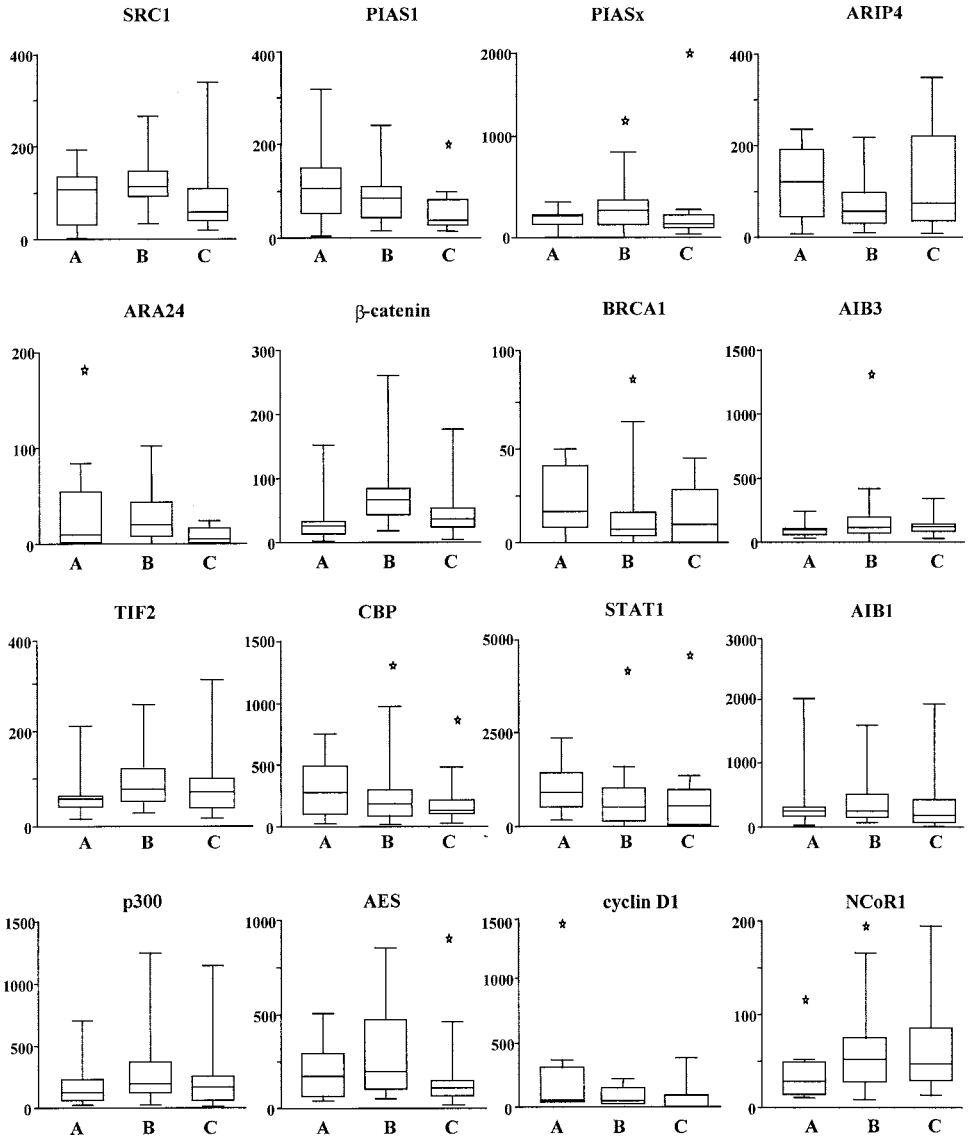

Fig. 2 *Box and whisker plots* displaying the expression of 16 putative AR coregulators in benign prostatic hyperplasias (group A; $n = 9$), primary untreated carcinomas (group B; $n = 30$), and hormone-refractory carcinomas (group C; $n = 12$) by real-time quantitative reverse transcription-PCR. The *boxes* indicate the area of 50% of samples, the *horizontal line in the boxes* indicates median value. The *whiskers* display the range. *Stars* depict the outlier values as evaluated by Grubb's test. Most of the genes were equally expressed in the three tumor groups. The expression of *PIAS1* and *SRC1* was significantly lower in hormone-refractory prostate carcinomas than in untreated prostate carcinomas (group C compared with group B, $P = 0.048$ and 0.017, respectively).

and they are able to recruit CBP/p300 (16). Here, the expression of *SRC1* was found to be lower in hormone-refractory prostate carcinomas than in the untreated prostate carcinomas ($P = 0.017$). The finding that *SRC1* expression is decreased in hormone-refractory prostate cancer is contradictory to the findings of Gregory *et al.* (23), who showed increased SRC1 protein expression in hormone-refractory tumors. Although the average *SRC1* expression was lower in our samples, the highest individual expression value was nevertheless found in a hormone-refractory tumor. This may explain, in part, the discrepancy between our results and those of the study by Gregory *et al.* (23). It should also be noted that Gregory *et al.* (23) studied protein levels, whereas mRNA expression was analyzed here. We also immunostained untreated and hormone-refractory, formalin-fixed, paraffin-embedded prostate carcinomas with polyclonal anti-SRC1 antibody (Affinity BioReagents Inc., Golden, CO). The tumors showed variable amounts of mainly nuclear staining. However, the quality of staining was not good enough for reliable quantitation (data not shown). Our finding of decreased expression of *SRC1* in hormone-refractory tumors is concordant with the findings of an earlier study by Nessler-Menardi *et al.* (26), who showed that an androgen-independent LNCaP subline has lower *SRC1* expression than androgen-sensitive LNCaP. Decreased expression of *SRC1* has also been suggested to be associated with tamoxifen resistance in breast cancers (38). Two recent studies have investigated the association of histological differentiation with *SRC1* expression in prostate cancer. Li *et al.* (28) found no association with *SRC1* expression and Gleason score in 45 primary prostate cancers by using mRNA *in situ* hybridization. In contrast, Fujimoto *et al.* (27) reported that *SRC1* expression, as measured by RT-PCR, was higher in poorly differentiated tumors and in cancers that responded poorly to therapy. Our data showed no correlation between *SRC1* expression and tumor grade or clinical stage in the untreated tumors.

In contrast to down-regulation of *SRC1* expression in hor-

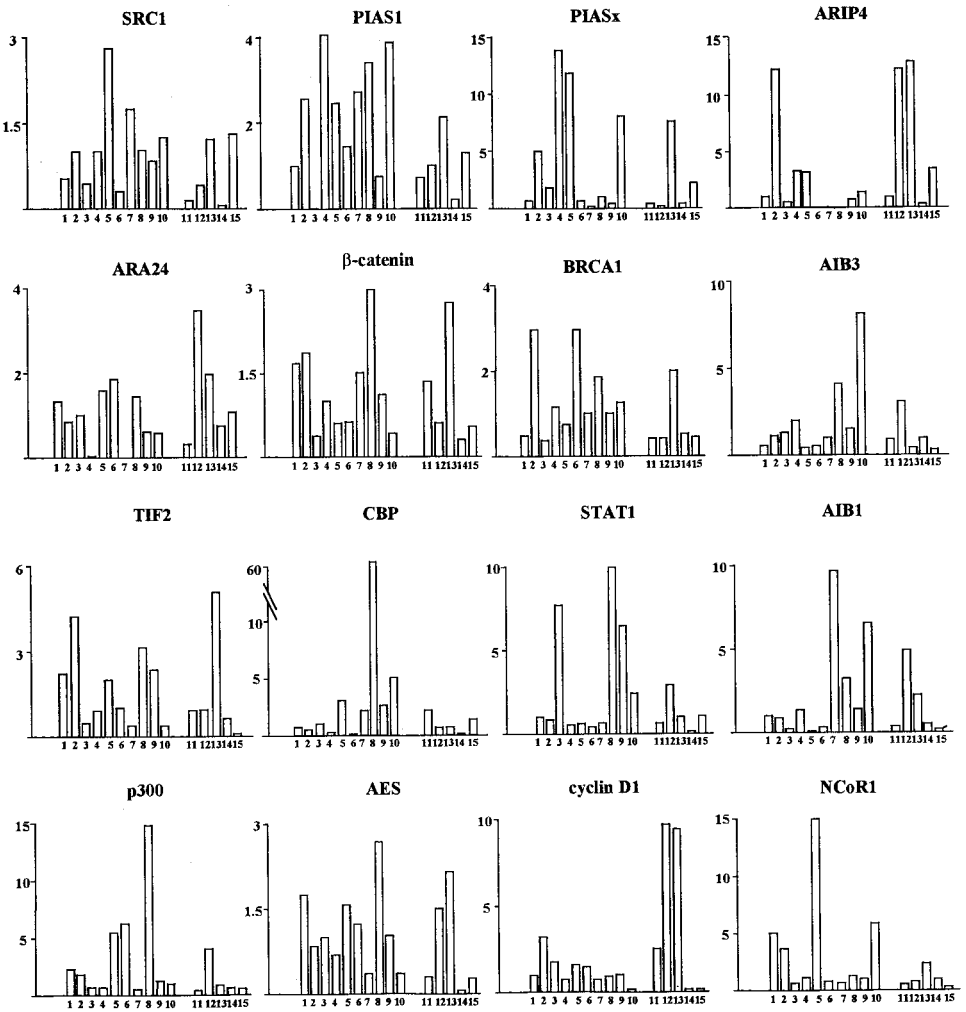

Fig. 3 Relative expression levels of AR coregulators in prostate cancer xenografts and cell lines: *1*, LuCaP 58; *2*, LNCaP; *3*, LuCap 73; *4*, LuCaP 41; *5*, LuCaP 70; *6*, LuCaP 23.1; *7*, LuCaP 23.12; *8*, LuCaP 23.8; *9*, LuCaP 35; *10*, LuCaP 69; *11*, 22Rv1; *12*, PC-3; *13*, DU145; *14*, NCI-H660; and *15*, LuCaP 49. Prostate cancer models 11–15 are considered androgen independent. The expression value of each individual case was normalized against the median expression value of the particular gene.

mone-refractory prostate tumors, amplification of the *SRC1* gene was detected in the LuCaP 70 xenograft. LuCaP 70 was originally established from a hormone-refractory liver metastasis but has been serially passaged in intact male mice. To our knowledge, this is the first description of *SRC1* gene amplification. The amplification seems to lead to up-regulation of the gene because LuCaP 70 expressed more *SRC1* than the other cell lines and xenografts. The other cell lines, xenografts, and clinical tumors analyzed did not exhibit *SRC1* gene amplification. A total of 66 tumors were analyzed for *SRC1* copy number, and only 1 (LuCaP 70) showed the amplification. Nevertheless, the amplification may, in rare cases, give a growth advantage to cancer.

Expression of the other p160/SRC family members was also analyzed. *AIB1*, which is amplified and overexpressed in ~10% of breast and ovarian cancers (39), showed no overexpression in prostate cancer. Interestingly, expression of *AIB1* was very low in xenograft LuCaP 70, which showed high expression of *SRC1*. This may indicate that *SRC1* has substituted the functions of AIB1 in the xenograft. Based on experiments with *SRC1* knockout mice, it has been suggested that p160 coactivator proteins could compensate for the functions of each other, at least to some extent (40). The third member of p160/SRC family, *TIF2*, showed constant expression in different prostate tumor types. This latter finding disagrees with the findings of a study by Gregory *et al.* (23), who showed overexpression of TIF2 protein in hormone-refractory tumors.

The activities of the p160 family members are dependent on CBP and p300 (41). For example, CBP has been shown to interact with AR and augment AR activity in a ligand-dependent manner (17). Recently, Comuzzi *et al.* (24) showed that the antiandrogen hydroxyflutamide enhanced AR activity in DU145 cells forced to express CBP. In the present study, the expression of *CBP* and *p300* was about equal in different groups of clinical prostate samples. However, the expression of *p300* was decreased in poorly differentiated, untreated prostate carcinomas. The data indicate that *CBP/p300* overexpression is unlikely during the progression of prostate cancer.

The PIAS (protein inhibitor of activated STAT) protein family members PIAS1 and splicing variants PIASxα (ARIP3) and PIASxβ (Miz1) have been reported to enhance transcriptional activity of AR (42–44). PIAS proteins exhibit E3 small

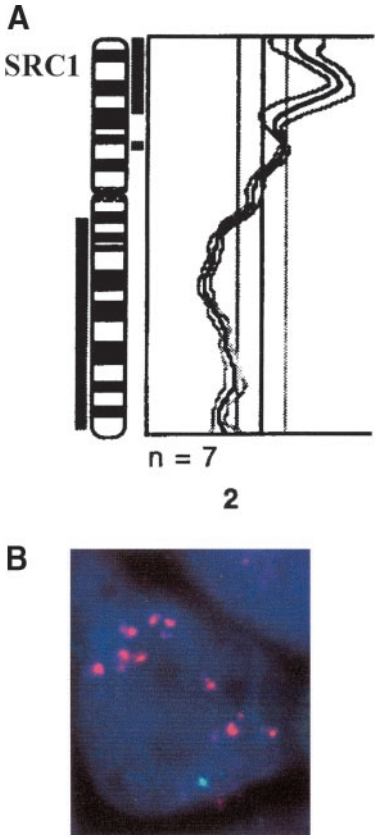

Fig. 4 *A*, comparative genomic hybridization profile (according to Ref. 34) of chromosome 2, indicating amplification of 2p16–2pter, in the prostate cancer xenograft LuCaP 70. The localization of the SRC1 (2p23) gene is shown along the chromosome ideogram. *B*, dual-color fluorescence *in situ* hybridization analysis. The clustering of *red signals* indicates amplification of the SRC1 gene. *Green signal* demonstrates one copy of human pHyde, located at chromosome 2q14.

ubiquitin like modifier (SUMO) ligase activity and modulate nuclear receptor and their coregulator activities through protein sumoylation (45–47). Recently, Li *et al.* (28) showed that expression of *PIAS1* was increased in 33% of primary prostate cancers compared with normal prostates as determined by *in situ* hybridization for *PIAS1* mRNA. Overexpression did not correlate with the Gleason score. Interestingly, we detected decreased *PIAS1* expression in hormone-refractory cancers compared with untreated primary cancers. There was no difference in *PIAS1* expression levels in BPH and untreated tumors. Biological significance of this finding requires additional studies, particularly because the E3 SUMO ligase activity of PIAS proteins is not restricted to steroid receptors and their coregulators (45, 48).

Several putative AR coregulators are mutated in cancers. In addition to *AIB1* (discussed above), *AIB3* (*RAP250/ASC-2*) is known to be amplified and overexpressed in some breast carcinomas (49). However, there was no difference in the expression levels of *AIB3* in BPH, untreated, and hormone-refractory prostate cancer specimens. In addition, *AIB3* expression was lower in poorly differentiated untreated tumors than in well-differentiated untreated tumors, suggesting that *AIB3* overexpression is not common in prostate cancer. BRCA1 has also been reported to enhance the transcriptional activity of AR in a ligand-dependent manner (21). Yeh *et al.* (50) demonstrated a contribution of BRCA1 to androgen-induced cell death via cyclin-dependent kinase inhibitor $p21^{waf/cip1}$, which is an androgen target gene as well. In the present study, we did not find any changes in the expression of *BRCA1* in the different prostate tissue and cancer samples studied. β-Catenin is a known downstream effector of the Wnt signaling pathway, and mutations in β-*catenin* have been described in numerous cancers, including prostate cancer (51). β-Catenin has also been shown to function as a transcriptional coactivator of AR (19). No β-*catenin* overexpression in prostate cancer was detected in this study.

Ran/ARA24 has been shown to interact with the polyglutamine region of AR and to enhance AR-dependent transcription. Recently, Li *et al.* (28) reported that *ARA24* is overexpressed in 81% of primary prostate tumor specimens by using *in situ* hybridization for *ARA24* mRNA. This did not correlate with the Gleason score of the tumors. In this work, we failed to find *ARA24* overexpression with quantitative real-time RT-PCR. This discrepancy could be due to the fact that although mRNA *in situ* hybridization is able to localize expression, it is poorly quantitative.

ARIP4 modulates AR function in a promoter-dependent manner in transient transfection assays: it enhances receptor activity on minimal promoters, but it does not activate more complex promoters. ARIP4 mutants devoid of ATPase activity fail to alter DNA topology and behave as trans-dominant negative regulators of AR function (52). Here, the expression levels of *ARIP4* were found to be higher in either locally (T_{3-4}) or distantly (N+ or M+) advanced disease than in localized cancer. However, *ARIP4* expression was about equal in hormone-refractory tumors and untreated primary tumors. Thus, overexpression of *ARIP4* is unlikely to be involved in the emergence of hormone-refractory disease.

Nuclear receptor corepressors were originally identified as proteins associated with nonliganded type II nuclear receptors that, unlike type I nuclear receptors such as AR, estrogen receptor (ER), glucocorticoid receptor (GR), and progesterone receptor (PR), can bind to DNA in the absence of ligand and mediate transcriptional repression (53). Two of the best-characterized corepressors, NCoR1 and SMRT (silencing mediator of retinoid and thyroid hormone receptor), do not interact with ER, GR, or PR in the absence of ligand (54). By analogy to other steroid receptors, one might expect that the interaction with these corepressors could only happen with antagonist-bound AR (55). Several other corepressors of AR, including cyclin D1 and AES (NH_2-terminal enhancer of split), have been identified (56, 57). We found that *cyclin* D1 was not overexpressed in prostate cancer, confirming the findings of a recent study by Gumbiner *et al.* (58). No alterations in the expression of the other corepressors were found here either.

It should be pointed out that the relative expression levels of coregulators did not correlate with those of *AR* in the clinical tumors. In addition, the AR-negative small cell carcinoma samples (cell lines NCI-H660 and LuCaP 49) and cell lines PC-3 and DU145 expressed the coregulators. This may be due to the fact that most of the coregulators have been shown to possess functions that are not related to the AR signaling pathway.

In conclusion, we demonstrated that several AR coregulators are ubiquitously expressed in AR-positive and -negative prostate tissues and cancerous samples. It appears that overexpression of these AR coregulators is not commonly associated with the progression of prostate cancer. However, other alterations, such as mutations, in these coregulator genes should now be investigated. In addition, the decreased expression of *PIAS1* and *SRC1* in hormone-refractory prostate cancer warrants additional studies.

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