Amplification and overexpression of prosaposin in prostate cancer

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Abstract

We identified prosaposin (PSAP) as a secreted protein expressed in androgen-independent (AI) prostate cancer cells by cloning/sequencing, after probing a PC-3 cDNA library expressed in the λTriplEx phagemid expression vector with a polyclonal rabbit antibody generated against pooled human seminal plasma. PSAP is a neurotrophic molecule; its deficiency or inactivation has proved to be lethal in man and mice, and in mice, it leads to

abnormal development and atrophy of the prostate gland, despite normal testosterone levels. We used Southern hybridization, quantitative real-time polymerase chain reaction, and/or single nucleotide polymorphism (SNP) array analysis, and we now report the genomic amplification of *PSAP* in the metastatic AI prostate cancer cell lines, PC-3, DU-145, MDA-PCa 2b, M-12, and NCI-H660. In addition, by using SNP arrays and a set of 25 punch biopsy samples of human prostate cancer xenografts (LAPC3, LuCaP 23.1, 35, 49, 58, 73, 77, 81, 86.2, 92.1, 93, 96, 105, and 115), lymph nodes, and visceral-organ metastases, we detected amplification of the *PSAP* locus (10q22.1) in LuCaP 58 and 96 xenografts and two lymph node metastases. In addition, AI metastatic prostate cancer cell lines C4-2B and IA8-ARCaP over-expressed *PSAP* mRNA without evidence of genomic amplification. Taken together with prior data that demonstrated the growth-, migration-, and invasion-promoting activities, the activation of multiple signal transduction pathways, and the antiapoptotic effect of PSAP (or one of its active domains, saposin C) in prostate cancer cells, our current observation of *PSAP* amplification or overexpression in prostate cancer suggests, for the first time, a role for this molecule in the process of carcinogenesis or cancer progression in the prostate.

INTRODUCTION

Prostate cancer is a leading cause of cancer deaths in men in the Western world. There is no cure for patients with advanced metastatic disease. In addition to the critical role of androgen(s) at any stage of the disease, polypeptide growth factors not only are implicated in prostate carcinogenesis, but also, as in other malignancies, the expression of a number of these growth factors and/or their receptors is upregulated throughout the course of disease progression (Hegarty et al., 1999; De La Taille et al., 2001; Feldman and Feldman, 2001; Kessler and Albertsen, 2003).

Prosaposin (PSAP) is a highly conserved glycoprotein (65–72 kDa; 527 amino acids) and is the precursor of four small lysosomal proteins, saposins A, B, C, and D, which are required for intracellular degradation of certain sphingolipids (Kishimoto et al., 1992; Huwiler et al., 2000; Sandhoff and Kolter, 2003; Fig. 1A). PSAP also exists as an extracellular secreted molecule having neurotrophic activities (O'Brien et al., 1994). Several reports have identified a 5–22 amino acid sequence with neurotrophic activity, located at the N-terminal portion of the saposin C domain of PSAP. Synthetic peptides derived from this region are characterized by their in vitro and in vivo biological activities, which are similar to those of saposin C or PSAP (Hiraiwa et al., 1997b). Although the receptor interacting with PSAP or its active derivatives (e.g., TX14A peptide, saposin C) has not been fully characterized or cloned, it is known that these effectors exert their activities, at least partially, by binding to a high-affinity G protein-coupled receptor (Vielhaber et al., 1996; Hiraiwa et al., 1997a, b; Campana et al., 1998).
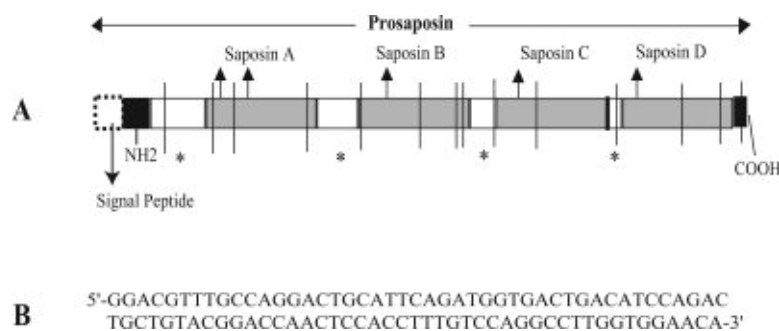

Figure1. Structure of human PSAP and cloning of PSAP from poorly differentiated AI prostate cancer cell line, PC-3. (A) Structure of the PSAP protein organization. Saposin domains and signal peptide are indicated; stars represent proteolytic cleavage sites in the intersaposin sequences; glycosylation sites and exon–intron boundaries are shown by arrows and vertical lines, respectively. (B) Partial sequence of a cloned cDNA fragment with complete sequence homology to the saposin B domain of human PSAP gene. Prosaposin was identified as a secreted protein expressed in AI prostate cancer cells by cloning/sequencing, after probing a PC-3 cDNA library expressed in the λTriplEx phagemid expression vector with polyclonal rabbit antisera generated against pooled human seminal

plasma.

Homozygous deletion of the PSAP gene proved to be lethal in mice (Fujita et al., 1996) and led to the development of distinct abnormalities in male reproductive organs, with gross pathologic features, including a reduction in size and weight of the testes, epididymis, seminal vesicles, and prostate gland and reduced spermiogenesis. In spite of these abnormal findings, the testosterone level was normal or elevated. Microscopic examination of the involuted prostate, seminal vesicles, and epididymis revealed the presence of undifferentiated epithelial cells, which did not show immunoreactivity for phosphorylated-MAPK (mitogen-activated protein kinase) and -Akt (Morales et al., 2000; Morales and Badran, 2003).

We have recently reported a higher expression of PSAP in androgen-independent (AI) prostate cancer cells (PC-3 and DU-145) than in androgen-sensitive (AS), LNCaP, or normal prostate epithelial cells. Immunohistochemical staining of benign and malignant prostate tissues also revealed an intense granular (extracytosolic) anti-PSAP immunoreactivity in tumor cells and in stromal, endothelial, and inflammatory mononuclear cells. In addition, we demonstrated that PSAP or its active derivatives (saposin C domain or TX14A-synthetic peptide) stimulates cell proliferation, migration, and invasion, upregulates the expression of urokinase plasminogen activator (uPA) and its receptor (uPAR), activates stress-activated protein kinase/c-Jun NH2-terminal kinase (SAPK/JNK) and p42/44 members of MAPK- and PI3K/Akt-signaling pathways, and functions as a survival and antiapoptotic factor for both AS and AI prostate cancer cells (Koochekpour et al., 2004c, 2005; Lee et al., 2004b).

On the basis of the cloning experiments described here, we identified PSAP as a molecule of potential interest in prostate cancer. For the first time, we demonstrated overexpression and/or genomic amplification of PSAP in a number of metastatic AI prostate cancer cells and punch biopsy specimens of human prostate cancer xenografts and lymph node metastases. PSAP overexpression without gene amplification was also observed in C4-2B (a bone metastatic AI subline of AS LNCaP cells) and IA8 (a highly invasive clone of ARCaP cell line derived from a patient with a terminal stage hormone-refractory prostate cancer) (Thalmann et al., 1994; Zhau et al., 1996). Upregulated expression of PSAP mRNA and its protein in metastatic or hormone-refractory prostate cancer cells suggests that a combination of androgen deprivation, genomic amplification, or transcriptional/posttranscriptional regulatory mechanism(s) of PSAP might at least partially contribute to the development of AI or metastatic phenotypes in prostate cancer.

Collectively, our observations of PSAP overexpression with or without genomic amplification in prostate cancer cells strongly suggest a role for this molecule in either carcinogenesis or cancer progression in the prostate.

MATERIALS AND METHODS

Cloning of PSAP from the AI Prostate Cancer Cell Line PC-3

Secreted prostatic proteins have the capacity to serve as tumor markers or potential therapeutic targets. Because AI disease is the major cause of mortality in prostate cancer, we sought to identify secreted proteins from AI cells, by the following methodology. After informed consent, we collected seminal fluids (which also contain prostate secretory products) from men on whom sperm analysis was performed as part of a couple-based infertility evaluation. None of these individuals had known prostate cancer.

Clarified seminal plasma was prepared by centrifugation and was frozen at –80°C until use. Pooled seminal plasma was used for immunization of rabbits, and pre- and postimmune sera were collected from these animals. To ascertain antibody recognition of potential antigens expressed by prostate cancer cells, we used these antisera for immunoblotting on protein samples derived from the AI PC-3 and DU-145 cell lines and from AS LNCaP cell lines. Using postimmune sera, we detected various distinct bands with differential expression levels in these cells. To identify these putative proteins, an expression/cloning strategy was then executed.

The λTriplEx™ phagemid cloning expression vector (Clontech Laboratories, Palo Alto, CA) was designed for cloning of genes by immunoscreening. The λTriplEX expresses cloned sequences in all three possible reading frames, and this feature increases the chance of productive clones in a λTriplEx library. Because we were focused on isolating secreted proteins from AI prostate cancer, mRNA derived from the PC-3 (as an example of poorly differentiated, metastatic, and AI prostate cancer) cell line was used as the template for obtaining a custom-made λTriplEx cDNA library (from Clontech). After plating and titrating in accordance with the manufacturer's instructions, library screening was optimized by presorbing of the antibodies with bacterial lysates, to reduce nonspecific background immunoreactivity. The libraries were screened with the selectively pooled rabbit antisera, on the basis of Western blot analyses of cancer cell-derived protein samples. Positive (recombinant) clones of λTriplEx were plaque-purified and converted to pTriplEx. The inserted cDNA fragments were then sequenced.

Tissue Culture and Cell Lines

AI (PC-3 and DU-145) and AS (LNCaP) prostate cancer cell lines were purchased from the American Type Culture Collection (Manassas, VA) and maintained in their defined medium. C4-2B, a bone metastatic AI subline of LNCaP cells, was obtained from UROCOR (Uroscience Group, Oklahoma City, OK), and IA8-ARCaP, a highly invasive clone of the parental ARCaP (androgen-repressed human prostate carcinoma) cell line, was derived from the ascites fluid of a patient with late-stage or advanced metastatic disease and cultured in T-medium or 5% FBS (Thalmann et al., 1994; Zhau et al., 1996). The MDA-PCa 2b cell

line was derived from a bone-metastatic AI prostate carcinoma and routinely cultured in BRFF-HPC1 medium (Athena Enzyme System, Baltimore, MD) supplemented with 20% FBS (Navone et al., 1997). Primary cultures of normal human prostate epithelial (Pr.Ep) and stromal cells (Pr.St) were purchased from Clonetics (BioWhittaker, Walkersville, MD) and used at passage numbers 5 and 10, respectively. These cells were maintained in their defined media (Pr.EGM for Pr.Ep and SCGM for Pr.St), as recommended.

For single nucleotide polymorphism (SNP) arrays, the following prostate cancer lines were used: PC-3, DU-145, LNCaP, MDA-PCa 2b, NCI-H660 (an extrapulmonary small-cell carcinoma of the prostate), WISH-PC2 (a neuroendocrine xenograft of prostate small-cell carcinoma), M12 (a metastatic subline of the SV40 T-antigen-immortalized human Pr.Ep cells), 22-Rv1 (a prostate cancer cell line derived from a hormone-refractory patient), and CA-HPV-10 (a non-tumorigenic immortalized prostate cancer cell line) (Weijerman et al., 1994; Hwa et al., 1998; Sramkoski et al., 1999; Abdul and Hoosein, 2000; Pinthus et al., 2000).

Prostate Cancer Xenografts and Metastatic Tissue Samples

Punch biopsy samples of lymph node (from patients with androgen-dependent (AD) prostate cancer) and visceral-organ (all from patients with AI hormone-refractory prostate cancer) metastases were provided by the Rapid Autopsy Program at the University of Michigan (Shah et al., 2004). Prostate cancer xenografts (LAPC-3 and LuCaP 23.1, 35, 73, 77, 81, 86.2, 92.1, and 105 derived from patients with AI hormone-refractory disease, LuCaP 49 and 115 from patients with AD tumor, LuCaP 58 derived from an untreated patient with clinically advanced metastatic disease, and LuCaP 96 established from a prostate-derived tumor growing in a patient with hormone-refractory disease) were also included in an SNP array analysis (Bav et al., 1994; Wainstein et al., 1994; Laitinen et al., 2002; True et al., 2002; Corey et al., 2003; Linja et al., 2004; Nupponen et al., 2004). LuCaP 49 (established from an omental mass) and LuCaP 93 are hormone-insensitive (androgen receptor [AR]-negative) prostate small-cell carcinomas. These two xenografts demonstrate a neuroendocrine phenotype. LAPC-3 and LuCaP 23.1 are maintained in SCID mice, and other xenografts are routinely maintained by implanting of small pieces of tumors in male BALB/c nu/nu mice. The xenografts designated as LuCaP and LAPC can be requested from Drs. Robert L. Vessella (vessella@u.washington.edu) and Charles L. Sawyers (CSawyers@mednet.ucla.edu), respectively.

Western Blot Analysis

Cells were cultured in their complete media up to 75% confluency, washed with PBS, and incubated in their respective basal media for 24 hr. Cell extracts and concentrated culture supernatants were prepared as described earlier (Koochekpour et al., 2004c). Protein samples (15 µg cell extracts or concentrated culture supernatants) were resolved by

SDS-PAGE under reducing conditions and subjected to Western blot analysis. PSAP was detected by a mouse monoclonal antibody against human saposin C. This antibody was generated through a contract with the antibody production unit at AnaSpec (San Jose, CA) and has been characterized in our laboratory. We obtained similar results using a goat antihuman saposin C as described before (Koochekpour et al., 2004c). Because actin expression is cell type-specific and varies among different cell types, normalization of protein loading for both culture supernatants and whole cell lysate was based on the total cell number as well as on the protein content.

RNA Isolation and Northern Blot Analysis

To analyze PSAP mRNA expression, we cultured the cells for 24 hr in their respective basal media. Total RNA was prepared from subconfluent culture of 11 different cell types, using 0.5 ml of RNA STAT-60 (TEL-TEST, Friendswood, TX) per 100-mm culture plate, as specified by the manufacturer. Northern blot analysis was performed as described previously (Koochekpour et al., 1999). Briefly, 15 μg of total RNA was denatured, electrophoresed, and capillary-transferred, and, after UV cross-linking, the membranes were prehybridized and hybridized. Autoradiography was performed, and after removal of the probe, the membranes were reprobed with a human glyceraldehyde 3-phosphate dehydrogenase for assessment of sample loading. We used a 1-kb PCR amplification product spanning exons 4–12 of the PSAP gene (accession number: M86181). To obtain this probe, we carried out RT-PCR in a gradient thermal cycler (Biometra, Horsham, PA), with normal human Pr.Ep-cell mRNA as a template, and the sense primer 5′-CCAGAGCTGGACATGACTGA-3′ (positions 516–535) and antisense primer 5′-CAGTTCCCAACAAGGGCTTA-3′ (positions 1496–1515) (accession number: NM-002778, as deposited at the NCBI/genome data bank). The PCR product was confirmed as a single band, by electrophoresis on a 1.2% agarose gel, and was gel-purified and sequenced. One hundred nanograms of this fragment was labeled with α32P-dCTP by random-priming, according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Unincorporated labeled nucleotides were removed on a G50–Sephadex spin column (Roche Applied Science, Indianapolis, IN). The relative level of PSAP transcript in different cell types was estimated by scanning of the Northern blot photographic plates with a Phosphorimage scanner and analyzer (Bio-Rad Molecular Imager FX, Hercules, CA). Differences in loading of the lanes were compensated for by comparison with the GAPDH-reprobed membrane. Data were compared with those of normal human Pr.Ep cells.

Genomic DNA Isolation and Southern Blot Analyses

High-molecular-weight DNA was prepared from the subconfluent cell cultures, according to standard procedures, with 1 ml of DNA STAT-60 (TEL-TEST) per 100-mm culture plate. For Southern blotting, 10 μg of genomic DNA was digested with EcoR1 for 24 hr at 37°C

and loaded on a 0.8% agarose gel. After electrophoresis, the DNA was transferred to nylon membrane and was UV cross-linked for 2 min. The blots were probed and reprobed with 32P-labeled probes (PSAP or GAPDH), washed once (under low-stringency conditions), and autoradiographed as described earlier.

Quantitative Real-Time Polymerase Chain Reaction

To evaluate PSAP expression and genomic amplification in the most frequently used prostate cancer cell lines, we performed a duplex (same tube) quantitative real-time PCR (Q-RT-PCR), using a TaqMan assay and the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) as previously described (Koochekpour et al., 2004a). PSAP primers and the TaqMan probe sequence (accession number: BC007612) were designed with Primer Express Software Version 1.5 (Applied Biosystems). PSAP primer sequences were: 5′–3′ forward AAGAGATGCCCATGCAGACTCT (positions 842–863) and reverse TCTTAATGGGCTCCACCAGTTC (positions 928–907). The TaqMan probe for PSAP was 5′-(FAM)-CCAAAGTGGCCTCCAAGAATGTCATCC-(TAMARA)-3′. As endogenous (reference) control gene, we used human GAPDH Control Reagents (Applied Biosystems; product number: 402869; includes a human GAPDH probe (JOE™ probe) and GAPDH forward and reverse primers).

Total RNA and genomic DNA from normal and malignant prostate cells were isolated as described earlier. To eliminate potential DNA contamination, we treated RNA samples with the DNA-free™ DNase treatment and removal reagents as recommended by the manufacturer (Ambion, Austin, TX). We used 5 μg of total RNA to synthesize the first strand of cDNA, with Superscript II reverse transcriptase and oligo (dT)12–18 primer, according to the manufacturer's protocol (Invitrogen Life Technologies, Carlsbad, CA). PCR mix (50 μl) contained 25 μl of TaqMan Universal PCR Master Mix (2×), 100 nm of each primer (PSAP and GAPDH), 10 ng of template cDNA or genomic DNA, and water. Thermal cycling consisted of 2 min at 50°C, 10 min at 95°C, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. Each test included a "no-template" control, and all samples were run in triplicate. Serial dilutions of templates were made corresponding to 100, 20, 4, 0.8, 0.16, and 0.032 ng concentrations. These samples were used to relate the threshold cycle (CT) to the log input amount of template.

After performing the validation experiment (relative efficiency plot for PSAP and GAPDH), we used the comparative CT method to quantitate the level of expression or amplification (User Bulletin, ABI Prism 7700 and GeneAmp 5700 sequence detection system, July 2004; Koochekpour et al., 2004a). The efficiency of the target gene (PSAP) amplification was found to be equal to that of the reference gene (GAPDH) amplification. The amount of target normalized to the endogenous reference and relative to a calibrator was obtained by the arithmetic formula: 2−ΔΔCT. ΔCT represents the differences between the average CT value of the target gene and the endogenous control GAPDH, and ΔΔCT indicates the

difference between the ΔCT values of a test sample and a calibrator. In our study, to compare all other cell types, we used normal human Pr.Ep cells (at passage five) as a calibrator sample. In addition to the melting curve information obtained from the cycler, the PCR products were subjected to agarose gel electrophoresis to ensure the amplification of the right-size product in the reaction tube. The intraassay coefficient of variation for PSAP was also determined (equal to 4.6%) by repetition of analyses of two of the test samples 10 times.

SNP Array Hybridization

Genomic DNA was isolated from a total of 34 samples (cell lines and punch biopsies of xenografts, lymph nodes, and visceral-organ metastases) according to the standard procedure. DNA was then hybridized with Affymetrix 10K SNP mapping arrays containing more than 460,000 probes covering more than 11,565 SNP loci, according to the manufacturer's protocol (Affymetrix, Santa Clara, CA; Kennedy et al., 2003). Briefly, 250 ng of DNA was fractionated with XbaI restriction enzyme digestion, ligated to an adapter, and subjected to single-primer PCR amplification. The PCR product was then fragmented, fluorophore-labeled, and hybridized with the array. Arrays were scanned with a GeneChip Scanner 3000 (Lin et al., 2004). Signal intensities for each probe were downloaded into the informatics platform dChipSNP (Lin et al., 2004; Rubin et al., 2004). Copy numbers were determined by comparison of these signal intensities with a composite reference representing germline DNA from 19 normal individuals, as described previously (Zhao et al., 2004).

RESULTS

Identification of PSAP as a Secreted Protein in Prostate Cancer Cells by Expression/Cloning Strategy

By using postimmune sera and Western blot analyses, we found several secreted proteins with differential expression patterns in conditioned media of the AI (PC-3 and DU-145) and AS (LNCaP) prostate cancer cell lines (data not shown). A cDNA library was constructed in λpTriplEx (a protein expression/cDNA cloning vector). We used PC-3 mRNA as the template. By using postimmune sera for screening, we plaque-purified positive clones. Analysis of the partial nucleotide sequences of one of the cDNAs (with 90 bases) with the Blastn-Nucleotide Sequence database revealed 100% homology to the saposin B domain of the human PSAP gene. A total of seven distinct clones containing PSAP sequences were subsequently identified. The nucleotide sequence presented (Fig. 1B) has been described previously and was deposited in Gene Bank and the EMBL Nucleotide Sequences databases (accession number: NM-002778) (Rorman and Grabowski, 1989).

PSAP Amplification/Overexpression in Prostate Cancer Cells

We examined both intracellular and extracellular expression of PSAP in a number of benign and malignant prostate cells. PSAP was detected as a strong band at approximately 64 kDa, when we used mouse monoclonal or goat antihuman saposin C antibody.

We found that, in each cell line, the expression of secreted PSAP (Fig. 2A, upper panel) was comparable to the intracellular levels (Fig. 2A, lower panel). In addition, in Northern blot analysis using total RNA, we detected PSAP mRNA transcript in all prostate normal and malignant cells under investigation (Fig. 2B). The size of PSAP mRNA closely matched that of Sgp-1, the rat homologue of human PSAP (Morales et al., 1996). The mRNA expression pattern of PSAP was comparable to the protein level in each cell line.
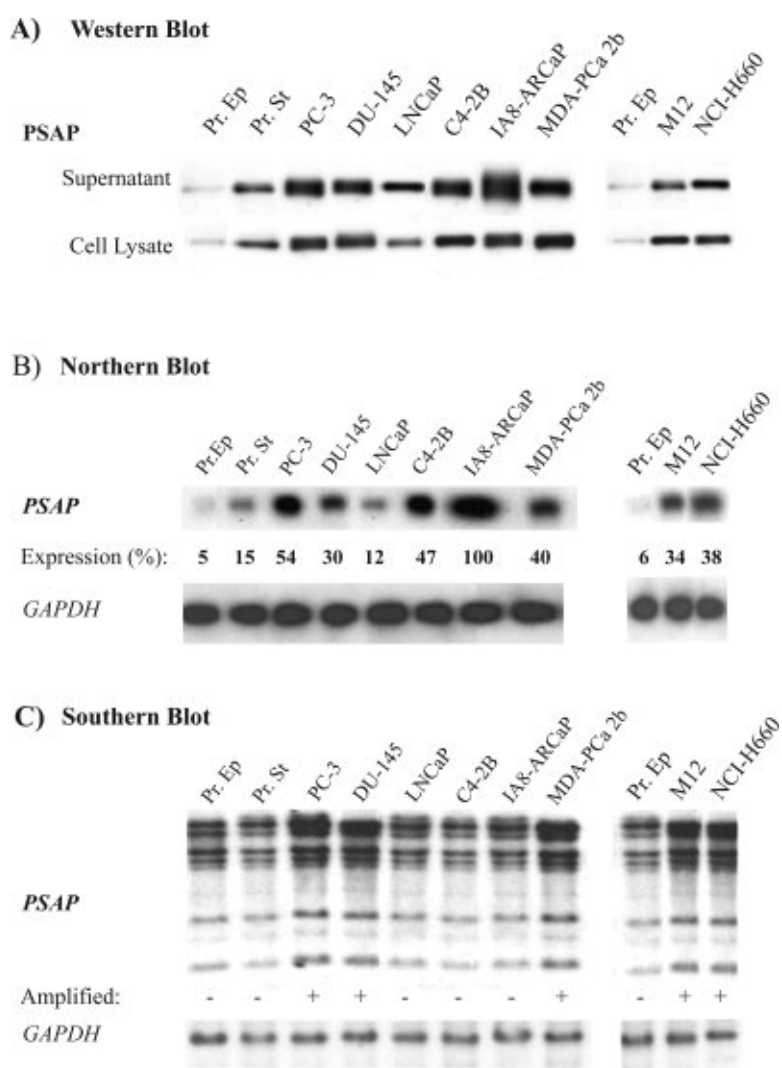

Figure2. Analysis of protein, mRNA, and genomic level of PSAP in prostate cancer cells. Cells were cultured in triplicate up to 75% confluency and then incubated for 24 hr in their respective basal medium. Cell-culture supernatant and whole-cell lysate were prepared from one plate, and the two other plates were used for total RNA and genomic DNA extraction.

(A) Western blot analysis was performed on concentrated conditioned media (upper panel) and whole-cell lysates (lower panel), by SDS-PAGE, in the presence of reducing agent. Mouse monoclonal antihuman saposin C antibody detected the 64 kDa PSAP, in conditioned medium, at a level similar to the intracellular amount. Normalization of protein loading for both culture supernatant and cell extract was based on total cell number as well as protein content. (B) Total RNA extraction and Northern hybridization were performed as described in Materials and Methods. The blots were probed with α32P-labeled-dCTP human PSAP cDNA, by random hexamer priming. An equal amount of loading per lane was demonstrated by reprobing of the membrane with a human GAPDH probe. The relative expression of the PSAP transcript was quantitated by scanning of the Northern blot photographic plates with a phosphorimage scanner and analyzer, and the values were normalized to the GAPDH-reprobed filters. The approximate level of PSAP mRNA was obtained by comparison of the samples and the average level in the reference cell lines. (C) High-molecular-weight genomic DNA was extracted as described in Materials and Methods. Ten micrograms of DNA was digested with EcoR1 for 24 hr at 37°C and was electrophoresed in a 0.8% agarose gel. Hybridization was carried out and the genomic content of PSAP was determined as described earlier. Presence (+) or absence (−) of amplification was confirmed by densitometric analysis as described earlier. Each of the above experiments was performed twice, independently. Pr.Ep, normal human prostate epithelial cells; Pr. St, normal human prostate stromal cells; PC-3 and DU-145, AI prostate cancer cell lines; LNCaP, AS prostate cancer cell line; C4-2B, a bone metastatic AI subline of LNCaP cells; IA8-ARCaP, a highly invasive clone of androgen-repressed metastatic human prostate carcinoma cells; MDA-PCa 2b, a bone metastatic AI prostate cancer cell line; M12, an AR-negative, highly invasive, and metastatic subline of the SV40 T-antigen-immortalized human prostate epithelial cells; NCI-H660, an AR-negative extrapulmonary small-cell carcinoma of the prostate.

PSAP protein and mRNA expression in normal Pr.Ep cells was less than that in prostate stromal or any other cell types (Figs. 2A and 2B). PSAP expression in LNCaP cells was slightly higher than that in prostate stromal cells (used at passage 10), but considerably lower than that in any other metastatic or AI prostate cancer cell lines. Among all cells under investigation, the highest expression of PSAP was observed in the IA8 cell line, representing the highly invasive clone of ARCaP (androgen-repressed metastatic hormone-refractory) prostate cancer cells. Interestingly, the expression level of PSAP mRNA and protein in AI C4-2B cells, a bone-metastatic AI subline of androgen-resposive (-sensitive) LNCaP cells, was considerably higher than that in the parental cells. Overall, these data demonstrate a differential expression pattern for PSAP mRNA and protein, with a higher level in malignant prostate cells originating from hormone-refractory patients or established under in vivo androgen-deprived conditions (C4-2B), compared with normal prostate epithelial and stromal cells (Fig. 2A).

To investigate whether the cell type-specific overexpression of PSAP transcript in the cells under investigation was due to gene rearrangement or amplification, we performed Southern blot analysis, using the same cDNA probe that was used for Northern hybridization. After enzymatic digestion, identical restriction patterns were detected in the genomic DNA extracted from the malignant cells as well as the two reference cell types (normal human Pr.Ep and Pr.St cells). Judging from the intensity of the bands (Fig. 2C), evidence of amplification was found in metastatic and/or AI prostate cancer cell lines (PC-3, DU-145, MDA-PCa 2b, M12, and NCI-H660). In spite of high mRNA and protein expression in C4-2B and IA8-ARCaP (and based on our densitometric analysis of the intensity of bands on Southern blot data), the genomic content of PSAP in these cell lines was comparable to that of the two normal cell types or the LNCaP cancer cell line.

Amplification of PSAP as determined by SNP Array Analysis

As an independent investigation, SNP array analysis was performed on genomic DNA extracted from a set of 14 xenografts, 9 cell lines, and 11 lymph node and visceral-organ metastases derived from patients with hormone-refractory prostate cancer or AD disease. The boundaries of the amplicons covering the PSAP gene were investigated and DNA copy numbers were estimated by measurement (for each SNP) of the intensity of hybridization to an oligonucleotide array with more than 460,000 probes to 11,565 SNPs across the genome (Rubin et al., 2004; Zhao et al., 2004). This analysis revealed amplification of the PSAP locus in seven of the samples investigated: PC-3, NCI-H660 (prostate small-cell carcinoma metastasized to lung), LuCaP 58 and 96 xenografts, and two of the lymph node metastases (Fig. 3). In addition, six other samples also displayed amplicons adjacent to, but not overlapping with the PSAP gene (Fig. 3; line A, 10q21.1–10q22.1). In contradiction to the Southern blot data, the SNP array analysis did not identify PSAP gene amplification in DU-145 or MDA-PCa 2b.
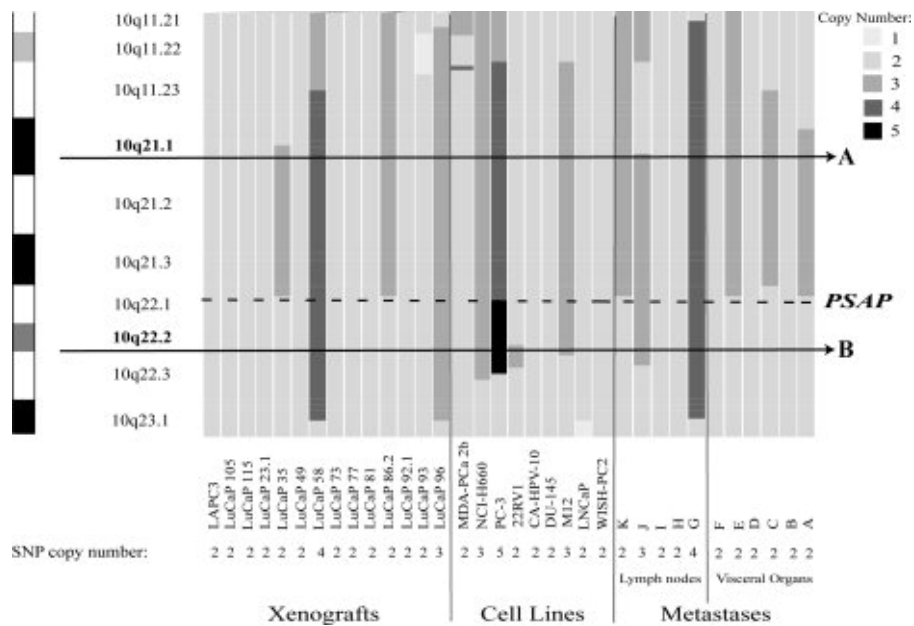
Figure3. SNP array analysis of human prostate cancer cell lines and punch biopsies of xenografts, as well as lymph node and visceral-organ metastases. Genomic DNA was extracted, fractionated with XbaI enzyme, ligated to an adapter, and subjected to single-primer PCR amplification. The PCR product was then fragmented, fluorophore-labeled, and hybridized to Affymetrix 10K SNP mapping arrays, and the arrays were scanned as described earlier. Copy numbers along 10q11.21–10q23.1 (cytoband reference on the left) are displayed as increasingly dark shades of gray. The PSAP locus is indicated by the dashed line. Two of 11 xenografts (LuCap 58 and LuCaP 96), 3 of 9 prostate cancer cell lines (M12, PC-3, and NCI-H660), and 2 of 11 lymph nodes (G and J) and visceral-organ metastases show an amplification stretch spanning the PSAP locus (region from cytoband 10q21.1 (line A) to 10q22.2 (line B)). Six other samples (two xenografts and four metastases) have an amplification stretch (from 10q21.1) adjacent to, but not overlapping with, the PSAP locus. Amplified samples: LuCaP58, xenograft established from an untreated patient who had advanced metastatic prostate cancer; LuCaP96, xenograft established from a prostate-derived tumor growing in a patient with hormone-refractory disease; M12, an AR-negative, highly invasive, and metastatic subline of the SV40 T-antigen-immortalized human prostate epithelial cells; PC-3, AI metastatic prostate cancer cell line; NCI-H660, an AR-negative extrapulmonary small-cell carcinoma of the prostate; G and J, lymph node metastases of prostate cancer. Nonamplified samples are defined in Materials and Methods.

Q-RT-PCR Analysis of PSAP mRNA and Genomic Copy Number in Prostate Cancer Cells

To confirm further the amplification of PSAP (as obtained by SNP array analysis and Southern hybridization) and its overexpression in the most common prostate cancer cell lines under investigation, we used Q-RT-PCR and found that PSAP mRNA was

overexpressed in all prostate cancer cell lines under investigation. The rank order of relative PSAP mRNA expression in cells, from the lowest to the highest, was normal human Pr.Ep, Pr.St, LNCaP, NCI-H660, M12, DU-145, MDA-PCa 2b, C4-2B, PC-3, and IA8-ARCaP cells (Fig. 4A). This ranking coincided with the Northern-hybridization profile (Fig. 2B). Q-RT-PCR also provides a great advantage in the identification of candidate gene amplification or copy number. This technique revealed the presence of PSAP amplification with the highest copy number for PC-3 (7.84 ± 0.61 (SD)), followed by MDA-PCa 2b (5.66 ± 0.4), M12 (4.8 ± 0.44), DU-145 (4.3 ± 0.36), and NCI-H660 (4.15 ± 0.21) (Fig. 4B). Compared with normal prostate cells, the elevated copy number in MDA-PCa 2b and DU-145 supported the Southern blot hybridization data (Fig. 2C). In addition, these results showed that PSAP mRNA overexpression in IA8-ARCaP, C4-2B, and LNCaP cells is not because of genomic amplification.
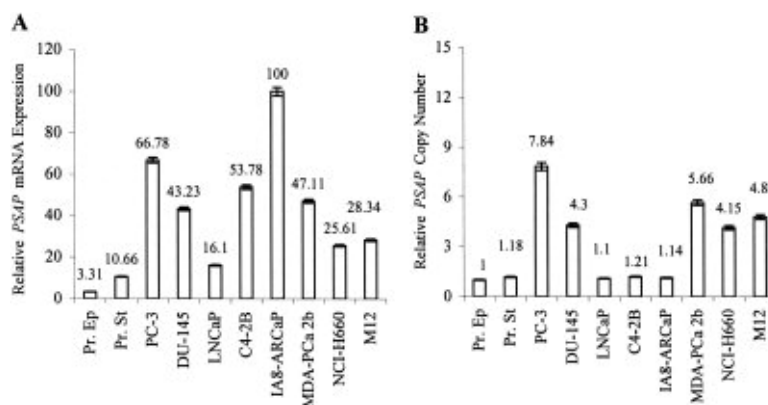

Figure4. Q-RT-PCR analysis of PSAP transcript level and genomic copy number in prostate cancer cell lines. Total RNA and genomic DNA were extracted from prostate normal and malignant cells and subjected to a duplex Q-RT-PCR for PSAP and GAPDH, as described in Materials and Methods. The relative PSAP mRNA expression level (A) and genomic copy number (B) were calculated by use of the comparative CT method, with the arithmetic formula, $2-\Delta\Delta CT$. Normal human prostate epithelial cells were used as the calibrator. Each assay included a "no template" control, and all samples were run in triplicate. Data represent mean ± SD.

DISCUSSION

We recently reported a higher expression of PSAP in AI PC-3 and DU-145 cells than in AS LNCaP and normal prostate epithelial cells (Koochekpour et al., 2004c). Here, we extended our expression analyses to other available metastatic and AI prostate cancer cells as well as punch biopsy samples of xenografts and metastatic lymph nodes and of visceral organs obtained from patients with AI hormone-refractory or AD prostate cancer. We discovered overexpression of PSAP protein in all metastatic AI prostate cancer cell lines examined,

compared with normal cell types. To investigate whether the overexpression of PSAP protein and/or mRNA in cells under investigation was due to PSAP gene rearrangement or amplification, we performed Southern blot analysis. On the basis of the intensity of bands and of densitometric analysis, we found amplification of PSAP in metastatic and AI PC-3, DU-145, MDA-PCa 2b, M12, and NCI-H660 prostate cancer cell lines. The above observation might at least partially explain the presence of high levels of PSAP mRNA and protein expression in these cells. On the other hand, the presence of the considerably high amounts of PSAP at both protein and mRNA levels in the highly invasive IA8 clone of ARCaP and C4-2B cells, with no evidence of gene amplification, raises the possibility that its overexpression may also be because of increased mRNA stability, reduced mRNA degradation, or posttranslational events.

SNPs present the most common type of polymorphism in the genome and provide a powerful diagnostic method for the molecular classification of a variety of pathologic conditions, including (but not limited to) malignancy. Previously, we have demonstrated the limitation and adequacy of detecting changes in DNA copy number (gain or loss) of the high-density SNP array (Rubin et al., 2004; Zhao et al., 2004). In those reports, by using Q-RT-PCR, we showed that our data obtained by dChip analysis of SNP array hybridization were valid, and we also demonstrated that 1.5- to 3-fold (three to six copies) changes in copy number could be predicted with reasonable accuracy, by quantitative SNP array. By using the same method for quantitation and analysis, in an independent investigation, we detected amplification of the PSAP locus in the PC-3, NCI-H660, and M12 cell lines, in punch biopsy specimens of prostate cancer xenografts LuCaP58 and LuCaP96, and in two lymph node metastases. Table 1 summarizes sources of the samples, their androgen sensitivity, and their amplification status. Quantitative SNP array hybridization data showed a relatively high frequency for PSAP amplification (altogether, 20.6% of samples under investigation: 4 out of 25 prostate cancer xenograft and metastatic tissues and three out of nine cell lines) that possibly excludes sporadic amplification for PSAP in prostate cancer and indicates that its locus may present a hot-spot for selection and genomic instability. In addition, PSAP amplification in prostate cancer xenografts and metastatic tissue samples indicates that this genomic alteration is not restricted to cell lines established or cultured under in vitro conditions.

Table1.PSAP Amplification in Prostate Cancer Cells, Xenografts, and Metastatic Tissues

Sample	Patient's tumor		Method of detection		
	AD_a	AI_b	SNP_c	$Q-PCR_d$	SB_e
Xenograft					
LAPC-3		+	−		

Sample	Patient's tumor		Method of detection		
	AD_a	AI_b	SNP_c	$Q\text{-}PCR_d$	SB_e
LuCaP 23.1		+	−		
LuCaP 35		+	−		
LuCaP 49$_f$	+		−		
LuCaP 58	+g		amph		
LuCaP 73		+	−		
LuCaP 77		+	−		
LuCaP 81		+	−		
LuCaP 86.2		+	−		
LuCaP 92.1		+	−		
LuCaP 93$_f$	+		−		
LuCaP 96	+i		amp		
LuCaP 105		+	−		
LuCaP 115	+		−		
Visceral mets$_j$					
A		+	−		
B		+	−		
C		+	−		
D		+	−		
E		+	−		
F		+	−		
L.N. mets$_k$					
G	+		amp		
H	+		−		
I	+		−		
J	+		amp		
K	+		−		

	Patient's tumor		Method of detection		
Sample	AD_a	AI_b	SNP_c	$Q\text{-}PCR_d$	SB_e

Table1.PSAP Amplification in Prostate Cancer Cells, Xenografts, and Metastatic Tissues

Sample	Patient's tumor		Method of detection		
	AD_a	AI_b	SNP_c	$Q\text{-}PCR_d$	SB_e
Cell lines					
MDA-PCa 2b		+	−	amp	amp
NCI-H660$_f$		+	amp	amp	amp
PC-3		+	amp	amp	amp
22RVI		+	−		
CA-HPV-10		+	−		
DU-145		+	−	amp	amp
M12		+	amp	amp	amp
LNCaP		+	−	−	−
C4-2B		+		−	−
WISH-PC2		+	−		
IA8-ARCaP		+		−	−

a Androgen-dependent or prior to treatment.

b Androgen-independent or hormone-refractory.

c Single-nucleotide polymorphism.

d Quantitative real-time PCR.

e Southern blot.

f Small cell carcinoma of the prostate.

g An utreated patient with metastatic disease.

h Amplification of *PSAP*.

i Prostate-derived tumor in a patient with hormone-refractory disease.

j Visceral-organ metastases of prostate cancer.

k Lymph node metastases of prostate cancer.

The size of the amplified DNA before and after the PSAP amplification locus (at 10q22.1; Fig. 3) is in the range of 20–25 Mb. Such a large size strongly raises the possibility that it may be coamplified with other genes whose products might function as biologically stabilizing factors. Our SNP array data also show an increased copy number spanning cytobands 10q21.1, 10q21.2, and up to the distal segment of 10q21.3 in two other xenografts (LuCaP 35 (AI) and LuCaP 86.2 (AI)) and four other lymph node (AD) and visceral-organ (AI) metastases that are in close proximity to the 10q22.1 locus. The

observed high frequency of PSAP amplification and the concurrent presence of other amplicons in this particular region strongly suggest that cell type-specific inductive forces for genomic amplification might exist in the prostatic epithelium.

Unlike our Southern blot analysis data, SNP array data did not show PSAP amplification in DU-145 and MDA-PCa 2b cells. Confirmation of PSAP amplification and its mRNA overexpression in the most frequently investigated prostate cancer cells (including MDA-PCa 2b and DU-145) was further assessed by Q-RT-PCR. Traditionally, Southern hybridization has been used to demonstrate gene amplification and to estimate gene copy number in malignant cells. Compared with the relative subjectivity or the semiquantitative nature of the Southern blot technique, Q-RT-PCR provides a very sensitive and quantitative approach to the detection of target gene amplification or loss. Based on this method, there seems to be a relative underestimation of the inferred copy number obtained by SNP array hybridization in M12, NCI-H660, and PC-3 cells. Similar differences in copy numbers of (amplified) genes have also been described with Q-RT-PCR and SNP array hybridization techniques (Zhao et al., 2004).

The absence of PSAP amplification in MDA-PCa 2b and DU-145 cells by SNP array hybridization may be due to the resolution of the array: none of the SNPs that are tiled on the array lie within the PSAP gene, and so small amplicons covering PSAP may not be resolved. Alternatively, the discordant results may also reflect differences in amplification profiles among different batches of the cell lines under investigation. In addition, amplified genes are frequently unstable, and it is not uncommon that, upon prolonged in vitro culture conditions, they may be lost (Schimke, 1986; Stark, 1986; Fukomoto et al., 1988).

Amplification of PSAP and its transcript overexpression in M12 and NCI-H660 prostate cancer cell lines was further confirmed by both conventional gene expression analysis (Western, Northern, and Southern blots) and Q-RT-PCR. M12 cells represent a subline of P69, a poorly tumorigenic cell line established by SV40 T-antigen immortalization of normal human Pr.Ep cells. Both parental P69 and M12 cells did not express AR mRNA. Therefore, these cells could be considered as AI or androgen-nonresponsive. Unlike P69, the M12 cell line is highly tumorigenic and metastatic after orthotopic injection into the athymic nude mice (Plymate et al., 2004). It is noteworthy that amplification of PSAP in the M12 subline of an immortalized prostate epithelial cell with a viral oncoprotein may raise the possibility that the PSAP gene may also be susceptible to genetic instability induced by transforming or oncogenic viruses. NCI-H660, an extrapulmonary small-cell carcinoma of the prostate gland, is also AR-negative and metastatic. This cell line represents a rare but highly invasive subtype of prostate carcinoma (Hwa et al., 1998; Linja et al., 2001).

Human prostate cancer xenografts provide a valuable model system in in vivo tumorigenesis studies and are especially useful in molecular-genetic characterization of prostate cancer (Linja et al., 2001, 2004; Laitinen et al., 2002; Zhao et al., 2004). All of the xenografts used in our study were obtained from patients who had advanced prostate cancer,

and most of them were established after an androgen-withdrawal period and during a hormone-refractory progressive phase of tumor growth (Table 1). Our data show that PSAP amplification occurs in LuCaP58 and LuCaP96 xenografts. The LuCaP58 was established from an untreated patient who presented with advanced metastatic prostate cancer. The growth rate of this xenograft in intact mice is slower than that in the castrated animal (Linja et al., 2001). The PSAP amplification identified in this xenograft may represent a genomic alteration that might be associated with natural progression of prostate cancer and the acquisition of metastatic phenotypes. The LuCaP96 xenograft was established from a prostate-derived tumor growing in a patient having hormone-refractory disease. PSAP amplification in this locally advanced prostate cancer tissue might indicate a role for this molecule in disease progression. Obviously, further in-depth investigation with relevant and comparable clinical samples from prostate or metastatic locations is required to elucidate the causal relationship between PSAP amplification and/or expression and disease progression in prostate cancer.

The PSAP gene has 15 exons, mapped to 10q22.1 (UCSC Genome Bioinformatics, Human May 2004 (hg16) assembly), and encodes the highly conserved saposins A–D (Henseler et al., 1996). So far, the only reported genetic alterations of PSAP and saposins are limited to point mutations leading to loss of the glycosylation sites, substituting a cytosine residue-generating a new splice site leading to a 33-base insertion into the mRNA, and an A-T transversion in the initiation codon leading to complete failure of PSAP synthesis and early death at 16 weeks of age (Schnabel et al., 1992). With the exception of total PSAP deficiency, because of the compensatory function of other saposin proteins, only some of the affected individuals with saposin deficiencies may develop lysosomal storage diseases (Huwiler et al., 2000).

Several genetic abnormalities in chromosomes 8, 10, and 16 are considered as early or late events in the course of prostate cancer from prostatic intraepithelial neoplasia to the advanced metastatic and aggressive stage (Cher et al., 1994; Lacombe et al., 1996; Nupponen et al., 1998a, b; Linja et al., 2001, 2004; Laitinen et al., 2002; Koochekpour et al., 2004b; Porrka and Visakorpi, 2004; Rubin et al., 2004; Savinainen et al., 2004; Wang et al., 2004). Chromosome region 10q21–10q24 is an interesting site. In one study focusing on chromosome 10, using microsatellite markers and 20 paired normal and primary tumors, 13 out of 20 cases were found with chromosomal changes, mostly deletion and rearrangements. Interestingly, the highest frequency of microsatellite abnormality was observed in two loci mapped to 10q21 and 10q23–10q24 (Achary et al., 2003). In addition, using comparative genomic hybridization (CGH; a molecular-cytogenetic technique that detects changes in DNA copy number in the whole genome), gain or amplification at 10q was reported in approximately 15% of hormone-refractory prostate carcinomas (Nupponen et al., 1998b). With the same technique, the presence of a high level of amplification at 10p12–10q23 has been demonstrated in the PC-3 cell line (Nupponen et al., 1998a). By CGH and comparison

of the chromosomal alterations in an in vivo (orthotopic) prostate cancer progression model (using the PC3M cell line), a gain of 10q21–10q22 was also detected in two sublines of these cells (PC3M-Pro4, which produces larger tumors, and PC3M-LN4, in which there is a high incidence of distant metastatic tumors, and larger metastatic tumors in lymph nodes; Chu et al., 2001).

Interestingly, after correctly mapping the UPA gene to the 10q22 locus (with only 2 Mb distance from PSAP), Helenius et al. (2001) discovered genomic amplification of UPA in the PC-3 cell line (see also Tripputi et al., 1985). It is noteworthy that, in addition to the highest level of PSAP amplification in this cell line (as demonstrated by Q-RT-PCR and SNP array analysis), we have also reported that saposin C (an active domain in the PSAP molecule) upregulates UPA/UPAR mRNA and protein expression in this cell line (Koochekpour et al., 2005). Considering the importance of UPA/UPAR as a major family of matrix-degrading proteolytic enzymes and its role in the invasion and metastasis of malignant cells (including prostate cancer), coamplification of PSAP and UPA in relatively the same chromosomal region—in agreement with other investigators—might strongly suggest and support the presence of a candidate target area for gene amplification. Considering the biological significance of UPA and PSAP, our data also suggest that such coamplifications might act synergistically and in favor of more aggressive malignant phenotypes in prostate cancer.

A number of cDNA microarray studies have also demonstrated upregulation of androgen-regulated genes after androgen deprivation in prostate cancer and during its progression to an advanced metastatic stage (Amler et al., 2000; Mousses et al., 2001). One important finding in our current study is the presence of a high level of PSAP transcript and protein in prostate cancer cells that are either AI or derived from patients with hormone-refractory disease. In AS LNCaP cells, our initial observations also indicate that PSAP is not only an AR-target gene, but also upregulates AR and PSA mRNA and protein expression under androgen-deprived culture conditions, indicating the presence of a bilateral cross-talk between PSAP and AR (Koochekpour et al., 2004b; Lee et al., 2004a; Koochekpour et al., unpublished data). In addition, we also have preliminary data that show PSAP mRNA and protein overexpression in some other endocrine-regulated tumor cell lines (e.g., breast, lung; Koochekpour et al., unpublished observations). Whether or not androgen or androgen deprivation may exert a selective pressure for PSAP overexpression or amplification remains an interesting subject for future investigations.

Overexpression of PSAP in the C4-2B cell line (an AI and bone-metastatic subline of LNCaP), without gene amplification, indicates that PSAP overexpression may be among those carcinogenesis events that are associated with bone metastasis in prostate cancer. The C4-2B cell line was generated by sequential coinoculation of the LNCaP cells with stromal cells in an in vivo androgen-deprived environment (Thalmann et al., 1994). Therefore, the high expression level of PSAP in these cells might be the result of a potential synergistic

effect caused by androgen deprivation and the presence of stroma-derived paracrine stimulation that eventually led to a permanently upregulated level for PSAP expression. The significance and influence of such stroma-derived paracrine regulatory factors has been demonstrated frequently in in vivo tumorigenesis models (Chung et al., 1991). Overexpression or amplification of PSAP might lead to the formation of autocrine/paracrine regulatory loops among various cellular components of stroma and cancer cells in the tumor microenvironment.

The biological significance of PSAP overexpression and its genomic amplification in prostate cancer lies within our previous findings that PSAP or its active derivatives (TX14A or saposin C) stimulate prostate cancer cell growth, motility, and invasion, upregulates UPA/UPAR exprerssion, activates p42/44 and SAPK/JNK members of the MAPK superfamily and PI3K/Akt signaling pathways, and protects cells from apoptotic cell-death induction by etoposide, via modulation of caspase-3, -7, and -9 expression/activity or the PI3K/AKT signaling pathway (Koochekpour et al., 2004b, c; Lee et al., 2004b).

In summary, in our search to identify prostatic proteins that might also be expressed by poorly differentiated prostate cancer cells, by using human seminal fluid and a cloning/expression strategy, we have identified PSAP as a secreted protein that is differentially expressed in AI versus AD prostate cancer cells. Our data, for the first time, provide evidence for PSAP amplification and/or overexpression in metastatic AI prostate cancer cell lines and punch biopsy samples of xenografts and lymph node metastases derived from patients with AI hormone-refractory or AD prostate cancer. Further studies are required to elucidate the regulatory mechanisms of PSAP expression and postreceptor signaling events in normal versus malignant cells, in particular, the relationship of PSAP overexpression to carcinogenesis. Analysis of PSAP expression levels in large clinical samples (serum and tissue) from patients with hormone-sensitive or -refractory prostate cancer is necessary for determining the diagnostic and therapeutic value of this pluripotent growth factor in prostate cancer.

Acknowledgements

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