PrLZ, a Novel Prostate-Specific and Androgen-Responsive Gene of the TPD52 Family, Amplified in Chromosome 8q21.1 and Overexpressed in Human Prostate Cancer


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Abstract

We report a previously unrecognized prostate-specific protein, PrLZ (prostate leucine zipper), a new member of the Tumor Protein D52 (TPD52) family. The gene for PrLZ was localized at chromosome 8q21.1, a locus most frequently amplified in human prostate cancer. Multiple tissue analyses demonstrated PrLZ predominantly in the prostate gland. Although its expression was enhanced by androgens in androgen receptor-expressing cells, PrLZ was detected in all of the human prostate cancer cell lines, regardless of androgen receptor status. Monoclonal anti-PrLZ antibodies were produced and intense immunohistochemical staining of PrLZ was observed in prostate epithelial cells in intraepithelial neoplasia and prostate cancer, whereas lower-level staining was detected in normal and benign epithelial components of the prostate gland. As the only prostate-specific gene identified in the most frequently amplified genomic region in prostate cancer, PrLZ may be the link between chromosome 8 amplification and malignant transformation of the prostate epithelia.

Introduction

Metastasis of prostate cancer (PCa) results from the malignant progression of localized tumor (known as prostatic intraepithelial neoplasia; PIN), and is the main cause of the morbidity and mortality of this disease. The mechanism for the development and progression of PCa has yet to be elucidated. To study the development and progression of PCa with a lineage-related cell model, we established a series of LNCaP sublines, including the C4, C4–2, and C4–2B cell lines, through tumor-stroma interaction and xenograft selection (1). The LNCaP/C4–2PCa model mimics the progression of clinical PCa. Whereas growth of the LNCaP is restrained male hosts, its derivative C4–2 and C4–2B sublines are androgen-dependent, proliferating only in intact but not in castrated male hosts, its derivative C4–2 and C4–2B sublines are androgen-independent, capable of growing in castrated male hosts. These sublines express androgen receptor (AR), secrete prostate specific antigen (PSA) in the absence of androgenic hormones, and metastasize to lymph node and bone. Furthermore, these cells exhibit bone-like properties, expressing osteoblastic factors and secreting bone matrix proteins (2, 3).

Received 10/22/03; revised 1/15/04; accepted 1/19/04.

Grant support: United States Department of Defense (DAMD 17–03–2–0033; L. W. K. Chung, H. E. Zhou, R. Wang; and DAMD PC991274; P. S. Nelson) and NIH (CA089812 and CA76620; W. K. Chung; CA82739, H. E. Zhou; and CA96994, J. A. Petros). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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We studied the LNCaP/C4–2 progression model for clues to genetic and expression changes, which may cause the transition from androgen-dependent to androgen-independent status. For this report, we isolated and characterized a novel prostate-specific transcript, PrLZ, based on its differentiated expression between LNCaP and lineage-related C4–2 cells. Our data suggest that overexpression of PrLZ is associated with PCa progression. PrLZ may function to promote prostatic epithelial proliferation and transformation.

Materials and Methods

Cell Lines and Reagents. PCa cell lines PC3 and DU145 were obtained from American Type Culture Collection (Manassas, VA). C4–2 and C4–2B cells were derived from chimeric LNCaP cell tumors (1). ARCaP was from a PCa metastasis (4). Cells were maintained in T-medium (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum and antibiotics (penicillin, 100 unit/ml and streptomycin, 100 μg/ml) at 37°C with 5% CO₂ in a humidified incubator. To study the effect of androgen, cells were plated on a 10-cm dish at ~50% confluence, subjected to androgen starvation in phenol red-free RPMI 1640 for 48 h, and treated with a synthetic androgen, methyltrienolone (R1881; Perkin-Elmer Life and Analytical Sciences, Boston, MA), in fresh phenol red-free RPMI 1640 with 10% dextran/charcoal absorbed fetal bovine serum for 12 h.

Microarray Expression Analysis. Total RNA samples from LNCaP and C4–2 cells were used for synthesizing fluorescence-labeled cDNA probes to screen an array of 1500 unique sequences derived from the prostate expression database (5). The detailed protocol used for analyzing prostate gene expression has been described previously (5). Two hybridizations were performed for each sample, and for each arrayed gene four data points were collected for statistical comparison.

DNA Cloning and Sequencing. A cDNA library of the C4–2 cells was constructed into the ZAPExpress phage (Strategene, La Jolla, CA) by the manufacturer’s recommended protocol. A 330-bp insert from the original expressed sequence tag clone, identified through cDNA microarray, was used as a probe to screen 2 × 10¹² plaque-forming units of the library. Positive clones rescued into pBK-CMV phagemid were subjected to restriction mapping and DNA sequencing. A human genomic library in bacterial artificial chromosome was screened with PCR with 5’-GGCTTAAGCTTTGATCCAGTACACGAGGTGGTCGAGATGAGATG-3’ as primers. Restriction fragments were subjected to nested deletion with the Erase-A-Base kit (Promega, Madison, WI). Manual DNA sequencing was performed with the ΔTaq Sequenase II kit (USB, Cleveland, OH), and automated sequencing was on an ABI sequencer (Applied Biosystems, Foster City, CA).

Expression and Multiple Tissue Expression Assays. The PrLZ-specific fragment was cloned to pGem-T easy (Promega) after amplification of the PrLZ cDNA with primers: 5’-GGCTGAACTGTTTGTTGCTGCTGCT-3’ and 5’-GAGTAGGTTGaCCTCCCCGGTGAGATGAGATG-3’. Similarly, coding se-
quences of the PSA and prostate-specific membrane antigen (PSMA) were cloned with primers 5′-ATGTTGCTCCCCGAGTCTCCCTCAC-CCTGTC-3′ and 5′-TCAGGGTTGCCACAGTGGTCTCCTGTC-3′ (PSA), and 5′-ATGATGATCCAGGCTGATCTCCCATCAGAAACC-3′ and 5′-CTAAGGTTTTAGGCATCTCCTACAAAGTCTC-3′ (PSMA) from prostate RNA (Clontech, Palo Alto, CA). Manufacturer-recommended protocols were used in hybridizations to the Multiple Tissue Expression array, the Multiple Tumor Expression array, and the Human Total RNA Master Panel (Clontech).

**Immunohistochemical Staining.** Polyclonal antibodies were produced by immunizing mice with synthetic peptides, followed by establishing and screening hybridoma cells for monoclonal antibody production (University of Virginia Hybridoma Core Facility). Antibody specificity was confirmed by Western blot and immunoprecipitation. Preimmune sera or control hybridoma fluid were used as negative control.

Multiple tumor arrays prepared from 100 radical prostatectomies performed at Emory University were subjected to immunohistochemical staining with the anti-PrLZ antibody. The data were evaluated by two pathologists (S. D. L. and M. A.). PrLZ staining was set as low if 0–15% of the cells were stained stronger than the normal level, and high if >15% cells were strongly stained. The data were subjected to Fischer’s exact test for cross-table analysis, with P < 0.05 considered statistically significant.

**PCR Analyses.** All of the PCR reactions were initiated with incubation at 94°C for 2 min, followed by 30 cycles of 94°C, 30 s; 55°C, 30 s; and 72°C, 2 min. Reactions were finished with a 72°C, 7-min extension. Primers were used with 5′-CTGAAATCTTTTTGATCTGGCCACATTTG-3′ and 5′-CAAATTTGTTGAGTATGTCGCGGTTGAG-3′ for exon 1 of the PrLZ gene; 5′-TCTAAGATGAGGGAGAAGAACACGTCGTTGG-3′ and 5′-ACTGATAGTGGATATTAACTGTTTCAATAGT-3′ for exon 7 of the PrLZ gene; 5′-CAAGTATCTGAGCTTTTCAATATTCCACGAG-3′ and 5′-GGGATTTAGGGTTTCCTGTTGT-3′ of the expression of the PrLZ and TPDS2 was done with gene-specific primer pairs of 5′-ATGGATTTGAGAGATGCTTTAATATAGG-3′, 5′-TCAAGGGCTCTCCGCAGTTTCTCAGG-3′ (PrLZ), and 5′-ATGGACGCCGCCGCGACCA-3′, 5′-TCACAGGCTCTCCCT TGTTCTTCTGAAGAGG-3′ (TPDS2).

**Fluorescence in Situ Hybridization.** The PrLZ-specific probe was labeled with either digoxigenin (Roche Diagnostics, Mannheim, Germany) or Alexa Fluor 594-dUTP (Molecular Probes, Eugene, OR) by nick-translation, and hybridized together with FITC-dUTP (DuPont, Boston, MA) -labeled chromosome 8 centromeric probe (pM128) to metaphase chromosomes as described (6). Slides were counterstained with 0.1 μm 4,6-diamidino-2-phenylindole in an antifade solution. Assignment of the gene to chromosome was based on 4,6-diamidino-2-phenylindole banding pattern.

**Fluorescence in situ hybridization was used to determine PrLZ gene amplification in PCA specimens. Forty formalin-fixed, paraffin-embedded, locally recurrent, hormone-refractory specimens (transurethral resection of prostate) from Tampere University Hospital were analyzed (6). PrLZ amplification was classified into three groups, no amplification (no in genomic signal), low-level amplification (3–4 copies of signals per cell), and high-level amplification (>5 copies of signals or clusters of signals per cell).

**Results.**

**PrLZ Isolation.** We studied 1500 arrayed genes for their expression in LNCaP and C4–2 cells. Comparative analysis and subsequent Northern blot hybridization identified 8 clones with >2-fold higher expression in C4–2 than in LNCaP (Fig. 1A). One of the clones showed a 9-fold higher expression in C4–2 (PrLZ; Fig. 1A). Using this expressed sequence tag clone (probe P; Fig. 1B) as probe, we screened a cDNA library of C4–2 cells and isolated 24 positive clones. DNA sequencing analyses revealed that these clones were from two transcripts that shared a homologous sequence in their 3′ half, but contained distinctive 5′ regions (Fig. 1B). Homology search through the GenBank revealed that one represented a previously reported TPDS2 (7), except the new isolate had a longer 5′ untranslated region and a shorter 3′ untranslated region. The other transcript contained a unique 502-bp sequence in its 5′ region, completely divergent from that of TPDS2. It encoded a polypeptide with a 41-residue unique NH2-terminus, 35 residues longer than the TPD52 protein (Fig. 1B; GenBank accession no. AF202897). We named this new isolate PrLZ, and characterized its transcript and protein product.

The full cDNA for PrLZ was 2573 bp in size, encoding a 224 amino acid residue polypeptide of 24.4 kDa, which was confirmed with in vitro transcription coupled translation, with the full cDNA as template (data not shown). PrLZ protein would be acidic (pI 4.64) and hydrophilic. Many structural features of the TPDS2 family were conserved in PrLZ. These included a coiled coil leucine zipper in the central region, two PEST domains flanking the leucine zipper, and multiple canonical serine/threonine phosphorylation sites, substrates for casein II kinase, protein kinase C, and cyclic AMP- and cyclic guanosine 3′,5′-monophosphate-dependent kinases. PrLZ harbored an additional N-glycosylation site and an NH2-terminal citron motif in its unique NH2-terminus. In cultured cells, the majority of the PrLZ-fusion protein was seen in cytoplasm, as determined by tagging PrLZ with green fluorescent protein and by Western blotting of the subcellular fractions (data not shown).
Prostate Specificity of PrLZ. The first 502 bp of the PrLZ cDNA, determined as unique and PrLZ-specific (probe PrLZ-502; Fig. 1B), was used as a probe to study the expression of PrLZ in various tissue and cell types. PrLZ was expressed predominantly in human prostate (Fig. 1C), with only minimal expression in the gastrointestinal tract and a few other glandular tissues with secretory functions (i.e., pituitary, salivary, and mammary glands, pancreas, and kidney). The expression in prostate was at least 12-fold higher than in any other tissues. Importantly, PrLZ was not detected in the rest of the 52 different embryonic and adult human tissues, nor in a list of human cell lines of extraprostatic origin. Similar prostate-specific expression of PrLZ was found in additional studies with the Multiple Tumor Expression array and with the Human Total RNA Master Panel (data not shown). In contrast, TPD52 was expressed in numerous human tissues without evidence of tissue specificity.

Chromosomal Localization and Amplification of the PrLZ Gene. Taking advantage of the gene specificity of the 502-bp fragment, we localized the PrLZ gene at human chromosome 8q21.1 (Fig. 2A). The PrLZ gene was isolated in a 180-kb genomic fragment in a bacterial artificial chromosome clone. Exons were mapped and corresponding restriction fragments subcloned for DNA sequencing. The PrLZ gene was also contained in the draft gene. Genomic structure of the PrLZ gene was also contained in the draft gene at human chromosome 8q21.1 and 8q21.3 with only minimal expression in the gastrointestinal tract and a few other glandular tissues with secretory functions (i.e., pituitary, salivary, and mammary glands, pancreas, and kidney). The expression in prostate was at least 12-fold higher than in any other tissues. Importantly, PrLZ was not detected in the rest of the 52 different embryonic and adult human tissues, nor in a list of human cell lines of extraprostatic origin. Similar prostate-specific expression of PrLZ was found in additional studies with the Multiple Tumor Expression array and with the Human Total RNA Master Panel (data not shown). In contrast, TPD52 was expressed in numerous human tissues without evidence of tissue specificity.

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Regulated Expression of PrLZ. Under regular culture conditions (T-medium with 10% fetal bovine serum), PrLZ was expressed in all of the tested PCa cell lines, independent of the AR status (Fig. 3A). This was in sharp contrast with PSA and PSMA, which were seen only in the AR-expressing LNCaP and its lineage derivatives. To investigate the regulation of PrLZ by androgens, we treated the PCa cells with an androgen analog, R1881 (1 nM), after androgen deprivation. After culturing the PCa cell lines under

Fig. 2. PrLZ gene amplification at chromosome 8q21.1. A, localization of PrLZ gene. Shown is a representative micrograph of the fluorescence in situ hybridization analysis (FISH), with red signals defining PrLZ at chromosomal 8q21.1 and green signals chromosome 8 centromeres. Inset is a magnification of the chromosome 8 and chromosome 8 ideogram. B, structure of the PrLZ gene. Genomic structure of PrLZ is shown at the top, with solid bars indicating exons. The corresponding region in cDNA is indicated in the lower portion. C, amplification of PrLZ gene in prostate cancer (PCa) specimens as detected by PCR. In this study, paired genomic DNA samples isolated from cancer cells (T), and unaffected glandular cells (N) of the same PCa specimen (depicted with case numbers), were used to detect the first and last exons of the PrLZ gene. Equal amounts (100 pg) of DNA were used in each sample. In the control lane (C), 10 ng of normal human genomic DNA (Clontech) was used. PrLZ gene was amplified in four of the seven cases tested (266T, 316T, 285T, and 320T). The equal amount of genomic DNA was controlled by PCR of 3'(HIF1A), which is rarely amplified in PCa (21). Mitochondrial DNA (mtDNA) was amplified for similar purposes. D, amplification of PrLZ gene in PCa specimens as detected by FISH. In contrast to the signal in normal prostate epithelial cells (top left panel) and high-level amplification (top right panel), nuclei of PCa cases showed low-level amplification (bottom left panel) and high-level amplification of the PrLZ gene. Amplification is seen as scattered (bottom left panel) or clustered (bottom right panel) hybridization signals. Green signals outside the nuclei were due to autofluorescence, typical for FISH analysis with tissue sections.
The constitutive expression in C4-2 compared with its regulation of PSA, androgen treatment seemed to enhance androgen deprivation nor by androgen stimulation (Fig. 3A). PrLZ was uniformly expressed (Fig. 3A), its expression neither affected by androgen deprivation nor by androgen stimulation (Fig. 3B). Compared with its regulation of PSA, androgen treatment seemed to have distinguishable effects on PrLZ expression. The effect of R1881 on PSA expression was seen only in LNCaP cells, whereas the constitutive expression in C4-2 and C4-2B was largely unaffected (Fig. 3B). In contrast, androgen-enhanced PrLZ expression was seen in both parental and LNCaP-lineage cells, with the highest up-regulation seen in C4-2B cells. The transition from androgen-dependent to androgen-independent state seemed a heterologous process; whereas LNCaP sublines lost the control for PSA expression, these cells still maintain the capability of controlling some other androgen responsive genes, such as PrLZ.

To study the expression of PrLZ at the protein level, we raised polyclonal and monoclonal antibodies by immunizing mice with a synthetic peptide, based on the unique NH2-terminal coding sequence of PrLZ. The antigen specificity of the antibodies was assessed by Western blotting against the endogenous PrLZ protein (Fig. 3C) and by immunoprecipitation analysis of the FLAG-tagged PrLZ (data not shown).

**PrLZ Expression in PCa Specimens.** Using the specific antibodies, we determined the expression of PrLZ in association with clinical PCa. Specimens from 100 PCa cases were used. PrLZ was generally low in unaffected secretory epithelia (75.3% being low) and in benign prostatic hyperplasia (BPH) (78.2% being low; Fig. 4B). It was highly expressed in high-grade prostatic intraepithelial neoplasia (PIN) (84.5% being high) and PCa (75% being high; Fig. 4B). Gleason grade 4 tumors stained more than Gleason grade 3 tumors. Remarkably, intense PrLZ staining was always limited to malignant cells, with the neighboring unaffected epithelial cells stained at the normal level (Fig. 4B). Enhanced expression of PrLZ was therefore, tumor cell specific.

**Discussion**

**PrLZ as a Prostate-Specific Protein and New PCa Marker.** Data from all of our expression studies strongly supported the finding that PrLZ is predominantly expressed in prostate, with minimal expression seen in a few other glandular organs. One unique feature of PrLZ expression is that, in contrast with other known prostate-specific proteins, which are found only in AR-expressing LNCaP and its lineage cells, PrLZ expression was detected in all of the PCa cell lines regardless of their AR status (Fig. 3A and B).

PrLZ appears to be a new member of the TPD52 family, a group of homologous proteins identified due to overexpression of D52 in tumors (7, 9, 10). Distinctively, PrLZ expression is prostate-specific and androgen-responsive, whereas TPD52 could be detected in many tissue and cell types (10), and is not affected by androgen (Fig. 1C and Fig. 3B). Using a gene-specific probe, we localized the PrLZ gene to chromosome 8q21.1 (Fig. 2A) and determined the gene structure by studying a bacterial artificial chromosome clone (Fig. 2B). Previously, other laboratories used the full cDNA as a probe to localize the TPD52 gene on 8q, proximate to the locus in which the PrLZ gene was localized (7, 8).
on AR presence (Fig. 2, B and C). Thus, enhanced PrLZ expression in PCa is correlated to malignancy rather than to AR activity.

**Oncogenic Property of PrLZ.** The biological function of PrLZ is unclear. Besides its unique NH$_2$-terminal structure, PrLZ contains conserved domains of the TPD52 family. Members of the TPD52 family are involved in protein-protein interaction (11–13). Annexin VI (14), MAL2 (13), syntaxin I, and VAMP2 (15) have been identified as interacting partners of TPD52 proteins. 14–3-3, a crucial player in Ras signaling, vesicular transport, and cytoskeletal organization, was revealed recently as another interacting partner (11). Also, TPD52 proteins could be modified post-translationally with phosphorylation, N-glycosylation, or protease digestion. In a separate study we found that PrLZ was highly expressed in the developing prostate gland (data not shown) with a spatial and temporal expression suggesting that PrLZ may help regulate growth, morphogenesis, and cytodifferentiation of glandular ducts during development (16).

The role of PrLZ in PCa development and progression has yet to be elucidated. The coding sequence of the PrLZ in PCA cell lines contained no mutations. PrLZ cDNA, when transfected to ARCAp cells, markedly stimulated their growth in immune-deficient mice (data not shown). Other TPD52 family members have been found to be associated with cancers in multiple tissues and organs (7, 10, 17). In addition, retroviral integration in avian TPD52 (R10) was accompanied by neuroepithelial proliferation (18). In a leukemic (HL-60 and K-562) cell differentiation model, expression of TPD52 members was associated with cell proliferation (9).

PrLZ gene is located in 8q21. Amplification of this gene in PCa is independent from 8q23–24 amplification (19). Within the 8q21 amplicon, we found previously that TCEB1 gene was ~6 mb pairs centromeric to the PrLZ (20). Fluorescence in situ hybridization analyses revealed that PrLZ and TCEB1 genes were coamplified in PCa specimens (data not shown). Additional investigation would clarify whether both of these genes are involved in malignancies of the prostate gland.

In summary, we have identified a prostate-specific and androgen-inducible gene, PrLZ, located at the most frequently amplified 8q chromosome region in human PCa. PrLZ was seen in the majority of the high-grade PIN specimens. Possibly up-regulation of PrLZ is an early sign of malignant transformation. PrLZ expression persisted in locally recurrent, hormone-refractory, and metastatic PCa. Additional elucidation of the function of PrLZ in PCa cells could provide insight into the malignant progression of PCa cells.

**Acknowledgments**

We thank Drs. Lara Harik, Fan Yeung, Yuanyuan Cui, and Zhihui Xie for technical assistance. We also acknowledge the technical support provided by Dr. Jay Fox of the Core Facilities at the University of Virginia for the chemical synthesis of PrLZ peptides.

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