PrLZ Is Expressed in Normal Prostate Development and in Human Prostate Cancer Progression

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Abstract Purpose: We previously reported the isolation and characterization of PrLZ, a novel prostate-specific and androgen-responsive gene of the tumor protein D52 family at chromosome 8q21.1. PrLZ is the only known gene in this locus with prostate specificity. Expression level of PrLZ was elevated specifically in cancer cells, suggesting its association with malignancy. Experimental Design: To define its biological function in the morphogenesis, development, and functional maturation of the prostate gland and to gain further insight into its role in prostate cancer, we examined PrLZ expression in prostate specimens during early embryonic development and in adult tissue. Results: PrLZ first appears in the nuclei of the prostate epithelia at 16 weeks of gestation before its distribution in the cytoplasm at later ages. Its expression peaks at 24 years of age, declines at 31 years of age, and maintains a minimal level in later age. On prostate cancer development, PrLZ expression is reactivated, and its expression increases from primary localized tumor to bone metastasis. Overexpression of PrLZ in prostate cancer cells accelerates their growth in vitro and tumor formation in vivo. Conclusion: This work identifies PrLZ as a marker for prostate cancer progression and metastasis, and its pattern of expression is suggestive of a proto-oncogene.

PrLZ was cloned and characterized after comparative gene array analysis (1). Contrary to its low expression in the androgen-dependent human prostate cancer cell line LNCaP, the level of PrLZ is differentially elevated in lineage-related but androgen-independent C4-2 human prostate cancer cells, which are capable of forming tumors in castrated mice (2). PrLZ is expressed specifically in human prostate in an androgen-responsive manner, mainly in the epithelial compartment. The COOH-terminal region of PrLZ is identical to tumor protein D52 (TPD52), which has been isolated due to its abnormally elevated expression in breast cancer (3) and lung cancer (4). In the NH2-terminal region, however, PrLZ extends with a unique sequence of 46 amino acid residues. Taking advantage of this structural feature, we showed that PrLZ was a novel member of the TPD52 family and was predominantly expressed in the prostate, with minimal expression in a few other glandular organs (1). Immunohistochemical assays revealed that expression of PrLZ was low in normal adult prostates, but prevalently high in prostate cancer specimens. In most cases, strong staining was seen in malignant cells but not in adjacent benign or normal cells, suggesting a direct involvement of abnormal PrLZ expression in prostate cancer oncogenesis (1). We mapped and isolated the PrLZ gene from chromosome 8q21.1, the most amplified genomic region in prostate cancer (5). PrLZ seems to be the only gene in this locus with prostate specificity, and concomitantly, it is amplified extensively in prostate cancer. Our published data strongly suggest a close relationship between PrLZ expression and prostate cancer development.

The biological function of PrLZ in the morphogenesis and functional maturation of the normal prostate gland was investigated in this report. We also determined the possible role of PrLZ in human prostate cancer progression. Because studies on the temporal and spatial distribution of the gene expression profile could provide valuable clues on the function of the gene, we examined PrLZ gene expression during morphogenesis and development of the prostate gland and upon oncogenesis, progression, and metastasis of human prostate cancer. Overexpression of PrLZ was done in prostate cancer cell lines in vitro and in xenograft tumors grown in vivo. These analyses indicate that, developmentally, PrLZ participates in early morphogenesis of the glandular prostate and in the mature function of the epithelial cells in adulthood. Aberrant expression of PrLZ in later adult life drives prostate cancer development, progression, and metastasis.
Materials and Methods

Cell lines. The human prostatic cancer cell lines used in this study were LNCaP and ARCaP. The latter was originally isolated from the ascites fluid of a patient with prostate cancer bone metastasis (6). These cells were cultured in RPMI 1640 (Invitrogen) with 5% fetal bovine serum. The transformed human embryonic kidney epithelial cell line HEK293 (American Type Culture Collection) was maintained in DMEM with 2 mmol/L l-glutamine, 0.1 mmol/L nonessential amino acids, 1 mmol/L sodium pyruvate, and 10% fetal bovine serum. All the cells were maintained in humidified incubators at 37°C with 5% CO₂.

Recombinant constructs. A mammalian PrLZ expression construct was prepared by fusing coding sequences at 5’ with FLAG tags in the p3×FLAG-myc-CMV-25 (Sigma). Coding sequences were produced with PCR. The PrLZ coding sequence was prepared by PCR reaction with the originally isolated cDNA clone as the template for PrLZ in the pBK-CMV phagemid (1) with primers 5’-GACAAGCTT-GATTGTAGAGAGATGGACTTATATGAGGAC-3’ and 5’-CGGGGATCCATCAAGCGCCCTCGTCTGTTCGAGGACG-3’. The coding sequence of TP52 was amplified with 5’-GACAAGCTT-GATCGTACGAGAGATGGACTTATATGAGGACG-3’ and 5’-CGGGGATCCATCAAGCGCCCTCGTCTGTTCGAGGACG-3’ from the originally isolated cDNA clone for TP52 in pBK-CMV. Reverse transcription-PCR reactions were done as previously reported (1). PCR products were cloned to the pGEM-T easy vector (Promega). Subsequently, inserts for the coding sequences were isolated with HindIII and BamHI digest and used in in-frame ligation to the 3×FLAG-myc-CMV-25. The resultant expression constructs were verified by DNA sequencing analysis.

Transfection. To transfet cultured cells with expression plasmids, the GenePORTER reagent (Gene Therapy Systems) was used according to the manufacturer’s recommended protocol. Briefly, cells in six-well plates at 70% confluence were transfected with 0.5 μg of plasmid DNA in 1 mL of serum-free medium containing the GenePORTER reagent for 5 h. An equal volume of medium containing 20% serum was added. Cells were allowed to recover for 48 h before further manipulation.

Confirmation of coding capability. The cDNA clones of PrLZ and TP52 in the original pBK-CMV phagemid (1) were used to confirm their coding potential with the in vitro transcription coupled translation method using the TNT T7/T3 Coupled Reticulocyte Lysate System (Promega) according to the manufacturer’s recommended protocol. The product was labeled with [35S]methionine and [35S]cysteine (Amersham Pharmacia Biosciences), fractionated on SDS-PAGE, and visualized by autoradiography.

Antibody production. A polypeptide (YLYLSPSGNSSPPGSPTLQKF) was synthesized according to the amino acid sequence in the unique NH₂-terminal region of PrLZ (GenBank accession no. AF202897). At the NH₂ terminus, the extra tyrosine residue was affixed to facilitate peptide coupling with keyhole limpet hemocyanin and bovine serum albumin. The peptide was used to immunize mice. Following two successive boostings, sera from the immunized animals were collected and characterized as polyclonal antibody. Splenocytes of the animals were used in fusion with thymoma. Hybridoma clones were screened by immunocytochemical detection of the PrLZ protein. LNCaP cells cultured on chamber slides (1 × 10⁴ per well) were subjected to androgen deprivation (cultured in phenol red–free and serum-free RPMI 1640 for 48 h), followed by treatment for 16 h with 1 nmol/L methyltrienolone (R1881, Perkin-Elmer Life Sciences), a synthetic and non-aromatizable androgen analogue. The cells were fixed with cold acetone, subjected to staining with the primary antibody, and detected with a previously reported protocol (1).

Human prostate specimens. The human primary prostate cancer specimens were from the archives of the Department of Pathology, Emory University, originally obtained from radical prostatectomy on prostate cancer patients at the Department of Urology, Emory University. Diagnosis of cancer at the primary site and metastatic sites was confirmed by histopathology. Specimens of bone metastasis from biopsy were decalcified for 48 h in the Decalcifying Solution (Richard-Allen Scientific). Use of the human specimens followed the regulations of the Institutional Review Board committee of Emory University. Embryonic prostate specimens used in this study were previously described (7). Age distribution (in weeks) and the total number of tissue specimens (in parentheses) were 12(2), 16(2), and 20(2).

A prostate cancer progression tissue microarray (TMA; courtesy of the University of Michigan Prostate Cancer Program of Research Excellence Tissue Core) was included in this study. Prostate cancer case selection and method for construction of the TMA has been described (8). This TMA represented samples from 216 patients, including 72 benign prostate samples, 72 clinically localized prostate cancer tumors, 36 hormone-naïve metastatic prostate cancers, and 36 hormone-refractory metastatic prostate cancers. The TMA was subjected to immunohistochemical examination for clinical association of the PrLZ level with prostate cancer development and progression.

Immunocytochemical assay and immunohistochemical analysis. The specificity and reactivity of antibodies produced were tested by immunocytochemical detection of the PrLZ protein. LNCaP cells cultured on chamber slides (1 × 10⁴ per well) were subjected to androgen deprivation (cultured in phenol red–free and serum-free RPMI 1640 for 48 h), followed by treatment for 16 h with 1 nmol/L methyltrienolone (R1881, Perkin-Elmer Life Sciences), a synthetic and non-aromatizable androgen analogue. The cells were fixed with cold acetone, subjected to staining with the primary antibody, and detected with a previously reported protocol (1).

Human prostate specimens were used to study the expression of PrLZ in clinical prostate cancer. Duplicate sections from each specimen were stained. To facilitate a semiquantitative comparison, all specimens were subjected to immunohistochemical analysis in parallel and processed simultaneously with an identical protocol. Paraformaldehyde-fixed paraffin-embedded sections and samples arrayed on the TMA were subjected to immunohistochemical analysis following a previously described protocol (1). Briefly, following deparfainization, the samples were treated for antigen retrieval by the microwave method. A monoclonal antibody to PrLZ (clone M50, 1:2, 500) was used in all immunohistochemical analyses. Immunohistochemical signals were detected with the Envision+ System (DakoCytomation) according to the manufacturer’s recommended protocol. The immunohistochemical stainings were treated for counterstaining with hematoxylin (BioGenex), fixed with Bluing reagent (Richard-Allen Scientific), mounted with Crystal/Mount (Fisher Scientific), and analyzed separately by two pathologists.

Cell proliferation and tumor formation assays. LNCaP and ARCaP cells were transfected with the FLAG-tagged PrLZ into the p3×FLAG-myc-CMV-25 vector. G418 (400 μg/mL) was added 48 h after transfection for selection of the stably transfected clones. Isolated clones were examined for PrLZ expression by Western blotting with the M2 anti-FLAG antibody (Sigma).
To assay for proliferation in vitro, cells were cultured in triplicates in multiple 24-well plates (1 × 10⁶ per well). At 24-h intervals, a plate was used to measure cell proliferation based on absorbance at A₅₉₀ by the crystal violet labeling method (9). In another set of experiments, cells were recovered from the plates, fixed in 75% ethanol, and stained with propidium iodide (50 µg/mL) in the presence of RNase A (10 µg/mL). The stained cells were examined for cell cycle profile by flow cytometric analysis on a FACSCalibur (Becton Dickinson).

Cell survival analysis was designed to assess capability to survive the apoptosis induction by androgen deprivation (10). Triplicate samples in six-well plates at 70% confluence were treated for 96 h with androgen-free medium (phenol red–free RPMI 1640; Invitrogen). The cells were then harvested for flow cytometric analysis following previously reported protocols (11, 12). To assay for proliferation in vitro, cells were grafted s.c. into severe combined immunodeficient mice (National Cancer Institute). We chose to use this strain over the nude mice because the xenograft growth of ARCaP tumor was well defined in the severe combined immunodeficient mice (13, 14). Two sites on both flanks on each mouse were inoculated (1 × 10⁶ cells per site). For each experimental group, five animals were used (10 sites). The animals were observed for tumor growth. Measurement of tumor size commenced 5 weeks after inoculation.

**Statistical analysis.** Criteria used to semiquantitatively score the immunohistochemical staining with the TMA specimens were based on staining intensity and frequency; negative (−), ≤15% of the cell population were stained; positive (+), 15% to 50% of the cells were stained; and strongly positive (++) >50% of the cells were stained. A specimen was excluded from the scoring because of the inherent low quality of that specimen but not because of the quality of the immunohistochemical staining. The scores were subjected to ANOVA analysis with the Scheffe post hoc test used for multiple comparisons between groups.

**Results**

As previously reported (1), the expression of PrLZ seems to be associated with androgen-independent progression and malignant behavior in both cultured cells and prostate cancer specimens. In this report, we further analyzed the expression of this gene to determine whether PrLZ is involved in the proliferation activity of prostate epithelial cells.

**Confirmation of the coding potential of PrLZ cDNA.** We isolated both cDNA and genomic clones and determined that PrLZ was a new member of the previously established TPD52 family. The cDNA for PrLZ contained an open reading frame for an acidic polypeptide (isoelectric point, 4.64) with a predicted molecular weight of 24.3 kDa. We confirmed the coding potential of the cDNA for PrLZ with in vitro transcription coupled translation. When the original phagemid containing this cDNA was examined, it yielded specific translated products of 28 and 30 kDa (Fig. 1A). We have also noticed similar migratory retardation and multiple banding in overexpression in human cell lines, either when the open reading frame was used alone (data not shown) or as tagged constructs. The retarded migration and multiple sized products are thus inherent features of the PrLZ protein. In this regard, similar features were observed in other members of the TPD52 family (15–17) and were attributed to posttranslational modifications because these proteins contain multiple consensus sites for N-glycosylation and phosphorylation.

**Production and characterization of antibodies to PrLZ.** Compared with other TPD52 members, the mRNA and deduced
amino acid sequence of PrLZ harbor distinctive structural features. In the 5' region of the transcript, for example, a 502-bp sequence of PrLZ is unique, not sharing homology with any other TPD52 members or known human genes. This region contains the 363-nucleotide 5' untranslated sequence and those coding for the first 47 amino acid residues. PrLZ thus contains a unique NH2 terminus (1). Taking advantage of this structural feature, we raised antibodies specific to the PrLZ protein but not reactive to any other members of the human TPD52 family.

Studies characterizing the reactivity and specificity of the anti-PrLZ antibodies revealed that both the polyclonal and selected monoclonal antibodies were highly reactive and specific to the PrLZ protein. We previously determined that in LNCaP cells, PrLZ expression was at a low level as determined with reverse transcription-PCR and Northern blot hybridization (1). Similarly, the anti-PrLZ antibody detected specific bands of 28 and 30 kDa in LNCaP cells (Fig. 1A) but not in HEK293, an immortalized kidney epithelial cell line that does not express PrLZ.

The identity of the detected signal was confirmed to be PrLZ protein by two methods. First, we confirmed that the specific detection could be blocked when the immunizing peptide (1 μg/mL) was added together with the antibody (data not shown). Second, immunoprecipitation analysis proved that the anti-PrLZ antibody detected purified PrLZ protein. In this study, FLAG-tagged PrLZ was stably transfected into HEK293 cells. Whole-cell lysates of the transfected cells were subjected to immunoprecipitation with the anti-FLAG antibodies or with the produced anti-PrLZ. The immunoprecipitates were fractionated on SDS-PAGE and subjected to Western blotting (Fig. 1B). This series of analyses showed that the anti-PrLZ antibody specifically detected the PrLZ protein but not the TPD52 protein, which was highly homologous to PrLZ except that it lacked the NH2-terminal region seen in the PrLZ protein (1). Similarly, the synthetic peptide used for immunizing the mice could completely block detection (data not shown). We concluded that these results confirmed the specificity of the monoclonal anti-PrLZ antibodies raised in this laboratory.

Expression of PrLZ in LNCaP cells could be stimulated by androgens when LNCaP cells were treated with the synthetic androgen R1881 (1). Correspondingly, the anti-PrLZ antibody detected an increased protein signal in the 28-kDa region in these experiments (Fig. 1C), a molecular size close to that of the predicted PrLZ protein.

To assess whether these antibodies could be used to detect PrLZ in clinical specimens, we first used the antibodies in immunocytochemical assays to detect elevated PrLZ proteins in LNCaP lineage cells following androgen stimulation (Fig. 1D). In this analysis, the anti-PrLZ antibody at 1:2,500 dilution clearly detected the intracellular PrLZ protein induced by R1881. With the specificity and sensitivity defined in cell lines

Figure 2. Developmental expression of PrLZ as determined by immunohistochemical staining. Representative immunohistochemistry results; magnification, ×100. A, PrLZ first appeared at 16 wk of gestation, mainly in nuclei of the prostate epithelia. B, both nuclear and cytoplasmic PrLZ were seen at 20 wk of gestation. C, maximal level of PrLZ was seen in epithelial compartment of the glandular prostate at 24 y of age, with both nucleus and cytoplasm stained. D, at 33 y of age, the level of PrLZ has declined compared with age 24 y. E, PrLZ staining was decreased further at 42 y of age. F, beyond 42 y, most of the normal and benign prostatic hyperplasia samples at older ages showed basal levels of PrLZ, similar to the staining of a benign prostatic hyperplasia gland from a 61-y-old.
and tissue specimens, we proceeded to the detection of PrLZ in clinical specimens. In this report, all the results presented were obtained with M50, one of the monoclonal anti-PrLZ antibodies.

**Developmental expression of PrLZ in glandular morphogenesis and functional maturation of the prostate.** We used the functionally characterized monoclonal antibodies to investigate the developmental expression of PrLZ. A panel of prostate specimens of different age groups were subjected to immunohistochemical staining. For each age group, two different specimens were used as previously reported (7). These included two embryonic prostate specimens at 12, 16, and 20 weeks of gestation and prostate specimens from men of ages 24, 33, 42, and 61 years. Eight prostate specimens from morphologically noncancerous prostate sections from eight benign prostatic hyperplasia patients 61 years or older were also studied. The quality of the immunohistochemical staining was confirmed to be high based on the finding that for each age group, intersample variations were small.

Results of the immunohistochemical analyses of embryonic prostate specimens revealed a temporal expression of PrLZ during morphogenesis of the glandular prostate. PrLZ expression was not detected at 12 weeks of gestation (data not shown), but specific staining for PrLZ was easily seen in specimens at 16 weeks of gestation (Fig. 2A). Importantly, this study revealed that the first appearance of PrLZ was in the nuclei of the prostate epithelial cells. PrLZ was mainly seen in the nuclei of a few cells within the epithelial layer. In comparison, the cytoplasmic stains were lighter but discernible, especially in portions close to the lumen (Fig. 2A). Accumulation of the PrLZ protein continued at 20 weeks of gestation and beyond, with the cytoplasmic staining becoming more pronounced and the nuclear staining less prominent (Fig. 2B). Along with embryonic development, progressive expression of PrLZ was found to extend to virtually all cells in the glandular epithelia, whereas little specific staining was seen in the fibromuscular stroma. Based on these results, we concluded that PrLZ was expressed during embryonic development upon morphogenesis and cytodifferentiation of the glandular epithelium, and that PrLZ protein is localized subcellularly in both the nuclear and cytoplasmic compartments.

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**Figure 3.** Correlation of PrLZ level with prostate cancer progression and metastasis. The M50 antibody was used in immunohistochemical assay for prostate cancer progression TMA. **A,** representative results of the staining of normal and benign specimens (the quadruplets on top left), localized prostate cancer (top right), hormone naive metastases (bottom left), and hormone-refractory metastases (bottom right). There is an incremental trend in staining intensity with the tumor stages. Magnification, ×40. **B,** ANOVA test of the immunohistochemistry scores is summarized. As shown in the histogram, not all of the 72 samples were used in the analysis in both the benign group and the localized primary prostate cancer group. The material quality of each specimen was determined based on H&E staining (data not shown). **C,** features of the subcellular location of PrLZ in prostate cancer cells. Shown are discrete distributions of PrLZ in the nuclei of prostate cancer cells. PrLZ could be seen preferentially in cytoplasm (first image), equally in both cytoplasmic and nuclear portions (second image), predominantly as nuclear staining (third image), or nodularly in a cluster of cell nuclei (fourth image). Magnification, ×200.
Expression of PrLZ in adulthood is similarly an age-related event. PrLZ expression was highest in the specimens from 24-year-old men (Fig. 2C) compared with the specimens from all other age groups analyzed. In these specimens, strong nuclear and cytoplasmic stains were seen throughout the entire epithelial compartment (Fig. 2C). In comparison, a similar staining pattern of PrLZ, but with less intensity, was observed in specimens at 33 years of age (Fig. 2D). Further decline in PrLZ expression became conspicuous and prevalent in specimens at 42 years of age (Fig. 2E), whereas beyond this age, only basal levels of PrLZ expression were seen in both the healthy prostates and the morphologically “normal” zones of benign prostatic hyperplasia glands (Fig. 2F, and data not shown). This latter result was in agreement with our previous finding (1) with a different set of prostate specimens.

Our immunohistochemical analyses of these prostate specimens provided an outline for the temporal pattern of PrLZ expression during prostate development. PrLZ first appears in nuclei of the prostate epithelia early in the second trimester, and its level increases during subsequent embryonic stages. In the adult prostate gland, expression of PrLZ reaches maximum in young adulthood. Expression declines progressively during middle age, reaching minimal levels in later adult life, during which enhanced PrLZ expression was seen only in malignantly transformed epithelial cells, as we previously reported (1). Comparing the temporal distribution pattern between normal...
and cancerous tissues, we conclude that in later adult life, abnormally enhanced PrLZ expression represents prostatic intraepithelial neoplasia and prostate cancer.

Correlation of abnormally enhanced PrLZ expression with progression and metastasis of the prostate cancer. We previously examined PrLZ levels in 100 clinical prostate cancer specimens representing primary and hormonal naive prostate tumors. Levels of PrLZ protein were elevated specifically in malignant prostate diseases including prostatic intraepithelial neoplasia and prostate cancer (1). To further determine the expression of PrLZ during the progression and metastasis of prostate cancer, we carried out additional immunohistochemical analyses on a prostate cancer progression TMA composed of 216 samples. This represented 72 cases of benign prostate, 72 clinically localized tumors, 36 hormone naive metastases, and 36 hormone-refractory metastases of prostate cancer.

In the current study using the prostate cancer progression TMA, we found that the level of PrLZ increased from benign to hormone-refractory and then to metastatic prostate cancer. The frequency of PrLZ positivity was ~23% (mostly +) in benign prostate tissues, 45% in localized primary prostate cancer, 38.9% in hormone-naive metastases, and 52.8% (mostly +) in hormone-refractory metastases. ANOVA test indicated that there was a significant difference of PrLZ levels between benign and hormone-refractory metastatic prostate cancer (P = 0.034; Fig. 3). This analysis supported the idea of a progressive enhancement of abnormal PrLZ expression during prostate cancer progression, and that the extent of the abnormal PrLZ expression was positively correlated to the clinical progression of prostate cancer.

We noticed that, in addition to the overexpression, the PrLZ signal was found to be distributed in both cytoplasmic and nuclear compartments (Fig. 3C). The nuclear staining of PrLZ was remarkably heterogeneous, with some specimens displaying uniform nuclear staining and others showing nodular nuclear PrLZ expression (Fig. 3C). Further immunohistochemical analysis of larger specimens is needed to characterize PrLZ subcellular distribution and the relationship of distribution to tumor progression.

Overexpression of PrLZ promoted growth of prostate cancer cell lines. The data from this study correlated the expression of PrLZ with proliferative activity of the prostate epithelia, both during physiologic proliferation and in cancerous growth. We tested whether PrLZ expression could promote prostate epithelial cell growth in vitro and in vivo. The LNCaP and ARCaP cell lines both express a minimal level of endogenous PrLZ without androgen stimulation (1). These were stably transfected with a plasmid expressing the FLAG-tagged PrLZ. Overexpression of PrLZ was confirmed by Western blotting (Fig. 4).

Effect of PrLZ on proliferation was determined in culture by flow cytometric analysis (Fig. 4A) and crystal violet staining (Fig. 4B). In this study, three LNCaP clones overexpressing the PrLZ fusion protein displayed accelerated growth as compared with the transfection controls and the parental cells. A representative result of three repeated cell cycle analyses is shown in Fig. 4A. Compared with LNCaP and vector controls, the stable clones display a reduced G0-G1 peak, which is indicative of accelerated cell cycle entry. The resultant effect on proliferation was confirmed by crystal violet staining (Fig. 4B). We are currently investigating molecular mechanisms by which PrLZ promotes proliferation.

To evaluate effect of PrLZ on cell survival, we cultured the clones under androgen deprivation condition. After being treated for 96 h, the samples were examined for apoptotic induction by flow cytometric analysis (11, 12). As expected, androgen deprivation induced death of LNCaP cells (Fig. 4A), and the death was due to apoptosis (10). After 96 h of starvation, a substantial amount of LNCaP cells underwent apoptosis. In comparison, significant decreases in apoptosis were observed from the three PrLZ-transfected clones studied. These results suggest that PrLZ may promote survival against cell death induced by androgen deprivation.

As shown in Fig. 4C, ARCaP clones overexpressing PrLZ were inoculated s.c. in athymic mice and the tumor volumes subsequently measured. All three ARCaP clones overexpressing the FLAG-tagged PrLZ exhibited enhanced growth rates compared with controls. Based on these studies, we concluded that elevated expression of PrLZ could indeed promote prostate cancer cell growth.

Discussion

In this report, we defined the patterns of PrLZ expression during prostate development and in clinically staged prostate cancers. Experimentally, PrLZ was found to promote proliferation of LNCaP in cell culture and tumor formation of ARCaP in mice (Fig. 4). Because androgens stimulate growth and PrLZ expression in LNCaP cells (1), PrLZ may play a mediating role in the proliferation. Conversely, although PrLZ promotes ARCaP tumor formation, growth of this cell line is known to be repressed by sex hormones (6, 18, 19). It is possible that the repressing effect of androgens may be exerted through additional mechanisms. Further investigation is needed to clarify the function of PrLZ in ARCaP prostate cancer cells.

In combination with our previous work, we have thus far examined more than 300 specimens for PrLZ expression in prostate cancers. A fraction of these specimens were conventionally prepared, but the majority were in the form of a TMA. Immunohistochemical analysis of the TMA specimens revealed the inherent limitations of the TMA method. TMA, for example, may hamper correlating gene expression with prostate cancer tumor staging because of the small sampling size for each specimen and the heterogeneous distribution of tumor cells within each specimen. It is possible that enhanced PrLZ occurs in a higher percentage of prostate cancer cases than we have estimated using the TMA method.

Our data support the notion that a reawakening of the normally latent PrLZ gene in later life could drive prostate oncogenesis. In the developmental process, human prostate epithelial cells undergo two waves of proliferation: at early embryonic morphogenesis and during early young adulthood (20, 21). Proliferative activity in the epithelial compartment is sustained during reproductive ages but declines with age, and in later life, only a basal level of epithelial proliferation is seen. The developmental expression of PrLZ correlates well with the proliferative activities of the prostate epithelia both in embryonic development and in adult life. The fact that it was first detected not at 12 weeks but at 16 weeks of gestation may suggest that PrLZ is not involved in initiation of morphogenesis but in the subsequent proliferative phase. This is supported by a separate study on the same embryonic prostate specimens (7), in which the highest levels of proliferative activity were detected.
at 16 weeks of gestation. Our experimental results also support the proliferation-promoting property of PrLZ because raising the PrLZ level by forced expression resulted in accelerated proliferation (Fig. 4). Previously, another member of the TPD52 gene family, the rat CRHSP-28, was studied in the context of gastrointestinal secretion (22). With its gastrointestinal expression and calcium sensitivity, this gene was proposed to be involved in the secretory function of eocrine cells. We have found no evidence whether PrLZ overexpression promotes similar biological activity by increasing prostate-specific antigen secretion (data not shown).

Another important aspect of the expression pattern is the general decline of PrLZ expression in later adult life. We previously identified that expression of PrLZ in prostate cancer cell lines was stimulated by androgens (1). Should the same hold true in vivo in humans, high levels of PrLZ expression during development may be a consequence of androgen stimulation. The decline of androgen level in later adult life is well known (23), and the reduction of PrLZ is in general paralleled by the age-related decline of circulating androgens. On the other hand, additional endocrinal hormones of the hypothalamus/pituitary origin are also produced in an age-related fashion (24, 25). Further investigation is needed to unveil hormonal factors controlling the developmental expression of PrLZ.

The expression of PrLZ in prostate cancers, however, does not coincide with declining serum androgens in older ages. Two separate mechanisms, gene amplification and transcriptional reactivation, may account for the abnormal expression. We have found that the PrLZ gene in chromosomal 8q21.1 is amplified in the majority of prostate cancer cases (1). On the other hand, we have detected no PrLZ gene amplification in a lineage-related prostate cancer progression model. The C4-2 cell line, for instance, is a derivative subline of LNCaP with a more advanced prostate cancer phenotype (2). Although PrLZ expression was enhanced, amplification of the 8q21.1 locus was not detected in C4-2 cells by comparative genomic hybridization (26). Furthermore, we detected no marked difference in PrLZ mRNA stability between LNCaP and C4-2 cells (data not shown). Transcriptional mechanism is likely attributable to abnormally enhanced PrLZ expression. Because the transcription of PrLZ is turned on early in embryonic development and off in later life, the enhanced expression in cancer cells should involve reactivation of the transcription.

In cancers, the reactivation of genes that are otherwise functioning in normal development is frequently seen. Organ-specific oncogenes play important biological functions at specified stages of morphogenesis or homeostasis, manifesting temporally controlled expression along with the developmental process. Many homeodomain proteins (27) and other well-established morphogens (28, 29) are abnormally expressed in cancer. Accordingly, we propose that PrLZ is one such proto-oncogene. Under normal conditions, it functions as a growth-promoting protein; once reactivated, PrLZ promotes tumor cell proliferation.

PrLZ can be found both in the nucleus and cytoplasm of the epithelial cells, although PrLZ does not contain a canonical nuclear localization signal. Instead, PrLZ contains a coiled coil leucine zipper, which has been known to be an interface for homodimerization and heterodimerization with other proteins within the cell and with proteins outside the family (30). The nuclear distribution of PrLZ may be through interaction with other proteins mediated by the leucine zipper. PrLZ contains a conserved phospho-serine/methionine motif that was first identified in TPD52L1, another member of the TPD52 family (31). On phosphorylation, this motif becomes a binding target by a specific member of the 14-3-3 proteins. The 14-3-3 proteins are known modulators in cell survival, proliferation, and differentiation, mainly by modulating other important client proteins to modulate their nuclear entry (32, 33). The observed subcellular distribution of PrLZ may be modulated in a similar mechanism.

Subsequent to our previously reported structural and expressional characterization, we have in this report defined the developmental and cancer-related expression of PrLZ gene. These works indicate that PrLZ could be developed as a marker for prostate cancer progression, whereas its abnormal expression may have transforming potential. Further investigation is warranted to unveil the pathologic role of PrLZ in prostate cancer progression and to evaluate this protein as a marker for prostate cancer diagnosis and therapy.

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