Identification and Characterization of Prostein, a Novel Prostate-specific Protein

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ABSTRACT

In this report, we describe the application of a systematic, genome-based approach to identify prostein, a novel prostate-specific protein expressed in normal and malignant prostate tissues. Characterization of the prostein gene shows that prostein cDNA encodes a 553-amino acid protein. The protein is predicted to be a type IIIa plasma membrane protein with a cleavable signal peptide and 11 transmembrane-spanning regions. The prostein gene is located on chromosome 1 at the WI-9641 locus between q32 and q42. Prostein mRNA is shown to be uniquely expressed in normal and cancerous prostate tissues using Northern blot, cDNA microarray, and real-time PCR analyses. Furthermore, prostein mRNA expression does not appear to be prostate tumor grade related and is restricted exclusively to prostate cell lines. Immunohistochemical staining using a mouse monoclonal antibody generated against prostein demonstrates that this protein is specifically detected in prostate tissues both at the plasma membrane and in the cytoplasm. Prostein expression is androgen responsive because treatment of LNCaP cells with androgen up-regulates prostein message and protein expression levels. These results validate prostein as a prostate-specific marker with potential utility in the diagnosis and treatment of prostate cancer.

INTRODUCTION

Prostate cancer is the second leading cause of death among men in the United States. The American Cancer Society has estimated that approximately 179,000 new cases would be diagnosed with prostate cancer and 37,000 deaths from prostate cancer would occur in 1999 (1). Little is known about the genetic events associated with the malignant transformation of prostatic cells. This is due in large part to the cellular heterogeneity of the prostate and the lack of systematic analysis of prostate tissue gene expression. The identification of novel prostate-specific gene products that can be used as therapeutic and diagnostic reagents for prostate cancer is of critical importance for prevention and treatment of the disease. Molecules that are uniquely expressed or overexpressed in prostatic tumors and/or prostate tissues are potential candidates to serve as therapeutic vaccine antigens or as novel cancer markers.

Numerous approaches have been used to identify cancer-specific and cancer-associated markers, including expressed sequence tag sequencing (2, 3), serial analysis of gene expression (4, 5), and differential display PCR (6). Expression cloning using sera (7) or T cells (8) reagents, and many antigens identified using these approaches are not cancer or tissue specific. Finally, none of the above-mentioned techniques provide a complete, systematic, and reliable comparison of the gene expression differences between two tissue types.

We have recently described a novel genome-based approach for the identification and characterization of tumor-specific proteins. This approach involves an initial cDNA library subtraction, followed by high-throughput microarray screening for tissue- and/or tumor-specific gene expression (9). In this report, we describe the application of this approach to isolate a novel prostate tissue-specific protein, prostein. The results presented in this report demonstrate that prostein is a prostate-specific marker with potential clinical utility for the diagnosis and treatment of prostate cancer.

MATERIALS AND METHODS

Tumor Samples. Cell Lines, Androgen Stimulation, and RNA Preparation. All clinical tissue samples used in this study were accompanied by clinical information and pathological reports and were histologically confirmed by pathologists. Prostate tumor cell lines LNCaP, PC-3 (metastatic adenocarcinoma), DU145 (carcinoma); breast tumor cell lines MDA-MB415, MCF7, SK-BR-3 (adeno/adenocarcinomas) MDA-MB435 (carcinoma), MDA-MB453, T-47D, and BT-474 (ductal carcinomas); colon tumor cell lines SW480 and COLO320 (adenocarcinomas); ovarian tumor cell lines SK-OV-3 (adenocarcinoma) and OV1063 (carcinoma); and lung tumor cell lines A549 (carcinoma), HTB-183, and HTB-177 (large cell lung carcinomas) were obtained from the American Type Culture Collection. Tumor cell lines 390T and 84T (squamous lung carcinomas) were kindly provided by Dr. Jill Siegfried (University of Pittsburgh Cancer Institute, Pittsburgh, PA); LT140-98 (lung adenocarcinoma) and TL1 (squamous lung carcinoma) were kindly provided by Dr. Elisabeth Repasky (Roswell Park Cancer Institute, Buffalo, NY); OT-39I-73 (large cell endometrial carcinoma) was kindly provided by Dr. Heather Secrist (Corixa Corp.). 391-06 (large cell lung adenocarcinoma) was kindly provided by Dr. Rob Henderson (Corixa Corp.).

For the androgen stimulation experiments, LNCaP cells were plated at 1.5 × 10^6 cells/T75 flask (for RNA isolation) or 3 × 10^6 cells/well of a 6-well plate (for FACs3 analysis) and grown overnight in RPMI 1640 containing 10% charcoal-stripped FCS (Life Technologies, Inc.). Cell culture was continued for an additional 72 h in RPMI 1640 containing 10% charcoal-stripped FCS, with 1 nM of the synthetic androgen methyltrienolone (R1881; New England Nuclear) added at various time points. Cells were then harvested for RNA isolation and FACs analysis at 0, 1, 2, 4, 8, 16, 24, 48, and 72 h after androgen addition.

For tissue sample RNA isolation, tissues were frozen in liquid nitrogen and homogenized with a Polytron (Kinematica), and total RNA was prepared using Trizol reagent (Life Technologies, Inc.). Polyadenylated RNA was isolated using a Qiagen oligotex spin column mRNA purification kit. For cell line RNA isolation, total RNA was prepared using Trizol reagent (Life Technologies, Inc.).

cDNA Library Subtraction. cDNA library subtraction was performed as described by Hara et al. (10), with modifications (9). Microarray. mRNA expression of prostein was determined using a microarray assay as described previously (9).

Northern Blot Analysis. Northern blot analysis was performed as described previously (9) using 32P-labeled prostein cDNA probe.

Quantitative Real-Time PCR. Quantitative real-time PCR assay was performed as described previously (9) using 300 nM each of prostein forward

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3 The abbreviations used are: FACs, fluorescence-activated cell-sorting; ORF, open reading frame; BPH, benign prostate hyperplasia; PSA, prostate-specific antigen.
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RESULTS

Identification, Cloning, Sequence Analysis, and Chromosome Location of Prostein. A prostate cDNA fragment was isolated using the cDNA library subtraction method described previously (9). A full-length prostein cDNA clone was isolated by colony hybridization from a prostate tumor cDNA library using the fragment identified above. The full-length prostein cDNA is 3410 bp in length and contains an ORF of 553 amino acids (Fig. 1). Multiple stop codons in frame with the prostein ORF were identified in the prostein cDNA upstream of the presumptive initiator ATG, indicating that the entire prostein ORF had been obtained. The full-length prostein sequence contains an ORF of 553 amino acids (Fig. 1). Multiple stop codons in frame with the prostein ORF were identified in the prostein cDNA upstream of the presumptive initiator ATG, indicating that the entire prostein ORF had been obtained. The full-length prostein sequence contains an ORF of 553 amino acids (Fig. 1).


A prostein-specific mouse monoclonal antibody 10E3-G4-D3 that recognizes an intracellular epitope of prostein was generated by standard hybridoma technologies and is described in detail elsewhere. For FACS analysis, cells were harvested by trypsin (which was shown not to affect the antibody epitope), fixed in 2% formaldehyde, permeabilized in 0.5% saponin, and incubated with either the anti-Prostein antibody 10E3-G4-D3 or a random primed prostein cDNA probe. The loading control is the RNA stained with methylene blue showing 18S and 28S rRNA bands.

Total RNAs (10 \( \mu \)g) were used in PCR reactions with DNA pools from the hybrid panel according to the manufacturer's instructions. After 38 cycles of amplification, the reaction products were separated on a 1.2% agarose gel, and the results were analyzed through the Whitehead Institute/MIT Center for Genome Research web server to determine the probable chromosomal location.

Immunohistochemical staining was performed on formalin-fixed, paraffin-embedded tissues by QualTek Molecular Laboratories using the prostein-specific mouse monoclonal antibody 10E3-G4-D3. Immunohistochemical Studies.

Northern blot analysis was initially used to determine the expression of prostein in prostate tumors, normal prostate, BPH, and a panel of normal tissues including colon, kidney, liver, lung, pancreas, skeletal muscle, brain, stomach, testis, small intestine, and bone marrow. As shown in Fig. 2, a 3.8-kb transcript was detected in four of four prostate tumors, one normal prostate, and one BPH sample; expression was undetectable in the other normal tissues tested. The prostate-specific expression was further analyzed by microarray analysis on a larger panel of tissues (Fig. 3). Similar to the results from Northern blot analysis, prostein mRNA expression was shown to be restricted to prostate tissues. Prostein expression levels in prostate tissues ranged from 2- to 66.5-fold higher than that of the paired normal tissues, with an average overexpression of 23.3-fold in prostate tissues compared to other tissues tested. Quantitative real-time PCR, a more sensitive and quantitative assay, was also used to examine prostein mRNA expression. As shown in Fig. 4A, prostein mRNA could be detected in 23 of 23 prostate tumors, 3 of 3 BPH samples, and 3 of 3 normal prostate samples. No significant expression was detected in any of the other normal tissues tested. In summary, the data generated from each of these three independent methodologies demonstrate that the prostein transcript is expressed in a prostate-specific manner by the majority of normal and malignant prostate tissues.

As shown in Figs. 2, 3, and 4A, prostein expression is not tumor specific because normal prostate, BPH, and prostate tumors all express prostein message. Prostein mRNA expression was further examined on a Gleason panel consisting of six normal prostate tissues, six BPH samples, and a panel of prostate tumors of various Gleason grades. As shown in Fig. 4B, prostein mRNA was expressed in all normal prostate, BPH, and prostate tumors of various Gleason grades. Additionally, no correlation was observed between the level of prostein mRNA expression and the Gleason grade of the tumors. PSA mRNA expression levels were also assessed on the same Gleason panel; similar to prostein, PSA expression levels were not Gleason grade dependent (data not shown).

We have also examined the expression of prostein in a cell line panel consisting of in vitro established tumor cell lines and pooled normal and tumor tissues. Prostein expression is specific for prostate...
Protein mRNA and Protein Are Expressed in Prostate Tumor Cell Lines. The expression of protein mRNA and protein in prostate tumor cell lines was determined using quantitative reverse transcription-PCR and FACS analysis. As shown in Fig. 5A, protein-specific transcripts could be detected in normal prostate and prostate tumors as well as in LNCaP cells and were detected at a very low level in PC-3 but not in DU-145 prostate tumor cells. These data also demonstrate that normal prostate and prostate tumor tissues express significantly higher levels of protein transcript than the prostate tumor cell lines tested. The FACS analysis shown in Fig. 5B confirmed the real-time PCR analysis because the protein-specific monoclonal antibody 10E3-G4-D3 specifically stained LNCaP cells and also stained PC-3 cells at a low level but failed to react with DU-145 cells. Because LNCaP cells expressed higher transcript levels and stained more intensely with the anti-protein monoclonal antibody when compared with PC-3 and DU145 cells, these results demonstrate that protein mRNA levels correlate with protein expression levels.

Protein Protein Expression Is Restricted to Prostate Tissues. To characterize the protein expression of protein in tissues, immunohistochemical analysis was performed using the monoclonal antibody 10E3-G4-D3. The specificity of 10E3-G4-D3 for protein was demonstrated initially by ELISA assays using protein-coated plates, and the 10E3-G4-D3 epitope was subsequently mapped to a peptide that is represented by protein sequence. Furthermore, FACS and Western analyses have shown that 10E3-G4-D3 reacted specifically with cell lines transduced to express protein (data not shown). Using 10E3-G4-D3, protein protein was shown to be specifically expressed in prostate tumors and normal prostate (Fig. 6). Expression was detected in all prostatic glandular cells. Furthermore, expression levels were not tumor grade related because similar staining intensity was seen in tumors of different grades. Prostate tumor tissues metastatic to lymph node, bone, and liver also expressed high levels of protein (data not shown). Protein expression was not detected in normal heart, kidney, liver, lung, or colon. The immunohistochemistry analysis also showed a punctate plasma membrane staining pattern. In many areas, the staining appeared to be clustered within the cytoplasm in a perinuclear location. The staining was typically polarized and located between the nucleus and the lumen in normal and benign prostate epithelial cells. In prostate carcinomas, the polarity of the staining was completely random from cell to cell but usually remained polarized within each cell.

To comprehensively examine protein expression in tissues, multitissue arrays were performed on human specimens representing major human normal organs as well as a wide range of human neoplasias. Approximately 4700 specimens were tested, including a total of 65 specimens from normal prostate and prostate carcinomas. All 65 prostate specimens stained positive using the protein-specific antibody 10E3-G4-D3, whereas the remainder of approximately 4635 non-prostatic tissue specimens were all negative (data not shown).

Expression of Protein Is Up-Regulated by Androgen. To determine whether protein expression was affected by androgen, LNCaP cells were grown overnight in androgen-depleted media and then supplemented with the synthetic androgen methyltrienolone. Cells were harvested after androgen stimulation for various time periods, and protein expression was measured both at the mRNA level using Northern blot analysis and at the protein level using FACS analysis. As shown in Fig. 7A, culture of LNCaP cells in the absence of androgen for 72 h (0 h time point) reduced protein mRNA levels. Culture of LNCaP cells in the presence of androgen resulted in the induction of protein mRNA, which was detectable as early as 2 h after androgen treatment and increased through 48–72 h of culture in the presence of androgen. As shown in Fig. 7B, protein protein levels were also affected by the presence of androgen. Culture of LNCaP in the absence of androgen for 72 h (0 h time point) resulted in a loss of detectable protein protein expression. Culture of LNCaP cells in the presence of androgen resulted in the induction of protein protein,
which was detectable as early as 8 h after androgen treatment and increased through 72 h of culture in the presence of androgen.

**DISCUSSION**

We have previously described a rapid, efficient, and systematic approach to identify prostate cancer- or tissue-specific genes (9). Here, we report the application of this approach to identify and characterize a novel prostate-specific protein, prostein. In this report, we demonstrate that prostein is abundantly and specifically expressed in prostate tumors and normal prostate tissues. Indeed, prostein was recovered multiple times from prostate libraries using the approach described in this report. Despite the relative abundance of prostein, it appears not to have been identified by other cloning methodologies. This observation may provide further evidence for the utility of our approach in identifying tumor-specific proteins not readily discovered by other methods.

Sequence homology comparisons suggest that the prostein gene product does not belong to a known family of proteins. Unlike a number of previously described prostate-specific genes such as PSA, glandular kallikrein, and the newly discovered serine protease prostate (11), prostein does not appear to be a serine protease and shows no homology to kallikrein family members. The closest identifiable homology to a known protein is to the human AIM-1 protein. Although the mechanisms for prostate-specific expression and the biological function of prostein are unknown, localization of the prostein gene to a region of chromosome 1 reported to be linked to prostate cancer susceptibility in hereditary prostate cancer (12, 13) suggests that the prostein gene may play a role in prostate cancer malignancy. The observation that the strongest homology of prostein was with a previously identified tumor antigen also provides the possibility that these molecules may be involved in the malignant phenotype.

The data presented in this report do not demonstrate correlation of
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prostein gene expression and prostate tumor Gleason grade. However, due to the cellular heterogeneity of prostate tumor tissue samples, it is likely that the percentage of actual cancer cells in each sample varies, and this may affect the levels of prostein gene expression detected. Immunohistochemical staining of a large panel of prostate tumors of various Gleason grades will further address the issue of prostein gene expression and Gleason grade.

Prostein appears to be expressed in an exclusively prostate-specific pattern because prostein mRNA and protein were not detected in any of the non-prostatic tissues tested. Recent reports have demonstrated similarities in gene expression between prostate and breast tissues (14). However, as shown in this manuscript, prostein mRNA expression could not be detected in normal and cancer breast tissues. Therefore, prostein could be useful in clinical diagnosis as an additional marker to both enhance the accuracy and reduce the false positive and negative rates of the currently used PSA test. Use of prostein in this setting may reduce or eliminate the issues of PSA cross-reactivity with other closely related serine proteases such as glandular kallikreins 1 and 2. Both protein-based assays (FACS, cytostain) and mRNA-based assays (reverse transcription-PCR) are currently being evaluated to determine the potential of prostein as a marker to detect prostate cancer cells circulating in the blood stream, which in turn may provide stage-related diagnostic and prognostic information. We anticipate that further characterization of the prostein genomic sequence will allow us to determine whether prostein is amplified in prostate cancers. The potential amplification of the prostein genomic sequence may be an important tool for prostate cancer diagnosis. Furthermore, the identification and characterization of the prostein promoter sequence may provide important information for the development of gene therapy approaches.

Expression of prostein on the cell surface was initially predicted by the bioinformatic algorithm PSORT. In support of this observation, immunohistochemical staining revealed a punctate surface-staining pattern. FACS analysis using prostein peptide-specific polyclonal antibodies to a predicted surface-expressed epitope showed surface staining of LNCaP cells (3). FACS analysis using the monoclonal antibody 10E3-G4-D3 only stained permeabilized prostate tumor cell lines, suggesting that the epitope recognized by 10E3-G4-D3 is intracellular. The potential expression of prostein on the cell surface suggests that prostein may be an excellent target for therapeutic antibodies. Antibody-mediated therapies toward cell surface proteins such as CD20 and HER2/neu are being used as treatments for non-Hodgkin’s lymphoma (15) and breast cancer (16), respectively. In prostate, antibodies against prostate-specific membrane antigens are being evaluated as a therapeutic approach to treat prostate cancers (17). Recently, a new prostate-specific cell surface antigen, STEAP, has been described (18). However, the expression of STEAP is not entirely specific for prostate because it has been found both at message and at protein levels in other tissues, such as normal bladder and colon and other types of tumors. In contrast, prostein is very specifi-
ically and highly expressed in almost all prostate tumors and normal prostate tissues. It is highly expressed in metastatic prostate cancers and is likely to be expressed on the cell surface. These features of prostate make it an ideal target for prostate cancer antibody therapy. Furthermore, the homogeneous expression pattern of prostein on all prostate glandular cells indicates that therapeutic regimens that use prostein could be very efficient and powerful because all prostatic cells can be targeted. Finally, prostein expression could potentially be used as a marker for in vivo imaging diagnosis to detect metastatic prostate cancer cells.

In addition to diagnostic and antibody therapeutic uses, immunotherapy strategies involving a vaccine targeting prostein are being evaluated. The immunological relevance of prostein and its potential as a vaccine candidate have been demonstrated by in vitro T-cell priming and stimulation experiments.

The homology of prostein with a shared melanoma antigen demonstrated to elicit immune response provides further evidence for the potential of prostein to serve as a tumor antigen. The development of adjuvant and delivery systems to generate and expand strong immune responses to prostein could allow this protein to be developed as an effective prostate cancer-specific vaccine.

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