Identification, Purification, and Subcellular Localization of Prostate-specific Membrane Antigen PSM' Protein in the LNCaP Prostatic Carcinoma Cell Line

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Abstract

An alternatively spliced variant of prostate-specific membrane antigen (PSMA) designated PSM' was originally described following identification of its mRNA in normal prostate. We have purified the PSM' protein from LNCaP cells using two immunoaffinity columns in tandem. The first column contained a monoclonal antibody (7E11) that was reactive with the NH2 terminus of PSMA, which specifically depleted the LNCaP lysate of full-length PSMA. The nonbinding fraction was then passed over a second column composed of a monoclonal antibody (PEQ226.5), the epitope of which was located within the 134–437 domain of PSMA and shared with PSM'. The protein eluted from the second immunoaffinity column produced a Mr 100,000 band on SDS-PAGE, which was slightly lower than the full-length PSMA at Mr 100,000. The band was NH2-terminally sequenced through 15 residues, and the assigned sequence coincided with the predicted sequence for PSM' protein minus the first two NH2 terminus amino acids. The PSM' protein, therefore, began with residue 60 of PSMA (alanine). LNCaP cells were fractionated, and PSM' was localized to the cytoplasm.

Introduction

Prostate cancer is the second leading cause of cancer death in men (1). Biomarkers of prostate cancer have been used in screening, diagnosis, and predicting disease progression. Currently, the most accepted of these markers is prostate-specific antigen (2, 3). A newer marker of prostate cancer, PSMA2 (2), was originally identified in LNCaP cells by its immunoprecipitation with the mAb, 7E11-C5 (4). PSMA is expressed in normal prostate (4–7) as well as a high proportion of prostate carcinomas (8). Expression of PSMA is enhanced in higher-grade cancers, metastatic disease (8), and hormone-refractory prostate carcinoma (7–9). Serum studies have suggested that PSMA levels may be linked to a more aggressive clinical phenotype (10). Low levels of the protein have also been reported in small intestine, colon (11), and the capillary endothelium of a variety of tumors (11, 12). A radioimmunoconjugate of the 7E11-C5 antibody designated CYT356 is currently being used as an imaging agent for prostate cancer (13). The gene for human PSMA has been cloned and sequenced (5) and mapped to chromosome 11q14 (14). The DNA sequence revealed that a portion of the coding region has a 54% homology to human transferrin receptor. The sequence also indicated that the gene coded for a putative type II transmembrane protein of Mr ~100,000 consisting of a short intracellular segment (amino acids 1–18), a transmembrane domain (amino acids 19–43), and an extensive extracellular domain (amino acids 44–750). Recently, an alternatively spliced variant of PSMA RNA called PSM' was described (15). PSMA and PSM' cDNAs are identical except for a 266-nucleotide region near the 5' end of PSMA cDNA (nucleotides 114–380) that is absent from PSM'. This region codes for the transmembrane domain of PSMA, so it was postulated that PSM' would be localized to the cytoplasm. To determine whether the PSM' mRNA is translated and expressed in prostate cancer cells and to identify its subcellular location, we used two mAbs that were specific to two different regions of the protein to affinity-purify PSM' and then sequence and characterize the protein.

Materials and Methods

Monoclonal Antibodies. The 7E11 antibody was obtained from Dr. Gerald Murphy (Pacific Northwest Cancer Foundation, Seattle, WA). Its development and clinical use with 111Indium has been described previously (4). 7E11 recognizes the NH2-terminal portion of PSMA (residues 1–7; Ref. 16). PEQ226.5 was developed from a BALB/c mouse inoculated i.p with plasma membranes prepared from prostate carcinoma tissue.

Purification and Sequencing of PSM'. LNCaP cells obtained from American Type Culture Collection (Rockville, MD) were grown in RPMI medium with 10% horse serum (Life Technologies, Inc., Grand Island, NY) and maintained in 5% CO2 at 37°C. Cells were grown in a T160 flask until 80% cell confluency was obtained. Cells were lysed following the addition of 2 ml of 1% Triton X-100, 10% glycerol, 15 mM MgCl2, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium vanadate, 10 μM aprotinin, and 50 mM HEPES (pH 7.5). The crude lysate was incubated with 1.0 ml of 7E11-AminoLink agarose beads for 4 h at 4°C. Antibodies were covalently linked to the agarose according to manufacturer's instructions (Pierce, Rockford, IL). The unbound fraction was then incubated with 1 ml of PEQ226.5-AminoLink beads for 4 h at 4°C. Beads were washed five times with 10 ml of 1% Triton X-100, 10% glycerol, 15 mM MgCl2, and 50 mM HEPES (pH 7.5) and then eluted with 1 ml of 2% SDS in 10 mM sodium phosphate-150 mM NaCl (pH 7.5). Aliquots of the eluents and unbound fractions were reduced and denatured and subjected to SDS-PAGE on 4–20% polyacrylamide gels (17). Following blotting to nitrocellulose paper, the proteins were probed with both the 7E11 and PEQ226.5 mAbs. The remaining eluent from the PEQ226.5-AminoLink beads was concentrated to a minimal volume and loaded onto a single lane of a 4–20% gel. Blotted to a polyvinylidene difluoride membrane, and stained with Coomassie Blue. The proteins were probed with both the 7E11 and PEQ226.5 mAbs. The remaining eluent from the PEQ226.5-AminoLink beads was concentrated to a minimal volume and loaded onto a single lane of a 4–20% gel, blotted to a polyvinylidene difluoride membrane, and stained with Coomassie Blue. The band was cut out of the blot and sequenced on a Procise Protein Sequencer (Perkin-Elmer Corp., Foster City, CA).

Western Blot. Blots were incubated with primary mAbs followed by incubation with goat antimouse IgG conjugated to horseradish peroxidase (Jackson Immunoresearch, West Grove, PA). Antibody reactivity was detected using the enhanced chemiluminescence reagent (Amersham, Arlington Heights, IL).

Cell Fractionation. Plasma membrane and cytosol fractions from LNCaP cells were prepared according to a previously published procedure (18), with minor modifications. Approximately 5 × 106 cells were disrupted by nitrogen cavitation (50 pounds per square inch for 20 min), and plasma membranes were purified by differential centrifugation. Protein was quantitated using the bicinchoninic acid assay (Pierce).

Cloning and Purification of Fusion Proteins. Three DNA fragments, including PSMA 1–173, PSMA 134–437, and PSMA 438–750 were amplified by PCR. Plasmids PACGHISNTA-PSMA1.9 and PDR2 were obtained from...
Dr. Gerald Murphy and were used as vectors to amplify PSMA. These fragments were cloned into PGEX (Pharmacia Biotech, Uppsala, Sweden) or pET5a (Novagen, Madison, WI) vectors to generate GST and T7 PSMA fusion proteins, respectively (19). Following transformation into *Escherichia coli* and induction, the fusion proteins were purified by high-performance liquid chromatography.

**Results and Discussion**

The identification of an alternatively spliced form of PSMA mRNA called PSM' (15) and the observed changes in ratios of PSMA:PSM' mRNA between normal prostate and prostate cancer tissue suggested that the PSM' protein might be a useful diagnostic or prognostic marker of prostate cancer. Although PSM' mRNA was identified in prostate tissues, the protein has not been identified to date. A two-step immunoaffinity chromatography procedure was used to purify PSM' from a crude lysate of the human prostate carcinoma cell line LNCaP. Because PSM'-specific mAbs are not currently available, we used two mAbs that recognize full-length PSMA. The first mAb (7E11) has previously been shown to bind to the NH2 terminus of PSMA (16). Because the PSM' cDNA is missing the 266-nucleotide region at the 5' end of PSMA (15), 7E11 presumably would not bind PSM'. We used a second PSMA mAb, PEQ226.5, which recognizes PSMA and could potentially bind PSM'.

A series of PSMA fusion proteins were synthesized to map the epitope of PEQ226.5. By Western blot analysis, PEQ226.5 reacted with fusion protein PSMA-GST-134-437 but did not bind fusion proteins PSMA-GST-1-173 or PSMA-GST-438-750. On the basis of these results, PEQ226.5 was mapped to the 173-437 domain of PSMA (Fig. 1). Both antibodies were covalently bound to agarose resin and used sequentially to first remove full-length PSMA from the lysate (7E11-agarose beads) and then capture the unbound PSM' on the second antibody resin (PEQ226.5-agarose beads). Eluted protein and unbound fractions from both columns were evaluated with 7E11 and by Western blot (Fig. 2). Both 7E11 and PEQ226.5 detect PSMA, which is captured and eluted from the 7E11-agarose beads (Lanes 1 and 3). In contrast, 7E11 fails to detect any protein in the PEQ226.5 eluent (Lane 2), whereas PEQ226.5 detects a protein that is migrating slightly faster than PSMA (Lane 4). Because 7E11 cannot detect PSM' but PEQ226.5 can, we speculated that the band in Lane 4 was PSM'.

To confirm the identity of the PEQ226.5-reactive protein, the entire PEQ226.5 eluent was concentrated and loaded in a single lane of a reduced SDS-polyacrylamide gel and blotted to a polyvinylidene difluoride membrane. The blot was stained with Coomassie blue and the 95,000 kDa band was cut out and NH2-terminally sequenced through 15 residues. The following sequence for PSM' was obtained: Ala-Phe-Leu-Asp-Glu-Leu-Lys-Ala-Glu-Asn-Ile-Lys-Lys-Phe-Leu. The sequence matched the predicted protein sequence for PSM' deduced from the cDNA sequence (11). The only deviation from the predicted sequence was the absence of residues 58 (Met) and 59 (Lys) at the NH2 terminus. Although the putative translation initiation site for PSM' was identified at residue 58 (Met), the actual NH2-terminal
amino acid by protein sequencing was identified as alanine at residue 60 of PSMA. NH$_2$-terminal methionine is usually not important for protein function and is often removed shortly after its incorporation by the limited proteolysis of a few residues that occurs at the amino terminus of many proteins in eukaryotes (20). Unlike full-length PSMA from LNCaP cells (5), PSM' was not NH$_2$-terminally blocked and, therefore, was sequenced directly without digestion. This result indicates that the two-column procedure is quite effective in first depleting the lysate of the majority of full length PSMA and then capturing the PSM' on the second column.

Because PSM' mRNA lacks the transmembrane domain expressed by PSMA, it was speculated that PSM' would be a cytosolic protein (15). To test this hypothesis, we prepared plasma membrane and cytosol fractions of LNCaP cells and examined each fraction by Western blot with the PSMA antibodies. The 7E11 antibody detected PSMA in the plasma membrane fraction (Fig. 3, Lane 2). In contrast, 7E11 detected only a minor band in the cytosol fraction. This subcellular location is consistent with PSMA being a transmembrane protein. When the cytosol was probed with PEQ226.5, the faster-migrating form (PSM') was detected (Lane 3). PEQ226.5 detected full-length PSMA in the plasma membrane fraction (Lane 4). A Western blot of LNCaP lysate probed with PEQ226.5 (data not shown) exhibited a strong PSMA band and a weak PSM' band, shown that PSMA is the predominant form in LNCaP cells. This result is in agreement with previous reports that have shown that, in LNCaP cells, the ratio of PSMA:PSM' RNA is ~10:1 (15). PSM' was not detected in the plasma membranes of LNCaP cells.

This report is the first study outlining methods to identify and purify PSM' protein from LNCaP cell lysates. NH$_2$-terminal sequence analysis has confirmed the identity of PSM' and shown that the protein begins at Ala-60 in the PSMA sequence. The protein has been shown to reside in the cytoplasm, as speculated previously. PSM' protein is a minor component in comparison to full-length PSMA in these cells. Studies are ongoing in our laboratory to determine whether ratios of PSMA:PSM' are of value in the detection and prognosis of prostate cancer.

References
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