Prostatic Acid Phosphatase Is Not a Prostate Specific Target

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Abstract
Prostatic acid phosphatase (PAP) is currently evaluated as a target for vaccine immunotherapy of prostate cancer. This is based on the previous knowledge about secretory PAP and its high prostatic expression. We describe a novel PAP spliced variant mRNA encoding a type I transmembrane (TM) protein with the extracellular NH2-terminal phosphatase activity and the COOH-terminal lysosomal targeting signal (Yxxφ). TM-PAP is widely expressed in nonprostatic tissues like brain, kidney, liver, lung, muscle, placenta, salivary gland, spleen, thyroid, and thymus. TM-PAP is also expressed in fibroblast, Schwann, and LNCaP cells, but not in PC-3 cells. In well-differentiated human prostate cancer tissue specimens, the expression of secretory PAP, but not TM-PAP, is significantly decreased. TM-PAP is localized in the plasma membrane-endosomal-lysosomal pathway and is colocalized with the lipid raft marker flotillin-1. No cytosolic PAP is detected. We conclude that the wide expression of TM-PAP in, for instance, neuronal and muscle tissues must be taken into account in the design of PAP-based immunotherapy approaches.

Introduction
Prostate cancer is the most common cancer in men and the second leading cause of cancer death in the Western countries. Although chemotherapy treatments have shown survival benefit for hormone-refractory prostate cancer, new and more effective therapies are needed (1). A novel approach refers to the activation of the immune system using an antigen loaded in antigen-presenting cells (2). Some of the new ongoing vaccine therapies in trial for prostate cancer treatment are based on the essential restriction of prostatic acid phosphatase (PAP; ACPP; EC 3.1.3.2) expression in prostate (3).

There are two forms of PAP, secretory and nonsecretory, with different isoelectric points and molecular weights (4). Only mRNA encoding the secretory form is described thus far (5). It is suggested to encode also a so-called cellular form (6). The physiologic substrate is still unknown.

It is claimed that PAP has growth-suppressing effect and it is due to its cellular protein tyrosine phosphatase activity (6). Within cells, the activity of PAP is lower in prostate carcinomas than in normal prostates (7), and both PAP mRNA and protein levels are decreased or absent in prostate carcinoma tissue (8). Our results show that PAP has two splicing variants encoding a secretory form and a type I transmembrane (TM) protein, which is in vesicles and membranes and is widely expressed in many nonprostatic cells and tissues.

According to our results, it is important to highlight the fact that the expression of PAP is not exclusive to prostatic tissue, and this issue has to be considered for the evaluation of unwanted side effects of PAP-based immunotherapy. This study suggests TM-PAP to have still unrevealed physiologic prostastic and nonprostastic functions.

Materials and Methods
Antibodies. Lysosomal associated membrane protein-2 monoclonal antibody was developed by J.T. August and J.E.K. Hildreth (Johns Hopkins University, Baltimore, MD) and obtained from the Developmental Studies Hybridoma Bank (developed under the auspices of the National Institute of Child Health and Human Resources and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA). Mouse monoclonal antibody against bis(monoacylglycerol)phosphate (BMP) was a generous gift from Prof. Jean Gruenberg (University of Geneva, Geneva, Switzerland). PAP polyclonal antibody was generated by our group (9). Flotillin-1 was from BD Biosciences.

PCR-based cloning of novel PAP variants. To obtain cDNA encoding rat TM-PAP, total RNA isolated from rat prostate was amplified by reverse transcription-PCR (RT-PCR) using primers 5′-ACCATGA-GAGCTGTCCCTCTCTG-3′ and 5′-TCAGATGTCGGCAGATACAC-3′ designed from the rat genomic region, which we found in silico based on our hypothesis of high similarity in rat and mouse PAP gene structure and the peptide sequence of a possible TM domain.

To obtain a cDNA encoding human TM-PAP, RT-PCR was done using total RNA from human prostate as a template and primers 5′-ATGAGAGCTGCACCC-3′ and 5′-GCTCTGGGCCAGATTCAAAAGG-3′. The resulting RT-PCR products were cloned into a pCRII-TOPO vector (Invitrogen) and sequenced.

Splice variant–specific RT-PCR. Reverse transcription of total RNA to cDNA and subsequent amplification were done using the GeneAmp RNA PCR kit (Applied Biosystems) according to the manufacturer’s instructions. Total RNA was isolated from human and mouse tissue specimens, mouse fibroblasts, SW-10, LNCaP, and PC-3 cells, and used as a template. Primers are described in Supplementary data.

Quantitative real-time RT-PCR to amplify cDNA fragments for the individual PAP variants in human prostate tissue specimens was done by using TaqMan chemistry on an ABI Prism 7700 sequence detection system (Applied Biosystems). Human tissue specimens were obtained as redundant material for diagnosis from hormonally untreated patients going through transurethral resection for benign prostatic hyperplasia or

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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total prostatectomy in the case of prostate cancer in the Oulu University Hospital. Histopathology was confirmed and cancer tissue dissected by the pathologist. The cDNA was synthesized from 0.5 μg total RNA derived from frozen prostate tissue samples, BHP (n = 27), and prostate cancer (n = 19). Primers are described in Supplementary data. TM-PAP mRNA variant, PAP mRNA secreted variant, and 18S RNA levels were measured. The results were normalized to 18S quantified from the same samples. Statistical analyses were done with Student’s two-tailed t test. Difference was considered significant at P < 0.05.

The permissions to use the human (prostate and muscle) and mice specimens for research purposes have been obtained from Oulu University Hospital Ethical Committee and National Authority for Medicolegal Affairs.
and from The Animal Experimentation Committee, University of Oulu, respectively.

**Cell culture.** The human prostate carcinoma cell lines PC-3 and LNCaP and mouse neuronal Schwann cells (SW-10) were obtained from American Type Culture Collection (ATCC) and grown according to the instructions of ATCC. Mouse skin fibroblasts were obtained by culturing of 2-day-old mouse skin explants and subcultured.

**Immunofluorescence and confocal microscopy.** Immunolabeling of human samples was done according to Andrejewski et al. (10). Samples were fixed in 4% paraformaldehyde/2.5% sucrose, mounted on sample holders, and frozen in liquid nitrogen. Cryosections for fluorescence and electron microscopy were cut at $-100^\circ$C. Fluorescent [Alexa Fluor 594 goat anti-mouse immunoglobulin G (IgG) and Alexa Fluor 488 goat anti-rabbit IgG, Molecular Probes] or gold-conjugated (5-nm gold goat anti-mouse IgG and 10-nm goat anti-rabbit IgG; British Biocell) secondary antibodies were used. The samples were analyzed using a confocal microscope (Leica Microscopy and Scientific Instruments Group) or a Jeol 1200 EX electron microscope.

LNCaP and SW-10 cells grown on Thermomax plastic coverslips (Nalgene-Nunc) were subjected to pre-embedding immunolabeling with anti-PAP antibody (9) as described earlier (11) and processed for Epon embedding. Sections were cut parallel to the coverslip, poststained with uranyl acetate and lead citrate, and imaged with Tecnai 12 (FEI Corp.) electron microscope at 80 kV.

LNCaP and PC-3 cells grown on coverslips were fixed with 4% paraformaldehyde and permeabilized with blocking buffer (1% bovine serum albumin/0.2% saponin in PBS). Primary antibodies were incubated for 1 h at room temperature. Fluorescent second antibodies were used [Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probes) and TRITC goat anti-mouse IgG (Sigma)] and 4,6-diamidino-2-phenylindole (Fluka Chemicals) was used to stain the nucleus. Cells were embedded in Immuno Mount (Thermo Sandon). Confocal imaging was done using Olympus FluoView 1000 confocal microscope.

**Transfection of PC-3 cells with TM-PAP-GFP.** cDNA for human TM-PAP was obtained by RT-PCR using human prostate total RNA as a template and primers 5'-TTAGGATCCACCATGAGAGCTGCACC-3' and 5'-AATGGATCCGATGTTCCCATAGGATTC-3'. The PCR product was cloned into pcR 2.1-TOPO plasmid (Invitrogen). From the recombinant plasmid, the BamHI restriction fragment containing the coding region of TM-PAP was cloned into a pEGF-N3 (BD Biosciences Clontech) plasmid. Restriction digestions and sequencing confirmed the direction of the insert and sequence of the construction. PC-3 cells were grown to 80% to 90% confluence and transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Transfection (DNA/Lipofectamine, 1:2) was done for 5 h at $37^\circ$C

**Figure 2.** PAP and BMP colocalization in human prostate cancer tissue. A, PAP was localized in vesicles both in the basal and apical cytoplasm, and strong labeling was observed in the lumen of the glands. PAP showed an almost complete colocalization with BMP (green, PAP; red, BMP; blue, nuclei; yellow, colocalization). B, PAP localized in the limiting and internal membranes of apical, electron-lucent vesicles (black arrow). PAP was also observed in the lumen of multivesicular endosomes. C, in addition, PAP labeling was observed in the membranous structures in the lumen. PAP (10-nm gold) colocalized with BMP (5-nm gold; arrowheads). Some BMP labeling was also observed in the endosomes (right, arrowheads).

AJ, adherent junction; L, lumen.
in Opti-MEM medium (Life Technologies, Inc.). Cells were incubated in normal growth medium for 24 h at 37°C before experiments.

Immunohistochemistry. Histopathologically normal skeletal muscle biopsies were chosen for the study (from the files of the pathology laboratory, Oulu University Hospital). The muscle biopsies were orientated and mounted in optimum cutting temperature compound, frozen in isopentane and liquid nitrogen, and cut for immunohistochemistry (10 μm). The immunohistochemistry procedure was done using an anti-PAP antibody (1:500; ref. 9), the EnVision (DAKO) staining kit, and diaminobenzidine as chromogen (DAKO).

Results and Discussion

Novel PAP isoform is encoded by alternative splice variant.

To generate human, mouse, and rat TM-PAP variants by RT-PCR (Fig. 1A), we used total RNA. Rat and human PCR products were cloned and sequenced. Sequences were highly homologous with cDNA of mouse TM-PAP variant. The rat cDNA (GenBank accession no. DQ826426) showed 91% and 81% identities with reported cDNA sequences of mouse (GenBank accession no. NM_207668; ref. 12) and human PAP (13), respectively. Comparison of the exon-intron junction suggests that rat, mouse, and human PAP variants are derived by alternative splicing. Position of the splicing of the 10th intron in the rat, mouse, and human gene is similar at the end of the 10th exon. In the rat and mouse gene, the splicing of the 10th intron results in the secreted variant mRNA, and in human in the TM variant. The splicing of the 10th intron, the 11th exon, and the 11th intron in the rat and mouse gene yields the TM variant mRNA. In the human secreted variant mRNA, the open reading frame continues over the splicing site until the stop codon (Fig. 1B).

The deduced 417-amino-acid sequence showed 89% and 95% homologies with human and mouse PAP, respectively. The NH2 terminus of PAP has a 32-amino-acid-long signal peptide. The COOH terminus of TM-PAP isoform contains the TM domain of 22 amino acid residues and an endosomal/lysosomal targeting Y[G/R][N] sequence separated by 10 amino acids from TM domain (Fig. 1C). The relative location of signal and TM peptides

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6 GenBank accession numbers: rat TM-PAP variant, DQ826426; mouse TM-PAP variant, NM_207668; human TM-PAP variant, BC007460; human secreted PAP variant, NM_001099; mouse LAP mRNA, BC023343; human LAP mRNA, BC093010; and human LPAP mRNA, AB031478.
determines the topology of the TM-PAP isoform as a type I TM protein. The present tyrosine signal Y[G/R]NI is similar to the lysosomal targeting sequence YxxΦ, where x can be any amino acid but tend to be hydrophilic and Φ is a hydrophobic residue. As reviewed by Bonifacino and Traub (14), this is evidence for lysosomal targeting. Moreover, the COOH-terminal residue of PAP, isoleucine, is the same as in lysosomal associated membrane protein 1, a type I TM protein known to be targeted to lysosomes (15). In conclusion, TM-PAP isoform can be efficiently internalized from plasma membrane due to the presence of the YxxI motif and delivered to lysosomes through the indirect endosomal pathway as confirmed by our observations. Alignment of human, mouse, and rat TM-PAP and lysosomal acid phosphatase (LAP) reveals similar TM domain and lysosomal targeting sequence (Fig. 1C). Human LAP presents two cleavage sites for two different proteases. When the COOH-terminal ends of human LAP and human PAP TM isoforms are compared, there is no identity of amino acids at the protease cleavage site or at the neighboring residues. Specificity requirements of these proteases suggest that TM-PAP is not a suitable substrate for them and it is possible that TM-PAP is recycling from lysosomes to plasma membrane.

**TM-PAP is widely expressed.** Based on the contradictory statements present in the literature about the tissue-specific expression of PAP (3, 16), we analyzed the expression of this novel splicing variant in both human and mouse. RT-PCR analyses showed that the TM-PAP variant was widely expressed in mouse tissues (e.g., in prostate lobes, salivary gland, thymus, lung, kidney, brain, spleen, thyroid, and mouse Schwann and fibroblast cells; Fig. 1D). Expression of the PAP secreted variant was clearly detected in salivary gland, thymus, and thyroid (data not shown). Secretory and TM-PAP variants were present in LNCaP cells, but they were not expressed in PC-3 cells (Fig. 1D).

**The ratio of PAP isoforms is changed in prostate cancer.** Because, immunohistochemically, PAP shows low expression in prostate carcinoma cells and tissues (8) although in the circulation of prostate cancer patients its concentration may be elevated (9), we further studied PAP expression in prostate. Both PAP variants were present in human benign prostatic hyperplasia and well-differentiated prostate cancer specimens (Fig. 1D). The abundance of PAP variants in benign prostatic hyperplasia (n = 27) and prostate cancer (n = 19) specimens was determined using quantitative real-time PCR. Values of TM-PAP mRNA variant were (mean ± SD) 0.5 ± 0.26 in benign prostatic hyperplasia and 0.45 ± 0.34 in prostate cancer, and values of PAP mRNA secreted variant were 0.60 ± 0.3 in benign prostatic hyperplasia and 0.31 ± 0.33 in prostate cancer. Both variants were expressed in all specimens, but expression of the PAP mRNA secreted variant was significantly decreased in prostate cancer (P < 0.05).

We used immunofluorescence and electron microscopy to further investigate the localization of PAP in prostate cancer tissue. Immunofluorescence revealed that PAP is localized in vesicles located both in the basal and apical cytoplasm (Fig. 2A, PAP). In addition, strong labeling was observed in the lumen of the glands (Fig. 2A, PAP). PAP showed an almost complete colocalization with BMP (Fig. 2A, BMP), a lipid highly enriched in late endosomes and multivesicular bodies (Fig. 2A, Merge). Immunoelectron microscopy was used to study the localization of PAP at higher resolution. PAP localized in the limiting and internal membranes of apical, electron-lucent vesicles (Fig. 2B, up) as well as in the internal membranes of multivesicular bodies.
In agreement with the immunofluorescence labeling, PAP colocalized with BMP in all these structures (Fig. 2C, right), and PAP was observed in the membranous structures in the gland lumen (Fig. 2C, left). This suggests that the luminal material may originate from PAP- and BMP-containing vesicles, which have fused with the plasma membrane and emptied their contents into the lumen. These observations suggest that at least some luminal PAP is associated with intracellular membrane structures. Normally, the internal vesicles of the multivesicular body are destined for degradation and are released into the lysosomal lumen. The residual membrane of the multivesicular body is believed to retain those proteins which will escape degradation. Multivesicular body can also recycle to the plasma membrane and release their contents (exosomes) in vivo into the circulation (17).

PAP is in vesicles and membranes. Similar pattern of distribution was detected in LNCaP cells, with a localization of PAP in endosome-like vesicles and lysosomes (Fig. 3A). The analysis of mouse Schwann cells showed PAP in the plasma membrane segments and filopodia-like structures (Fig. 3B). In the human skeletal muscle fibers, PAP expression was present in the sarcolemma (Fig. 4A).

We examined the subcellular localization of TM-PAP-GFP in transfected PC-3 cells that do not express PAP endogenously. Subcellular localization of TM-PAP-GFP in PC-3 cells was analyzed by confocal microscopy. TM-PAP-GFP was seen on the plasma membrane, as expected, and also observed in intracellular vesicles (Fig. 4B). In transfected control cells with an empty EGFP-N3 vector, the signal was evenly distributed throughout the cells.

We studied further the colocalization of endogenous PAP with flotillin-1, which is associated with membrane lipid raft, and showed that both proteins colocalize in plasma membrane and intracellular vesicles (Fig. 4C). Flotillins have been implicated in signaling, regulation of actin cytoskeleton, and membrane transport processes (18). It is still open if PAP is involved in these processes.

Considering the alignment results showing that PAP contains the lysosomal targeting sequence Yxx0, we studied the colocalization of PAP with a lysosomal associated type I membrane protein, lysosomal associated membrane protein 2 (Fig. 4D). Our findings showed a clear colocalization between these proteins.

In conclusion, there are two widely expressed isoforms for PAP, secreted and TM. The importance of these two isoforms in prostate cancer development remains to be addressed, but the presence of a novel TM-PAP isoform in plasma membrane would suggest it to be involved in crucial cellular functions.

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References

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