Trp-p8, a Novel Prostate-specific Gene, Is Up-Regulated in Prostate Cancer and Other Malignancies and Shares High Homology with Transient Receptor Potential Calcium Channel Proteins

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ABSTRACT

We have identified and cloned a novel gene, trp-p8, by screening a prostate-specific subtracted cDNA library. The 5694-hp cDNA has a 3312-bp open reading frame, which codes for a 1104 amino acid putative protein with seven transmembrane domains. The predicted protein revealed significant homology with the transient receptor potential (trp) family of Ca\(^{2+}\) channel proteins. Northern blot analysis indicated that trp-p8 expression within normal human tissues is mostly restricted to prostate epithelial cells. In situ hybridization analysis showed that trp-p8 mRNA expression was at moderate levels in normal prostate tissue and appears to be elevated in prostate cancer. Notably, trp-p8 mRNA was also expressed in a number of nonprostatic primary tumors of breast, colon, lung, and skin origin, whereas transcripts encoding trp-p8 were hardly detected or not detected in the corresponding normal human tissues.

INTRODUCTION

Prostate carcinoma is the most common cancer diagnosed in men in the United States and has the second highest cancer death rate yielding only to lung adenocarcinoma (1). Although it is possible to effectively treat organ-confined prostate cancer, there are very limited treatment options for metastatic disease. Thus, it is of great importance to find novel ways to diagnose early stage disease and to closely monitor both progression and treatment of the disease, as well as to develop new therapeutic approaches.

To achieve this, it is important to understand the molecular mechanisms of prostate cancer development and to identify new biochemical markers for disease diagnosis and progression. To date, there are very few prostate-specific markers available. The best-known and well-characterized markers of proven prostate cancer diagnostic value are the proteins PAP2, PSA (2–8), and prostate-specific membrane antigen (9–13). Each of these proteins has also become the target for novel immunotherapy approaches to the treatment of the disease (14–16).

In this study, we report the identification of a novel gene, designated trp-p8, which is preferentially expressed in prostate. Expression of trp-p8 is mostly restricted to normal prostate epithelial cells and is up-regulated in prostate carcinomas. Cloning of a full-length human trp-p8 cDNA revealed a transcript corresponding to 1104 amino acid polypeptide sharing homology with the trp family of calcium channels (17). Trp-p8 showed particularly high homology with the human TRPC7 gene, a putative Ca\(^{2+}\) channel protein of the trp family, which is highly expressed in brain tissue (18). Trp-p8 also showed significant homology to human melastatin, another trp-family-related protein expressed in melanocytes and believed to be a tumor suppressor gene (19, 20). Perhaps of greatest interest is the observation that the trp-p8 gene appears to be expressed in a large spectrum of nonprostatic neoplastic lesions.

Trp-p8 represents a novel, mostly prostate-restricted as well as pantumor-expressed marker with significant potential use in disease diagnosis and monitoring of disease progression during treatment. It may also serve as a novel target for cancer therapy.

MATERIALS AND METHODS

Isolation of Original Partial cDNA Insert

Subtractive hybridization was carried out using a cDNA library prepared from human prostate mRNA (tester cDNA library), and cDNAs prepared from 10 different human mRNA (designated driver cDNAs). Spleen, thymus, brain, heart, kidney, lung, ovary, placenta, and skeletal muscle were used as driver cDNA to generate a prostate-specific subtracted cDNA library. The subtractive hybridization was performed according to the methods described in the PCR-Select cDNA Subtraction Kit (Clontech Laboratories, Inc., Palo Alto, CA). This method is based on the selective amplification of differentially expressed sequences, which overcomes technical limitations of traditional subtraction methods (21). All of the mRNAs used in the preparation of the prostate-specific subtracted library were obtained from Clontech Laboratories.

The subtracted prostate cDNA, highly enriched for prostate-specific partial cDNA sequences, was amplified using the primer pair NP1 (5-TCGAGCGGCGCCCGGGGGAGGTCGAGTGAAG)-3/ NP2 (5-AGGGCCGGTCGTCGAGGGGGAGGTCGAGTGAAG) on a Perkin-Elmer GeneAmp 9600 Cycler for 11 cycles with the following thermal cycling profile: 94°C for 10 s; 68°C for 30 s; and 72°C for 5 min. This was followed by one additional round at 72°C for 5 min. The products were ligated into the pCR2.1 TA cloning vector (Invitrogen, Carlsbad, CA) and transformed into Escherichia coli strain TOP10F” (Invitrogen). Individual ampicillin-resistant bacterial transformants were screened by PCR for the presence of inserts using the M13 Reverse (5-CACGGAACACGGTAAGAT) and the following thermal cycling profile: 94°C for 15 s; 55°C for 30 s; and 72°C for 1 min for 30 cycles. This was followed by one additional round at 72°C for 6 min. The original trp-p8 insert in the pCR2.1 plasmid was sequenced using the M13 reverse primer (5-CACGGAACACGGTAAGAT) and the M13–20 primer (5-GTAACGCGGCGCGAATC) on an ABI 373 DNA Sequencer. DNA sequences were analyzed using Sequencher 3.0 software (Gene Codes Corporation, Ann Arbor, MI).

Isolation of Complete Trp-p8 cDNA

The full-length cDNA encoding trp-p8 was isolated from a cDNA library prepared from normal human prostate poly(A) RNA using both the 5’ and 3’ RACE method (Marathon cDNA amplification Kit; Clontech Laboratories). 5’ RACE was carried out with the API (5-CCATCCTATATCGTCACTCATATTAGGCG-3)/(5-GCCGAGTAATAGGAGACACGCGTGGTGGG-3) primer pair. 3’ RACE amplification reaction was carried out with the API (5-CCATCCTATATCGTCACTCATATTAGGCG-3)/(5-TGGAAACTGTGTTGCGA-3).
ACTTCCCG-3) primer pair. The thermal cycling profile for all of the RACE reactions was 94°C for 15 s and 68°C for 4 min for 30 cycles using Advantage cDNA Polymerase Mix (Clontech Laboratories). 5’ and 3’ RACE products of approximately 4 kb and 1.6 kb, respectively, were isolated by agarose gel electrophoresis, ligated into the pCR2.1 TA vector (Invitrogen), and transformed into competent E. coli cells. Individual ampicillin-resistant bacterial colonies were screened by PCR for the presence of an insert with the primer pair AP2 (5’-ACTCATAATGGGCTCGACGGC-3’)/(5’-GGCAGGATAATAG-GAGACAGCCTGTTG-3’) for the 5’ RACE products and the primer pair AP2 (5’-ACTCATAATGGGCTCGACGGC-3’)/(5’-CAAGTCATTTGGCAAGCAGACC-3’) for the 3’ RACE products using a thermal cycling profile of 94°C for 15 s, 55°C for 30 s, and 72°C for 1 min for 35 cycles, followed by one additional round at 72°C for 6 min. PCR products were resolved by agarose gel electrophoresis, and individual bacterial transfectants were grown in liquid culture (Luria-Broth supplemented with ampicillin 100 μg/ml) before preparation of plasmid DNA. The complete DNA sequence of individual RACE products was determined by automated fluorescent sequencing using an ABI 373 DNA sequencer in conjunction with custom DNA primers and the Primer Island Transposition Kit (PE Applied Biosystems, Foster City, CA). The near full-length trp-p8 cDNA was amplified and cloned using primers that correspond to the ends of the 5’ and 3’ RACE sequences.

Expression of Recombinant Trp-p8

Expression in Bac-to-Bac Baculovirus System (Life Technologies, Inc.). Trp-p8 cDNA was amplified using AdvanTaq DNA Polymerase (Clontech) and primers TACTACGATATCATGAGGAACAGAAGGAATGACACTCTGGACAGCAC and TTATATTAAATACCTGGCGACACCCTTATGATGTTGATGATGATGAGGTGGATGAGGTGGTTTGATTTATACATGGGGAACAGAAGGAATGACACTCTGGACAGCAC and digested with NotI and EcoRV and inserted into NotI- and SalI-digested pFAST-Bac1 plasmid. All of the subsequent procedures were performed according to the manufacturer’s protocols. Briefly, pFASTBac1 plasmid carrying trp-p8 insert was used to transform competent DH10Bac E. coli cells. White colonies were screened for transposition event by PCR, and high molecular weight DNA of selected clones was used to transfect S21 insect cells grown in SF-900 II medium. Resulting viral stock was amplified twice. Infected cells were collected 24–72 h after infection, lysed in PBS containing 8 M urea and protease inhibitors cocktail (Roche Molecular Biochemicals), diluted in SDS-PAGE sample buffer, and separated on 8–16% polyacrylamide gradient SDS-PAGE gels (Novex/Invitrogen). The separated proteins were transferred on nitrocellulose membrane (Protran; Bio-Rad), blocked, and developed with COOH-terminal oligohistidine-specific mouse monoclonal antibody (Invitrogen), followed by horseradish peroxidase-conjugated sheep antibodies specific for mouse immunoglobulin (Amersham/Pharmacia). The blots were visualized by enhanced chemiluminescence (Amer- sham/Pharmacia).

Expression in E. coli (pTriEx-1 System; Novagen). Trp-p8 cDNA was amplified using AdvanTaq DNA Polymerase (Clontech) and primers ATATATATACGATATCATGAGGAACAGAAGGAATGACACTCTGGACAGCAC and TTATATTAAATACCTGGCGACACCCTTATGATGTTGATGATGATGAGGTGGATGAGGTGGTTTGATTTATACATGGGGAACAGAAGGAATGACACTCTGGACAGCAC and digested with NcoI and NotI, and cloned into pTriEx-1 vector, which was previously cut with the same restriction enzymes. The resulting DNA was used to transform (DE3) pLacI expression hosts (Novagen). Overnight bacterial cultures were inoculated into fresh Luria-Bertani medium supplemented with ampicillin and chloramphenicol, grown for 3–5 h, and induced with 1 mM isopro- pyl-1-thio-β-D-galactopyranoside for 3 h. Bacterial pellet was collected and lysed, and the expression was verified as described above for baculovirus system.

Transient Expression in Mammalian Cells. EcoRV- and NotI-digested trp-p8 cDNA, prepared as described above, was cloned into EcoRV and NotI sites of the pcR3.1 plasmid (Invitrogen). The resulting DNA (5–10 μg) was transfected into COS-7 cells (80% confluent in 6-well tissue culture plates) using 2 μl of Lipofectamine2000 (Life Technologies, Inc.) well. DNA-liposome complexes were formed for 15 min in 100 μl of serum-free Iscove’s modified Dulbecco’s medium. Eight h after the transfection, the cells were injected with vaccinia virus carrying T7 RNA polymerase, harvested, and lysed as described for the baculovirus-infected cells.

Poly(A)+ RNA Preparation

Total RNA was extracted from cells using Trizol Reagent (Life Technologies, Inc.), following the manufacturer’s instructions. Poly(A)+ RNA were isolated using mRNA Purification Kit (Pharmacia, Palo Alto, CA). The final RNA preparation was resuspended in 10 mM TrisCl, 1 mM EDTA (pH 8.0), prepared with diethylpyrocarbonate-treated water, and quantitated by light absorbency at 260 nm.

RT-PCR

First strand cDNA was prepared using human poly(A)+ RNAs and the SMART PCR cDNA Synthesis Kit (Clontech). Briefly, 5 μl of template-primer mixture, containing 1 μg of Poly(A)+ RNA, were incubated at 70°C for 2 min. Superscript II reverse transcription mix (5 μl) was added, and reverse transcription was carried out at 42°C for 1 h. The reaction mix was diluted with 45 μl of 10 mM TrisCl, 1 mM EDTA (pH 8.0) buffer, and reaction was terminated at 72°C for 7 min. The primer pair (5’-GGGTTTCCCGAGTGTTGATGTTGAGGTGGATGAGGTGGTTTGATTTATACATGGGGAACAGAAGGAATGACACTCTGGACAGCAC and 5’-CCCCAGACGGCATTGATGTCG-3’) was used to assess expression with a thermal cycling profile of 94°C for 15 s, 65°C for 15 s, and 72°C for 30 s (30 cycles), followed by one additional round at 72°C for 6 min. Amplification products (503 bp) were resolved by agarose gel electrophoresis and visualized by ethidium bromide staining and UV light.

Northern and Dot Blot Analysis

Northern blots, in which 2 μg of Poly(A)+ RNA from the tissue indicated were loaded into each lane, and human RNA master blot, in which the amount of RNA+ was normalized (Clontech), were used. Trp-p8 342 bp (“original fragment”) and human G3PDH were used as probes. The filters were prehybridized in ExpressHyb solution (Clontech) for 30 min at 65°C and for 2 h in the same solution containing 1 × 106 cpm/ml 32P-labeled probe at 65°C. The filters were washed in 2× SSC, 0.1% SDS for 15 min at room temperature and then with a solution containing 0.1 × SSC, 0.1% SDS for 15 min at room temperature and for 1 h at 68°C. Filters were exposed to a Kodak XR film for 1 h to 3 days at ~70°C.

Virtual Northern Blots

A SMART PCR cDNA Synthesis Kit (Clontech) was used to generate SMART cDNAs from Poly(A)+ RNA samples isolated from different tissues. Then, cDNAs were electrophoresed on an agarose gel, denatured, transferred onto a nylon membrane, and hybridized to the 32P-labeled probes as described above.

Cell Culture

The human prostate cancer cell lines LNCaP (ATCC CRL 1740), PC 3 (ATCC CRL 1435), and DU145 (ATCC HTB 81), as well as the melanoma G361 (ATCC CRL1424), the colorectal adenocarcinoma SW480 (ATCC CCL228), and the lung carcinoma A549 (ATCC CCL185) cell lines, were obtained from American Type Culture Collection. The cells were propagated in DMEM containing 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml fungizone (Life Technologies, Inc.)

In Situ Hybridization

The tissues used in this study were formalin-fixed, paraffin-embedded archival blocks donated by regional hospitals to the tissue bank of LifeSpan BioSciences in Seattle, Washington. The tissues had been removed at surgery, fixed in 10% neutral buffered formalin for 16–24 h, processed, and embedded in paraffin. Serial 4-μm sections were then stained with H&E or used in the in situ hybridization studies. Two pathologists independently evaluated the samples for diagnostic verification and grading. Adjacent serial sections were then screened for the presence of preserved RNA by hybridization with an antisense collagen control riboprobe. Only tissues that passed the hybridization test with similar levels of signal were used in subsequent hybridization analyses for trp-p8. After hybridization, the slides were then independently evaluated by two pathologists for interpretation of the in situ hybridization signal.
Serial tissue sections from paraffin samples were hybridized with probes that had been synthesized from linearized plasmids (pCR 2.1; Invitrogen) containing the original partial cDNA fragment of trp-p8 gene (342 bp) isolated from the subtracted library. In these studies, digoxigenin-labeled riboprobes transcribed from the T7 site (antisense and sense probes) were used. After transcription, the probes were subjected to electrophoresis onto a 1% agarose gel to determine the size and purity of the riboprobes. In addition to the antisense trp-p8 probes, adjacent tissue sections were hybridized with both sense and antisense α-(1) Type I collagen riboprobes, and a subset of the slides was also hybridized with the sense orientation trp-p8 riboprobe. Tissue sections from paraffin blocks were digested with proteinase K for 3 to 4 min and then hybridized with the antisense probe at a concentration of 1 μg/ml at 60°C. The hybridization signals were visualized with NBT/BCIP substrates using two cycles of an alkaline phosphatase TSA amplification system (NEN Life Sciences). The specimens were then counterstained with methyl green. The signal was developed within 30–40 min at room temperature. The slides were then imaged using a Sony digital photo camera and a Nikon microscope.

RESULTS

Prostate-specific candidate clones were isolated as described in “Materials and Methods.” A total of eight candidate cDNA sequences were found to be novel, as evidenced by the lack of significant database matches. These cDNAs were selected for further analysis. As a confirmation for this approach to enrich prostate-specific genes, we also detected the PAP gene three times, PSA eight times, semenogelin I, II, III fifteen times, and prostate-specific transglutaminase five times of a total 200 clones.

The eight chosen clones were further analyzed for prostate-specific expression pattern by Northern and dot blots. One clone that displayed the most restricted prostate-specific expression pattern was chosen. The full-length cDNA was isolated and sequenced as described in “Materials and Methods.” This cDNA was designated trp-p8.

Trp-p8 Nucleic Acid Sequence and Predicted Protein Structure Analysis. The entire 5694-bp nucleotide sequence designated as trp-p8 cDNA is shown in Fig. 1. The sequence contains a single open reading frame with an apparent translational initiation site at nucleotide positions 41–43 and with a stop codon at nucleotide positions 3353–3355 (22). The trp-p8 cDNA has a 2338-bp 3′-untranslated region, which includes a poly(A) tail.

The putative protein sequence of trp-p8 protein consists of 1104 amino acid residues and does not have any apparent eukaryotic signal peptide at its NH₂-terminal end. Hydrophilicity analysis of the predicted trp-p8 peptide sequence revealed seven putative hydrophobic transmembrane α-helices at the COOH-terminus of the protein and a large NH₂-terminal hydrophilic domain (Fig. 2). Analysis of trp-p8 protein sequence for various post-translational modification consensus sites revealed eight potential Asn-linked glycosylation sites, all of which reside on the hydrophilic portions of the polypeptide. An amino acid homology search with NTBLAST algorithm against the National Center for Biotechnology Information database revealed that trp-p8 shared homology to Caenorhabditis elegans, Drosophila, and human trp (Htrp) proteins. Comparison of amino acid sequence between putative transmembrane domains of the trp-p8, Drosophila, and human trp proteins is shown on Fig. 3. Sequence analysis showed significant homology among trp-p8 putative transmembrane domains with human and Drosophila trp proteins. All of the proteins showed significant homology within a highly conserved motif in the trp family (Fig. 3, bold font). The distribution and conservation of six of seven transmembrane domains (domains 2–7) corresponding to the Drosophila S1 to S6 domains strongly suggest the function of trp-p8 protein as a Ca²⁺ channel, similar to trp proteins. The phylogenetic relationship between trp-p8, Drosophila, and human trp proteins is shown in Fig. 4. Results from a multiple sequence alignment using CLUSTAL W program (23) suggest that evolutionary trp-p8 is the most closely related to the recently discovered TRPC7 protein, expression of which is highly restricted to human brain (18). Trp-p8 also showed significant homology to melastatin, a protein that is differentially expressed in human melanocytes (19).

Trp-p8 Recombinant Protein Expression. When trp-p8 expression constructs, supplemented with the COOH-terminal 6-histidine epitope tags, were used to produce recombinant protein in mammalian and insect cells, a single protein band of M₀ 130,000 was detected by visualizing the presence of the 6-histidine tag (Fig. 5). This closely matches the predicted molecular weight of trp-p8 gene product, M₀ 128,300 (M₀ 127,500 without the epitope tag).

Genomic Organization of Trp-p8. The results of a recent (1999) sequence similarity search of the National Center for Biotechnology Information using BLASTX searches of the nonredundant database revealed a match to the cosmid clone NH0332L11 containing human genomic sequence (locus AC005538). The analysis of genomic data indicated that trp-p8 gene spans AC005538 locus in the direction opposite of the published genomic sequence orientation. The data suggest that trp-p8 is composed of 24 exons that are spread across approximately 95 kb (nucleotides 1 through 95 kb of the AC005538 locus; Fig. 6).

Trp-p8 Expression in Human Cell Lines and Tissues. To study the expression of trp-p8, we used the original 342-bp fragment to probe multiple human tissues on Northern blots from Clontech containing mRNA from eight normal human tissues (skeletal muscle, uterus, colon, small intestine, bladder, heart, stomach, and prostate), as well as mRNA from various cell lines and cancer tissues. As shown in Fig. 7, the trp-p8 probe detected two transcripts of approximately 6.2 kb and approximately 5.2 kb in normal prostate only, but not in the other tissues. We were also able to detect two similar transcripts in the melanoma cell line G361. Lower rows show hybridization of the blot to a control human β-actin cDNA probe.

Using the same 342-bp probe, we have performed a dot blot analysis of a human RNA blot (Clontech), containing a panel of mRNAs from numerous tissues of both adult and fetal origin, as shown on Fig. 8. The blot was normalized using eight housekeeping cDNA probes as standards: ribosomal protein S9, M₀ 23,000 highly basic protein, tubulin, phospholipase, ubiquitin, hypoxanthine phosphoribosyltransferase, β-actin, and GAPDH (Clontech). The results indicate that trp-p8 mRNA is only expressed in prostate tissue. The remaining samples remained negative even after prolonged exposure of the radiogram.

The tissue distribution of trp-p8 mRNA was also examined in a panel of human normal and cancer tissues and cell lines using RT-PCR. As shown in Fig. 9, numerous samples were amplified, and trp-p8-specific mRNA expression was confirmed in testis and prostate, as well as in a melanoma cell line, G361, a colorectal adenocarcinoma cell line, SW480, and a prostate carcinoma cell line, LNCaP. A trace amount of the signal was also detectable in breast and thymus tissues.

Trp-p8 mRNA Expression in Human Tissue Sections from Various Tumors. Trp-p8 mRNA expression was examined by in situ hybridization in the following tissue sections: 7 normal prostate samples, 4 BPH samples, and 16 prostate cancer samples.

In normal prostate sections, the epithelial cells showed moderately positive hybridization reaction (Fig. 10E). The strongest signal was seen within the reserve (basal) cell layer and also at the luminal surface of the epithelium. Vascular smooth muscle tissue and endo-
The expression of trp-p8 was variable among the patients, ranging from moderate in normal prostate, moderate to high in BPH, and high in prostate cancer.

In addition to the normal prostate and prostate cancer, we have examined tissue sections from neoplasms of different origin, such as breast adenocarcinoma (14 samples), melanoma (4 samples), colorectal adenocarcinoma (14 samples), and lung carcinoma (10 samples; Table 1). Trp-p8 transcripts were present throughout all of the types.

Fig. 1. Nucleotide sequence of trp-p8 cDNA. The predicted S' and 3' untranslated region is depicted in lowercase, with the open reading frame from position 41 to 3352 shown in uppercase. The predicted protein sequence encoded by trp-p8 gene has seven putative hydrophobic membrane-spanning domains, which are underlined. Boxed letters show the highly conserved region characteristic for the trp family.
were moderately positive for hybridization signal within Type II pneumocytes and some bronchial epithelial cells, although the dot blot analysis of the normal lung mRNA yielded negative results. The bronchoalveolar carcinoma was strongly positive in areas displaying a Type II pneumocyte differentiation pattern. Overall, although results were variable, depending on the type and progression of the tumor, 8 of 10 samples revealed weak to very strong positive signals.

Three of four melanomas were positive for hybridization signals, although the melanoma cells showed a high degree of staining variability. The strongest hybridization signal was seen toward the deeper areas of invasion, and often-superficial melanoma cells were negative for Trp-p8 expression.
hybridization. The single case of melanoma that appeared negative was a superficially spreading melanoma, a less malignant type of melanoma (Fig. 11, F and G).

Hybridization of the control (sense orientation) trp-p8 RNA probe to various human tissues was negative (Fig. 12).

Fig. 7. Northern blot analysis of trp-p8. A 342-bp trp-p8 fragment was \(^{32}\)P-labeled and hybridized to the multiple Northern blots of RNA extracted from normal human tissue samples, as well as human tumor cell lines and human cancers (Clontech). Two bands of 5.1 kb and 6.2 kb were visible in normal prostate and melanoma cell lines but not in numerous other cell and tissue samples. Lower rows show hybridization of the blot to a control human \(\beta\)-actin cDNA probe.

Fig. 8. A, dot blot analysis of trp-p8 mRNA in various human tissues. A 342-bp trp-p8 fragment was \(^{32}\)P-labeled and hybridized to the Human mRNA Master Blot from Clontech containing normalized loading of poly(A)\(^+\) RNA from 50 different human tissues and six different control RNAs and DNAs. Strong signal was observed only in prostate (c7), a1, whole brain; a2, amygdala; a3, caudate nucleus; a4, cerebellum; a5, cerebral cortex; a6, frontal lobe; a7, hippocampus; a8, medulla oblongata; b1, occipital lobe; b2, putamen; b3, substantia nigra; b4, temporal lobe; b5, thalamus; b6, subthalamic nucleus; b7, spinal cord; c1, heart; c2, aorta; c3, skeletal muscle; c4, colon; c5, bladder; c6, uterus; c7, prostate; c8, stomach; d1, testis; d2, ovary; d3, pancreas; d4, pituitary gland; d5, adrenal gland; d6, thyroid gland; d7, salivary gland; d8, mammary gland; e1, kidney; e2, liver; e3, small intestine; e4, spleen; e5, thymus; e6, peripheral leukocyte; e7, lymph node; e8, bone marrow; f1, appendix; f2, lung; f3, trachea; f4, placenta; f5, fetal brain; f6, fetal heart; g1, fetal kidney; g2, fetal liver; g3, fetal spleen; g4, fetal thymus; g7, fetal lung; h1, yeast total RNA; h2, yeast tRNA; h3, E. coli tRNA; h4, E. coli DNA; h5, vPoly p(A); h6, human C\(_0\)t1 DNA; h7, human DNA (100ng); h8, human DNA (500ng). B, same blot as A hybridized to the control human ubiquitin cDNA probe.

Fig. 9. The expression pattern of mRNA for the trp-p8 gene in numerous tissue types and cell lines. The PCR products (~500 bp) were observed in normal testis, normal prostate, and the following cancer cell lines: SW480, G361, and LNCaP. A trace of product was also detected in the normal mammary gland.

Overall, these results indicate that trp-p8 is most abundantly expressed in prostate. It appears to be up-regulated in prostate cancer and expressed in other nonprostatic primary neoplasms. Very low expression in other tissues is limited to certain epithelial cell types and is too weak to be detected by Northern or dot blotting.

DISCUSSION

A novel gene, designated trp-p8, was cloned, and its expression in normal human tissue was found to be predominantly restricted to the prostate with only trace expression found in testis, as detected by Northern blot. When the most sensitive RT-PCR method was used to detect message, trace levels could also be found in breast, in thymus, and occasionally in lung tissues. An interesting attribute of this gene is that it appears to be not only overexpressed in prostate carcinomas but also expressed in different nonprostatic primary cancers, such as melanoma, colorectal carcinoma, and breast carcinoma.

The deduced protein sequence revealed seven transmembrane domains in the COOH-terminal region of trp-p8. Database alignments of the predicted protein structure showed a strong homology to the trp family of proteins. The trp family consists of a number of members of the gene superfamily. Although the function of these trp proteins is not yet known, it has been shown that Drosophila trp proteins, which also suggests that trp-p8 likely belongs to the trp gene superfamily. Although the function of C. elegans trp proteins is not yet known, it has been shown that Drosophila trp proteins, which highly resemble human trp proteins, act as Ca\(^{2+}\) channels (29).
Functional studies to confirm the putative role of trp-p8 as a calcium channel, as well as to elucidate its role in regulating metastatic potential of prostate cancer, are currently underway.

Analysis of trp-p8 mRNA expression in different prostate cancer cell lines revealed expression in the LNCaP cell line and the lack of expression in the DU 145 and PC-3 cell lines. The LNCaP cell line is derived from a lymph node metastases, DU145 is derived from brain metastases, and PC-3 is derived from bone metastases of prostate cancer patients (33–35). The LNCaP cell line has been reported to produce prostate-specific proteins such as PAP, PSA, and prostate specific membrane antigen, whereas expression of these antigens in DU145 and PC-3 cells was not detectable or very low (36). Of the three studied cell lines, only LNCaP, the sole trp-p8 expresser, was found to be androgen-dependent. However, conclusions based exclusively on patterns of expression of proteins in vitro by established cell lines have to be drawn with considerable caution. The expression can be significantly altered by culture conditions and may vary from expression by a tumor in vivo. Therefore, we have conducted extensive in situ mRNA hybridization studies of the primary human cancers and corresponding normal tissues.

We have analyzed a number of radical prostatectomy cases for trp-p8 expression via in situ hybridization. Moderate mRNA expres-
sion was observed in normal prostate, whereas the expression in prostate cancer appears to be elevated. Reserve (basal) epithelial cells were usually the most intensively stained in the normal prostate; however, the stain was observed throughout all of the neoplastic cells in the cancerous tissue. In addition to the normal prostate and prostate cancer, we have examined tissue sections of neoplasm of different origin such as breast adenocarcinoma, melanoma, colorectal adenocarcinoma, and lung adenocarcinoma. We have found that trp-p8 mRNA is expressed in all of these types of cancerous tissues. It appears that trp-p8 gene is most abundantly expressed in a prostate; however, it is also ectopically re-expressed in different forms of cancer of epithelial histogenesis.

Normal, nonmutated genes that encode shared tumor antigens have been classified in two major groups: (a) differentiation antigens shared on melanoma and melanocytes (melanocyte/differentiation antigens: MART-1 and MART-2); and (b) differentiation antigens shared on a variety of tumors as well as normal testis (cancer/testis antigens: MAGE, GAGE, BAGE, and NY-ESO-1; Refs. 37, 38). Although expression of this gene needs further evaluation on the protein level, trp-p8 does not seem to fall in either of these groups. It is expressed predominantly in the normal prostate, as well as in melanoma and in a variety of tumors. Only a trace amount of trp-p8 has been identified in testis.

The trp-p8 gene sequence revealed the highest homology to TRPC7, a gene primarily expressed in human brain. The trp-p8 cDNA also showed homology to melastatin, normally expressed in melanocytes. Thus, contrary to melastatin, which is thought to be a tumor suppressor gene, trp-p8 could be an oncogene or tumor promoter gene. Although a limited number of melanoma samples were analyzed, the intensity of trp-p8 expression showed a direct correlation to melanoma aggressiveness, which distinguishes it from melastatin. The expression of melastatin was inversely correlated with melanoma aggressiveness and metastatic potential (19, 20).

The adult prostate is maintained through equilibrium between cell growth and cell death rates. Recent reports (39) show the link between elevation of intracellular Ca\(^{2+}\), androgen levels, and apoptotic prostatic cell death. Although it is premature to draw conclusions about the role of trp-p8 in the function of the normal prostate, the possibility exists that the trp-p8 gene product is involved in the regulation of intercellular Ca\(^{2+}\). An overexpression of trp-p8 in prostate cancer could suggest the role in the protection of the prostate cancer cells from apoptosis in malignancy.

Considering its mostly prostate-restricted expression, its suggested association with tumor, and its presumed membrane-bound nature, trp-p8 could be used for in vivo imaging, as well as a target for immunotherapy. Its potential function as a Ca\(^{2+}\) channel protein also

Fig. 11. In situ hybridization of trp-p8 RNA probe to multiple cancer tissues. Serial 4-μm sections were stained with H&E (A, B, C, and D) or used in the in situ hybridization with the trp-p8 probe (E, F, G, and H). A and E, colon carcinoma; B, F, and C, G melanoma; D and H, breast carcinoma. Magnifications: A, B, C, D, E, G, and H, ×200; F, ×40.
raises the possibility of pharmacological intervention with specific Ca\(^{2+}\) channel blockers. This use could extend not only to prostate carcinomas but also to diagnosis and treatment of other, nonprostatic tumors. Overall, trp-p8 could prove a clinically useful marker of carcinomas and serve as a target against which novel therapies can be directed.

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