The T Cell Receptor γ Chain Alternate Reading Frame Protein (TARP), a Prostate-specific Protein Localized in Mitochondria*

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We previously showed that mRNA encoding TARP (T cell receptor γ chain alternate reading frame protein) is exclusively expressed in the prostate in males and is up-regulated by androgen in LNCaP cells, an androgen-sensitive prostate cancer cell line. We have now developed an anti-TARP monoclonal antibody named TP1, and show that TARP protein is up-regulated by androgen in both LNCaP and MDA-Pca-2b cells. We used TP1 to determine the subcellular localization of TARP by Western blotting following subcellular fractionation and immunocytochemistry. Both methods showed that TARP is localized in the mitochondria of LNCaP cells, MDA-Pca-2b cells, and PC-3 cells transfected with a TARP-expressing plasmid. We also transfected a plasmid encoding TARP fused to green fluorescent protein into LNCaP, MDA-Pca-2b, and PC-3 cells and confirmed its specific mitochondrial localization in living cells. Fractionation of mitochondria shows that TARP is located in the outer mitochondrial membrane. Immunohistochemistry using a human prostate cancer sample showed that TP1 reacted in a dot-like cytoplasmic pattern consistent with the presence of TARP in mitochondria. These data demonstrate that TARP is the first prostate-specific protein localizing in mitochondria and indicate that TARP, an androgen-regulated protein, may act on mitochondria to carry out its biological functions.

Prostate cancer is the most frequently occurring solid cancer in men and the second cause of death from cancer in the United States. Current statistics estimate that 220,900 new cases were diagnosed in 2003 and that about 28,900 males die of this disease (1). Most forms of prostate cancer are initially androgen-dependent, but the response to androgen ablation therapy is transient. After a few years, most cases of metastatic prostate cancer relapse to the status of androgen independence, resulting in death. No effective treatment options exist against androgen-independent prostate cancers. Improvement in this clinical situation requires novel therapies based on a better understanding of the biochemical basis of prostate cancer initiation and progression.

One approach for understanding the progression mechanism of prostate cancer is to identify new prostate-specific genes whose expression is altered in prostate cancer. These prostate-specific genes should enable us to understand more about normal prostate function and contribute to our understanding of prostate cancer progression. Prostate-specific genes also can be useful targets for molecular based therapies using antibodies, vaccines, and small inhibitory molecules. Because the prostate is not a vital organ, its destruction by antibodies or vaccines that target prostate-specific antigens should not adversely affect the prognosis of the patient. Also, prostate-specific genes can be useful diagnostic markers to predict the presence, the spread, and the prognosis of prostate disease using samples such as blood or urine. These three aspects: cancer progression, pharmacology, and diagnostics, have encouraged many researchers to try and identify prostate-specific genes.

More than 20 genes have been reported in the literature as prostate-specific, although many of these prostate-specific genes have been found to be expressed in other organs including essential organs (2–19). Prostate-specific membrane antigen (PSMA) is a plasma membrane protein that is being clinically evaluated as an immunotherapy target. Its expression is not entirely specific, because it has been detected in brain and nervous tissue (20, 21). Prostate-specific antigen (PSA) is a secretory protein that is widely used as a serum tumor marker for the diagnosis of prostate cancer and is being developed as a target for prostate cancer vaccines (22). Although these prostate-specific genes have made contributions to prostate cancer diagnosis, the function of these two genes and that of other prostate-specific genes remains unknown. Additional prostate-specific genes that play significant roles in prostate cancer need to be identified, and their functions established for the development of novel therapies.

TARP1 is an unusual prostate-specific gene that was identified by a computer-based analysis of the data base of expressed sequence tags (ESTs) (3, 23). The TARP gene is embedded within the TCRγ locus. The TARP mRNA transcript originates within an intron just upstream of the Jγ 1.2 segment of the T cell receptor γ (TCRγ) gene and consists of four exons. The TARP protein is encoded by the second exon: it consists of 58

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† The abbreviations used are: TARP, T cell receptor γ chain alternate reading frame protein; CS, charcoal-stripped; COX, cytochrome c oxidase; DHT, dihydrotestosterone; EGFP, enhanced green fluorescent protein; EST, expressed sequence tag; FCS, fetal calf serum; mAb, monoclonal antibody; MBP, maltose-binding protein; PDI, protein-disulfide isomerase; ROS, reactive oxygen species; TCRγ, T cell receptor γ; VDAC, voltage-dependent anion-selective channel; HSP, heat shock protein.
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amino acids and is 7 kDa in size. The open reading frame for TARP differs from that used to make the TCRγ chain (3, 24). Therefore, the amino acid sequences of the two proteins are different even though cDNA or oligonucleotide probes that detect TCRγ mRNA also detect TARP mRNA. We discovered TARP when we observed that there were many ESTs for TCRγ mRNA but none for TCRδ mRNA in EST libraries prepared from prostate cancer samples. Because these two receptor chains are only found in T cells and because they are always expressed together, we assumed the TCRγ signal detected in prostate was not coming from T cells present in prostate tissue but had another explanation. The finding that mRNA hybridizing with a TCRγ probe was present at high levels in LNCaP cells and that the mRNA in LNCaP cells differed in size from TCRγ transcripts found in T cells led us to clone and sequence this mRNA and demonstrate its unusual properties (3). Since our initial observations, several groups have performed cDNA microarray analyses of prostate samples and reported that TCRγ mRNA, but not TCRδ mRNA, is expressed in prostate tissue and that TCRγ is more than 3-fold higher in prostate cancer specimens than in normal prostate (25–27). For the reasons cited above we believe that the transcript measured encodes TARP and not TCRγ.

In our previous analysis, TARP mRNA was exclusively shown to be present in normal prostate and prostate cancer in males and in breast cancer in females (3, 24). Furthermore, we reported two findings that suggested an important role for TARP in prostate cancer. One is that TARP mRNA is up-regulated by androgen in the LNCaP cell line, an androgen-sensitive prostate cancer cell line. The second is that the growth rate of PC-3 cells, an androgen-independent prostate cancer cell line that does not make TARP, is increased when TARP is introduced and expressed in these cells. We previously reported that TARP appeared to be a nuclear protein from the analysis of subcellular fractions using a rabbit polyclonal antibody and from its homology to a portion of the yeast transcription factor Tup1 (24, 28). However, these studies did not include other methodologies to verify the localization and the antibody used in these studies was a polyclonal antibody that showed high background binding to other proteins in addition to TARP. These findings prompted us to prepare a highly specific monoclonal antibody (mAb) to clarify the subcellular localization of TARP.

In this study we have used three different approaches to determine the intracellular location of TARP, and all three show that TARP is a mitochondrial protein. This is the first study that demonstrates the existence of a prostate-specific protein that is present in mitochondria and implies that TARP regulates prostate function via these organelles.

EXPERIMENTAL PROCEDURES

Plasmids—The TARP open reading frame was generated by PCR and was subcloned between the SacI and HindIII sites into pCMV96, a pMal-C2 vector (New England Biolabs, Inc., Beverly, MA) containing Gateway Cloning Cassette (Invitrogen, Carlsbad, CA) provided by Dr. David Waugh (NCI, National Institutes of Health) (29). The resulting plasmid, pMBP-TARP, encodes TARP protein fused to the C′ terminus of maltose-binding protein (MBP). Eukaryotic expression plasmids were obtained from Invitrogen; pTetOn, coding a reverse tetr transactivator of pTRE-TAR, pTRE2-Luc, coding a tetracycline-responsive element; pTRE2-Luc, luciferase inserted into multicloning sites of pTRE2-hyg; and pEGFP-N1, coding enhanced green fluorescent protein (EGFP) and multicloning site to the N′ terminus of the EGFP. TARP open reading frame fragments, generated by PCR, were subcloned into multicloning sites of pTRE2-hyg and pEGFP-N1. Constructed plasmids were designated as pTRE-TARP and pTARP-EGFP, respectively.

Production of Monoclonal Antibodies—pMBP-TARP was expressed in Escherichia coli BL21-CodonPlus™(DE3)-RIL cells (Strategene) for 2 h at 37 °C in the presence of 1 mM isopropylthigalactoside. The pellets were resuspended in amylase column buffer (20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1 mM EDTA). The suspension was then sonicated on ice to lyse the cells and centrifuged for 30 min at 9000 × g. The resulting supernatant was loaded onto a 15-ml amylase-resin column (New England Biolabs, Inc.). MBP-TARP was eluted with 4 column volumes of amylase-column buffer containing 10 mM maltose. Purified MBP protein expressed in E. coli was obtained from New England Biolabs, Inc. His-tagged TARP protein was made as described previously (24).

Anti-TARP mAbs were generated by A&G Pharmaceuticals (Balti-
more, MD). Three female mice (4–7 weeks old) were immunized multiple times with MBP-TARP. On the 14th day after initial immunization, an enzyme-linked immunosorbent assay (ELISA) was performed to test the titer of the specific antibody in the serum against the antigens. Splenocytes were harvested and fused to mouse myeloma cells. Fourteen days after fusion, the supernatant was harvested and screened for mAb production by ELISA. The initial screening was done by ELISA using MBP-TARP, His-tagged TARP, MBP, and E. coli BL21 proteins as antigens. The second screening was performed by Western blot analysis for these antigens. The selected hybridomas were grown in a bioreactor, and the antibodies were purified using a protein G column.

Cell Culture and Treatment with Androgen—LNCaP, PC-3, and DU145 cells were cultured in RPMI plus 10% fetal calf serum (FCS) at 37 °C in 5% CO2 as previously reported (24). HeLa, LNCaP prostate cancer cells, and 293T, human embryonal kidney cells, were cultured in Dulbecco’s modified Eagle’s medium plus 10% FCS at 37 °C with 5% CO2. MDA-PCa-2b cells were obtained from American Tissue Culture Collection and grown in BRFF-HPC1 medium (Biological Research Faculty and Facility, Inc., Jamsville, MD) plus 20% FCS. For androgen stimulation, 5 × 105 LNCaP cells per well were grown in a 6-well plate for 48 h in either regular culture medium or steroid-depleted culture medium, phenol red-free RPMI 1610 supplemented with 5% charcoal-stripped (CS)-FCS (HyClone, South Logan, UT), 2 mM l-glutamine, 10 mM HEPES, and 1 mM sodium pyruvate. Cells were then treated with medium with or without dihydrotestosterone (DHT) at indicated concentrations for 24 h. For MDA-PCa-2b cells, steroid withdrawal was done by phenol red-free RPMI 1610 plus 20% CS-FCS for 24 h.

Transfection—Co-transfection of pTetOn with pTRE-TARP or pTRE2-Luc was done with LipofectAMINE 2000 (Invitrogen) to subcon- fluent PC-3 cells, followed by treatment with 1 μg/ml doxycycline (In- vitrogen) 24 h after transfection. These cells were used for Western blotting and immunocytochemistry 48 h after transfection. For living cell observation, transfection of 1.0 μg of pTRE2-EGFP or pEGFP-N1 was carried out with LipofectAMINE 2000 to 2 × 105 cells in a 2-well chamber coverglass slide (Nalge Nunc International, Naperville, IL).

Western Blotting—Radioimmune precipitation assay buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, pH 8, 0.1% Triton X-100, 0.5% mouse pheynylmethylsulfonyl fluoride, 1 μM leupeptin, and 1 μg/ml pepstatin) containing 2% SDS was used to solubilize cells on the dish or the cell pellet in the tube. Protein concentration was determined by bicinchoninic acid (BCA) protein assay (Pierce) according to the manufacturer’s protocol. The protein sample was blotted using a standard method. Each blot was treated with primary antibodies at the following concentrations: 1 μg/ml anti-cytochrome c oxidase (COXIV) mouse monoclonal IgG2a (Molecular Probes, Eugene, OR), 2 μg/ml anti-catalase mouse monoclonal IgG1, 3.7 μg/ml anti-Golgi 58K mouse monoclonal IgG1 (Sigma Chemical Co.), anti-Na-K-ATPase mouse monoclonal IgG1 (Upstate, Charlottesville, VA), 3.5 μg/ml anti-protein-disulfide isomerase (PDI) monoclonal IgG1 (probed by Dr. Sy Sone, National Institutes of Health) (30), 1 μM anti-β-tubulin, and 0.2 μg/ml anti-HSP60 rabbit polyclonal (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and 1 μg/ml anti-β-actin (BD Biosciences, San Diego, CA). Primary antibodies were detected by secondary antibodies conjugated with horseradish peroxidase. Antibody detection was performed with enhanced chemiluminescence detection system (Amersham Biosciences).

Subcellular Fractionation—Subcellular fractionation by differential centrifugation was done at 4 °C. Subconfluent cells were scraped after washing with PBS, and were centrifuged at 200 × g for 5 min. The cell pellet was resuspended with a buffer containing 10 mM NaCl, 1.5 mM MgCl2, and 7.5 mM Tris-HCl, pH 7.5. After incubation, the swollen cells were broken open with 10 strokes of Dounce homoge-
nizer. Immediately 2.5% MS buffer (525 mM mannitol, 175 mM sucrose, 12.5 mM Tris-HCl, pH 7.5, and 2.5 mM EDTA, pH 8) was added to the cell suspension to give a final concentration of 1× MS buffer. The
homogenate was centrifuged at 600 × g for 5 min, and the pellet was designated as nuclear fraction. The supernatant was centrifuged at 17,000 × g for 15 min, and the pellet was designated as the mitochondrial fraction. The supernatant was ultracentrifuged at 100,000 × g for 1 h and the resultant pellet was designated as the microsomal fraction. The supernatant, precipitated with trichloroacetic acid, was designated as cytosol fraction. All fractions were solubilized in radioimmuno precipitation assay buffer plus 2% SDS.

Analytical subcellular fractionation using continuous density gradient was carried out at 4 °C as follows. After obtaining nuclear fraction from 1 × 10^6 LNCaP cells using the same method as described above, the supernatant was centrifuged at 3000 × g for 10 min. Furthermore, the supernatant was centrifuged at 17,000 × g for 15 min. The pellet designated as mitochondrial fraction was suspended with 1× MS buffer. The cell suspension was added by using 50% iodoxan to give a final concentration of 35% iodoxan (Axis-Shield, Oslo, Norway). Continuous gradient from 10–50% iodoxan was made in the ultracentrifuge tube by spontaneous diffusion overnight. The cell suspension was loaded to the bottom of this continuous gradient, and was ultracentrifuged at 100,000 × g for 3 h. After ultracentrifugation, the sample was divided into 16 fractions, 1 ml per fraction. One percent of each fraction, 10 μl, was applied to SDS-PAGE. The density of each fraction was obtained from the calibration curve measured by a refractometer according to the manufacturer’s instructions.

**Immunocytochemistry**—Cells in a 4-well chamber coverglass slides were fixed for 20 min in 3.7% formaldehyde, treated for 20 s with methanol for permeability on ice, blocked for 30 min with 10% normal goat serum (Sigma Chemical Co.) in phosphate-buffered saline, then incubated at room temperature for 1 h with the first antibodies at 3 μg/ml mouse monoclonal anti-TARP (Santa Cruz Biotechnology, Inc.), and other antibodies for organellar markers as described in Western blotting. Subsequently, the cells were incubated with the following secondary antibodies at 2 μg/ml: chicken anti-rabbit IgG Alexa 488, goat anti-mouse IgG Alexa 555, and/or goat anti-mouse IgG1 Alexa 488 (Molecular Probes) for 1 h at room temperature. The slides were mounted in ProLong Gold Antifade with 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA). Labeled cells were analyzed by laser confocal microscopy using a ×63 oil immersion objective lens.

** Observation of Living Cells**—48 h after transfection, cells on the chamber slides were incubated in the medium containing 100 ng Mitotracker Red 580 (Molecular Probe) for mitochondrial staining and 1 μg/ml Hoechst 33342 (Molecular Probe) for nuclear staining at 37 °C for 30 min. After washes with cell culture medium, cells were observed by laser confocal microscopy as described above.

** Submitochondrial Fractionation**—The mitochondrial fraction was obtained by subcellular fractionation using differential centrifugation as described above. For Triton X-114 separation, the pellet of mitochondrial fraction was suspended in buffer including 10 mM Tris, pH 7.5, 150 mM NaCl, 1 mM ethylenediamine-N,N,N,N′,N′-pentamethylenediamine (EDTA), 10 μg/ml leupeptin, and 1% Triton X-114. The suspension was treated according to the method of Bordier (31). Mitochondrial fraction proteins were distributed into the detergent and the aqueous phases. Each phase protein equivalent to 5 × 10^6 cells was examined by Western blotting after trichloroacetic acid precipitation. For sucrose density-gradient separation, a pellet of 1 mg of mitochondrial fraction was suspended in 2 ml of 0.45 M sucrose and 0.05 mM EDTA (32). The suspension was incubated on ice for 10 min. After sonication, the suspension was centrifuged at 17,000 × g for 10 min at 4°C to remove unbroken mitochondria. The supernatant of submitochondrial membrane layers was layered onto continuous sucrose gradient (15 ml, 0.85–1.8 M in 10 mM KCl, 10 mM Tris-HCl, pH 7.5). After ultracentrifugation at 100,000 × g for 16 h at 4°C, the sample was divided into 17 fractions (1 ml per fraction). 200 μl of each fraction was applied to SDS-PAGE after trichloroacetic acid precipitation.

** Immunohistochemistry**—One formalin-fixed, paraffin-embedded prostatectomy specimen was acquired for analysis. Serial sections were cut from a paraffin-embedded specimen and were processed on glass slides. Immunohistochemistry was performed with slight modifications to the ABC technique (33). Briefly, sections were deparaffinized and rehydrated. Antigen was unmasked by heating in the presence of “Nuclear Decloaker” (Biocare Medical, Walnut Creek, CA). Sections were then pretreated with a background suppressant followed by an endogenous biotin-blocking agent (Background Sniper and Avidin Biotin Blocking Kit from Biocare Medical). Antibody to TARP was applied at 30 μg/ml for 60 min at room temperature.

**RESULTS**

**Specificity of TP1, the Anti-TARP mAb**—mAbs to TARP were prepared by immunizing mice with a MBB-TARP fusion protein. One of 300 hybridoma clones screened specifically reacted on an immunoblot with a purified His-tagged TARP protein (data not shown) and with MBB-TARP (Fig. 1, lane 1) but not with the MBP protein (lane 2). This clone was named TP1 and was used for the studies described here. To determine whether TP1 can recognize recombinant TARP in human cells, we expressed TARP in PC-3 cells by transfection of a plasmid encoding TARP and analyzed the cell extract on a Western blot. Fig. 1A shows that TP1 reacts with TARP expressed in PC-3 cells (lane 3) but not with untransfected PC-3 cells (lane 4). These results indicate that TP1 can recognize recombinant TARP expressed in mammalian cells. We next used Western blotting to examine whether TP1 can recognize endogenous TARP protein in prostate cancer cell lines. Our previous analysis with reverse transcriptase-PCR showed a relatively high expression of TARP mRNA in androgen-sensitive LNCaP cells (24, 25) and in MDA-PCa-2b cells (data not shown), and no expression in HeLa or 293T cells (data not shown). Western blotting using cell lysates showed a 7-kDa band in both LNCaP (Fig. 1B, lane 5) and MDA-PCa-2b (lane 6) cells grown in regular medium with FCS, but no band in HeLa (lane 1) or 293T cells (lane 2). We conclude that mAb TP1 specifically reacts with the TARP protein and is suitable to use to examine the intracellular location of TARP and to identify TARP-expressing cells.

** Up-regulation of Endogenous TARP Protein by Androgen**—We have previously shown that androgen treatment increases TARP mRNA levels in LNCaP cells (28). To determine whether androgen would also up-regulate TARP protein, we treated LNCaP and MDA-PCa-2b cells with DHT for 24 h and analyzed the cell extract on a Western blot. In cells grown in medium containing charcoal-treated serum to remove endogenous androgens, the TARP protein is up-regulated by DHT in a dose-dependent manner in both LNCaP and MDA-PCa-2b cells (Fig. 2, upper panels). In cells grown without DHT, TARP protein is barely detected, and it is increased in cells treated with 10^-8 M and 10^-10 M DHT (Fig. 2, upper panels). In cells grown under normal conditions the level of TARP was higher than in cells grown in the steroid-free medium, and the level was also elevated by treatment with 10^-8 M DHT (Fig. 2, lower panels).
These results demonstrate that androgen up-regulates TARP at the protein level in androgen-sensitive prostate cancer cell lines.

Subcellular Localization of TARP—To determine the location of endogenous TARP, we prepared subcellular fractions from LNCaP cells by differential centrifugation and examined these fractions by Western blotting. In LNCaP cells, we found the strongest signal in the mitochondrial fraction, faint signals in nuclear and microsome fractions, and no signal in the cytosolic fraction (Fig. 3A). As expected, we found a strong signal for COXIV in the mitochondrial fraction. There was a weak signal in the nuclear fraction probably caused by mitochondrial contamination (Fig. 3A). To improve the purification of the mitochondria we divided the light mitochondrial fraction from LNCaP cells into 16 additional fractions using a continuous density gradient formed by iodixanol and examined these fractions by Western blotting using antibodies for various organelle markers. Fig. 3B shows that TARP and COXIV are present in the same fractions. There is a slight overlap between TARP and catalase, a peroxisome marker (Fig. 3B). Overlap between peroxisomal and mitochondrial markers has been shown to occur using this method (34).

We next performed immunocytochemistry using two cell lines that express endogenous TARP. In both LNCaP (Fig. 4A) and MDA-PCa-2b (Fig. 4B) cells labeled with TP1, we observed a dot-like pattern throughout the cytoplasm. In the cells labeled with anti-HSP60 antibody, the same cytoplasmic distribution was observed and the merged images showed marked yellow signals, indicating co-localization of TARP and HSP60. This result demonstrates that the endogenous TARP protein is localized in mitochondria. To confirm that the red fluorescence is specific and because of the reaction between TP1 and its secondary antibody conjugated with Alexa 555, we observed cells labeled with anti-HSP60 antibody alone and did not observe the red signal characteristic of TARP in MDA-PCa-2b cells labeled with anti-HSP60 antibody alone, (Fig. 4C) or in LNCaP cells (data not shown). In both PC-3 and DU145 cells where TARP mRNA was not detected by reverse transcriptase-PCR, we did not observe a fluorescence signal for TARP (data not shown). Subcellular fractionation using gradient density suggested the possibility that some TARP may be located in peroxisomes (Fig. 3B). To determine if some endogenous TARP is localized in peroxisomes, we carried out immunocytochemistry using an antibody for catalase, a peroxisome marker. We did not find any co-localization of TARP and catalase in LNCaP cells (Fig. 4D) or MDA-PCa-2b cells (data not shown). In other organelles, including the endoplasmic reticulum, Golgi, and plasma membrane, we also did not detect a TARP signal co-localizing with markers for those organelles (data not shown).

We conclude that endogenous TARP is exclusively located in mitochondria. Mitochondrial localization of TARP was also observed by immunocytochemistry with PC-3 cells transiently transfected with a TARP expression plasmid (data not shown).

Furthermore, we constructed a TARP-EGFP fusion gene as described under “Experimental Procedures” and expressed it in several different cell lines. In this construction EGFP is fused to the carboxyl end of TARP. The fusion protein has the expected molecular mass of 35 kDa as determined by Western blotting of transfected cell extracts with anti-GFP and anti-TARP antibodies (data not shown). To determine the intracellular location of TARP-EGFP we transfected pTARP-EGFP into cells, and 48 h later observed the living cells under confocal fluorescent microscopy. In both LNCaP and MDA-PCa-2b cells, we found that the cytoplasmic distribution of TARP-EGFP, which gives a green signal, is identical to the signal produced.
by cells incubated with Mitotracker Red 580, which is located in the mitochondria of living cells and gives a red signal. The organelles showed the characteristic worm-like patterns of mitochondria. In cells stained with both dyes, bright yellow signals due to co-localization of the green and red signals were observed clearly showing that TARP is located in the mitochondria of living cells (Fig. 5, A and B). Mitochondrial localization of TARP-EGFP was also observed in TARP-negative PC-3 cells (Fig. 5C) showing that the presence of endogenous TARP is not required for the localization of TARP-EGFP in cells. EGFP alone was detected in the cytosol of PC-3 cells but not in mitochondria as expected (Fig. 5D). Similar results were observed with LNCaP and MDA-PCa-2b cells (data not shown). The finding that TARP-EGFP is localized in the mitochondria, but EGFP alone is not, indicates that the TARP protein has a specific sequence that causes it to become associated with mitochondria. Furthermore, this sequence is not located at the carboxyl end where TARP is attached.
Mitochondria are composed of four compartments: matrix, inner membrane, intermembrane space, and outer membrane. To determine the location of TARP in mitochondria, submitochondrial fractionation was carried out. First we separated membrane-associated proteins of LNCaP cells by phase separation using the nonionic detergent Triton X-114 and then carried out Western blotting using membrane-associated proteins from the Triton X-114 and aqueous phases. The method completely resolved the membrane proteins CoxIV and VDAC from the soluble protein HSP60 (Fig. 6A). We found that TARP was mainly present in the Triton X-114 phase, which contains the membrane proteins, although some TARP was present in the aqueous phase. This finding indicates that TARP is a membrane-associated protein. To determine if TARP localizes in the inner or outer membrane of mitochondria, we fractionated submitochondrial membrane particles by sucrose density gradient to determine if TARP localizes in the inner or outer membrane of mitochondria. After trichloroacetic acid precipitation, 200 μl of each fraction from 1.4–0.45 M sucrose was examined by Western blotting using antibodies as described above.

**Association of TARP with the Outer Membrane of Mitochondria**—Mitochondria are composed of four compartments: matrix, inner membrane, intermembrane space, and outer membrane. To determine the location of TARP in mitochondria, submitochondrial fractionation was carried out. First we separated membrane-associated proteins of LNCaP cells by phase separation using the nonionic detergent Triton X-114 and then carried out Western blotting using membrane-associated proteins from the Triton X-114 and aqueous phases. The method completely resolved the membrane proteins CoxIV and VDAC from the soluble protein HSP60 (Fig. 6A). We found that TARP was mainly present in the Triton X-114 phase, which contains the membrane proteins, although some TARP was present in the aqueous phase. This finding indicates that TARP is a membrane-associated protein. To determine if TARP localizes in the inner or outer membrane of mitochondria, we fractionated submitochondrial membrane particles by ultracentrifugation with a sucrose-density gradient and examined the fractions by Western blotting. The result of Western blotting showed that the distribution of TARP is almost the same as that of VDAC, an outer membrane protein, and is different from that of CoxIV, an inner membrane protein (Fig. 6B). Taken all together, these results demonstrate that TARP is a mitochondrial protein associated with the outer mitochondrial membrane.

**Cytoplasmic Localization of TARP in Immunohistochemistry**—Our previous RNA in situ hybridization showed that, in human prostate tissue, TARP mRNA is highly expressed in epithelial cells within the acinar ducts of the prostate whereas stromal cells and other cell types in the prostate are negative (3). To determine if TARP protein can be detected in human prostate tissue, we performed immunohistochemistry using formalin-fixed, paraffin-embedded human prostate cancer specimens obtained from radical prostatectomy. A typical result is shown in Fig. 7. In the section labeled with TP1, malignant epithelial cells showed a positive signal that was manifest as a dot-like pattern throughout the cytoplasm (Fig. 7, closed arrowheads). The normal epithelial cells, stromal cells, and infiltrating lymphocytes were entirely negative (Fig. 7, open arrowheads). These results indicate that the TARP protein is clearly detected in the cytoplasm of malignant epithelial cells in human prostate tissue and suggests that the TARP protein is elevated in malignant epithelial cells.

**DISCUSSION**

We have previously shown that TARP mRNA was detected in LNCaP cells and mRNA levels were elevated after treatment with androgen. In the current study we describe the production of a mAb (TP1) that specifically recognizes TARP protein and have used it to determine the induction of TARP protein by androgen and the subcellular localization of TARP. We found that androgen up-regulates endogenous TARP protein in both LNCaP and MDA-PCa-2b cells (Fig. 2). We then analyzed the subcellular location of TARP and found it located in mitochondria. The mitochondrial localization of TARP surprised us not only because TARP is the first prostate-specific protein localized in mitochondria but also because our previous study using a polyclonal antibody detected TARP in a crude nuclear fraction. The previous results could be explained by the contamination of the crude nuclear fraction with mitochondria. The present study using a specific monoclonal and several different experimental approaches clearly show that TARP is located in mitochondria.

To precisely determine the subcellular localization of TARP we used several different and complementary methods. These are subcellular fractionation (Fig. 3), staining of fixed cells with the mAb to TARP (Fig. 4) and analysis of living cells transfected with a plasmid expressing TARP with EGFP fused to its C terminus (Fig. 5). All three methods are in complete agreement.

Our previous data using reverse transcriptase-PCR to analyze TARP expression showed that in males TARP was only detected in the prostate (3) and the current results show it is only located in mitochondria. To date 25 genes have been described that are selectively expressed in the prostate. A few of these are only expressed in prostate while the expression of...
others has also been detected at various levels in other organs. Table I summarizes the subcellular localization of the proteins expressed by these 25 prostate-specific genes (2–19). These data show that TARP is the only prostate-specific protein that is found in mitochondria.

Our results from submitochondrial fractionation showed that TARP is associated with the outer membranes of mitochondria (Fig. 6). The outer membrane contains proteins known to play essential roles in metabolism and in apoptosis. The concentrations of citrate and zinc are extremely high in prostate, possibly due to a unique citrate-related metabolic pathway regulated by testosterone and prolactin (35, 36). Mitochondria produce large amounts of reactive oxygen species (ROS) that include hydrogen peroxide, superoxide, hydroxyl radicals, and nitric oxide. ROS are generated through the respiratory chain of mitochondria and are scavenged by antioxidant systems. An intracellular imbalance of ROS is harmful and can cause several diseases, including cancer and inflammation. Ripple et al. (37) reported that in LNCaP cells physiology levels of androgens induced a pro-oxidant anti-oxidant shift toward a more oxidative state in which intracellular levels of ROS, oxygen consumption, and lipid-per-oxidation were increased. One possible role of TARP could be to control ROS generation in prostate mitochondria under androgen regulation. Several groups have provided evidence that lipid metabolism is related to the biology of prostate cancer (38, 39). It is also known that dietary fat can be a risk factor for prostate cancer and lipid per-oxidation may have a role in this process (40). Another possible role of TARP could be in the regulation of fat metabolism in mitochondria. We are now investigating the effect of TARP on ROS generation and lipid metabolism.

Since the discovery that mitochondria are the control center for apoptosis, interest in prostate mitochondria has been focused on apoptosis of prostate cancer (41, 42). Several drugs have been reported to induce apoptosis through alterations of mitochondria in prostate cancer (43, 44). TARP, like Bcl-2, Bcl-xL, and Bad, is located in the outer membrane. These Bcl-2 family members control apoptosis by regulating release of cytochrome c, and TARP could have a role in this regulation. In the biology of the prostate, androgen is an essential anti-apoptotic, survival factor for prostate cells. Androgen depletion causes apoptosis of prostate epithelium, and androgen blocks the apoptosis produced by various apoptosis inducers such as Fas or tumor necrosis factor (TNF-α) (45, 46). TARP could control apoptosis through its interaction with the protein or proteins to which it binds in mitochondria just as Bcl-xL is functionally regulated by binding to Bad (41, 47). We are planning to identify proteins that bind to TARP. We believe that two approaches will help clarify the possible role of TARP in apoptosis.

Another interesting question is whether TARP plays a role in prostate cancer progression. Several recent reports show that TARP mRNA levels are elevated in prostate cancer using cDNA microarrays (25–27). Stamey et al. (25) showed a 5-fold induction of TARP in cancers with a high Gleason grade, compared with benign prostate hyperplasia. Ernst et al. (26) found a 3.1-fold induction of TARP in cancer when compared with areas of normal prostate adjacent to the prostate cancer specimen. Rhodes et al. (27) performed meta-analysis and demonstrated TARP as one of the representative genes that are overexpressed in prostate cancers. Results from these cDNA microarray studies consistently show that TARP is overexpressed in prostate cancers. As shown in Fig. 7, mAb TPI can detect TARP protein in prostate cancer cells using a clinical prostate cancer specimen fixed with formalin. We have initiated immunohistochemical studies to determine if TARP protein is elevated in prostate cancer and if it is to determine at which stage in cancer progression TARP becomes elevated. In conclusion, we have demonstrated that TARP is the first prostate-specific protein localizing in mitochondria. These results indicate that mitochondria may play an important role in prostate-specific function.

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TABLE I
Subcellular localization of endogenous proteins from 25 prostate-specific genes reported in the literature

| Identified | Non-secretory | Mitochondria | Endoplasmic reticulum | Cytoplasm | Nucleus | Plasma membrane | Secretory |
|-----------|---------------|--------------|----------------------|-----------|---------|----------------|----------|
| 14         | 10            | 1            | 1                    | 2         | 2       | 4              | 4        |
| TARP      |               |              |                      |           |         |                |          |
| PDDR1     |               |              |                      |           |         |                |          |
| PSM A 4    |               |              |                      |           |         |                |          |
| AIPC       |               |              |                      |           |         |                |          |
| NIKX1.1     |               |              |                      |           |         |                |          |
| PSDR1      |               |              |                      |           |         |                |          |
| PSCA        |               |              |                      |           |         |                |          |
| STEAP       |               |              |                      |           |         |                |          |
| Prostein    |               |              |                      |           |         |                |          |
| DD3         |               |              |                      |           |         |                |          |
| PCGEM-1     |               |              |                      |           |         |                |          |
| PTI-1       |               |              |                      |           |         |                |          |
| PART-1      |               |              |                      |           |         |                |          |
| PSGR        |               |              |                      |           |         |                |          |
| Trp-p8      |               |              |                      |           |         |                |          |
| GDEP        |               |              |                      |           |         |                |          |
| HPG-1       |               |              |                      |           |         |                |          |
| PMEPA1     |               |              |                      |           |         |                |          |
| AlhZIP      |               |              |                      |           |         |                |          |
| STAMP1      |               |              |                      |           |         |                |          |
| Total       | 9             |             | 2                    | 2         |         | 1              | 1        |
| 25          |               |              |                      |           |         |                |          |

* Of 25 prostate-specific genes, subcellular localization of endogenous proteins have been identified in 14 proteins; 4 secretory and 10 non-secretory proteins. Subcellular localization was not determined in 11 other genes.

PDEF hPSE. PDEF and hPSE are the same protein but with different names.

Of 11 genes in which subcellular localization was not determined, nine genes were expected to encode proteins, but two other genes were not.

These proteins fused with GFP were shown to localize in the Golgi apparatus.
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The T Cell Receptor γ Chain Alternate Reading Frame Protein (TARP), a Prostate-specific Protein Localized in Mitochondria

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