Tocopherol-Associated Protein Suppresses Prostate Cancer Cell Growth by Inhibition of the Phosphoinositide 3-Kinase Pathway

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Abstract

Epidemiologic studies suggested that vitamin E has a protective effect against prostate cancer. We showed here that tocopherol-associated protein (TAP), a vitamin E–binding protein, promoted vitamin E uptake and facilitated vitamin E antiproliferation effect in prostate cancer cells. Interestingly, without vitamin E treatment, overexpression of TAP in prostate cancer cells significantly suppressed cell growth; knockdown of endogenous TAP by TAP small interfering RNA (siRNA) in nonmalignant prostate HPr-1 cells increased cell growth. Further mechanism dissection studies suggested that the tumor suppressor function of TAP was via down-regulation of phosphoinositide 3-kinase (PI3K)/Akt signaling, not by modulating cell cycle arrest or androgen receptor signaling. Immunoprecipitation results indicated that TAP inhibited the interaction of PI3K subunits, p110 with p85, and subsequently reduced Akt activity. Constitutively active Akt could negate the TAP-suppressive activity on prostate cancer cell growth. Moreover, stable transfection of TAP in LNCaP cells suppressed LNCaP tumor incidence and growth rate in nude mice. Furthermore, TAP mRNA and protein expression levels were significantly down-regulated in human prostate cancer tissue samples compared with benign prostate tissues as measured by reverse transcription-PCR, in situ hybridization, and immunohistochemistry. Together, our data suggest that TAP not only mediates vitamin E absorption to facilitate vitamin E antiproliferation effect in prostate cancer cells, but also functions like a tumor suppressor gene to control cancer cell viability through a non–vitamin E manner. Therefore, TAP may represent a new prognostic marker for prostate cancer progression. (Cancer Res 2005; 65(21): 9807-16)

Introduction

Tocopherol-associated protein (TAP) was first identified as a tocopherol-binding protein from bovine liver cytosol using α-tocopherol (α-vitamin E) as the bait. The dissociation constant of α-vitamin E to TAP is around 0.46 μmol/L (1), lower than the physiologic concentration of α-vitamin E (19-29 μmol/L; ref. 2), suggesting that TAP can bind to α-vitamin E with reasonable affinity in vivo. TAP is universally expressed, with the highest expression in liver, brain, and prostate (1), and TAP has a sequence identical to that of supernatant protein factor, which is involved in cholesterol synthesis, stimulating squalene monooxygenase and oxidosqualene cyclase (3). However, results from the in vitro ligand-binding assay indicated that TAP/supernatant protein factor has a low affinity with many compounds that are involved in the cholesterol synthesis (4). The crystal structure suggested that TAP/supernatant protein factor has an NH2-terminal CRAL-TRAL domain containing a small lipid ligand-binding cavity and a COOH-terminal domain GOLD (Golgi dynamics), which is involved in protein-to-protein interaction (5). Nevertheless, functions of TAP in prostate cancer remain unclear.

Prostate cancer is the second most fatal carcinoma in American men (6). The epidemiologic study indicated that supplementation of α-vitamin E has a beneficial effect for preventing the incidence and mortality of prostate cancer (7). Besides its antioxidant function, α-vitamin E and its analogues can inhibit transforming growth factor-β (8) and androgen receptor/prostate-specific antigen pathways (9), regulate cell cycle distribution (10, 11), arrest DNA synthesis (8), induce apoptosis (12–14), and reduce invasiveness (15) in prostate cancer cells. α-Tocopherol (α-vitamin E) is the major vitamin E isof orm that is preferentially bound by tocopherol transfer protein (TTP) to transport from liver to serum in the human body. However, how vitamin E enters the peripheral tissue and cells and how it influences cell function are not well understood.

We hypothesized that TAP could play a critical role in the vitamin E–mediated effect in prostate cancer cells. Therefore, our study was aimed at characterizing the function of TAP in prostate cancer. Here, we show for the first time that TAP can facilitate vitamin E retention in the prostate cancer cells and promote vitamin E antiproliferation effect. Moreover, the expression level of TAP significantly governs prostate cancer cell growth in vitro and in vivo independent of its vitamin E–related action. Further characterization indicated that the tumor suppressor function of TAP is via modulating the phosphoinositide 3-kinase (PI3K)/Akt pathway, not by disturbing cell cycle arrest or induction of apoptosis in LNCaP cells. Consistently, the fact that TAP, but not other TAP family genes, has much lower expression in human prostate cancer tissues supported our hypothesis that TAP may have a tumor suppression function in prostate cancer and could represent a new prognostic marker for prostate cancer.

Materials and Methods

Cell culture. LNCaP, PC-3, DU-145, and RWPE-1 cells were obtained from the American Type Culture Collection (Manassas, VA). CWR22R cells were from Dr. Ching-Hai Kao (Indiana University, Indianapolis, IN). HPr-1 cells were from Dr. Yong-Chuan Wong (University of Hong Kong, Hong Kong, China). LNCaP, PC-3, and CWR22R cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS). DU-145 cells were
maintained in DMEM supplemented with 10% FBS, RWPE-1 and HPV-1 cells were maintained in the KSF medium (Life Technologies, Carlsbad, CA).

**Chemicals and reagents.** α-Tocopherol, γ-tocopherol, δ-tocopherol, and ascorbic acid were purchased from Sigma (St. Louis, MO). Anti-Flag M2 monoclonal antibody and antithemagglutinin antibody were from Sigma. Anti-PI3K p110, anti-phospho-Janus-activated kinase 1 (JAK1), anti-phospho-FOXOA3a, anti-Akt, and antiactin antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-FOXOA3a and anti-PI3K p85 antibodies were from Upstate Biotechnology (Lake Placid, NY). Anti-phospho-mitogen-activated protein kinase (MAPK), anti-phospho-signal transducers and activators of transcription 3 (STAT3), and anti-phospho-Akt (Ser473) antibodies were from Cell Signaling Technology (Beverly, MA). Anti-androgen receptor antibody and pCDNA3-Akt plasmids were described previously (16).

**Construction of tocopherol-associated protein and tocopherol-associated protein small interfering RNA expression plasmid.** Full-length TAP was isolated from cDNA of benign prostate hyperplasia (BPH) and then subcloned into the pCM3A-FLAG TAP small interfering RNA (siRNA) was constructed using a DNA-based vector pMSCV/U6, which was a gift from Dr. P. Silver (Harvard Medical School, Boston, MA) that contains the puromycin resistance marker. The oligonucleotide QCATGTTGAGTCC- GAAAGTTCAGAGATCTGAACTCACATGTTT was subcloned into the Aapt-ECO RI site of pMSCV/U6 vector to generate pMSCV/1-6-TAP-siRNA. All the constructions were verified by sequencing.

**Transfection.** Transfections were done by either SuperFect (Qiagen, Chatsworth, CA) or electroporation. For electroporation (Gene Pulser, Bio-Rad, Hercules, CA), exponentially growing LNCaP cells were resuspended at 1 × 10⁶/mL cell density in 2.5% FBS (no antibiotics). Electroporation settings were voltage 280 mV and capacitance 950 μF. Sample volumes were 400 μL and total DNA amounts were 10 μg.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was done as previously described (10). Briefly, 5,000 cells were seeded on 24-well plates. At the indicated time points, 0.5 mL MTT (0.5 mg/mL; Sigma) was added into each well for 3 hours at 37°C. Then, the MTT crystals were dissolved in 1 mL of 0.04 mol/L HCl in isopropyl alcohol. The absorbance was read at a wavelength of 595 nm. Data were conducted in triplicate.

**Western blot analysis.** Western blot analyses were done as previously described (9). Briefly, 50 μg of protein was resolved by SDS-PAGE gel and transferred to nitrocellulose membranes. After blotting with blocking buffer (PBS containing 0.1% Tween 20 and 10% serum) for 1 hour, the membrane was incubated with primary antibody for 1 hour at room temperature. The membranes were then incubated with alkaline phosphatase–conjugated secondary antibodies for another 1 hour at room temperature. The proteins were detected by alkaline phosphatase reagents (Bio-Rad).

**Northern blot analysis.** Northern blot analyses were done as previously described (10). Briefly, total RNA was extracted by Trizol (Life Technologies) according to the instructions of the manufacturer. Fifteen micrograms of total RNA were electrophoresed on a 1% agarose formaldehyde gel and transferred to Hybond-N+ membrane (Amersham Pharmacia Biotech, Piscataway, NJ). TAP cDNA was labeled with [γ-32P]dCTP using Random labeling kit (Amersham Pharmacia Biotech). The membranes were then prehybridized and hybridized using Rapid-Hyb system (Amersham Pharmacia Biotech). Expression signal was detected by PhosphorImage analysis (Molecular Dynamics, Piscataway, NJ).

**Commincoprecipitation assay.** Five hundred micrograms of cell lysate protein were incubated with either 1.5 μg p110 antibody or PBS for 2 hours at 4°C with constant rocking. Twenty-five microliters of protein A/G beads were then added into each tube and incubated for another 2 hours with constant rocking. After four washes with PBS, the beads were resuspended in protein loading buffer. After boiling for 5 minutes, the samples were separated by gel electrophoresis.

**Colony-forming assay.** Cells were plated (5,000 per well) in triplicate in a six-well plate. Cells were incubated and supplied with fresh medium every 4 days. After 15 days, they were fixed with methanol and stained with 1% crystal violet and the colonies were counted.

Semiquantitative reverse transcription-PCR and real time quantitative reverse transcription-PCR. One microgram RNA was subjected to reverse transcription using Superscript II (Invitrogen, Carlsbad, CA). The specific primers designed were as follows: TAP, 5′-CCAGCCAGAAG- GAGGCTATTG-3′ (forward) and 5′-TGGAGGAGCAAGCAGAG-3′ (reverse); TTP, 5′-TACGCGAAGATGAACTCAAG-3′ (forward) and 5′-ATCCGGA- TACAGAGCAATCT-3′ (reverse); cellular retinaldehyde-binding protein (CRALBP), 5′-CACGGCTCCCAATGATGATG-3′ (forward) and 5′-CCAGGA- CATGTAGAGAG-3′ (reverse); 18S rRNA, 5′-TGCCTCCTCTGATGTG- GTAG-3′ (forward) and 5′-CTGCTGCTCATACATTTTG-3′ (reverse).

Real-time PCR was performed with SYBR Green PCR Master Mix (Bio-Rad). PCR was done at 94°C for 3 minutes, and 40 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds on an iCycler Q Multicolor real-time PCR detection system (Bio-Rad). Each sample was run in triplicate. Data were analyzed by an iCycler iQ software (Bio-Rad).

**Vitamin E extraction and high-performance liquid chromatography analysis.** The cell pellets were lysed with 0.3 mL 1% ascorbate in 0.1 mol/L SDS plus 1 nmol δ-tocopherol as internal control. Vitamin E isoforms were extracted with 0.8 mL hexane (17). The hexane extract was taken to dryness under N₂ in the TurboVap LV concentration workstation (Zymark, Hopkinton, MA). The residue was dissolved in 2.5% ascorbate in methanol (1 mL) and analyzed (50 μL) by high-performance liquid chromatography (HPLC).

Measurements of vitamin E were done on XTerraRP18 (5 μmol/L, 4.6 × 150 mm) column eluted with 96% methanol at a flow rate of 1.8 mL/min. The signals were detected with Waters 2475 multi-λ fluorescence detector and data were analyzed by Empower software (Waters, Milford, MA).

**Tumor formation in vivo.** Each flank of male athymic nude mice (5-6 weeks old) were injected s.c. with 2 × 10⁶ LN-TAP, LN-V, or LNCaP cells resuspended in Matrigel (BD Biosciences, San Jose, CA). Mice were then monitored weekly for tumor formation. Tumor volumes were calculated using the formula (length × width²) / 2 (18). All the animal experiments were done in accordance with NIH animal use guidelines and the protocol approved by the University Committee on Animal Resources at the University of Rochester.

**Tissue samples.** Frozen or paraffin-embedded prostate cancer tissue blocks were obtained from patient surgeries at the University of Rochester Medical Center (Rochester, NY). All histologic diagnoses were confirmed by staining parallel sections with H&E staining. Specimens were reviewed by two independent pathologists.

**In situ hybridization.** Sense- or antisense-oriented RNA probes labeled with digoxigenin were generated by in vitro transcription of TAP NH2-terminal fragments cloned in pGEM-T-easy vector (Promega, Madison, WI) using a digoxigenin RNA labeling kit (SIG/T7; Roche, Indianapolis, IN) according to the instructions of the manufacturer. Ten nanograms of digoxigenin-labeled RNA probes in 100 μL of hybridization buffer (40% denoized formamide, 4 × SSC) were incubated with tissue sections at 42°C in a humidified chamber overnight. Bound RNA was detected using sheep antidigoxigenin Fab fragments coupled to alkaline phosphatase (1:500 dilution) and visualized using nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate, 4-toluidine salt (Bio-Rad) and 1 mmol/L levamisole (Sigma). Slides were counterstained in the nuclear fas-red (Vector Laboratories, Burlingame, CA), mounted in Immum-Mount (Shandon, Pittsburgh, PA).

**Polycyclonal tocopherol-associated protein antibody production.** pET-TAP-NH2-terminal (1-79 amino acids) plasmids were transformed into Escherichia coli BL21-CodonPlus(DES)-RIIL (Stratagene, La Jolla, CA). Purification of His-TAP-NH₂ terminus was achieved using Ni-NTA agarose column by stepwise increasing imidazole concentrations. Purified TAP-NH2 terminus was injected into rabbits to produce the polyclonal antibodies (Alpha Diagnostic, San Antonio, TX). The quality and specificities of antibody were confirmed by Western blotting assay and immunohistochemistry.

**Prostate tissue microarray construction.** Prostatic adenocarcinoma cases over a 2-year period (2002-2003) were reviewed at the University of Rochester Medical Center Strong Memorial Hospital and 80 cases were selected. The slides were reviewed for accuracy of Gleason scores and that adequate areas of each component Gleason grade pattern were available. None of the cases used to construct the tissue microarray received any preoperative therapy, including hormone therapy. Areas for sampling were
designated as normal, hyperplastic (BPH), high-grade prostatic intraepithelial neoplasia (PIN), low-grade adenocarcinoma (Gleason pattern 1, 2, and 3), and high-grade adenocarcinoma (Gleason pattern 4 and 5). The decision to divide the tumors into low- and high-grade adenocarcinoma was based on literature that show worse prognosis for patients with Gleason grade patterns 4 and 5 (5 is the highest), compared with lower Gleason grade patterns (19). A total of 50 normal, 82 BPH, 35 PIN, 104 low-grade adenocarcinoma, and 82 high-grade adenocarcinoma areas were chosen for sampling, averaging four to six cores per case.

The tissue microarray was constructed using a manual tissue arrayer (Beecher Instruments, Sun Prairie, WI). The instrument consists of thinned-walled, stainless-steel needles with an inner diameter of 0.6 mm and stylet used to empty and transfer the needle contents. The XY coordinate displacement of the arrayer was adjusted manually with the aid of digital micrometers for approximation. Core samples were retrieved from the selected region in each donor paraffin block and transferred to a receiver paraffin block. Tissue cores are 0.6 mm in diameter and 1.0 to 3.0 mm in length, depending on the tissue thickness in the donor block. Blank cores were removed from the 45 × 20 × 12 mm (standard size) recipient paraffin block by the same procedure that donor cores were obtained and inserted. The cores in the recipient block are spaced 1.0 mm apart. The array blocks were then incubated 10 minutes at 37°C to improve adhesion between the cores and paraffin of the recipient block. A section from each block was cut, stained, and reviewed to ensure quality control. Sections for immunostaining were placed on charged glass slides (20).

**Immunohistochemical staining.** Deparaffinized tissue sections were heated in 10 mmol/L sodium citrate solution for 20 minutes in a steamer. TAP antibody diluted 1:1,600 in blocking buffer (1:50 dilution of goat serum in PBS) was incubated on slides overnight at 4°C. Sections were then incubated with 1:300 diluted biotinylated secondary antibody (Vector Laboratories) and ABC solution (Vector Laboratories). Then, the tissues were stained by AEC (DAKO, Carpinteria, CA), followed by Meyor’s hematoxylin counterstaining (DAKO).

**Immunostaining interpretation and statistical analysis.** Each core was examined under a light microscope and separately scored. Cores that had <50% of original tissue present were disregarded. Scoring included percentage of staining and staining intensity (0, 1+, 2+, 3+). Results were recorded as positive (5% or more, 2+ to 3+) or negative (<5% staining or staining intensity 0 to 1+). Data obtained were analyzed using Fisher's exact test (21).

### Results

**Tocopherol-associated protein expression levels are reduced significantly in human prostate cancer cells.** Prostate is one of the tissues with the highest TAP expression (1). To understand TAP function in prostate cancers, we first examined the expression pattern of TAP in normal and prostate cancer cell lines. Using the Northern blotting assay, our results indicated that TAP has three isoforms, sized 4.2, 2.8, and 1.5 kb. The 2.8 kb fragment is the major isoform (Fig. 1A, arrow), which is consistent with previous findings (1). In addition, we used real-time PCR to quantitate the amount of TAP mRNA. Both Northern blotting (Fig. 1A) and real-time PCR (Fig. 1B) show that TAP mRNA expression was high in nonmalignant prostate epithelial cells (RWPE-1 and HPr-1), moderate in PC-3 cells, and low in several prostate carcinoma cell lines, including LNCaP, DU-145, and CWR22R. Androgen and estrogen receptor activity are important for the initiation and progression of the prostate and prostate cancer (22, 23). We also examined whether TAP expression was regulated by hormone or hormone receptor. However, dihydrotestosterone (10 nmol/L) and 17β-estradiol (10 nmol/L) treatment did not significantly influence TAP expression in LNCaP (androgen receptor positive, estrogen receptor β positive), CWR22R (androgen receptor positive, estrogen receptor β positive), or DU-145 (androgen receptor negative, estrogen receptor β positive) cells (refs. 24, 25 and data not shown), indicating that down-regulation of TAP is independent of the function of androgen and estrogen receptor.

Taken together, these data indicated that TAP mRNA expression levels are down-regulated in several prostate cancer cell lines. This reduced TAP expression might be due to multiple mechanisms and might be cell type specific.

**Tocopherol-associated protein facilitates vitamin E uptake into the cells and promotes vitamin E-mediated antiproliferation.** A daily supplement of α-vitamin E was reported to prevent the incidence and mortality of prostate cancer. TAP is a vitamin E-binding protein (1) and has a low expression in prostate cancer cells. Therefore, we asked whether restoration of TAP in prostate cancer cells could enhance the uptake of vitamin E or sensitize vitamin E-mediated antiproliferation function. Results from prostate cancer LNCaP cell growth assays showed that α-vitamin E marginally inhibited prostate cancer LNCaP cell growth, whereas γ-vitamin E and the α-vitamin E analogue, α-vitamin E succinate, inhibited LNCaP growth by ~40% and 55% (Fig. 2A). We stably transfected TAP cDNA with flag-tag into LNCaP cells, and four independent stable clones with up-regulated TAP in LNCaP cells (LN-TAP5, LN-TAP7, LN-TAP9, and LN-TAP10) were identified (Fig. 2B). Next, LN-TAP and LN-V cells were treated with 20 μmol/L α-vitamin E and the cellular concentrations of α-vitamin E were measured by HPLC. Although the additional uptake of vitamin E was not exactly proportional to the increased protein level of TAP, LN-TAP cells (LN-TAP5, LN-TAP7, LN-TAP9) contained 50% more α-vitamin E than control LN-V cells (LN-V1and LN-V2) after 48 hours of treatment (Fig. 2C). Similar data were obtained for γ-vitamin E, α-vitamin E succinate (Fig. 2C), and δ-vitamin E (data not shown). Therefore, our data indicated that TAP can facilitate the uptake of vitamin E into cancer cells.

To study whether enhanced uptake of vitamin E by TAP can influence the antitumor effect of vitamin E, we used LN-TAP5 and LN-V1 as representative cells to perform MTT assays. Surprisingly, without vitamin E treatment, expression of TAP notably suppressed prostate cancer cell growth (Fig. 2D, white columns). Therefore, we examined the effect of vitamin E on the growth of LN-V and LN-TAP cells. As shown in Fig. 2D, 20 μmol/L α-vitamin E suppressed LN-TAP5 cell growth by 43% after 6 days treatment as opposed to 21% in the LN-V1 cells. γ-Vitamin E and α-vitamin E succinate suppressed LN-TAP5 cell growth by 83% and 85%, respectively, but
only suppressed LN-V1 cell growth by 38% and 45%, respectively. This increased growth inhibition by vitamin E in LN-TAP cells is correlated with better absorption of vitamin E in LN-TAP cells compared with that in LN-V cells, suggesting that TAP plays a role in vitamin E–induced cell growth inhibition. These cell growth results have been reproducible in other LN-TAP cell clones (data not shown).

Non-vitamin E–mediated tumor suppression functions of tocopherol-associated protein in prostate cancer cells. Interestingly, we found that restoring the TAP protein in LNCaP by TAP cDNA transfection can significantly inhibit the growth of LNCaP prostate cancer cells without vitamin E treatment (Figs. 2D and 3A). Without adding an extra amount of it, vitamin E is barely detectable in the medium by a highly sensitive HPLC method (data not shown).

To further characterize the tumor suppressor function of TAP, colony-forming assays were applied. As shown in Fig. 3B, LN-V1 cells had a similar colony-forming ability as the parental LNCaP cells, whereas LN-TAP cell clones (LN-TAP5, LN-TAP7, and LN-TAP9) had much less ability to form colonies with up to 95% inhibition. These results indicated that TAP inhibited prostate tumorigenesis in vitro.

To test if TAP-mediated tumor suppression function can be extended to other prostate cancer cells in the absence of vitamin E, we up-regulated TAP in prostate cancer DU-145 cells, which has low endogenous TAP expression (Fig. 1B). Three clones (DU-TAP2, DU-TAP4, and DU-TAP7) were identified by neomycin selection and the growth results of those clones were suppressed by 38% to 59% compared with that of vector control cells (DU-V1 and DU-V4; Fig. 3C). In contrast, we knocked down endogenous TAP expression by siRNA strategy in HPr-1 cells, the nonmalignant prostate epithelial cells with high endogenous TAP expression. As seen in Fig. 3D, the pooled TAP siRNA–transfected HPr-1 cells had reduced TAP expression and ~2-fold increase in cell growth compared with vector-transfected cells.

Taken together, our results suggested that prostate cancer cells have relatively low expression of TAP and benign cells have a high level of TAP. Elevated TAP can suppress the growth of prostate cancer LNCaP and DU-145 cells and decreasing TAP expression up-regulating TAP in LNCaP cells (LN-TAP5, LN-TAP7, and LN-TAP9) did not significantly change cell morphology, cell cycle distribution, or induce apoptosis as determined by TUNEL assay (data not shown). The androgen receptor is required for normal prostate development and prostate cancer cell proliferation and survival (23, 26, 27), yet our results showed no significant difference of androgen receptor protein expression level between TAP stable clones (LN-TAP5, LN-TAP7, LN-TAP9) and its vector control LN-V1 or parental cells LNCaP (Fig. 4A).

Others and we have shown that MAPK, JAK/STAT3, and PI3K/Akt signaling pathways are involved in prostate cancer cell growth (28–35). We next examined if TAP influenced these signaling networks in prostate cancer cells.
TAP Inhibits Prostate Cancer Cell Growth

TAP reduces prostate cancer cell growth in the absence of vitamin E. A, TAP suppresses LNCaP cell growth. MTT assays were done to detect cell growth of the LN-TAP clones for 6 days. The result of day 0 was set at 1. B, TAP reduces the colony-forming ability of LNCaP cells. LNCaP, LN-V1, and LN-TAP clone cells were cultured at 2,000 cells/35 mm dish for 14 days. A group of cell numbers ≥15 cells were counted as a colony. The result of parental LNCaP was set at 100%. Columns, mean of triplicate samples; bars, SD. C, TAP reduces the growth of DU-145 cells. DU-145 cells were stably transfected with pCDNA3-flag-TAP or vector plasmids. After 500 μg/mL G418 selection, three DU-TAP clones (DU-TAP2, DU-TAP4, and DU-TAP7) were confirmed by Western blotting assay (bottom). MTT assays were done to detect growth rate at the indicated times. The absorbance of day 0 was set at 1. Points, mean of triplicate samples; bars, SD.

We then applied multiple approaches to confirm whether TAP mRNA expression is altered in prostate cancer. First, we used in situ hybridization to examine whether TAP mRNA expression is altered in prostate cancer tissue. As shown in Fig. 6B, TAP mRNA signal was easily detected in the epithelial cells of normal glands, ducts, and some basal cells in BPH, but not in stroma, blood vessels, nerves, etc. TAP mRNA signal in the adjacent tissue
was low or undetectable. Results of nine different prostate samples revealed significant differences between the normal/BPH and tumors. The mean of intensity scoring data in the normal/BPH was 2.4, whereas that in the tumor tissues was 1.8. Together, our results from RT-PCR and in situ hybridization indicated that TAP mRNA expression is low in the prostate cancer.

To obtain more compelling evidence, we further evaluated changes and expression patterns of TAP protein in human prostate samples by immunohistochemical staining. TAP polyclonal antibody was produced in the laboratory and used to detect TAP protein expression pattern on a tissue microarray with a large number of prostate carcinoma cases (36). Five different types of prostatic tissue were included in the tissue array: normal prostate (40 cores), BPH (69 cores), PIN (26 cores), low-grade adenocarcinoma (65 cores), and high-grade adenocarcinoma (45 cores). Consistent with the in situ hybridization data, TAP protein was detected in prostatic epithelial cells but not in prostatic stromal cells. TAP expression was located in both the nucleus and cytoplasm in benign tissue (Fig. 6 C). Interestingly, TAP protein can be predominantly detected in the nuclei in neoplastic tissue, including PIN, low-grade adenocarcinoma, and high-grade adenocarcinoma. Staining for TAP was positive in 92% (100 of 109) of benign tissue cores, 73% (19 of 26) of PIN cores, and 44% (49 of 110) carcinoma cores (low- and high-grade adenocarcinoma). Statistical analyses revealed a significant loss of TAP protein expression in carcinoma tissues compared with benign tissues (P < 0.01). There were also significant reductions in PIN tissues compared with BPH tissues (P < 0.05) and reductions in carcinomas compared with PIN tissues (P < 0.01; Fig. 6 C).

Taken together, TAP expression is profoundly reduced in prostate carcinoma and could be used as a clinical marker to correlate with cancer incidence. The reduced TAP expression may allow prostate cancer cells to gain proliferation advantages and suggests an antiproliferation role of TAP.

**Discussion**

Mutations or abnormal expression of genes have been linked to the prostate cancer development, including the Ras association domain family 1 gene (37, 38) and promyelocytic leukemia gene (39–41). Here, we report that TAP expression levels are significantly reduced in prostate cancer tissues and its reduction is associated with prostate tumorigenesis. Moreover, TAP suppresses prostate cancer cell growth in vitro and in vivo. This suggests that TAP might contribute to prostate cancer initiation and progression.
TAP is also named supernatant protein factor and is universally expressed in all the tissues examined, with highest expression in liver, brain, and prostate (1). TAP contains the cellular retinal and TRIO guanine exchange factor (CRAL-TRIO) domain that is common to the small lipid-binding protein. The deficiency or mutation of caytaxin, CRALBP, and TTP in the CRAL-TRIO family has been well linked to specific human diseases, including Cayman ataxia (42), autosomal recessive retinitis pigmentosa (43), and ataxia with vitamin E deficiency (44). It has been found that TAP/supernatant protein factor regulates cholesterol synthesis in liver (3). To our knowledge, no previous study has linked TAP function to the prostate. Here, we report that TAP expressions were down-regulated in several tested prostate cancer cell lines, including LNCaP, CWR22R, and DU-145 cells, although TAP expression in PC-3 cells is slightly reduced when compared with that in RWPE-1 cells; yet, it is significantly lower compared with HPt-1 cells. The more compelling data come from the analyses of clinical human prostate cancer samples. In this study, we have compared the mRNA level of TAP with CRALBP and TTP in seven pairs of clinical specimens (Fig. 6A). Although more clinical specimen were needed to confirm the unchanged mRNA level of CRALBP or TTP, our results indeed suggested that TAP has a differential down-regulation in cancer compartments. The subsequent immunohistochemistry (protein level) and in situ hybridization (mRNA level) analyses have confirmed our observation. TAP down-regulation in prostate cancer may represent a specific cellular event and diverse functions for members of the CRAL-TRIO family.

It has been reported that genetic mutation (e.g., gene loss or mutation) and epigenetic alterations (e.g., DNA methylation and histone modification) contribute to down-regulation of tumor suppressor genes (45, 46). However, the TAP mRNAs in LNCaP, CWR22R, and DU-145 cells were still detectable. Moreover, we did not identify any somatic mutation in the TAP-coding region in these cell lines (data not shown), suggesting that gene loss may not be a key contributory factor for down-regulation of TAP. We also examined whether DNA methylation and histone modification contribute to the reduced expression of TAP in prostate cancer cells. Indeed, 5-azadeoxycytidine, a demethylation reagent, significantly increased TAP expression in LNCaP cells, but not in DU-145 and CWR22R cells. TSA, a histone deacetylation reagent, could restore TAP expression by 2- to 3-fold in CWR22R and LNCaP cells, but not in DU-145 cells (data not shown). These results suggested that down-regulation of TAP in prostate cancer cells might be due to multiple mechanisms and could be cell type specific. Due to the complexity, further characterization is needed and will be conducted in the future.

Our study also found that TAP enhanced vitamin E function in prostate by improving vitamin E uptake (Fig. 2C). There are eight isoforms of vitamin E: α, β-, γ-, and δ-tocopherol and α-, β-, γ-, and δ-tocotrienol. The human body has a preferred absorption for natural α-tocopherol. Liver TTP is responsible for this preference due to the higher binding affinity of TTP toward α-tocopherol (47). In contrast, TAP has a similar binding affinity for those isoforms of vitamin E and some phospholipids (1, 4, 48). It was reported that the α-tocopherol metabolite, δ-tocopherolquinone, has a higher affinity to TAP compared with α-tocopherol (49). Together, these studies suggested that vitamin E functions could be modulated by TAP in prostate cells. In normal prostate tissue, TAP locates in the epithelial cells with high expression, suggesting that TAP may facilitate vitamin E transport into the prostate tissue from the plasma and retain the high concentration of vitamin E within the cells. Indeed, we found that TAP can facilitate the retention of α-, γ-, δ-vitamin E, and α-vitamin E succinate in the prostate cancer cells (Fig. 2C) and increase the antiproliferation effects of α-vitamin E, γ-vitamin E, and α-vitamin E succinate (Fig. 2D). In rat prostate, vitamin E deficiency disrupts some differentiation functions, such as delaying the secretion of 26 kDa protease in ventral prostate (30). Thus, the high expression of TAP protein in prostate may have a physiologic role in normal prostate development and vitamin E–related functions.

PI3K/Akt signaling is the major survival pathway in prostate cancer cells and plays a variety of physiologic roles, including cell growth, cell cycle regulation, migration, and survival (31–35). Akt regulates the cell cycle and apoptosis via many target genes, including the FOXO family proteins. Accumulating evidence suggests that TAP/supernatant protein factor not only binds to vitamin E, but also binds to other small lipid molecules. It was reported that the binding affinity of TAP/supernatant protein factor to phosphatidylinositol is even better than that to α-vitamin E (4). In this work, our results revealed that TAP disrupted the
PI3K/Akt pathway independent of vitamin E action. TAP might regulate intracellular and plasma membrane phosphoinositide homeostasis by transferring the lipid ligand to the membranes to serve as the substrate for PI3K and remove the phospholipid from the membranes for degradation. Here, our data suggested that TAP may control the homeostasis of phospholipid, interfering with p110α-p85 complex formation and low Akt activity. Supportively, Kempna et al. (48) also reported that TAP reduced PI3K/p110α activity in leukemia cells. Furthermore, TAP suppresses Akt downstream target, FOXO3a phosphorylation, confirming our observation that TAP suppresses PI3K/Akt signaling. Remarkably, constitutively active Akt could rescue TAP-mediated growth inhibition. These results indicated that TAP suppresses prostate cancer cell growth, at least partly, via the PI3K/Akt signaling. Although it has been speculated that Akt/FOXO3a pathway may associate with cell cycle arrest or apoptosis (51), we did not observe high TAP expression and inhibition of Akt/FOXO3a pathway, resulting in any cell cycle arrest or apoptosis in our condition. One explanation is that this biological event could have cell type specificity. For example, Nakamura et al. observed that up-regulation of FKHR (FOXO1), another FOXO family gene and Akt target gene, in LNCaP cells, reduced the cell viability using MTT assay. However, this FKHR up-regulation in LNCaP cells cannot induce cell cycle arrest or apoptosis, which has been observed in prostate cancer specimens. A, TAP mRNA expression in prostate cancer by semiquantitative RT-PCR. mRNA for TAP and its family genes (TTP, CRALBP) was detected in benign and adjacent prostate cancer tissues. 18S was used as the internal control. CRALBP or TTP mRNA expression levels showed no significant changes among those normal/tumor pairs, whereas five of seven pairs show that TAP mRNA expression was significantly reduced. One set of representative data was shown here. B, TAP mRNA expression level in human prostate tissues by in situ hybridization. a, typical TAP expression signal in prostate normal and cancer tissue using antisense probe (blue, ×40); b, normal glands/BPH (×100); c, adjacent adenocarcinoma malignant prostate glands (high Gleason grade; ×100); d, sense probe showing negative mRNA signal (negative control, ×40); e, summary of TAP mRNA expression level by in situ hybridization. C, TAP protein expression in human prostate tissues using tissue microarray. a, normal prostate tissue stained with TAP antibody showing 3+ cytoplasmic and nuclear staining (×400); b, BPH tissue stained with TAP antibody showing 3+ cytoplasmic and nuclear staining (×400); c, PIN stained with TAP antibody showing 2+ nuclear staining only (×400); d, low-grade adenocarcinoma stained with TAP antibody showing 2+ nuclear staining (×400); e, high-grade adenocarcinoma stained with TAP antibody showing 1+ nuclear staining (×400); f, summary of TAP protein expression level by immunohistochemistry (IHC) in five different stages of human prostate. Data were analyzed using Fisher’s exact test (21).

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**Figure 6.** Decreased expression of TAP in human prostate cancer specimens. A, TAP mRNA expression in prostate cancer by semiquantitative RT-PCR. mRNA for TAP and its family genes (TTP, CRALBP) was detected in benign and adjacent prostate cancer tissues. 18S was used as the internal control. CRALBP or TTP mRNA expression levels showed no significant changes among those normal/tumor pairs, whereas five of seven pairs show that TAP mRNA expression was significantly reduced. One set of representative data was shown here. B, TAP mRNA expression level in human prostate tissues by in situ hybridization. a, typical TAP expression signal in prostate normal and cancer tissue using antisense probe (blue, ×40); b, normal glands/BPH (×100); c, adjacent adenocarcinoma malignant prostate glands (high Gleason grade; ×100); d, sense probe showing negative mRNA signal (negative control, ×40); e, summary of TAP mRNA expression level by in situ hybridization. C, TAP protein expression in human prostate tissues using tissue microarray. a, normal prostate tissue stained with TAP antibody showing 3+ cytoplasmic and nuclear staining (×400); b, BPH tissue stained with TAP antibody showing 3+ cytoplasmic and nuclear staining (×400); c, PIN stained with TAP antibody showing 2+ nuclear staining only (×400); d, low-grade adenocarcinoma stained with TAP antibody showing 2+ nuclear staining (×400); e, high-grade adenocarcinoma stained with TAP antibody showing 1+ nuclear staining (×400); f, summary of TAP protein expression level by immunohistochemistry (IHC) in five different stages of human prostate. Data were analyzed using Fisher’s exact test (21).
other cells (51). The precise mechanisms of cell type–specific response await additional investigation.

Although TAP was shown to act as a transcription factor (52), no TAP target genes have yet been identified. Interestingly, the TAP cellular distribution pattern is different between benign prostate, PIN, and prostate cancer. TAP is located both in the cytosol and nucleus in the normal and benign prostate tissue, whereas TAP is mainly located in the nucleus in prostate carcinoma, suggesting that TAP moves into the nucleus during prostate cancer progression. Nevertheless, the mechanism and function of this TAP redistribution remains to be elucidated.

Vitamin E and TAP have distinct pathways to modulate prostate cancer cell growth and we hypothesize that vitamin E and TAP can work together to elicit better tumor suppression effects. Surprisingly, vitamin E does not significantly further suppress the growth of LN-TAP xenograft tumor in vivo (data not shown). Without i.p. administration of vitamin E, α-vitamin E concentration in LN-TAP tumor is higher than that in LN and LN-V tumor in xenograft nude mice, supporting our findings that TAP can enhance the α-vitamin E uptake into tumors. However, administration of α-vitamin E into the mice did not further increase α-vitamin E concentration in LN-TAP tumors. It is possible that the ability of tumors to uptake or retain α-vitamin E is limited in s.c. implanted xenografts. Therefore, extraneous α-vitamin E (i.p. administration) cannot efficiently increase vitamin E concentrations of xenograft nor does it enhance the tumor-suppressing effect. A parallel observation was reported about cellular retinoic acid–binding protein type II (CRABP-II). In the xenograft tumor model, CRABP-II can significantly suppress breast cancer cell growth. However, there was no significant difference of tumor growth with and without retinol acid injection (53).

In summary, our study found that TAP suppressed prostate cancer cell growth in vitro and in vivo. Further study revealed that TAP expression level was significantly down-regulated in the PIN stage and prostate cancer compared with normal prostate, suggesting that TAP expression pattern might represent an escape mechanism for prostate cancer cell survival. Therefore, TAP may serve as a new diagnostic marker for prostate cancer progression, and reintroducing TAP expression in prostate cancer may have a therapeutic benefit.

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Tocopherol-Associated Protein Suppresses Prostate Cancer Cell Growth by Inhibition of the Phosphoinositide 3-Kinase Pathway

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