TARP: A nuclear protein expressed in prostate and breast cancer cells derived from an alternate reading frame of the T cell receptor γ chain locus

Curt D. Wolfgang, Magnus Essand*, James J. Vincent, Byungkook Lee, and Ira Pastan

Laboratory of Molecular Biology, Division of Basic Sciences, National Cancer Institute, National Institutes of Health, Building 37, Room 4E16, Bethesda, MD 20892

Contributed by Ira Pastan, June 12, 2000

Previously, we identified the expression of a prostate-specific form of T cell receptor γ chain (TCR$\gamma$) mRNA in the human prostate and demonstrated that it originates from epithelial cells and not from infiltrating T lymphocytes. Here, we show that this prostate-specific transcript is also expressed in three breast cancer cell lines and breast cancer tissues. Analysis of the cDNA sequence predicts that this transcript can encode two protein products of 7 and 13 kDa, and in vitro translation experiments showed that both proteins were made. The longer ORF encodes a 13-kDa truncated version of TCR$\gamma$, whereas the shorter alternative reading frame encodes a 7-kDa protein with five leucine residues in heptad repeats followed by a basic region. Studies with specific antibodies against each protein product revealed that both prostate and breast cancer cells contain only the 7-kDa protein, which is located in the nucleus. We have named this protein TCR$\gamma$ alternate reading frame protein (TARP). These results demonstrate that an alternative protein product is encoded by the TCR$\gamma$ locus in cells other than T lymphocytes.

P

Disease prevention and treatment, and it has been demonstrated that it originates from epithelial cells and not from the prostate. Here, we show that this prostate-specific transcript is also expressed in three breast cancer cell lines and breast cancer tissues. Analysis of the cDNA sequence predicts that this transcript can encode two protein products of 7 and 13 kDa, and in vitro translation experiments showed that both proteins were made. The longer ORF encodes a 13-kDa truncated version of TCR$\gamma$, whereas the shorter alternative reading frame encodes a 7-kDa protein with five leucine residues in heptad repeats followed by a basic region. Studies with specific antibodies against each protein product revealed that both prostate and breast cancer cells contain only the 7-kDa protein, which is located in the nucleus. We have named this protein TCR$\gamma$ alternate reading frame protein (TARP). These results demonstrate that an alternative protein product is encoded by the TCR$\gamma$ locus in cells other than T lymphocytes.

P

Disease prevention and treatment, and it has been demonstrated that it originates from epithelial cells and not from the prostate. Here, we show that this prostate-specific transcript is also expressed in three breast cancer cell lines and breast cancer tissues. Analysis of the cDNA sequence predicts that this transcript can encode two protein products of 7 and 13 kDa, and in vitro translation experiments showed that both proteins were made. The longer ORF encodes a 13-kDa truncated version of TCR$\gamma$, whereas the shorter alternative reading frame encodes a 7-kDa protein with five leucine residues in heptad repeats followed by a basic region. Studies with specific antibodies against each protein product revealed that both prostate and breast cancer cells contain only the 7-kDa protein, which is located in the nucleus. We have named this protein TCR$\gamma$ alternate reading frame protein (TARP). These results demonstrate that an alternative protein product is encoded by the TCR$\gamma$ locus in cells other than T lymphocytes.
The prostate-specific TCR\textsubscript{y} transcript. (A) Schematic of the TCR\textsubscript{y} locus and how the prostate TCR\textsubscript{y} is transcribed and spliced in prostate cells. The transcript consists of a J\textsubscript{y}1.2 segment, three C\textsubscript{y}1 exons, and an untranslated region. (B) Nucleotide and amino acid sequences of the prostate-specific TCR\textsubscript{y} transcript. The full-length transcript is shown starting with the transcription start site and ending with the polyadenylation signal. Arrows above the corresponding nucleotides indicate exon boundaries. The predicted amino acid sequences for two potential ORFs are noted in bold or italics.

Fig. 1. Isolation of poly(A) RNA. Isolation of poly(A) RNA was performed as described (7).

In Vitro Transcription-Coupled Translation. In vitro transcription-coupled translation reactions were described (7). pBSKK-TCR\textsubscript{y}, pBSKK-TCR\textsubscript{y}mutATGdown, pBSKK-TCR\textsubscript{y}mutATGup\textsubscript{1}, and pBSKK-TCR\textsubscript{y}mutATGup\textsubscript{2} were used as templates.

Cell Culture. LNCAp, PC3, MCF7, BT-474, and SK-BR-3 cells were maintained in RPMI medium 1640 (Quality Biologicals, Gaithersburg, MD) at 37°C with 5% CO\textsubscript{2}. The medium contained 10% FBS (Quality Biologicals), 1 mM sodium pyruvate, and penicillin/streptomycin. Hs57Bt cells were maintained in RPMI medium 1640 at 37°C with 5% CO\textsubscript{2}. The medium contained 10% FBS, 30 ng/ml epidermal growth factor (Harlan Laboratories, Cincinnati, OH), 2 mM L-glutamine, 1 mM sodium pyruvate and penicillin/streptomycin.

Antibody Production. Polyclonal PE-TARP antibodies were made as follows. pVC4D-TARP, which contains the entire TARP ORF fused to the C\textsubscript{y} terminus of a catalytically inactive form of the Pseudomonas exotoxin (APE) (9), was expressed in E. coli BL21-CodonPlus (DE3)-RIL cells (Stratagene). Preparation of inclusion bodies and rabbit immunization were described (10). The antiserum was purified by using the Immunopure IgG (protein A) Purification Kit according to the manufacturer’s instructions (Pierce).

TCR\textsubscript{y} antibodies were made as described above by using PET-TCR\textsubscript{y}, an expression plasmid containing the extracellular domain of TCR\textsubscript{y} fused to a C\textsubscript{y}-terminal six-His tag. Before immunization, the His-tagged TCR\textsubscript{y} protein was purified by using a Ni-NTA agarose column according to the manufacturer’s instructions (Qiagen, Chatsworth, CA).

Preparation of Cell Extracts. Whole-cell protein extracts were prepared as follows. Growing cells (5 \times 10\textsuperscript{6}) from each cell line were harvested and resuspended in 1× RIPA buffer containing protease inhibitors (50 mM Tris-HCl, pH 7.5/150 mM NaCl/1 mM EDTA/0.1% Triton X-100/1 mM PMSF/1 µg/ml aprotinin/1 µg/ml leupeptin). The extracts were sonicated briefly and clarified by centrifugation. Protein concentrations were determined by using the Coomassie Plus Protein Assay reagent according to the manufacturer’s instructions (Pierce). Protein extracts from prostate tissue were prepared by grinding 0.5 g of prostate cancer tissue frozen at ~80°C into a fine powder by using a cold mortar and pestle. The powdered tissue was collected, resuspended in 1× RIPA, and processed as described above.

Nuclear, membrane, and cytoplasmic extracts from prostate and breast cell lines were prepared based on protocols published (11, 12).
Western Blot Analysis. Protein extract (20 or 40 μg) and 1 μg of recombinant His-TARP or 100 ng of recombinant His-TCRγ were run on a 16.5% polyacrylamide gel buffered with Tris/Na-tris(hydroxymethyl)methylglycine (Tricine) (Bio-Rad) and transferred to a 0.2-μm Immun-Blot poly(vinylidene difluoride) membrane (Bio-Rad) in transfer buffer [25 mM Tris/192 mM glycine/20% (vol/vol) methanol, pH 8.3] at 4°C for 4 h at 30 V. Filters were probed with either 10 μg/ml ΔPE-TARP antiserum or 1 μg/ml TCRγ antiserum, and their respective signals were detected by using a chemiluminescence Western blotting kit according to the manufacturer's instructions (Roche Molecular Biochemicals).

Results

In Vitro Translation of the Prostate-Specific TCRγ Transcript. Previously, we identified the expression of TCRγ mRNA in the human prostate and demonstrated that it originates from epithelial cells of the prostate and not from infiltrating γδ T lymphocytes (7). Analysis of this transcript revealed two potential reading frames. One encodes a protein with a calculated molecular mass of 7 kDa (Fig. 1B; bold amino acids), and one encodes a protein with a calculated molecular mass of 13 kDa (Fig. 1B; italicized amino acids). To determine the protein product encoded by this transcript, we analyzed its translational activity by in vitro transcription-coupled translation with the full-length prostate TCRγ cDNA as template. As demonstrated (7), two protein products of about 7 and 13 kDa were obtained (Fig. 2; lane 3). No specific protein products were obtained when the reactions were done without the TCRγ cDNA template or with vector only (Fig. 2; lanes 1 and 2). Therefore, the in vitro translation results correlate well with the predicted protein sizes.

To verify that the two protein products observed in Fig. 2 were caused by the use of two independent ORFs, site-directed mutagenesis was performed to mutate individual initiation ATG codons to ATA. Mutation of the third ATG, corresponding to the predicted start of the second ORF, resulted in the loss of the 13-kDa protein product (Fig. 2; lane 4). However, mutation of the first ATG, corresponding to the predicted start of the first ORF, did not result in the loss of the 7-kDa protein product (Fig. 2; lane 5). Analysis of the first ORF revealed a second in-frame ATG codon 2 aa downstream of the first ATG. Surprisingly, mutation of the second ATG did not result in the loss of the 7-kDa protein product (Fig. 2; lane 6). However, mutation of both the first and second ATGs in the first reading frame resulted in the loss of the 7-kDa protein. These results indicate that the first reading frame can start at either ATG.

Inspection of the amino acid sequence encoded by the second ORF indicates that it is derived from one of the ATG codons in the original TCRγ reading frame. Therefore, this protein will be referred to as TCRγ in the rest of this paper. On the other hand, the protein product encoded by the first ORF shows no resemblance to any published protein sequence in GenBank. Therefore, this protein will be referred to as TCRγ alternate reading frame protein (TARP).

TARP is a Nuclear Protein Expressed in Prostate Cancer Cells. To determine whether TARP or TCRγ exists in prostate cancer cells, we generated antibodies against both proteins and performed Western blot analyses on different prostate cancer cell extracts. As shown in Fig. 3A Upper, TARP was detected in the prostate cancer LNCaP cell line and a prostate cancer tumor extract. The 7-kDa band comigrates with the recombinant His-TARP, suggesting that the product detected in the LNCaP and cancer extracts is TARP. Previously, we demonstrated that the prostate-specific TCRγ transcript is not expressed in the prostate cancer PC3 cell line (7). Therefore, we used PC3 cell extracts as a negative control and demonstrated that the 7-kDa band was absent in these extracts (Fig. 3A Upper).

Importantly,
no 7-kDa bands were detected when the prebleed antiserum or an antiserum against the *Pseudomonas* exotoxin (PE; see Materials and Methods) was used (data not shown). TCRγ was not detected in any of these extracts even though the recombinant protein showed a very strong signal with the antibody used (Fig. 3A Lower). These data indicate that the prostate-specific TCRγ transcript encodes TARP.

To determine the cellular localization of TARP, we prepared nuclear, cytoplasmic, and membrane fractions from LNCaP cells. As shown in Fig. 3B, TARP was detected in the nucleus and not in the cytoplasm or membrane fraction. Similar results were obtained by using nuclei purified by fractionating the cell extracts through a sucrose cushion (12) (data not shown).

**The TARP Transcript Is Expressed in Breast Cells.** We reported that the TCRγ EST cluster also contains some ESTs from brain libraries (5). After this initial report, additional ESTs have been deposited into the database and the cluster now contains ESTs from breast, colon, kidney, and gastric libraries as well. To determine whether the existence of these ESTs indicates the expression of the TARP transcript in these cells or it was caused by the presence of infiltrating γδ T lymphocytes when these libraries were made, we performed RT-PCR on various cell lines to test for the presence of the TARP transcript. As shown in Fig. 4A, expression of the TARP transcript was detected in the breast cell lines MCF7, BT-474, SK-BR-3, and CRL-1897. No signals were detected in the neuroblastoma cell line A172, glioblastoma cell line IMR32, colon cell line COLO 205, gastric cell line KATO III, or kidney cell lines COS7 and 293 (Fig. 4A and data not shown). To determine whether the TARP transcript is expressed in human breast tissues in addition to cell lines, we tested 12 different normal breast and 12 different breast cancer cDNAs by using a RAPID-SCAN panel (OriGene Technologies, Rockville, MD). TARP mRNA was shown to be abundant in some of the breast cancer samples (Fig. 4B Upper), whereas it was barely detectable in the normal breast samples after 35 rounds of PCR (data not shown). Significantly, no signals were detected in reactions lacking cDNA. *actin* was used to show that similar amounts of cDNA were present in each lane (Lower). The weak signals in the normal breast samples correlate well with the lack of TARP signal shown in Figs. 4A and 5 for the Hs578Bst cell line, a breast cell line derived from normal breast tissue. These results suggest that expression of the TARP transcript in the breast is increased after oncogenic transformation. However, more studies are needed before any definitive conclusions can be made.

To determine whether the TARP transcript observed in the breast cell lines is the same as the transcript found in the prostate cell line, we performed RT-PCR using primers against different regions of the TARP transcript. As shown in Fig. 5A, the TARP transcript in prostate contains a portion of the Jγ1.2 gene segment, three Cγ1 exons, and some untranslated sequence followed by a poly(A) tail (7). Primer set 1 and 3 amplifies the entire TARP transcript (Fig. 5B Top), whereas primer set 2 and 3 amplifies the Cγ1 region only (Fig. 5B Bottom). As shown in Fig.
signals were detected in the reactions lacking cDNA (dH2O) and cell line (LNCaP) by using either primer set. Importantly, no (MCR7, BT-474, and SK-BR-3) as compared with the prostate

However, as shown in Fig. 3, our initial hypothesis was incorrect. The TARP actin control (Fig. 5) showed similar amounts of cDNA were used as demonstrated by the TARP peptide in the cell because most are usually secreted. Second, because TCR

To determine whether TARP protein exists in the breast cancer cell lines, we performed Western blotting with breast cancer nuclear extracts by using an antibody against TARP. As shown in Fig. 6, TARP-reactive bands were detected in three breast cell lines (MCF7, BT-474, and SK-BR-3) when compared with the prostate cell line (LNCaP), although at a weaker intensity. Therefore, we conclude that TARP mRNA is expressed in prostate and breast cancer cells.

To determine whether TARP protein exists in the breast cancer cell lines, we performed Western blotting with breast cancer nuclear extracts by using an antibody against TARP. As shown in Fig. 6, TARP-reactive bands were detected in three breast cell lines (MCF7, BT-474, and SK-BR-3) when compared with the prostate cell line (LNCaP), although at a weaker intensity. Therefore, we conclude that TARP mRNA is expressed in prostate and breast cancer cells.

Discussion

We report the identification of a 7-kDa nuclear protein encoded by a specific transcript derived from the TCRγ locus expressed in prostate and breast cancer cells. Because the protein is encoded from a reading frame different from TCRγ, we name it TARP for TCRγ alternate reading frame protein. Besides being translated from an alternate reading frame of a transcript originating within an intron of the TCRγ locus, TARP has two other unusual features. First, it is surprising to find such a small peptide in the cell because most are usually secreted. Second, TARP lacks a good Kozak sequence (13). Because the TCRγ reading frame contains a good Kozak sequence, we initially hypothesized that a truncated TCRγ protein was encoded. However, as shown in Fig. 3, our initial hypothesis was incorrect.

5B, similar-sized bands were detected in three breast cell lines (MCR7, BT-474, and SK-BR-3) as compared with the prostate cell line (LNCaP) by using either primer set. Importantly, no signals were detected in the reactions lacking cDNA (dH2O) and similar amounts of cDNA were used as demonstrated by the control (1 μg of His-tagged TARP (His-TARP) and 100 ng of His-tagged TCRγ (His-TCRγ) were run on the gels. Size markers in kDa are indicated on the left.

Fig. 6. TARP exists in the nuclei of breast cancer cells. Western blot of nuclear extracts derived from LNCaP, MCF7, BT-474, SK-BR-3, and HisTARP, and His-TCRγ cells. Each nuclear extract (40 μg) was run on a Tris/Tricine 16.5% polyacrylamide gel and probed with an antibody against TARP (Upper) or TCRγ (Lower). As a positive control, 1 μg of His-tagged TARP (His-TARP) and 100 ng of His-tagged TCRγ (His-TCRγ) were run on the gels. Size markers in kDa are indicated on the left.
TARP

The TARP antibody recognizes a doublet in prostate and breast nuclear extracts (Fig. 6 Upper). The faster 7-kDa band comigrates with the His-TARP recombinant protein, whereas the weaker band runs at a larger molecular mass. One possible explanation for the 9-kDa band is posttranslational modifications. To determine if TARP contains any known posttranslational modification sites, we analyzed the TARP amino acid sequence by using the PROMfite program of the Swiss Institute of Bioinformatics ExPaSy proteomics server (http://www.expasy.ch) (22, 23). As shown in Fig. 7A, many possible phosphorylation sites were found, including cAMP- and cGMP-dependent protein kinase phosphorylation sites (RRAT and RRRG) and protein kinase C phosphorylation sites (SSR and STRR). Phosphorylation has been shown in many cases to cause transcriptional regulation. Therefore, it is necessary to identify TARP-interacting proteins to determine its function.

The TARP antibody was used to determine the true nature of the 9-kDa band and whether TARP is regulated by estrogen. This hypothesis is strengthened by the existence of an estrogen response element (ARE) within the prostate-specific antigen promoter. It is believed that this mutation leads to the loss of androgen-regulated prostate-specific antigen expression in breast tumors (25). Molecular analysis of the aberrant expression of prostate-specific antigen leads to the discovery of a single point mutation in one of the AREs found within the prostate-specific antigen promoter. It is unclear at this time whether a similar mutation in the TARP promoter occurs in the three breast cell lines tested.

The prostate is dependent on androgens for maintenance of its structure and function. When prostate cells become malignant, they often lose their androgen dependence. In this study, we used two prostate cell lines that differ in their dependence on androgens for growth: LNCaP and PC3 cells. The androgen receptor is present in the androgen-dependent LNCaP cell line, but is absent in the androgen-independent PC3 cell line (26). As shown in Fig. 3, TARP is expressed in LNCaP cells but not in PC3 cells. This result suggests that TARP expression may be regulated by androgen stimulation. The identification of an ARE-like element within the TARP promoter strengthens the idea that TARP is induced by androgens. Further experiments are needed to determine whether androgens induce TARP mRNA expression. Expression in LNCaP cell but not in PC3 cells may indicate that TARP is important in regulating androgen-dependent responses.

We thank Drs. Charles Vinson, Xiu Fen Liu, Kristi Egland, Pär Olsson, and Tapan Bera for their helpful comments and discussions, Robb Mann for editorial assistance, and Dr. Alfred Johnson for RNA samples.