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D-GPCR: a novel putative G protein-coupled receptor overexpressed in prostate cancer and prostate

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Abstract

The use of molecular targets in novel strategies of tumor treatment largely depends on the identification of proteins with a tumor- or tissue-restricted expression. We identified the novel protein D-GPCR that is selectively overexpressed in human prostate cancer and prostate and belongs to the subfamily of odorant-like orphan G protein-coupled receptors. Quantification of *D-GPCR* transcripts in different human tissues by real-time PCR demonstrated 27-fold overexpression in prostate compared to skeletal muscle, the organ with second highest transcript numbers in males. Investigation of tumor/normal cDNA pairs obtained from 241 cancer patients including four prostate tumors confirmed the preferential expression in prostate. When comparing the mean transcript level of 15 prostate cancer tissues to their non-tumorous counterparts, *D-GPCR* was almost 6-fold upregulated. Coupled in vitro transcription and translation of *D-GPCR* cDNA produced a protein band of approximately 28 kDa. Recombinant, His-tagged protein was expressed in transfected HEK293 cells and gave rise to a 30 kDa band specifically detected by anti-His antibody. These data provide the basis for future studies evaluating the diagnostic potential of D-GPCR and its utility as a novel target in immunotherapy of prostate cancer.

Keywords: G protein-coupled receptor; Prostate cancer; Real-time PCR

Treatment of localized prostate cancer (PCa) is still limited to radical prostatectomy, radiotherapy, and androgen ablation. About one-third of patients suffers from recurrent disease despite optimization of therapies. Although PCa starts as androgen-dependent tumor, the beneficial effects of androgen ablation are often temporary and the development of hormone-refractory PCa that is essentially incurable seems to be almost inevitable during later stages [1]. This underlines the need for novel therapeutic modalities like monoclonal antibodies, T cell-mediated immunotherapy or novel chemical compounds with higher target selectivity [2,3]. Therefore, the identification of genes overexpressed in prostate tissue and prostate tumors is a crucial prerequisite for diagnosis and treatment of PCa.

Gene chips have been widely used for the identification of genes associated with the progression of PCa by assessing expression profiles of clinical samples and cell lines [4–9]. In addition, cDNA arrays have been successfully used for the identification of genes preferentially expressed in normal and malignant prostate tissues by comparing their transcription profiles to a broad variety of normal tissues [10,11]. In addition to the well-known markers of proven diagnostic value in

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PCa, such as prostate-specific antigen (PSA), prostatic acid phosphatase (PAP), and prostate-specific membrane antigen (PSMA) [12–14], several novel prostate-restricted proteins were identified, for example, the G protein-coupled receptor (GPCR) prostate-specific G protein-coupled receptor (PSGR) [15,16].

Transmembrane (TM) receptors coupled to heterotrimeric G proteins represent the largest family of cell surface mediators of signal transduction. They are linked to a broad range of physiological functions and therefore, understanding their regulation can provide the basis for intervention in a variety of disease states (see, for example [17]). Most of the known human GPCRs do not have any other conserved functional domain apart from their seven TM helices. The sequence of a GPCR does not necessarily provide information about the nature of its cognate ligand and, therefore, such receptors are termed as *orphan* GPCRs (oGPCRs). They are probably the most important group of proteins that represents molecular targets for pharmacological therapeutics and have been widely used in ligand screening assays to discover novel endogenous receptor–ligand systems. Many oGPCRs, like PSGR, belong to the family of odorant-like GPCRs, a classification that is defined merely by sequence homology as specific odorant ligands have been identified only for a few of the more than 300 putative GPC-odorant receptors (ORs) described so far. A growing number of ORs are expressed in tissues with no known odor recognition function, such as human testes, erythroid, and prostate cells [18].

There is also growing evidence that GPCRs might be involved in neoplastic transformation of the prostate [19]. For example, increased levels of PSGR, bradykinin 1 receptor [20], and endothelin 1A receptor [21] are expressed in PCa. Recently, it was demonstrated that inhibition of Gβγ signaling retards growth of prostate tumor xenografts by inducing apoptosis [22]. This makes prostate-specific GPCRs and their respective signaling pathways attractive candidate targets for the treatment of PCa.

Here, by mining a large expression database, we identified the expressed sequence tag (EST) AI694767 that is overexpressed in human PCa and prostate. Analysis and extension of the EST sequence led to the identification of a 3045 bp cDNA containing an open reading frame of 318 amino acids (aa) coding for a protein with homology to GPCRs of the OR subfamily. We termed the novel protein Dresden-GPCR (D-GPCR).

Materials and methods

Identification of D-GPCR cDNA sequences and dot blot analyses. The EST AI694767 was identified by screening the GeneExpress database [23] (Gene Logic, Gaithersburg, MD, USA) for ESTs with a prostate-specific expression pattern and an association with PCa. The data were analyzed using the GeneExpress Software System and tools available on public web sites (www.ncbi.nlm.nih.gov).

A major part of the EST corresponding to nucleotides 87-488 of AI694767 was amplified from pooled prostate cDNA (BD Clontech, Heidelberg, Germany) using the primers D-GPCR_N1(5′-TGAC AAAGGTTTGGCTCTGCACAGTT-3′) and D-GPCR_C1(5′-GGA CTGTAAGCCCATGAGGGCACTG-3′) with the cycling profile: 95 °C for 3 min; 30 cycles: 95 °C for 30 s, 70 °C for 30 s, and 68 °C for 30 s followed by one round at 68 °C for 5 min. The 402 bp product was ligated into pCRII-TOPO (Invitrogen, Karlsruhe, Germany) and sequenced. After excision with *Eco*RI and isolation from an agarose gel, the cDNA fragment was labeled with [^{32}P]dCTP by random priming (Megaprime DNA labeling system; Amersham Biosciences, Freiburg, Germany) and hybridized to the multiple tissue expression (MTE) array 2, to the multiple tissue Northern blot II (MTN II), and to the cancer profiling array I (CPA I; all from BD Clontech). The MTE 2 array contains mRNA from 58 pooled normal human tissues, 7 pooled fetal tissues, and 8 cell lines normalized to 8 housekeeping genes, the MTN II blot represents normalized mRNA from 8 different tissues, and the CPA I contains paired samples of normalized cDNA from 241 tumor and corresponding normal tissue from individual patients. The hybridizations were performed according to the provider's instructions using 2×10^6 cpm/ml of the radioactively labeled probe and signals were visualized by phosphoimaging after 72 h exposure (Amersham Biosciences).

The sequence of AI694767 was extended by custom EST clustering using the "EST clustering" tool from the Heidelberg unix sequence analysis resources (HUSAR; http://genome.dkfz-heidelberg.de). The resulting consensus sequence was used for reiterative BLAST searches against the HONEST (non-EST) database leading to the identification of GenBank clones AK023643 and AB065787, extending the consensus sequence and comprising the D-GPCR ORF cDNA, respectively. During our ongoing investigation, a 3077 bp full-length cDNA clone comprising the originally identified EST and both cDNA clones, AK023643 and AB065767, was published in GenBank (Accession No. AL833127).

In vitro transcription and translation. For in vitro translation of the D-GPCR cDNA, the ORF cloned into pCRII-TOPO (see Western blot analysis) was used in an in vitro transcription-coupled translation system (TNT, rabbit reticulocyte lysate system; Promega, Mannheim, Germany). [^{35}S]Met (Amersham Biosciences) was incorporated in the reaction for visualization of translated products. The reaction mixture was analyzed on a reducing 15% SDS–PAGE together with a protein molecular weight marker (Bio-Rad). The gel was dried and subjected to autoradiography.

Prostate cancer patients, tissue samples, and cell lines. All primary tumor samples were obtained from prostatectomized PCa patients with informed consent (Table 1). We analyzed pairs of tissue samples (primary PCa specimens and autologous non-malignant prostate tissue) from 15 patients. Histopathological examination of the tumors was performed according to the UICC classification system from 1997 [24].

The PCa cell lines LNCaP, DU 145, 22RV1, and PC-3 (all from American Type Culture Collection, Manassas, VA) were cultured according to the provider's instructions.

RNA isolation and cDNA synthesis from tissue samples and cell lines. Total RNA was extracted by standard procedures (Trizol LS Reagent; Invitrogen) and quality was analyzed by agarose gel electrophoresis. After DNA digestion (DNase I; Amersham Biosciences), absence of traces of genomic DNA was checked by PCR using β-actin-specific primers prior to reverse transcription. The cDNA synthesis was performed using 4 μg of total RNA and random hexamer primers in a standard 32-μl reaction (Ready to Go You Prime First Strand Kit; Amersham Biosciences).

Quantitative real time-PCR. Tissue specificity of mRNA expression was analyzed by a quantitative PCR assay in a panel of cDNAs derived from 16 human tissues (Human MTC Panels I and II; BD Clontech). The number of *D-GPCR* transcripts was determined by

Table 1
Pathological and clinical parameters and percentage of tumor cells in the tissue samples analyzed by real-time PCR

Patient	Age[a]	TMN classification	GS	G	Percentage of tumor cells (%)[b]malignant/non-malignant sample
1	57	pT2b pN0 cM0	6	IIb	90/0
2	78	pT2b pN0 cM0	5	lla	90/0
3	54	pT2b pN0 cM0	6	IIb	90/0
4	72	pT2a pN0 cM0	6	IIa	80/0
5	61	pT2b pN0 cM0	7	IIb	90/0
6	64	pT2b pN0 cM0	6	IIb	90/0
7	62	pT2b pN0 cM0	6	llb	90/0
8	67	pT2b pN0 cM0	6	IIa	90/0
9	65	pT2b pN0 cM0	6	llb	80/0
10	72	pT2a pN0 cM0	8	*	75/0
11	58	pT2a pN1 cM0	8	IIIa	80/2
12	57	pT2a pN0 cM0	7	IIb	80/0
13	70	pT2a pN0 cM0	6	IIb	70/0
14	67	pT2a pN0 cM0	6	IIb	75/0
15	59	pT2a pN0 cM0	7	IIb	100/0

G, tumor grade; GS, Gleason score; N, lymph node metastases; p, pathological examination; and T, tumor stage. (*) Grading could not be determined as this patient was hormonally pretreated.
[a] Age at the time of radical prostatectomy.
[b] Percentage of tumor cells within the epithelial cells estimated by a pathologist on a representative specimen of the investigated sample.

applying a real-time PCR protocol based on fluorescence resonance energy transfer (FRET) hybridization probes (LC-Fast Start DNA Master Hybridisation Probes; Roche Diagnostics, Mannheim, Germany) using the intron-spanning primer pair D-GPCR_N78 (5′-GGTCACACATTCCTTCCATAC-3′)/D-GPC R_C342 (5′-AA GAAATATATACATGGGCTCATGCA-3′) for amplification of a 239 bp fragment comprising a part of the D-GPCR ORF without homology to PSGR. FRET donor and acceptor probes were D-GPCR_FL (5′-T CAGTTCTGGTTGGCCTTCCCATT-3′-F ITC) and D-GPCR_LC (LC_Red_640-5′-TGCTCCCTCTACCTTATTG CTGTGCTp-3′), respectively (TibMolbiol, Berlin, Germany). The PCR protocol for the *D-GPCR* LC-assays consisted of a predenaturation step (10 min at 95 °C) and 45 amplification cycles (10 s at 95 °C, 10 s at 52 °C, and 11 s at 72 °C).

To quantify the transcript levels in matched malignant and non-malignant prostate samples and in the PCa cell lines DU-145, LNCaP, PC3, and 22RV1, 2 μl of the 1:5 diluted cDNA products was used for the same amplification protocol.

The *D-GPCR* transcript number was normalized to the quantity of β-actin. SYBR Green I-based quantification of β-actin was performed using the primers Act_N1 (5′-GCCGTCTTCCCCTCCATCGTG-3′) and Act_C1 (5′-GGAGCCACACGCAGCTCATTGTAGA-3′) applying the following PCR protocol: 10 min at 95 °C and 40 amplification cycles (15 s at 95 °C, 5 s at 70 °C, and 12 s at 72 °C).

Serial dilutions of plasmid DNA containing the β-actin fragment or LC capillaries coated storageable with HPLC calibrated *D-GPCR* fragments (Roboscreen GmbH, Leipzig, Germany) over eight log scales (10^1–10^8 molecules per capillary) were used as internal template standards (calculation via LC quantification software version 3.5; Roche). Each determination was carried out twice for each cDNA sample as independent PCR runs. The molecule ratios of *D-GPCR* to β-actin transcripts were calculated from the mean values.

Western blot analysis. The *D-GPCR* coding sequence was amplified from pooled prostate cDNA (BD Clontech) with the primers D-GPCR_N8 (5′-CACGAATTCATGATGGTGGATCCCAATGGCA ATGAA-3′) and D-GPCR_C9 (5′-CACCTCGAGGGGCTCTGAA GCGTGTGTGGC-3′), thereby introducing *Eco*RI and *Xho*I sites, respectively. After ligation into vector pCRII-TOPO and subcloning into pcDNA6-HisA (both from Invitrogen), the construct was sequenced. HEK293 cells were transfected with vector encoding D-GPCR or with empty vector, respectively, using lipofectamine (Invitrogen) and cells were harvested after 48 h. For detection of recombinant protein expression, cells were lysed in boiling Laemmli buffer and subjected to SDS–PAGE and Western blotting. Immunodetection was performed with an anti-penta-His antibody (Qiagen, Hilden, Germany) using the ECL plus Detection System (Amersham Bioscience).

Generation of anti-D-GPCR antiserum and fluorescence-activated cell sorter analysis. The *D-GPCR* coding sequence was amplified with the primers D-GPCR_N6 (5′-CACAGATCTATGATGGTGGATCCCA ATGGCAATGAA-3′) and D-GPCR_C6 (5′-CACGAATTCTCA ATGGTGATGGTGATGGGGCTCTGAAGCGTGTGTGGC-3′), thereby introducing *Bgl*II and *Eco*RI sites, respectively. After ligation into vector pCRII-TOPO and subcloning into pIRES-EGFP (BD Clontech), the resulting construct pIRES-D-GPCR was sequenced. BALB/c mice were immunized with 1×10^7 γ-irradiated (100 Gy) P-815 mouse mastocytoma cells stably transfected with pIRES-D-GPCR according to standard procedures. After three immunizations, PC-3 cells stably transfected with the same construct as well as PC-3 wild-type cells were incubated with the antiserum diluted 1:50 in the absence of a permeabilizing agent. Cells were washed three times and were subsequently incubated with a 1:200 dilution of R-phycoerythrin labeled goat anti-mouse IgG $F(ab')_2$ (DAKO, Hamburg, Germany). After the staining procedure, cells were washed twice and were evaluated by fluorescence-activated cell sorter (FACS) analysis. Flow cytometry was performed on a FACScan cytometer (BD Pharmingen, Heidelberg, Germany). Fluorescence was displayed in the single parameter histogram plot and statistical analysis was performed using WINMIDI2.8 software.

Results

D-GPCR is a novel GPCR overexpressed in prostate tissue

The GeneExpress database (Gene Logic, Gaithersburg, MD, USA) contains a wide variety of clinical samples, cell lines, and normal tissue specimens profiled on commercially available Affymetrix DNA chips [23]. By

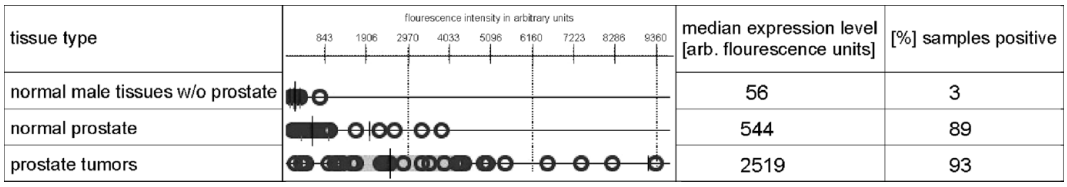

Fig. 1. Electronic Northern data of EST AI694767 expression in various tissues obtained from the transcriptome database GeneExpress. Each circle represents a positive sample with its fluorescence intensity. Median expression is illustrated by vertical bars.

screening this database for prostate-specific gene products, we identified the Affymetrix HG-U95E chip element 74420, indicative for a 559 bp EST (GenBank Accession No. AI694767) bearing an oligo(dT) tail. Element 74420 displayed readily detectable expression in 89% of normal prostate samples and in 93% of PCa specimens with median expression levels increasing from 544 fluorescence units in normal prostate tissue to 2519 fluorescence units in PCa (Fig. 1). Expression of AI694767 in all other major male normal tissues was significantly lower reaching only median levels of 56 fluorescence units in 3% of the samples.

In order to further prove the tissue-restricted expression of AI694767, the cDNA fragment was amplified from pooled prostate cDNA by PCR, cloned, and sequenced. Hybridization of the radioactively labeled PCR product with a multiple tissue expression (MTE) array representing pooled mRNA samples from 58 different human adult tissues, 7 fetal tissues, and 8 cell lines confirmed the tissue-specific expression in the prostate (Fig. 2).

By custom EST clustering with the web based ESTCluster tool of the Heidelberg unix sequence analysis resources (HUSAR; http://genome.dkfz-heidelberg.de), we extended the sequence information in silico. BLAST searches [25] of the generated consensus sequence against the HUSAR non-EST cDNA database (HONEST) identified a putative full-length cDNA clone (GenBank Accession No. AL833127) of 3077 bp (see Materials and methods). Analysis of the sequence revealed an open reading frame (ORF) of 954 nucleotides (nt) encoding a 318 amino acid (aa) protein (Fig. 3A). The "simple modular architecture research tool" (SMART; http://smart.embl-heidelberg.de) predicted 7 TM domains within the putative protein and the PSORT II algorithm (http://psort.nibb.ac.jp) suggested its localization at the cell membrane. The *D-GPCR* ORF showed great degree of similarity to the GPCR family with highest similarity to ORs (Fig. 3B). Specifically, BLASTP analysis of human SwissProt annotated proteins showed closest homology (57% identity, 73% similarity) of D-GPCR to PSGR, a previously characterized prostate-specific oGPCR [15,16]. D-GPCR seemed to be conserved during evolution as gorilla, mouse, and rat homologs displaying aa identities varying from 92% to 88% were detected by BLASTP search (GenBank Accession Nos. AAR19522, XP_145749, and XP_344930, respectively).

To demonstrate expression of the full-length *D-GPCR* transcript in prostate, Northern blot was performed using a multiple tissue Northern blot II (MTN II), representing pooled, normalized mRNA from eight different tissues including prostate. Hybridization to the same radioactively labeled cDNA probe as used for the MTE array confirmed the tissue-specific expression

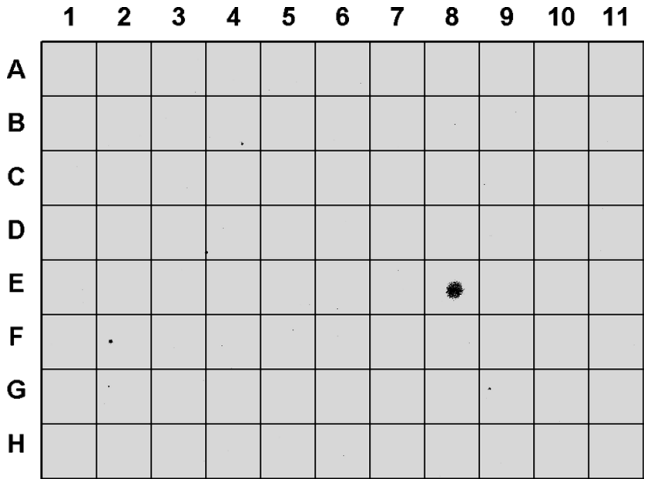

Fig. 2. Dot blot analysis of *D-GPCR* mRNA expression pattern in normal tissues. A 402 bp cDNA fragment of the *D-GPCR* transcript was radioactively labeled and hybridized to the human multiple tissue expression (MTE) array 2 that provides poly(A)$^+$ RNA from 58 human adult tissues, 7 human fetal tissues, and 8 tumor cell lines normalized to 8 housekeeping genes. After 72 h, strong hybridization signals were only revealed in prostate tissue. A1, whole brain; B1, cerebral cortex; C1, frontal lobe; D1, parietal lobe; E1, occipital lobe; F1, temporal lobe; G1, paracentral gyrus of the cerebral cortex; H1, pons; A2, cerebellum left; B2, cerebellum right; C2, corpus callosum; D2, amygdala; E2, nucleus caudatus; F2, hippocampus; G2, medulla oblongata, H2, putamen; B3, nucleus accumbens, C3, thalamus; E3, spinal cord; A4, heart; B4, aorta; C4, atrium left; D4, atrium right; E4, ventricle left; F4, ventricle, right; G4, interventricular septum; H4, apex of the heart; A5, esophagus; B5, stomach; C5, duodenum; D5, jejunum; E5, ileum; F5, ilocecum; G5, appendix; H5, colon ascendens; A6, colon transversum; B6, colon descendens; C6, rectum; A7 kidney; B7, skeletal muscle; C7, spleen; D7, thymus; E7, peripheral blood leukocyte; F7, lymph node; G7, bone marrow; H7, trachea; A8, lung; B8, placenta; C8; bladder; D8, uterus; E8, prostate; F8, testis; G8, ovary; A9, liver; B9, pancreas; C9, adrenal gland; D9, thyroid gland; E9, salivary gland; A10, leukemia, HL-60; B10, HeLa S3; C10, leukemia, K-562; D10, leukemia, MOLT-4; E10, Burkitt's lymphoma, Raji; F10, Burkitt's lymphoma, Daudi; G10, colorectal adenocarcinoma, SW480; H10, lung carcinoma, A549; A11, fetal brain; B11; fetal heart; C11, fetal kidney; D11; fetal liver; E11, fetal spleen; F11, fetal thymus; and G11, fetal lung.

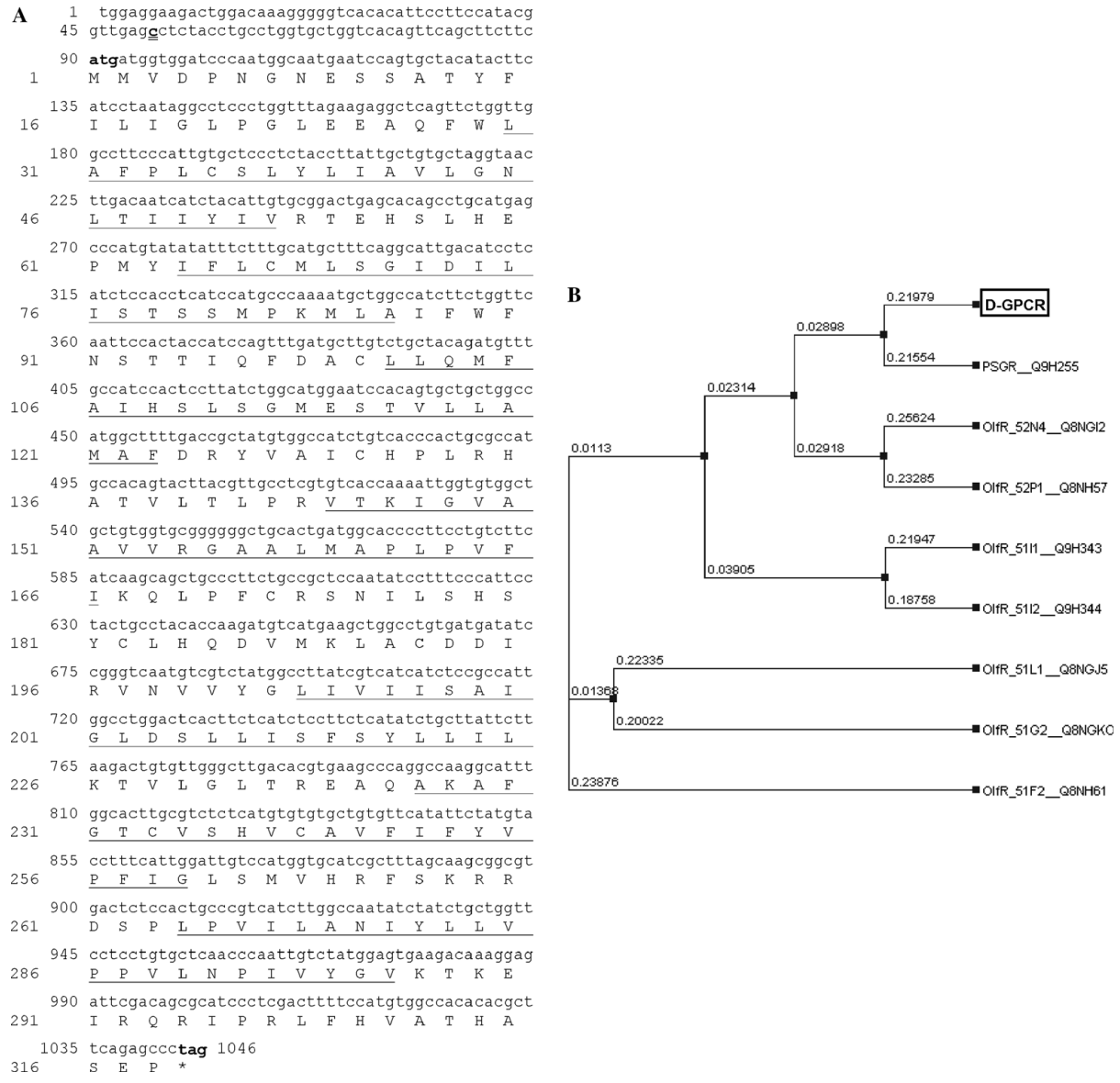

Fig. 3. Characterization of the *D-GPCR* cDNA sequence (GenBank Accession No. AY698056). (A) Nucleotide sequence and conceptual translation of *D-GPCR*. The first nucleotide of exon 2 is in bold and is doubly underlined. The potential initiation codon and the translation termination codon are indicated in bold. The stop codon is indicated by an asterisk (*). The transmembrane domains 1–7 are underlined. (B) Multiple alignments of the predicted protein sequence of D-GPCR and other human GPCRs like PSGR shown as phylogenetic tree. Multiple alignment was performed with the CLUSTALW program [37]. Names of the proteins and their Swissprot accession numbers are indicated at the right. Branch lengths are drawn to equal length and evolutionary distance is indicated by numbers.

of the 3.2 kb full-length *D-GPCR* transcript in the prostate (Fig. 4).

For the evaluation of *D-GPCR* expression in different tissues with sensitivity higher than that afforded by dot blot, transcripts were quantified in a cDNA panel generated from 16 different human tissues by quantitative real-time PCR (Q-RT-PCR) and normalized to actin. Using an amplicon with no homology to PSGR, this assay confirmed selective overexpression of *D-GPCR* in prostate tissue. However, the high sensitivity of Q-RT-PCR revealed expression in additional tissues such as placenta, skeletal muscle, heart, ovary, and testis (Fig. 5). In male tissues, expression of *D-GPCR* was decreased by 27.1-fold (skeletal muscle), 30.5-fold (heart), and 62.9-fold (testis) compared to prostate.

In vitro transcription and translation

The *D-GPCR* cDNA isolated from prostate has a predicted ORF of 318 aa with a calculated molecular mass of 34 kDa. To determine the actual size of the protein, coupled in vitro transcription and translation was performed. SDS–PAGE analysis and autoradiography of the translated product showed that the D-GPCR protein has an apparent molecular mass of about 28 kDa (Fig. 6). The discrepancy between

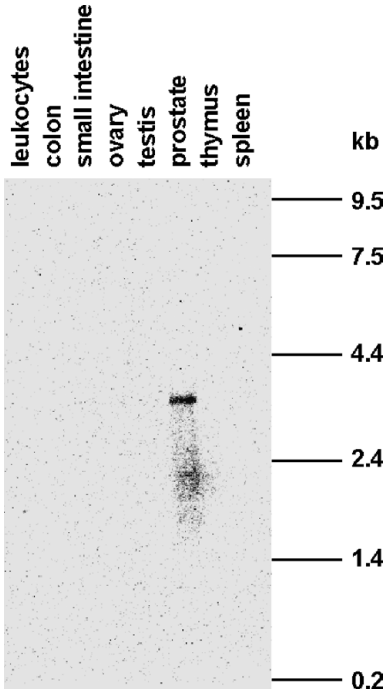

Fig. 4. Multiple tissue Northern blot analysis of the D-GPCR transcript. The same radioactively labeled 402 bp cDNA fragment of the *D-GPCR* transcript as used for Fig. 2 was hybridized to the human multiple tissue Northern blot II (MTN II) that provides pooled, normalized mRNA from eight different tissues including prostate. After 72 h of exposition, a transcript of approximately 3.2 kb was detected specifically in prostate.

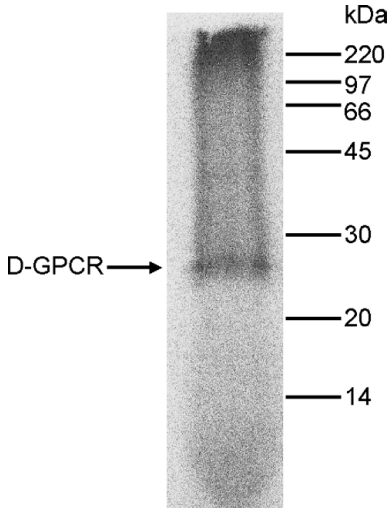

Fig. 6. In vitro transcription and translation of D-GPCR. The *D-GPCR* cDNA was transcribed with T7-RNA polymerase and translated with rabbit reticulocyte lysate in the presence of [^{35}S]methionine. The reaction mixture was analyzed on a reducing 15% SDS–polyacrylamide gel together with a protein molecular weight marker. After autoradiography, a band of approximately 28 kDa was detected.

calculated and apparent molecular masses is probably due to the high amount of small hydrophobic (36%) and aromatic (10%) aa in the D-GPCR protein. In proteins with a high proportion of TM helices, for example in connexins, considerable differences between calculated molecular masses and electrophoretic mobilities are frequently observed (see for example [26]).

Expression of the D-GPCR protein

To analyze the D-GPCR protein in intact eukaryotic cells, the cDNA was cloned into a mammalian expression vector upstream of a C-terminal His-tag. HEK293

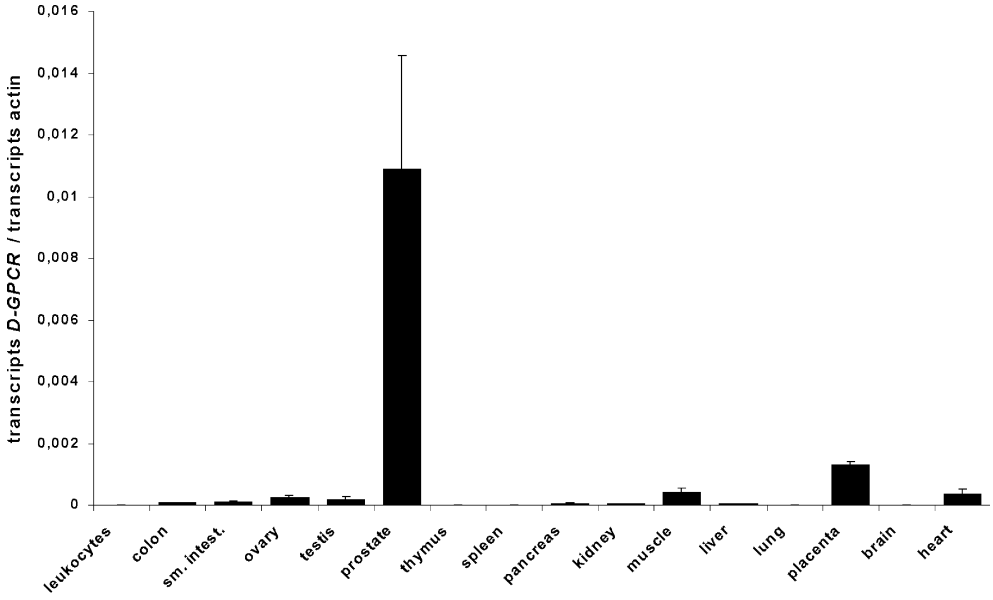

Fig. 5. Real-time PCR analysis of *D-GPCR* expression in human tissues. The tissue specificity of *D-GPCR* mRNA was determined by real-time PCR of pooled cDNA samples derived from 16 different human tissues. The *D-GPCR* transcript quantity was normalized to β-actin mRNA quantity that was also measured by real-time PCR. The results represent means of two independent LC runs, bars indicate SE.

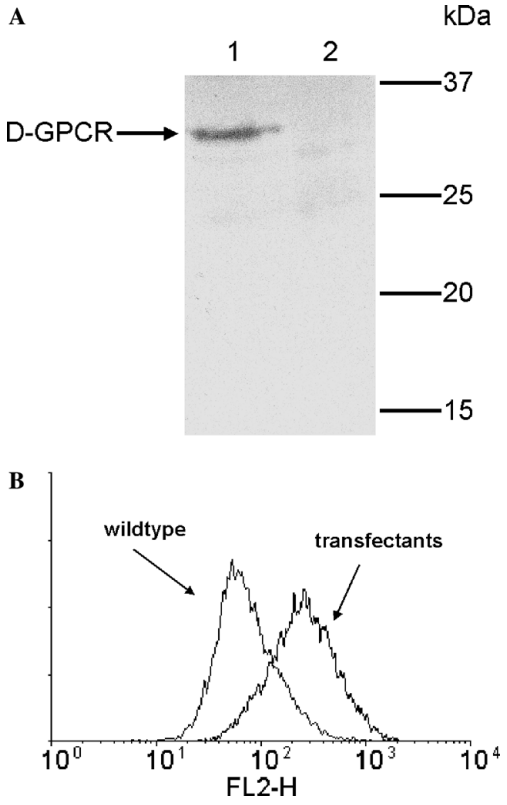

Fig. 7. Analysis of the D-GPCR protein. (A) The His-tagged D-GPCR was detected at about 32 kDa on a reducing 12.5% SDS–PAGE. Lane 1 shows the lysate from HEK293 cells transfected with His-tagged D-GPCR, whereas lane 2 contains the mock-transfected control lysate. Immunodetection was performed with anti-penta-His antibody. (B) FACS analysis of D-GPCR expression in stably transfected PC-3 prostate cancer cells. Single parameter histograms for the wild-type PC-3 cells (solid line; left peak) and D-GPCR transfected PC-3 cells (solid line; right peak) are shown. Cells were incubated with the mouse anti-human D-GPCR antiserum, followed by R-phycoerythrin labeled goat anti-mouse IgG. Mean fluorescence intensities (MFI) were 96 for the wild-type cells and 325 for the transfectants, respectively. Incubation of the transfectants with preimmune serum resulted in an MFI of 100 (data not shown).

cells were transfected with vector encoding D-GPCR or with empty vector, respectively, and lysates were analyzed after 48 h by Western blotting. Immunodetection revealed the presence of the His-tagged 32 kDa D-GPCR protein only in the transfectants but not in the mock-transfected parental cell line (Fig. 7A).

Membrane localization of D-GPCR in prostate cells

We determined the subcellular localization of D-GPCR in transfected PC-3 prostate cancer cells stably expressing the protein by FACS analysis. Transfectants as well as wild-type PC-3 cells were incubated with a D-GPCR-specific antiserum in the absence of a permeabilizing agent and subjected to FACS analysis as described in Materials and methods. The signifcantly higher fluorescence intensity of the transfectants indicates that the D-GPCR protein is associated with the plasma membrane of the cells (Fig. 7B).

Genomic localization of D-GPCR

Based on in silico genomic analysis, the gene for *D-GPCR* is located on chromosome 11p15 at a distance of 30 kb from *PSGR* but in opposite orientation. *D-GPCR* and *PSGR* represent members of a large cluster of GPCR genes and pseudogenes on chromosome 11p15. Analysis of a 170 kb genomic clone containing the *D-GPCR* sequence (GenBank Accession No. AC090719) with the Genscan 1.0 algorithm (http://genes.mit.edu/cgi-bin/genscanw.cgi) predicted the same ORF as found experimentally for *D-GPCR*. Its mRNA is coded by two exons located at nucleotide positions 64854–64806 and 56350–53354, respectively, on the reverse strand of clone AC090719.

Expression in matched samples of malignant and non-malignant prostate tissues and tumor cell lines

Because gene expression is often differentially regulated in normal and the corresponding malignant tissue, expression of *D-GPCR* was further analyzed by hybridization of the 402 bp probe to a cancer profiling array I (CPA I). On the CPA I, normalized cDNA from 241 individual cancer patients is immobilized as paired dots from the tumor and corresponding normal tissue. *D-GPCR* expression was detected in all four malignant prostate specimens and in two of the corresponding normal samples (Fig. 8). Expression was downregulated in tissue pair No. 4 and was upregulated in the other three pairs of samples. In addition, strong hybridization signals were obtained in one colonic adenocarcinoma and in one renal tumor (samples #1 and #2 in Fig. 8, respectively). Interestingly, overexpression of the highly prostate-specific transcript *D-PCa-2* [27] was previously demonstrated in the latter one, characterized as a benign tumor of the kidney (oncocytoma). Very weak expression of *D-GPCR* was also detected in six colon cancer samples, three kidney tumor specimens, and three tumors of the rectum, but in none of the corresponding normal tissues.

Expression of *D-GPCR* was further characterized in a group of PCa patients. In order to evaluate a possible correlation of clinical or pathological parameters of tumor progression with *D-GPCR* expression, transcripts were quantified in paired malignant and non-malignant prostate tissue specimens from 15 PCa patients using a Q-RT-PCR assay. As documented in Fig. 9, *D-GPCR* transcripts could be detected in all normal and in 14 out of 15 malignant prostate tissue samples. In the tumor specimen originating from patient #6, no *D-GPCR* expression was found. Staging and grading of the analyzed tumor specimens are summarized in Table 1. The

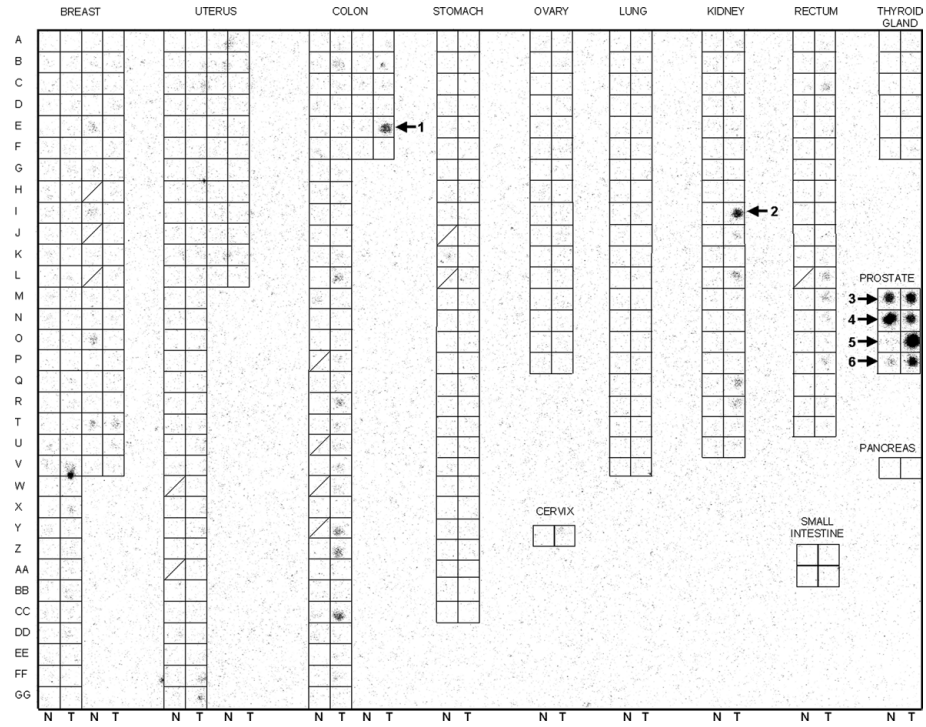

Fig. 8. Analysis of *D-GPCR* expression in paired samples of normal and tumorous tissues. The 402 bp cDNA fragment of *D-GPCR* was labeled radioactively and hybridized to a cancer profiling array I that contains paired samples of normalized cDNA from 241 tumor and corresponding normal tissues from individual patients. High expression was detected in all prostate tumor specimens and in some of the corresponding normal prostate samples (patients #3 and #4). In addition, strong hybridization signals were obtained in one colonic adenocarcinoma (patient #1) and in one renal tumor (patient #2). N, non-tumorous samples; T, tumorous samples. Fields with a diagonal bar did not contain cDNA. The fields to their right display metastatic specimens from the patients above.

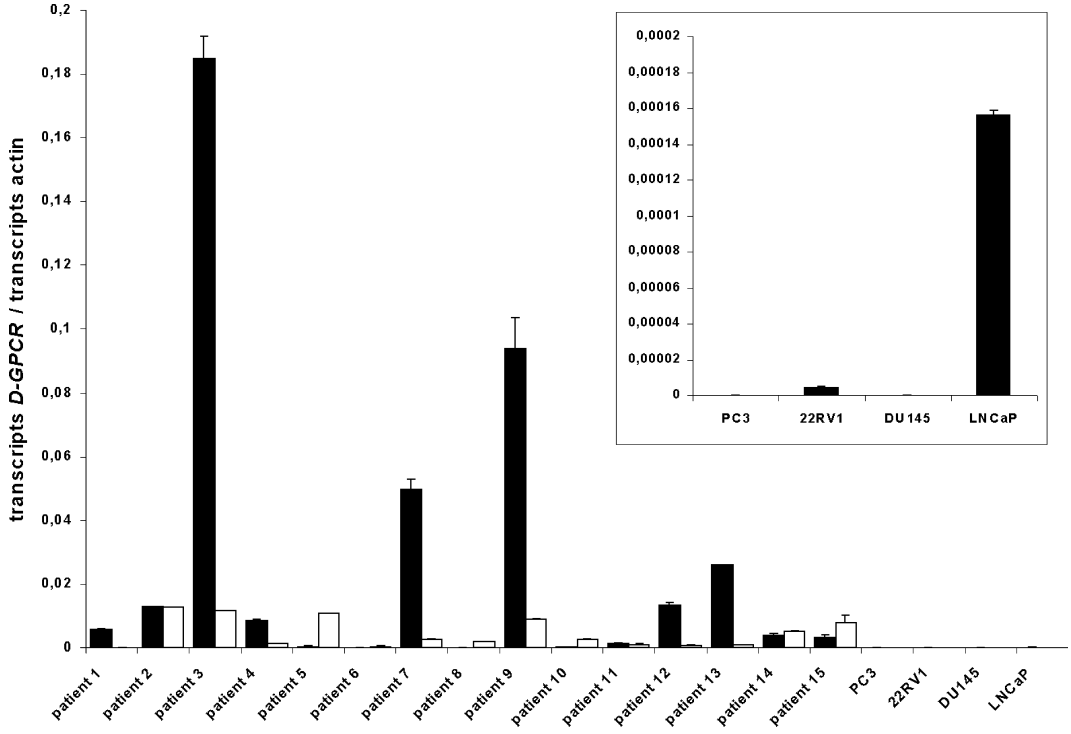

Fig. 9. Real-time PCR analysis of *D-GPCR* mRNA expression in matched samples of malignant and non-malignant prostate tissues. The *D-GPCR* mRNA was quantified in paired cDNA samples of tumorous (black columns) and non-tumorous (white columns) prostate tissue from PCa patients by applying the same real-time PCR protocol as described in Fig. 5. The transcript quantity was normalized to the expression level of β-actin. For clarification, expression in PCa cell lines is also shown at a different scale (see inset). The results represent means of two independent LC runs. Bars reflect SE.

relative transcript levels were found to be highly variable among patients' samples and the relation of transcript quantity in the tumorous tissues to that in the non-malignant samples did not show obvious correlation with pathological and clinical parameters of cancer progression (Fig. 9 and Table 1). However, in concordance with the data from GeneExpress, mean expression of *D-GPCR* was almost 6-fold higher in tumorous specimens (0.0269 transcripts *D-GPCR* per transcript actin) compared to the non-tumorous samples (0.0045 transcripts *D-GPCR* per transcript actin).

Quantitative analysis of *D-GPCR* transcripts in PCa cell lines demonstrated low expression in LNCaP, very weak expression in 22RV1, and the lack of *D-GPCR* transcripts in DU145 and PC-3. Remarkably, the relative expression in LNCaP is about two orders of magnitude lower when compared to the malignant prostate samples (Fig. 9). Hormone-induction experiments in the androgen-sensitive cell line LNCaP were conducted according to [28] using the synthetic androgen R1881 (methyltrienolone; 1 nM). The level of *D-GPCR* transcripts did not increase significantly within 48 h whereas PSA transcript levels increased dramatically, indicating that *D-GPCR* is not an early response androgen-inducible gene (data not shown).

Discussion

Here we describe the identification and characterization of a novel putative GPCR, D-GPCR, that is overexpressed in PCa and prostate. The tissue-restricted expression pattern was demonstrated by RNA-dot blot analysis and was quantified by Q-RT-PCR. Examination of 15 paired tissue samples from PCa patients demonstrated expression in all normal prostate specimens and in almost all PCa samples.

GPCRs have attained growing attention in the context of tumor formation since they have been shown to participate in the Rho/Cdc42/Rac and MAPK signaling pathways implicating a role in cell growth and proliferation [29,30]. For example, the receptor for serotonin 1C as well as adrenergic α1 receptors and muscarinic m1, m3, and m5 receptors have been demonstrated to transform contact-inhibited cultures of rodent fibroblasts when persistently activated, thereby suggesting that GPCRs can behave like agonist-dependent oncogenes (reviewed in [31]). Many GPCRs, including those with transforming capacity, can stimulate Rho-dependent pathways by acting either on G_q or on specific members of the G_{12} family of heterotrimeric G proteins [30]. In fact, increasing evidence argues for a direct correlation between aberrant GPCR signaling and the development and progression of human cancers (reviewed in [32]). In PCa, endothelin 1A receptor, lysophosphatidic acid receptor, PSGR, and bradykinin 1 receptor are overexpressed (reviewed in [19]). In advanced, androgen-independent PCa higher levels of endothelin-receptors are detected in comparison to localized PCa [21]. Although the significance of these elevated GPCRs in the context of PCa formation and/or progression is not yet clearly explained, the finding that inhibition of Gβγ signaling retards growth of prostate tumor xenografts [22] contributed to the increasing belief that there exists a functional link between GPCRs and PCa. In a phase II clinical trial atrasentan, a selective endothelin receptor antagonist, delayed progression in patients with metastatic, hormone-refractory PCa [33].

D-GPCR shows high homology to PSGR and represents the second example of a mainly prostate-localized GPCR. Future studies are required to determine the significance of ORs in the prostate or in other tissues outside the nasal epithelium. PSGR has been demonstrated to interact with $G\alpha_{12}$ in the yeast two-hybrid system and it will be interesting to investigate if D-GPCR partners with the same protein [16]. Recently, Gαolf, a G protein expressed not only in the olfactory neuroepithelium, but also in peripheral tissues, has been demonstrated to promote cellular invasion and neuroendocrine differentiation of LNCaP cells, leading to a more aggressive tumor phenotype [34]. The recent finding that targeting GPCR signaling by inhibitory peptides inhibits prostate tumor formation and growth emphasizes the significance of GPCRs and their concomitant signaling networks as potential molecular therapy tools [22].

When comparing the mean transcript levels of 15 prostate cancers to their non-tumorous counterparts, *D-GPCR* was almost 6-fold upregulated although we could not detect a general upregulation of *D-GPCR* in each individual malignant specimen of the paired samples. This reflects the fact that, during our initial screening of the expression database, we put emphasis on tissue selectivity of expressed transcripts rather than on upregulation of expression in tumorous versus normal prostate tissue.

The tissue-restricted expression pattern makes D-GPCR a potential candidate for novel therapies in PCa treatment, for example cell-mediated or antibody-based immunotherapeutic approaches or other approaches using molecular targets. Vaccination of tumor patients with tumor-associated antigen (TAA) loaded dendritic cells has been proven useful for the treatment of advanced PCa in animal models and in phase I/phase II clinical studies (reviewed in [35]). Furthermore, it is well known that a tumor vaccine comprising different TAAs is more effective than using a single antigen. Therefore, the identification of additional genes overexpressed in a large proportion of PCa is required to enable the development of a widely applicable polyvalent vaccine [36]. In this light, D-GPCR may be a useful molecular target, although D-GPCR expression in each sample has to be quantified upfront because of the inter-individual variation.

Critical issues in the evaluation of target genes are the degree of tissue-specificity and the expression in a high percentage of certain tumor entities. Here we demonstrated that *D-GPCR* is expressed in a very high percentage of PCa specimens (14/15) and that expression in normal prostate is about 27-fold higher than in skeletal muscle and about 31-fold higher than in heart, the two non-prostatic male tissues with highest expression. If one takes into account that overall mean expression in the 15 PCa samples was almost 6-fold higher compared to the mean transcription level in the respective non-tumorous samples, the overexpression of *D-GPCR* in prostate tumors in comparison to non-prostatic normal tissues is even more pronounced. We therefore conclude that D-GPCR may be a well-suited target for cellular immunotherapy provided that immunogenic, MHC class I binding peptides can be identified.

The biological function of D-GPCR has not yet been elucidated. Future studies seeking to determine the signals transduced by D-GPCR in the prostate gland will address this issue.

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