Expression of CaT-like, a Novel Calcium-selective Channel, Correlates with the Malignancy of Prostate Cancer*

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The regulation of intracellular Ca\(^{2+}\) plays a key role in the development and growth of cells. Here we report the cloning and functional expression of a highly calcium-selective channel localized on the human chromosome 7. The sequence of the new channel is structurally related to the gene product of the CaT1 protein cloned from rat duodenum and is therefore called CaT-like (CaT-L). CaT-L is expressed in locally advanced prostate cancer, metastatic and androgen-insensitive prostatic lesions but is undetectable in healthy prostate tissue and benign prostatic hyperplasia. Additionally, CaT-L is expressed in normal placenta, exocrine pancreas, and salivary glands. New markers with well defined biological function that correlate with aberrant cell growth are needed for the molecular staging of cancer and to predict the clinical outcome. The human CaT-L channel represents a marker for prostate cancer progression and may serve as a target for therapeutic strategies.

The link between ion channels and disease has received widespread attention in the last few years as mutations in several ion channels have been shown to be responsible for various forms of neurological disorders (1, 2). Whereas many of these mutations affect well characterized channels of the nervous system, little is known about the situation in non-excitable cells. One new superfamily of channels of widespread expression and function include channels of the Trp family. The prototypical members of this family of six transmembrane domain channel subunits come from the visual system of Drosophila where they have been shown to be responsible for the light-activated cationic conductance changes (3). Other members of these growing family of ion channels include osmo- and mechanosensitive ion channels (4, 5), channels responsible for pain and heat perception like the vanilloid receptors (6, 7), and channels involved in agonist/receptor activated cation influx (8) into cells such as Trp-1 to Trp-7. Also new on the scene are the epithelial Ca\(^{2+}\) channel, ECaC\(^{-1}\) (also ECaC1 (9)), and the Ca\(^{2+}\) transport protein CaT1 (also ECaC2, (10)), implicated to play a role in the reabsorption of Ca\(^{2+}\) by the kidney (ECaC) and intestinal epithelial cells (ECaC and CaT1).

Two other identified members of this family of Trp-related proteins, p120 and melastatin, have not yet been demonstrated to function as ion channels. One of these genes, p120 (11), when overexpressed, appears to interfere with normal cell growth, whereas the second, melastatin (12), is abundantly expressed in benign cutaneous nevi but appears to be down-regulated in primary melanomas and, especially, in metastatic lesions.

Here we report the cloning of a new human gene product that is structurally related to the rat CaT1 cDNA and that we tentatively called Ca\(^{2+}\) transport protein-like (CaT-L). Unlike CaT1 and ECaC, CaT-L is not expressed in the small intestine (CaT1, ECaC (10, 13)), in colon (CaT1 (10)) and in the kidney (ECaC (9, 13)). CaT-L is abundantly expressed in the placenta, pancreatic acinar cells, and salivary glands. So far, little is known of the CaT-L entry pathways in these tissues. The Ca\(^{2+}\)-permeation properties of the CaT-L channel, shown here, renders CaT-L as a good candidate for secretion coupling in these tissues. Most interesting, the CaT-L transcripts are undetectable in benign prostate tissue but are present at high levels in locally advanced prostate cancer, metastatic lesions, and recurrent androgen-insensitive prostatic adenocarcinoma. Hence, molecular classification of prostate cancer subclasses and class prediction by monitoring the level of human CaT-L gene expression is feasible. In addition, functional characterization of the new Ca\(^{2+}\) channel suggests a possible link between Ca\(^{2+}\) signaling and prostate cancer progression.

EXPERIMENTAL PROCEDURES

Cloning of the CaT-L cDNA from Human Placenta—Total RNA was isolated from human placenta as described (14), and poly(A)\(^{+}\) RNA was obtained using poly(A)\(^{+}\) RNA spin columns (New England Biolabs, Beverly, MA) according to the manufacturer’s instructions. To obtain an oligo(dT)-primed cDNA library, placenta poly(A)\(^{+}\) RNA was reverse-transcribed using the cDNA choice system (Life Technologies, Inc.), and the resulting cDNA was subcloned in l-Zap phages (Stratagene, La Jolla, CA). After screening the library with the human expressed sequence tag 1404042 (GenBank\(^{TM}\)), several cDNA clones were identified, isolated, and sequenced. Additional cDNA clones were isolated from two specifically primed cDNA libraries from a second placenta using primers corresponding to amino acids 677-680LPM and 271-GPLTSL of the CaT-L sequence (Fig. 1a) and the 345-bp Ncol/BamHI and 596-bp EcoRI/SalI cDNA fragments of CaT-L as probes. Thirteen independent cDNA clones were sequenced on both strands. In addition the complete coding region of the CaT-L protein was amplified by PCR, using human cDNA isolated from placenta as template, and eight independent cDNA clones were sequenced on both strands. The nucleotide sequences of CaT-La and CaT-Lb have been deposited in DDBJ/EMBL/GenBank\(^{TM}\) under the accession numbers AJ243500 and AJ243501, respectively.

Northern Blot Analysis—For Northern blot analysis 5 \(\mu\)g of human poly(A)\(^{+}\) RNA from human placenta and from prostate (obtained from patients undergoing transurethral prostatectomy because of benign prostatic hyperplasia) were separated by electrophoresis on 0.8% agarose gels and thereafter transferred to Hybond N nylon membranes (Amersham Pharmacia Biotech) as described (14). The membranes were hybridized in the presence of 50% formamide at 42 °C overnight.

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FIG. 1. Primary structure of human CaT-L. a, alignment of the deduced amino acid sequence of CaT-L with the sequences of human ECaC, rat CaT1, and the rat vanilloid receptor Vr1 (GenBank™ accession numbers AF160798, AJ401155, and T09054). CaT-La and CaT-Lb arise due to polymorphism of the CaT-L gene (see “Results”). Amino acid residues are numbered on the right. Residues within CaT-L identical to ECaC, CaT1, and Vr1, the putative transmembrane segments (S1–S6), the putative pore region, and the partial CaT-L2 sequence obtained by reverse transcriptase-PCR (see “Results”) are indicated. b, hydropathy profile (46) of CaT-L. Transmembrane segments S1–S6 were defined as regions with a hydropathy index ≥1.5 using a window of 19 amino acids. c, predicted membrane topology of the CaT-L protein. Putative ankyrin repeats (A), an N-glycosylation site (branched circles) and protein kinase C phosphorylation sites (circled p) are indicated. The positions of amino acid exchanges of CaT-La and CaT-Lb are symbolized by M. d, phylogenetic tree based on the full-length cDNA sequences of CaT-L-related mammalian gene products ECaC (32), CaT1 (10), SIC (47), Vr1 (6), VRL (7), and GRC (48), respectively.
Alternatively, a human multiple tissue RNA blot (CLONTECH) was hybridized under the same conditions. The probe was a 345-bp EcoRI/BamHI fragment spanning the protein coding region of amino acid residues 528–643 of the CaT-L protein (Fig. 1a), labeled by random priming with [a-32P]dCTP. Filters were exposed to x-ray films for 4 days.

Construction of Expression Plasmids and Transfection of HEK Cells—To obtain the recombinant dicistronic expression plasmid pdiCaT-L carrying the entire protein-coding regions of CaT-Lb and the GFP (15), the 5′- and 3′-untranslated sequences of the CaT-Lb cDNA were removed, and the consensus sequence for initiation of translation in vertebrates (16) was introduced immediately 5′ of the translation initiation codon; and the resulting cDNA was subcloned into the pcAGGS vector (17), downstream of the chicken β-actin promoter. The internal ribosomal entry site derived from encephalomyocarditis virus (18), followed by the GFP cDNA containing a Ser-65 → Thr mutation (19), was then cloned 3′ to the CaT-Lb cDNA. The internal ribosomal entry site sequence allows the simultaneous translation of CaT-Lb and GFP from one transcript. Thus, transfected cells can be detected unequivocally by the development of green fluorescence. Human embryonic kidney (HEK) 293 cells (ATCC CRL 1573) were transfected with pdiCaT-L using lipofectamine (Qiagen, Hilden, Germany) as described (20).

For measuring [Ca2+]i, HEK cells were cotransfected with the pcDNA3-CaT-Lb and pcDNA3-GFP (21) in a ratio of 4:1. To obtain pcDNA3-CaT-Lb the entire protein coding region of CaT-Lb including the consensus sequence for initiation of translation in vertebrates (16) was subcloned into the pcDNA3 vector (Invitrogen, Groningen, Netherlands). Measurements of [Ca2+]i, and patch clamp experiments were carried out 2 days and 1 day after transfection, respectively.

Chromosomal Localization of the CaT-L Gene—The chromosomal localization of the human CaT-L gene was performed using NIGMS somatic hybrid mapping panel 2 (Coriell Institute, Camden, NJ) described previously (22, 23) and primers corresponding to amino acids 115VEGQTA and 139NLIVYG of the CaT-L sequence (Fig. 3a).

Electrophysiological Recordings—Patch clamp recordings on single transfected cells were performed at 22–25 °C in the tight seal whole-cell configuration using fire-polished patch pipettes (3–10 MΩ uncompensated series resistance) 2 days after transfection. Pipette and cell capacitance were electronically canceled before each voltage ramp. Membrane currents were filtered at 1.5 kHz and digitized at a sampling rate of 5–10 kHz. To analyze transfected cells, currents were recorded with an EPC-9 patch clamp amplifier controlled by Pulse 8.3 software (HEKA Electronics). The pipette solution contained (in mM) the following: 140 aspartic acid, 10 EGTA, 10 NaCl, 1 MgCl2, 10 Hepes (pH 7.2 with NaOH). The bath solution contained (in mM) the following: 110 NaCl, 10 CaCl2, 2 MgCl2, 50 mannitol, 10 glucose, 20 Hepes (pH 7, 4 with CsOH) and 2 CaCl2 (–Ca2+ solution). Divalent free bath solution contained (in mM) the following: 116 NaCl, 10 CsCl, 50 mannitol, 10 glucose, 20 Hepes, 1 EGTA (pH 7, 4 with CaOH) and bath solution without Na+ contained 110 N-methyl-D-glucamine instead of NaCl. Whole-cell currents were recorded every second by applying 200-ms voltage clamp ramps from –100 to +100 mV from a holding potential of either –40 or +70 mV. The holding potential of +70 mV, which reduces Ca2+ influx, in combination with high internal EGTA was used to minimize Ca2+ dependent feedback mechanisms. Data are given as mean ± S.E. Values were not corrected for liquid junction potentials. Measured currents were normalized to cell capacitance, i.e. –25.3 ± 0.4 pA/pF for CaT-L-transfected cells (n = 12) and 1.56 ± 0.54 pA/pF for GFP controls (n = 6) at −80-mV ramp potential in normal bath solution.

Measurements of [Ca2+]i in Transiently Transfected HEK Cells—Measurements of [Ca2+]i in single HEK cells were performed with a digital imaging system (T.L.L. Photonics). Cells grown on coverslips were loaded with 4 μM fura-2/AM (Molecular Probes, Eugene, OR) for 60 min at 37 °C in minimal essential medium containing 10% fetal calf serum. Cells were washed three times with 300 μl of buffer containing 115 mM NaCl, 2 mM MgCl2, 5 mM KCl, 10 mM Hepes (pH 7.4). Nominal Ca2+-free solutions contained ~2 μM Ca2+. [Ca2+]i was calculated from the fluorescence ratios obtained at 340 and 380 nm excitation wavelengths as described (24). Experiments were repeated three times.

In Situ Hybridization Analysis—Sense and antisense oligodeoxynucleotides corresponding to the amino acid residues 115LILCLWSK, 139QDLNRKQR, and 159FFHRGSED of the CaT-L sequence (Fig. 1a) were synthesized. Using the BLAST sequence similarity search tool provided by the National Center for Biotechnology Information (Bethesda, MD), the antisense sequences show maximal similarity of ~71% to sequences in the GenBank™ data base. The oligodeoxynucleotides used for hybridization were biotinylated at the 3′ end.

Fig. 2. Expression of CaT-L mRNA in human tissues. a, autoradiogram of blot hybridization analysis (lower panel, signals after hybridization of human β-actin cDNA as control to the same filters). B–i, in situ hybridization reveals high steady state levels of CaT-L mRNA in trophoblasts and syncytiotrophoblasts of the normal placenta (b). Original magnifications are as follows: × 25 (left) and ×100 (right). Strong expression of CaT-L transcripts are detected in acinar structures of the normal pancreas, whereas pancreatic ductal epithelial cells (arrow) and Langhans islets (asterisk) lack CaT-L mRNA. Original magnifications are as follows: × 25 (left) and × 200 (right). In salivary glands CaT-L mRNA expression is restricted to subsets of myoepithelial cells (d). Original magnification, ×100. In situ hybridization analyses performed in adjacent tissue sections with the sense probe were distinctly negative (e–g). CaT-L mRNA expression was undetectable in other human tissues investigated, including the colon mucosa (h) and the normal kidney (i). Original magnifications are as follows: h, ×100; i, × 25 (left) and ×100 (right).
**FIG. 3.** Polymorphism of the human CaT-L gene. a, three of the five nucleotide substitutions result in changes of the encoded amino acid (aa) residues yielding the CaT-L variants a and b (see “Results”). b, PCR amplification of a 458-bp CaT-L fragment from human genomic DNA and expected fragments after cutting with Bsp1286I (b). c, genotyping of 12 human individuals. Both classes of DNA were amplified. Primers are indicated by arrows. The nucleotide a1080g substitution in the CaT-Lb DNA generates a recognition site for the restriction enzyme Bsp1286I. The resulting Bsp1286I fragments were separated by polyacrylamide gel electrophoresis. nt, nucleotide; aa, amino acid; M, methionine; C, cysteine; V, valine; T, threonine, A, arginine.

**RESULTS**

Primary Structure of Human CaT-L—In search of proteins distantly related to the Trp family of ion channels, a human expressed sequence tag (EST 1404042) was identified in the GenBank™ database using BLAST programs (29). This EST was used as a probe to screen oligo(dT) and additional specifically primed human placenta cDNA libraries. Several positive cDNA clones were isolated, sequenced, and found to contain the complete sequence of the EST 1404042 clone as well as additional 5′-sequences. These clones cover an mRNA of about 2.9 kb with an open reading frame of 2175 bases (Fig. 1a) encoding a protein of 725 amino acid residues that we tentatively called human Ca2+ transport protein-like (CaT-L). Downstream of the CaT-L coding sequence an additional open reading frame has been postulated (GenBank™ accession number X83877) to represent a zinc finger type DNA-binding protein. The functional significance of this putative gene product is not known.

Hydropathy analysis reveals a hydrophobic core in the CaT-L protein with six peaks likely to represent membrane-spanning helices (S1 to S6) and a putative pore region between S5 and S6 (Fig. 1b). The hydrophobic core is flanked by long presumptive cytoplasmic domains at the N and C termini (Fig. 1c). A similar topology has been proposed for the light-activated ion channels in the Drosophila compound eye, Trp and TrpL, and related nematode and mammalian gene products (21, 30).

As shown in Fig. 1, a and d, amino acid sequence comparisons place human CaT-L in close relationship to the rat intestine Ca2+ transport protein (CaT1 (10)) and the human renal epithelial Ca2+ channel (ECaC (9, 32)), sharing 90% (rat CaT1) and 77% (human ECaC) overall amino acid sequence identity. More distantly related members of this gene family include non-selective cation channels such as the rat vanilloid receptors VR1 and VRL that share common amino acid sequence motives (21), although overall sequence identity is low (VR1, 28%; VRL, 27%).

The non-radioactive in situ hybridization method was carried out as described (25) using formalin-fixed slices of 6–8 μm thickness. Briefly, the slices were deparaffinized, rehydrated in graded alcohols, and incubated in the presence of PBS buffer including 10 μg/ml proteinase K (Roche Molecular Biochemicals) for 0.5 h. After prehybridization, the slices were hybridized at 37 °C using the biotinylated deoxyoligonucleotides (0.5 pmol/μl) in the presence of 33% formamide for 12 h. Thereafter, the slices were rinsed several times with 2× SSC and incubated at 25 °C for 0.5 h with avidin/biotinylated tyramide peroxidase complex (ABC, Dako). After several washes with PBS buffer, the slices were incubated in the presence of biotinylated tyramide and peroxide (0.15% w/v) for 10 min, rinsed with PBS buffer, and additionally incubated with ABC for 0.5 h. The slices were then incubated with PBS buffer and incubated in the presence of DAB solution (diaminobenzidine (50 mg/ml), 50 mM Tris/EDTA buffer, pH 8.4, 0.15% H2O2 in N,N-dimethylformamide, Merck). The reaction was stopped after 4 min by incubating the slides in water. Biotinylated tyramide was obtained by incubating NHS-LC biotin (sulfosuccinimidyl-6-[biotinimid]-hexanoate, 2.5 mg/ml, Pierce) and tyramine-HCl (0.75 mg/ml, Sigma) in 25 mM borate buffer (pH 8.5) for 12 h. The tyramide solution was diluted 1000-fold (v/v) in PBS buffer before use.

**Tissue Selection**—Normal human tissue included placenta (n = 2), prostate tissue (n = 2), colon (n = 2), stomach (n = 2), lung (n = 2), kidney (n = 2), endometrium (n = 2), salivary glands (n = 2), pancreas (n = 2), and parathyroid glands (n = 2). Transurethral resections with benign prostatic hyperplasia were obtained from three patients without clinical and pathological evidence of malignancy. Prostate cancer tissue from five radical prostatectomy specimens was submitted for study. The pathological stages and grades included pT2a (n = 2), pT2b (n = 2), pT2c (n = 1), and primary Gleason grades 5 (n = 2), 4 (n = 2), and 3 (n = 2). Four foci of high grade prostatic intraepithelial neoplasia were identified in the radical prostatectomy specimens. Lymph node metastases were obtained from five staging lymphadenectomies without subsequent prostatectomy. The material further contained palliative transurethral resection specimens from five patients with recurrent androgen-insensitive adenocarcinomas after orchietomy. All specimens were available as formalin-fixed paraffin-embedded tissue sections.

**Miscellaneous Methods**—Sequences were analyzed using the Heidelberg Unix Sequence Analysis Resources of the biocomputing unit at the German Cancer Research Center, Heidelberg. The phylogenetic distances of proteins were calculated with the ClustalW algorithm (28). Photographs were scanned and processed using Corel Photo-Paint/Corel Draw and Adobe PhotoShop.

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2 N. Tomilin and V., Boyko, unpublished results.
Expression of CaT-L Transcripts in Human Tissues—To investigate CaT-L expression, Northern analysis was performed using poly(A)⁺ RNA from different human tissues and a 345-bp EcoRI/BamHI fragment of CaT-L cDNA as a probe (Fig. 2a).

We found that CaT-L transcripts of 3.0 kb are expressed in placenta, pancreas, and prostate. The size of these transcripts corresponds to the size of the cloned CaT-L cDNA (2902 bp). In addition, a shorter transcript of 1.8 kb is detectable in poly(A)⁺ RNA isolated from human testis, which may result from alternative mRNA processing in this tissue. No CaT-L transcripts were detected in heart, lung, liver, skeletal muscle, spleen, ovary, and leukocytes. Interestingly, no CaT-L transcripts could be detected in small intestine, where both CaT1 and ECaC transcripts have been detected, nor in colon and brain (CaT1) or in kidney (ECaC) where these transcripts are predominantly expressed. The lack of CaT-L expression in human kidney and intestine suggests that CaT-L does not serve the physiological functions in these tissues that have been associated with the ECaC and CaT1 proteins and that include intestinal and renal Ca²⁺ absorption. Therefore, CaT-L is unlikely to represent the human ortholog of rat CaT1. A human cDNA sequence of 446 bp has been deposited to the GenBank™ data base (accession number AJ277909) that is identical to the corresponding sequence reported here. This sequence has been postulated to represent part of human CaT1, but no data are available that support this suggestion. Interestingly a 115-bp fragment, tentatively called CaT-Like2 (CaT-L2), was amplified from human genomic DNA and sequenced. It encodes an amino acid sequence (Fig. 1a) that shares 92% sequence identity with human CaT-L, 95% with human ECaC, and 81% with rat CaT1 sequences and may represent a part of an additional ECaC/CaT1-related channel.

To characterize further the cell-specific expression of CaT-L transcripts, in situ hybridization experiments were performed, using various human tissue sections (Fig. 2, b–i) obtained from placenta (taken from a 10-week-old abort), pancreas (removed from patients with pancreatic cancer), salivary gland, colon, and kidney. In the placenta (Fig. 2b) CaT-L transcripts are expressed in trophoblasts and syncytiotrophoblasts. In the pancreas (Fig. 2c) CaT-L transcripts are restricted to acinar cells and are not detectable in ductal epithelial cells and Langerhans islets. No CaT-L expression was found in regions of pancreatic carcinomas (data not shown). In salivary glands, CaT-L expression occurs in subsets of myoepithelial cells (Fig. 2d).

Corresponding to the results obtained by Northern blot analysis, no CaT-L transcripts could be detected in tissue sections of human colon (Fig. 2h) and human kidney (Fig. 2i). In addition no transcripts could be detected in stomach, endometrium, lung, and parathyroid gland (data not shown).

Polymorphism and Chromosomal Localization of the Human CaT-L Gene—Comparison of the DNA sequences of the various CaT-L cDNA clones obtained from a human placenta revealed that the sequences could be grouped into two classes, which differ in five nucleotide substitutions (Fig. 3a). Three of the five substitutions resulted in changes of the encoded amino acid residues, whereas two nucleotide substitutions were silent (a1080g and g1878a). The resulting two protein sequences that differ in three amino acid residues (R157C, V378M, and D390V) were called CaT-La and CaT-Lb (Figs. 1a and 3a). This finding was reproduced by isolating CaT-La and b cDNA clones from a second placenta. In addition, PCR amplification of the full-length CaT-L cDNA from a third placenta yielded only the b variant when eight amplified full-length CaT-L cDNAs were subcloned and sequenced.

The nucleotide substitutions may reflect a coupled polymorphism; alternatively, the underlying mRNAs of the two cDNA classes may be products of different gene loci or may arise by RNA editing. To distinguish between these possibilities, we first designed a primer pair common to both CaT-L a and b isoforms that flanked the silent substitution a1080g and the substitution that leads to the amino acid exchange V378M. We then PCR-amplified a DNA fragment of 458 bp from genomic DNA isolated from human T-lymphocytes. Both classes of DNAs were amplified, and both amplification products contained a common intron sequence of 303 bp (Fig. 3b).

The a1080g substitution in the CaT-Lb DNA generates a new rec-
ognition site for the restriction enzyme Bsp1286I (Fig. 3b). Accordingly, genomic DNA isolated from blood cells of 12 healthy male human individuals was used as template to amplify the 458-bp DNA fragment, and the amplified DNAs were then incubated in the presence of Bsp1286I. In 11 out of 12 individuals the expected DNA fragments of the CaT-Lb variant could be identified, whereas one individual contained both a and b variants (Fig. 3c). In summary, these findings suggest that the two CaT-L variants may be due to a coupled polymorphism of one gene locus. By using a monochromosomal hybrid mapping panel, this locus was assigned to human chromosome 7 (data not shown).

**CaT-L Is a Ca**$$^{2+}$$**-selective Cation Channel**—To characterize the electrophysiological properties of CaT-L, CaT-L and GFP were co-expressed in HEK cells using the dicistronic expression vector pdiCaT-L. Whereas only small background currents were observed under control conditions (GFP alone), large inwardly rectifying currents could be recorded in CaT-L-transfected HEK cells after establishing the whole-cell configuration (Fig. 4, a–e), indicating that CaT-L forms constitutively active ion channels. Switching the holding potential from the initial −40 to +70 mV, currents increased dramatically in size (Fig. 4, a and e). This increase in current size with a change in holding potential was not observed for sodium currents (at zero extracellular divalent ions) and indicates that CaT-L may be partially inactivated by intracellular Ca$$^{2+}$$. For the following experiments voltage ramps were applied from a holding potential of +70 mV. Although the initial characterization of CaT-L currents was reminiscent of currents mediated by ECaC (33–35), the sequence differences led us to a more detailed investigation of CaT-L selectivity. CaT-L-specific currents were completely abolished following removal of external Ca$$^{2+}$$ (Fig. 4, a and c) but slightly increased when external Na$$^{+}$$ was removed (summarized in Fig. 4e). The ion exchange experiments and the inwardly rectifying current-voltage relationship with the rather positive reversal potential (E$$\text{rev}_\text{in}$$) provide strong evidence that CaT-L forms Ca$$^{2+}$$-selective ion channels (Fig. 4, a, c, and e). The Ca$$^{2+}$$ selectivity, as defined by the E$$\text{rev}_\text{in}$$, becomes even more evident (Fig. 4e, inset) if the background current is subtracted (background current defined as the remaining current

**FIG. 5. CaT-L is expressed in malignant lesions of the prostate but not in benign prostate tissues.** a, autoradiogram of blot hybridization analysis using poly(A)$^{\text{+}}$ RNA isolated from human placenta and prostate tissue obtained from 20 patients with benign prostate hyperplasia and without clinical and pathological evidence of malignancy (*, upper panel). CaT-L transcripts are present in placenta but, in contrast to Fig. 2a, are absent in prostate (lower panel, signals after hybridization of human β-actin cDNA as control to the same filters). The absence of CaT-L mRNA expression in normal adult prostate tissue (b) and benign prostatic hyperplasia (c) was confirmed by in situ hybridization analyses. Original magnifications are as follows: × 25 (left) and × 100 (right). Primary prostatic adenocarcinoma (Gleason score: 3 + 4 = 7) with extraprostatic extension (pT3a) reveals high steady state levels of CaT-L mRNA in subsets of tumor cells (d). Extensive CaT-L mRNA expression is detected in lymph node metastasis (e) and hormone refractory, recurrent lesions (f). Original magnifications are as follows: × 25 (left) and × 100 (right).
in the absence of Ca$^{2+}$). The slight but consistent increase of current size in the absence of Na$^+$ (Fig. 4e) is largely due to a local perfusion effect as perfusion of unaltered bath solution (puft in Fig. 4a) revealed a similar increase in current size and could indicate an activation mechanism partially mediated by shape changes.

A feature of non-voltage-operated Ca$^{2+}$-selective ion channels is their ability to conduct Na$^+$ only if all external divalent cations, namely Ca$^{2+}$ and Mg$^{2+}$, are removed from the extracellular solution (34–36). To test whether CaT-L channels conform with this phenomenon, normal bath solution was switched to a solution containing no divalent cations with 1 mM EGTA added. As can be seen in Fig. 4, b, d, and e, CaT-L channels can now conduct very large Na$^+$ currents. Interestingly, immediately after the solution change, the current size first becomes smaller (Fig. 4b) before increasing rapidly, indicating that the pore may initially still be blocked by Ca$^{2+}$ suggesting an anomalous mole fraction behavior. Inactivation of CaT-L currents is mediated in part by binding of Ca$^{2+}$/Calmodulin (37). Interestingly, this inactivation can be counteracted by phosphorylation of the calmodulin-binding site of CaT-L by protein kinase C (37).

The high Ca$^{2+}$ selectivity of CaT-L channels together with its spontaneous activity leads to the assumption that the resting [Ca$^{2+}$], of CaT-L-transfected cells should be rather high and strongly dependent on the extracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]). This prediction was tested in fura-2-loaded CaT-L-transfected HEK cells. In the presence of 1 mM [Ca$^{2+}$], [Ca$^{2+}$], in CaT-L-expressing cells was typically above 200 nm (Fig. 4f), whereas in non-transfected control cells or in cells expressing GFP alone, [Ca$^{2+}$], was less than 100 nm. Following removal of extracellular Ca$^{2+}$, the [Ca$^{2+}$], of CaT-L-expressing cells decreased, whereas readdition of 1 mM Ca$^{2+}$ to the bath led to a significant rise of [Ca$^{2+}$], in CaT-L-transfected cells but not in control cells. Thus, the measurements of [Ca$^{2+}$], are in very good agreement with the electrophysiological recordings, making CaT-L an excellent candidate as a selective Ca$^{2+}$ uptake channel in tissue where it is usually expressed.

**Differential Expression of CaT-L Transcripts in Benign and Malignant Prostate Tissue**—CaT-L transcripts are abundantly expressed in human prostate as shown by Northern blot analysis using a commercially available human multitissue RNA blot (Fig. 2a). To characterize further CaT-L expression, we prepared poly(A)$^+$ RNA from prostate tissues obtained from patients with histologically proven benign prostate hyperplasia. Northern blot analysis with poly(A)$^+$ RNA extracted from benign prostate tissue and human placenta showed CaT-L expression in the latter but failed to demonstrate any CaT-L mRNA in benign prostate tissue (Fig. 5a). This observation was confirmed by in situ hybridization analysis performed in tissue sections. We were unable to demonstrate detectable levels of CaT-L mRNA in normal prostate tissue (Fig. 5b) and benign prostatic hyperplasia (Fig. 5c), and the high grade prostatic intraepithelial lesions were investigated. Conversely, high steady state levels of CaT-L mRNA were detectable in primary prostatic adenocarcinoma (Fig. 5d). Thus, we can conclude that the commercially available RNA blot contains mRNA from prostate cancer patients, although this has not been specified by the manufacturer. In primary prostate adenocarcinoma the most significant levels of CaT-L mRNA were detected in high grade (primary Gleason grades 4 and 5) tumors with extraprostatic extension (pT3a/b) ranging from 10 to 30% of positive tumor cells (Fig. 5, c, e, and g). Conversely, in the organ-confined primary Gleason grade 3 tumor, no levels of CaT-L mRNA were detectable. All lymph node metastases (Fig. 5e, n = 5) and recurrent lesions (Fig. 5f, n = 5) examined revealed CaT-L expression in 10–60% of tumor cells and 10–55% of positive tumor cells, respectively. This indicates that the presence of CaT-L in human prostate cancer is rather a late event in tumor progression.

**DISCUSSION**

The present study has identified CaT-L as a novel Ca$^{2+}$-selective cation channel that is highly expressed in the human placenta, pancreatic acinar cells, salivary glands (Fig. 2), and in malignant prostatic lesions but not in healthy and benign prostate tissues (Fig. 5). Human CaT-like shares 75 and 77% amino acid sequence identity with rabbit and human ECaC, respectively, and the cation permeation properties of the recombiant CaT-L channel resemble those of ECaC (33–35). CaT-L is unlikely to represent the human version of CaTI as its expression is undetectable in the small intestine and colon, tissues where CaTI is abundantly expressed. If, however, CaT-L is the human version of rat CaTI, a second gene product appears to be required for Ca$^{2+}$ uptake in human small intestine and colon attributed to CaTI in rat small intestine and colon. Most interesting, the CaT-L gene, like the human ECaC gene, is localized on human chromosome 7.

In the trophoblasts and syncytiotrophoblasts of the human placenta, CaT-L channels might be involved in transcellular Ca$^{2+}$ transport (38), supplying the fetal circulation with Ca$^{2+}$ from the maternal blood. This transcellular Ca$^{2+}$ transport includes Ca$^{2+}$ influx from maternal plasma across the microvillus plasma membrane into the trophoblasts, Ca$^{2+}$ translocation across the cytosol of the trophoblast cell, and Ca$^{2+}$ efflux from cytosol across the fetal-facing membrane of the trophoblast and entry into the fetal circulation. The Ca$^{2+}$ efflux might be due to the activity of a high affintiy Ca$^{2+}$ ATPase identified in the fetal-facing plasma membrane of trophoblasts (39). Ca$^{2+}$ uptake could be accomplished by CaT-L in a similar way as it has been suggested for ECaC in the kidney (9) and CaT-L in the intestine (10).

In pancreatic acinar cells, the activation of exocytotic secretion of digestive enzymes primarily depends on release of Ca$^{2+}$ from stores in the endoplasmic reticulum (40). Exocytosis can be triggered by hormones and neurotransmitters via intracellular messengers such as inositol 1,4,5-trisphosphate, cyclic adenosine 5'-diphosphate-ribose, and NAADP (41). When the cytosolic Ca$^{2+}$ concentration rises, the plasma membrane Ca$^{2+}$-ATPase pump is invariably activated to extrude Ca$^{2+}$, and in the absence of compulsory Ca$^{2+}$ entry from the extracellular space, cells would inevitably run out of stored Ca$^{2+}$. The molecular structures of the channels responsible for Ca$^{2+}$ entry are not known, but Ca$^{2+}$-selective and non-selective cation influx pathways have been described (42, 43). It will be interesting to study the contribution of CaT-L to these pathways and its role in Ca$^{2+}$ secretion coupling in pancreatic acinar cells.

The most striking feature of CaT-L expression is its complete absence in healthy and benign prostate tissue but its presence at high steady state levels in malignant prostatic lesions (Fig. 5). Prostate cancer is the most commonly diagnosed malignancy in men and is the second leading cause of cancer-related death in Western countries (44). When organ-confined at the time of diagnosis, prostate cancer can be cured by radical prostatectomy. Unfortunately, more than 50% of cancers that are considered clinically confined prior to surgery show extracapsular extension upon pathological analysis and thus represent a high risk of progression (45). In fact, locally advanced cancer is still a fatal disease for which presently no curative treatment is available. There is a great need for new molecular markers predicting tumor progression and the clinical outcome. The observation that CaT-L is undetectable in most of
normal human tissue including the prostate, but present at high levels in locally advanced, metastatic, and recurrent prostatic lesions suggests that CaT-L is a promising marker for the molecular staging and detection of prostate cancer. Interestingly, up-regulation of CaT-L expression has not yet observed in other malignancies such as pancreatic carcinoma (data not shown), arguing against CaT-L being a general marker of cell proliferation. The high levels of CaT-L expression in subsets of tumor cells in advanced stages of the disease suggests a specific function of these cells. It will be of interest to determine the regulating impact of these cells and of the polymorphic variants of the CaT-L protein on the process of tumor progression and hormone therapy failure. Furthermore, the function of CaT-L as $Ca^{2+}$-selective ion channels may offer novel therapeutic strategies interfering with the uptake of $Ca^{2+}$ and its not yet established downstream events in prostatic cancer cells.

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Expression of CaT-like, a Novel Calcium-selective Channel, Correlates with the Malignancy of Prostate Cancer

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