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Mammalian Protein Homologous to VAT-1 of *Torpedo californica:* Isolation From Ehrlich Ascites Tumor Cells, Biochemical Characterization, and Organization of Its Gene

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Abstract Recently, interest has focused on the human gene encoding the putative protein homologous to VAT-1, the major protein of the synaptic vesicles of the electric organ of the Pacific electric ray *Torpedo californica*, after it has been localized on chromosome locus 17q21 in a region encompassing the breast cancer gene *BRCA1*. Chromosomal instability in this region is implicated in inherited predisposition for breast and ovarian cancer. Here we describe isolation and biochemical characterization of a mammalian 48 kDa protein homologous to the VAT-1 protein of *Torpedo californica*. This VAT-1 homolog was isolated from a murine breast cancer cell line (Ehrlich ascites tumor) and identified by sequencing of cleavage peptides. The isolated VAT-1 homolog protein displays an ATPase activity and exists in two isoforms with isoelectric points of 5.7 and 5.8. cDNA was prepared from Ehrlich ascites tumor cells, and the murine VAT-1 homolog sequence was amplified by polymerase chain reaction and partially sequenced. The known part of the murine and the human translated sequences share 97% identity. By Northern blots, the size of the VAT-1 homolog mRNA in both murine and human (T47D) breast cancer cells was determined to be 2.8 kb. Based on the presented data, a modified gene structure of the human VAT-1 homolog with an extended exon 1 is proposed. VAT-1 and the mammalian VAT-1 homolog form a subgroup within the protein superfamily of medium-chain dehydrogenases/reductases. J. Cell. Biochem. 69:304–315, 1998.

Key words: VAT-1; Pacific electric ray *Torpedo californica;* ATPase; *Mus musculus;* gene structure; Ehrlich ascites tumor

VAT-1 is a 41 kDa protein which has been isolated from the electric organ of the Pacific electric ray *Torpedo californica*, where it is a major component of the synaptic vesicles [Linial et al., 1989]. Although little biochemical data of VAT-1 is known, including its ability to bind both ATP and calcium as well as its ATPase activity, VAT-1 has not yet been assigned to a specific function [Linial and Levius, 1993a; Levius and Linial, 1993]. It has the highest homology to members of the protein superfamily of medium chain dehydrogenases/reductases [Persson et al., 1994]. So far, no mammalian protein closely related to VAT-1 is known.

Inherited predisposition to breast and ovarian cancer was shown to be linked to a 650 kilobase region on chromosome locus 17q21 [Hall et al., 1990; Friedman et al., 1995a]. Recently, the breast cancer gene 1 (*BRCA1*) was identified in the middle of this region as a strong candidate gene for breast and ovarian cancer susceptibility [Miki et al., 1994]. Further 22 genes of the 17q21 region were mapped by positional cloning and identified [Friedman et al., 1995b]. Among them is the gene coding for the putative human homolog of the VAT-1 protein of *Torpedo californica*. Since allelic imbalances and deletions centromeric to the *BRCA1* gene as well as somatic mutations in genes of this region other than *BRCA1* were

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suggested to be linked also with inherited predisposition to breast and ovarian cancer [Aberle et al., 1995; Munn et al., 1996; Tangir et al., 1996], the possible involvement of the *VAT-1 homolog* gene should be considered (cf. Discussion).

In our effort to isolate the heat shock protein 25 kinase (HSP25-kinase) from Ehrlich ascites tumor (EAT) cells [Hayess and Benndorf, 1997] we observed a novel 48 kDa protein. Here we describe its isolation, its catalytic activity, the expression of its gene in tumor cell lines, and, by partial sequencing, its identification as the murine homolog of the VAT-1 protein of *Torpedo californica*. Additionally, a modified gene structure of the human VAT-1 homolog with an extended exon 1 is proposed.

MATERIALS AND METHODS

Purification of the 48 kDa Protein (Murine VAT-1 Homolog)

Thirty gram EAT cells (derived from a murine breast cancer) were grown in the intraperitoneal cavity of mice and harvested as described [Benndorf et al., 1988]. After disintegration, the crude cell extract was fractionated by ammonium sulfate precipitation to yield the 0.45S fraction [Benndorf et al., 1992]. To further fractionate the proteins, 15 ml of the 0.45S fraction was loaded on a phenyl-Sepharose CL-4B column (22 × 1.5 cm) (Pharmacia, Freiburg, FRG) equilibrated with buffer A (20 mM Tris-HCl, pH 7.4; 1 mM DTE; 1 mM EDTA; 1 mM benzamidine; 0.5 mM Na_3VO_4; 5 mM sodium pyrophosphate; 50 mM NaF; 160 mM NaCl, 10% glycerol). Proteins were eluted with buffer B (20 mM Tris-HCl, pH 7.4; 1 mM DTE; 1 mM EDTA; 1 mM benzamidine; 0.5 mM Na_3VO_4; 5 mM sodium pyrophosphate; 50 mM NaF; 0.05% Triton X-100) followed by a 0–2.5% Triton X-100 gradient in buffer B. Fractions 50–61 were pooled and loaded on a hydroxyapatite Biogel HT column (5.6 × 1.5 cm)(Bio-Rad, München, FRG) equilibrated with buffer B. After washing with buffer B, a 0–250 mM potassium phosphate gradient in buffer B was applied. Fractions 10–24 were dialyzed overnight with 5 l of buffer C (20 mM HEPES-NaOH, pH 7.4; 1 mM DTE; 1 mM EDTA; 1 mM benzamidine; 0.5 mM Na_3VO_4; 5 mM sodium pyrophosphate; 0.05% Triton X-100). Thereafter, the proteins were loaded on the cationic-exchanger SP-Sepharose Fast Flow column (8.5 × 1.5 cm) (Pharmacia) equilibrated with buffer C. Unbound proteins were washed off the column with buffer C, and bound proteins were eluted with a 0–0.4 M NaCl gradient in buffer C followed by 1.0 M NaCl in buffer C. To exchange the buffer, fractions 58–63 were concentrated ten times by ultrafiltration, diluted with buffer D (25 mM Bistris–iminodiacetic acid, pH 7.1; 1 mM DTE; 1 mM EDTA; 1 mM benzamidine; 0.5 mM Na_3VO_4; 5 mM sodium pyrophosphate; 0.05% Triton X-100), and proteins were separated on a chromatofocussing Mono P HR5/5 FPLC column (Pharmacia) equilibrated with buffer D. After loading, the column was washed with buffer D, and proteins were eluted with buffer E (Polybuffer 74–iminodiacetic acid, pH 4.0; 1 mM DTE; 1 mM EDTA; 1 mM benzamidine; 0.5 mM Na_3VO_4; 5 mM sodium pyrophosphate; 0.05% Triton X-100) according to the manufacturer's instruction followed by 2 M NaCl in buffer D. Pooled fractions 8–15 were diluted five times with buffer F (20 mM Tris-HCl, pH 7.4; 1 mM DTE; 1 mM EDTA; 1 mM benzamidine; 0.5 mM Na_3VO_4; 5 mM sodium pyrophosphate; 0.05% Triton X-100) and loaded onto a Mono Q HR5/5 FPLC column (Pharmacia) equilibrated with buffer F. The column was washed with buffer F, and elution was performed with a 0–0.4 M NaCl gradient in buffer F followed by 1 M NaCl in buffer F.

Electrophoretic Methods

Proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [Laemmli, 1970] or by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) [O'Farrell, 1975]. Reference proteins from Amersham (Braunschweig, FRG) were used for estimation of the molecular mass by SDS-PAGE and reference proteins from Bio-Rad for estimation of the isoelectric points by 2D-PAGE.

Protein Blotting Followed by In Situ Digestion by Lys-C

For identification, the 48 kDa protein was transferred from an SDS gel to a high retention polyvinylidene difluoride (PVDF) membrane (ProBlott™ Applied Biosystems, Foster City, CA) for 2 h at 1 mA/cm^2 in a transblot apparatus (Bio-Rad) using buffer G (10 mM 3-cyclohexylamino-1-propanesulfonic acid-NaOH, pH 11.0; 10% methanol, 0.07% SDS). The blotted protein was visualized by staining (1 min) with Coomassie brilliant blue and destaining with 50%

methanol. The membrane area carrying the 48 kDa protein was cut into small pieces (1–2 mm^2) and immersed in 0.04 ml solution H (0.1 M Tris-HCl, pH 8.5; 10% acetonitrile; 1% reduced Triton X-100) as described [Fernandez et al., 1994]. One microgram of the endoproteinase Lys-C (Boehringer, Mannheim, FRG) was added, and digestion was allowed to proceed for 20 h at 37°C. After sonication (5 min) and centrifugation, the supernatant was collected, and the membrane pieces were washed with 50 µl of solution H and twice with 100 µl 0.1% trifluoroacetic acid. At each step, the sample was sonicated for 5 min. After removal of acetonitrile in a SpeedVac concentrator, the collected supernatants were injected onto the HPLC column.

Reversed-Phase HPLC Purification of Peptides and Edman Degradation

Peptides eluted from the PVDF membrane were separated on the SMART™ System (Pharmacia) with a dual-syringe solvent delivery system and a µPeak Monitor set at 214, 260, and 280 nm for peak detection. The sample was applied to a µRPC C2/C18 SC 2.1/10 column (Pharmacia), and peptides were separated by 5–45% acetonitrile (in 0.1% trifluoroacetic acid) gradient elution (0.1 ml/min flow rate, 65 min duration). Edman degradation of peptides was performed using the Applied Biosystems pulsed-gas-liquid sequencer 477A connected to an online narrow HPLC system 120A for detection of the amino acids.

Homology Analysis

Amino acid sequences of the peptides were compared to known protein sequences in the databases SWISSPROT and PIR using the programs TBLASTX and TBLASTN for comparison of all translated reading frames of the nucleotide databases GenBank and EMBL as described previously [Altschul et al., 1990].

Isolation and Sequencing of a Murine VAT-1 Homolog cDNA Clone

Total cellular RNA was isolated from EAT cells in a single-step procedure [Chomczynski and Sacchi, 1987] by homogenization of frozen pellets using Trizol (Life Technologies, Gaithersburg, MD) according to the manufacturer's instruction. The concentration and integrity of RNA were determined spectrophotometrically at an absorbance of 260 nm and by agarose gel electrophoresis. Total cellular RNA (5 µg) was reverse-transcribed into cDNA using oligo(dT) as primer and Superscript reverse transcriptase (Life Technologies). A 5' primer (5'-ACGAGGCGGGCTAACTTCGC-3') and a 3' primer (5'-AGTCGTGTGATAGTCGATGGG-3') homologous to the human VAT-1 homolog sequence [Friedman et al., 1995b] were used for RT-PCR. PCR amplification was performed at an annealing temperature of 55°C for 35 cycles. The resulting PCR fragments were directly ligated into pCRII (Invitrogen, Leek, The Netherlands) and transformed into *E. coli*. Plasmid inserts from resulting colonies were sequenced in both directions (sense and antisense strand) using M13 and T7 primers with the Sequenase Terminator Double-Stranded DNA Sequencing Kit (Perkin-Elmer, Weiterstadt, FRG) according to the manufacturer's instruction. The resulting reactions were analyzed on an ABI 373A DNA sequencer.

Isolation of RNA From Cell Cultures

Total cytoplasmic RNA was isolated from each 10^6 murine (EAT, propagated as described above) and human breast cancer cells (T47D cells, propagated in RPMI medium supplemented with 10% fetal calf serum, 10 µg/ml insulin, 20 µg/ml hydrocortison, and 50 µM β-mercaptoethanol) as described elsewhere [Gough, 1988]. Concentration and integrity of RNA were determined spectrophotometrically and by agarose gel electrophoresis. Poly(A+) RNA was obtained using the Oligotex-mRNA kit (Qiagen, Hilden, FRG) according to the supplier's protocol.

Northern Blotting

Total RNA (15 µg) was heat-denatured at 55°C for 15 min, electrophoretically separated on an 1.2% agarose/1.1% formaldehyde gel, transferred to a Hybond nylon membrane (Amersham) by vacuum blotting with 20× SSC, and cross-linked to the membrane by UV fixation (Stratalinker; Stratagene, La Jolla, CA) for 1 min. The [^{32}P]phosphate-labeled 478 bp VAT-1 homolog cDNA probe used for hybridization was generated by amplification (PCR) in the presence of [^{32}P]-dCTP (3,000 Ci/mmol) (Amersham). Hybridization and stripping of Northern blots were carried out according to standard procedures [Kroczek, 1993]. After stripping, hybridization with a β-actin cDNA

probe was performed as loading control. The membranes were evaluated with a phosphoimager (Molecular Dynamics, Sunnyvale, CA) using the ImageQuaNT software.

ATPase Assay

Reactions were performed at 30°C in 10 µl reaction mixture containing 20 mM HEPES-NaOH, pH 7.4, 20 mM $MgCl_2$, 1 mM dithioerythritol, 10 µM ATP, 60 kBq [γ-^{33}P]ATP, and 0.4 µg VAT-1 homolog from fraction 49 (Fig. 2). One microliter aliquots were removed, 1:10 diluted with water, and immediately frozen in liquid nitrogen at the time points marked. For thin layer chromatography, 0.5 µl samples were loaded on PEI-cellulose sheets (Merck, Darmstadt, FRG), and the chromatogram was run in 0.75 M potassium phosphate buffer at pH 3.5. The amount of released inorganic [^{33}P]phosphate was determined with a phosphoimager (Fuji, Berlin, FRG).

RESULTS

Isolation of the 48 kDa Protein

The murine 48 kDa protein later on identified as the homolog of the VAT-1 protein of the Pacific electric ray *Torpedo californica* was observed during our effort to isolate the HSP25 kinase from EAT cells [Hayess and Benndorf, 1997]. An extract of these cells was fractionated by ammonium sulfate precipitation and sequential chromatography as described in Materials and Methods. The elution profiles of protein separations using phenyl-Sepharose, hydroxyapatite, SP-Sepharose Fast Flow, Mono P, and Mono Q columns are shown in Figure 1A–E, respectively. For the first time, the 48 kDa protein was observed after analysis of fractions 8–15 of the Mono P column by SDS-PAGE (Figs. 1D, 2A). Proteins of these fractions were further separated on a Mono Q column yielding the 48 kDa protein in fractions 46–50 (Figs. 1E, 2B). Fraction 49 contains the 48 kDa protein in a highly purified form, although most of the protein is contained in fraction 50 together with three contaminating proteins (Fig. 2B). Two-dimensional PAGE of proteins of fraction 49 reveals two isoforms of the 48 kD protein (Fig. 2C) with estimated isoelectric points of 5.8 (major isoform, a) and 5.7 (minor isoform, b).

Partial Sequencing of Cleaved Peptides

For identification, the 48 kDa protein was processed for proteolytic cleavage and partial

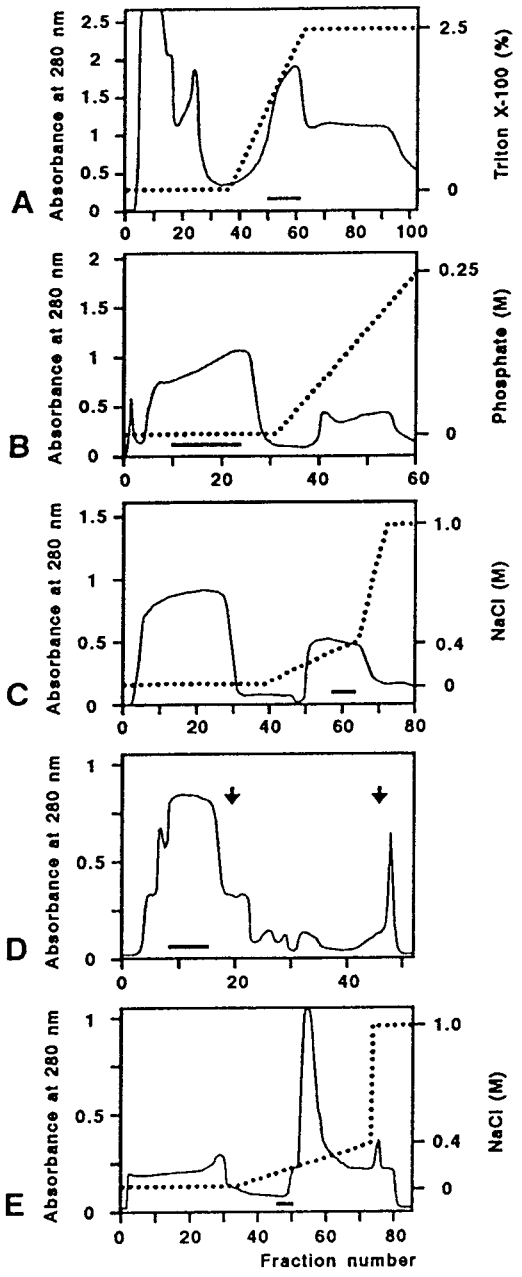

Fig. 1. Purification of the 48 kDa protein (VAT-1 homolog) by sequential chromatography. A cell extract of EAT cells was fractionated by hydrophobic (phenyl-Sepharose), hydroxyapatite (Biogel HT), cationic exchange (Fast Flow S), chromatofocussing (Mono P), and anionic exchange (Mono Q) chromatography (**A–E,** respectively). Absorbance at 280 nm (solid line) and the elution gradients (dotted line) are indicated. The position of the 48 kDa protein is indicated by bars. In D, the left arrow indicates the onset of the pH gradient and right arrow the onset of elution with 2 M NaCl.

sequencing. After SDS-PAGE, the protein was electrotransferred onto a PVDF membrane and digested by the endoproteinase Lys-C as is described in Materials and Methods. The ob-

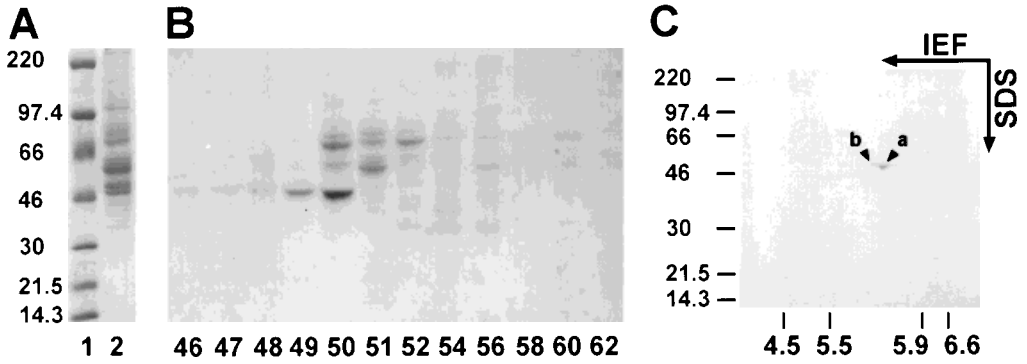

Fig. 2. Electrophoretic detection of the 48 kDa (VAT-1 homolog) protein. **A:** Separation by SDS-PAGE of molecular mass reference proteins **(lane 1)** and of proteins from pooled fractions 8–15 of the Mono P column (**lane 2**). The bar indicates the position of the VAT-1 homolog. **B:** Separation by SDS-PAGE of proteins from fractions 46–62 of the Mono Q column. Numbers refer to fractions. **C:** Separation by 2D-PAGE of the 48 kDa protein of fraction 49. The position of the major and minor isoform of the 48 kDa protein is indicated by letters a and b, respectively. At the bottom, the scale of the determined isoelectric points is given using reference proteins. Numbers at the left of panels A and C refer to the molecular mass of reference proteins in kDa.

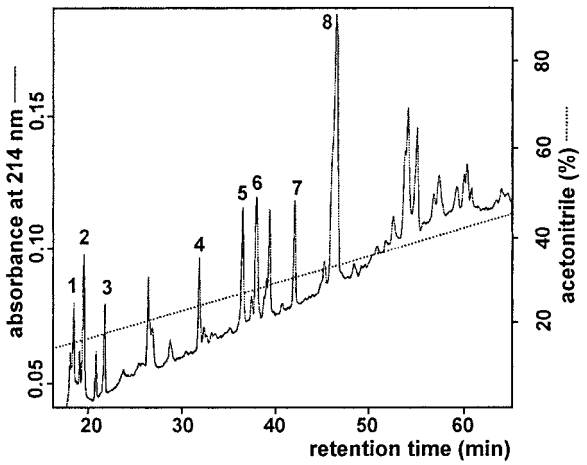

Fig. 3. Reversed-phase HPLC of the Lys-C digest of the 48 kDa protein. Numbers refer to peaks subjected to Edman sequence analysis.

TABLE I. Sequences Determined by Edman Degradation of Lys-C Peptides of the 48 kDa Protein

Peak number[a]	Peptide number	Sequence
1	P1a	P M G K
	P1b	Q M Q E K
2	P2	K I S P K
3	P3	(H) E V L K[b]
4	P4	G Y X L L *K*[b,c]
5	P5	V L L V P G P E K
6	P6	E N G V T(H) P I D Y(H) T T D Y[b]
7	P7	G V D I V M D P L G G S D T A *K*[c]
8	P8a	V V T Y G M A N L L T G P K
	P8b	P(H) I D S V W P F E K[b]

[a]Peak numbers refer to Fig. 3. Peaks 1 and 8 each consisted of two peptides resulting in two signals at every degradation step. The given peptide sequences were derived from alignment to the translated sequence of the human VAT-1 homolog.

[b]Residues not identified or not identified unambiguously are indicated by X or are in parenthesis, respectively.

[c]C-terminal lysine residues assumed on the basis of the cleavage specificity of Lys-C are in italics.

tained peptides were separated by HPLC as shown in Figure 3, and the peptides corresponding to peaks 1–8 were sequenced by Edman degradation. The obtained peptide sequences are presented in Table I. Peaks 2–7 each contained one peptide, while peaks 1 and 8 each contained two peptides resulting in two signals at each sequencing step. Based on the alignment with the translated sequence of the human *VAT-1 homolog* gene, the sequences of the peptides P1a, P1b, P8a, and P8b also could be established with sufficient certainty (cf. following section).

Homology Search Analysis

The primary structures of the peptides were compared to known protein sequences and to all translated reading frames of the nucleotide databases as described in Materials and Methods. Peptides 2, 4, 5, 6, and 7 were found to be contained in three translated sequences (EMBL/GenBank accession numbers U18009, U25779, L78833) all originating from human chromosome locus 17q21 and describing the same human gene being approximately 53% identical to the gene of the Pacific electric ray *Torpedo californica* encoding VAT-1, a major protein of the synaptic vesicles of the electric organ

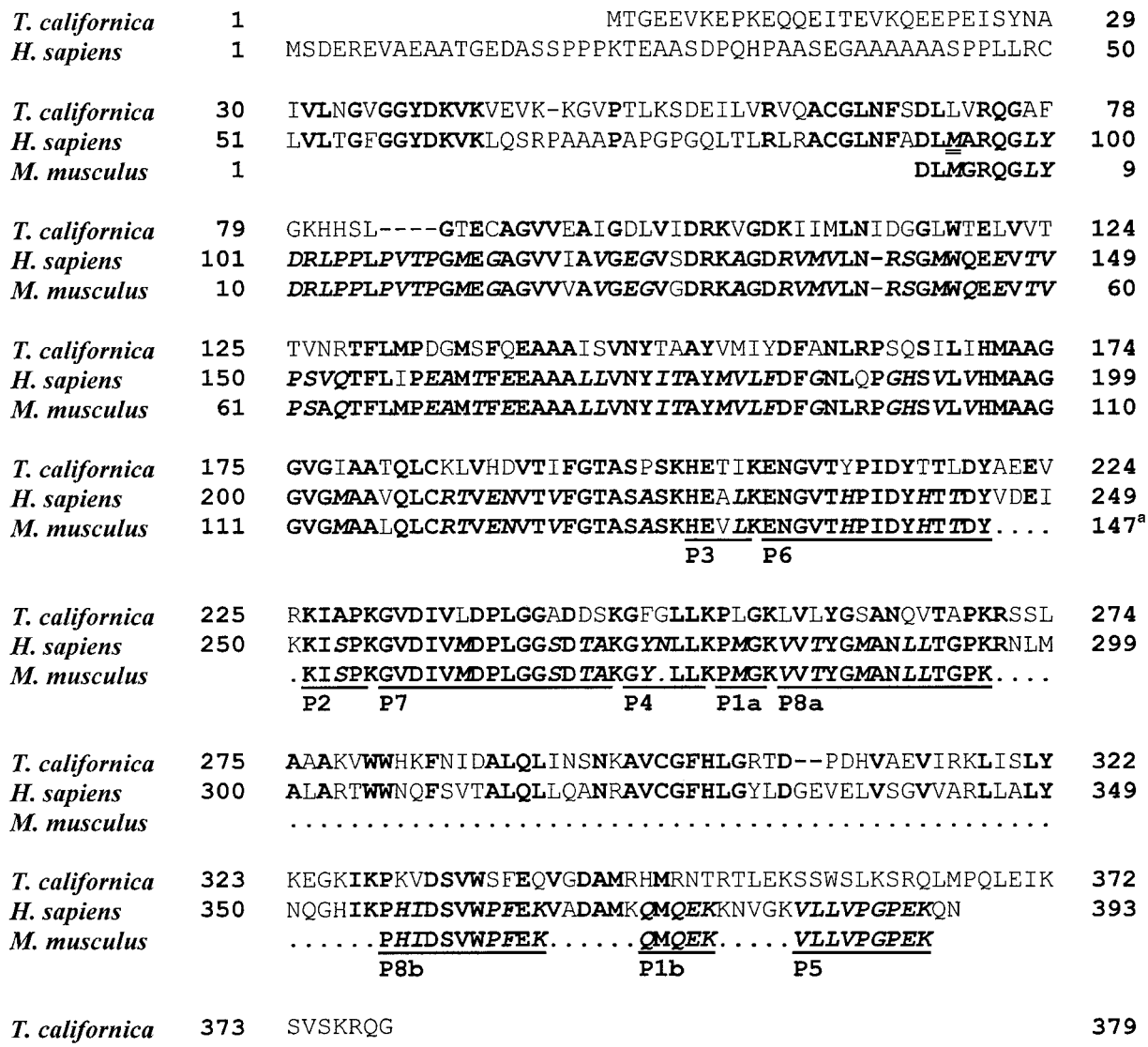

Fig. 4. Sequence alignment. The sequences of the cleavage peptides P1a, P1b, P2, P3, P4, P5, P6, P7, P8a, and P8b (indicated by bars) of the VAT-1 homolog protein of *Mus musculus* are aligned with the translated amino acid sequence of the murine VAT-1 homolog cDNA (cf. Fig. 5), with the translated genomic sequence of the *Homo sapiens* VAT-1 homolog DNA (EMBL/GenBank accession number L78833) and with the translated sequence of the *Torpedo californica* VAT-1 cDNA (EMBL/GenBank accession number P19333). In the given sequence of the human VAT-1 homolog, amino acid residues 1–93 result from a proposed upstream extension of exon 1 beyond the methionin residue 94 (doubly underlined) (cf. Results). Amino acid residues which are identical among the sequences of *T. californica* and at least one of the mammals are in bold. Amino acid residues which are identical amongst the two mammals only are in bold italics. Deletions are indicated by dashes; missing parts of the murine sequence between or within cleavage peptides are indicated by dots. [a]Position 147 is the last position of the murine sequence obtained by cDNA sequencing.

(cf. Fig. 4) [Linial et al., 1989]. The locus 17q21 is known for its chromosomal instability and carries the susceptibility gene for breast and ovarian cancer *BRCA1* (cf. Discussion). The sequence L78833 contains the complete genomic sequence encompassing *BRCA1*. Comparison of the translated sequences with the sequencing information obtained from peaks 1 and 8 enabled the sequences of peptides P1a, P1b, P8a, and P8b to be established with sufficient certainty (Fig. 4). Peptide P3 (HEVLK) derived from the murine protein corresponds to the human sequence HEALK and indicates a sequence difference between the murine and human proteins, as confirmed by cDNA sequencing (cf. following section). The three human sequences U18009, U25779, and L78833 differ slightly probably due to sequencing errors. Peptides P1a–P8b fit better to the translated sequences U18009 and L78833 than to U25779

since no frame shift manipulations are needed for alignment (not shown).

Isolation of a Murine VAT-1 Homolog cDNA Clone and Sequencing

To isolate the murine VAT-1 homolog cDNA, we used two primers homologous to the human VAT-1 homolog sequence for amplification by PCR. After subcloning, the obtained PCR fragments were sequenced. The obtained murine sequence (deposited under EMBL/Genbank accession number X95562) is 89% identical with the corresponding part of the human sequence L78833 (Fig. 5). Based on both the predicted amino acid sequences of the clones of human and mouse origin and on the peptide sequencing data, the human and murine VAT-1 homolog proteins share approximately 97% sequence identity (Fig. 4).

Expression and Size of the Mammalian VAT-1 Homolog

Expression of the *VAT-1 homolog* gene was analyzed each in a murine (EAT) and a human (T47D) breast cancer cell line by Northern blot hybridization using 2 µg poly(A^+) RNA, as described in Materials and Methods. Expression was detected in both cell lines as shown in Figure 6 (upper part). The size of the VAT-1 mRNA was estimated to be 2.8 kb. For control, a probe specific for β-actin was used as internal standard (lower part).

From the published human sequences U18009, U25779, and L78833, the molecular mass of the putative VAT-1 homolog protein was predicted to be approximately 30 kDa using the putative transcription start site of exon 1 at position 106659 and the putative translation start site at position 106682 (Fig. 5) [Friedman et al., 1995b; Smith et al., 1996]. However, this is in contrast to the estimated molecular mass of 48 kDa of the murine protein. Indeed, a reexamination of the *VAT-1 homolog* gene structure (sequence L78833) using XPOUND of the GCG program package suggests an upstream extension of exon 1 with the translation start site being at position 106403. Translation of this sequence would result in an N-terminally extended human VAT-1 homolog protein with a molecular mass of approximately 42 kDa, as shown in Figure 4. An additional argument for the extended exon 1 and thus the extended N-terminus is provided by the existence of an expressed sequence tag (EST05479, GenBank accession number T07589) which covers this part of the sequence. In Figure 5 the proposed transcribed sequence of the human *VAT-1 homolog* gene consisting of six exons is shown as well as the alignment with part of EST05479.

Using the extended exon 1, we calculated the size of the VAT-1 homolog mRNA to be approximately to 2.8 kb (assuming a poly(A^+) tail of 200 bp), which matches well the size determined in Figure 6. The predicted molecular mass of the corresponding protein (42 kDa) is similar to the molecular mass of VAT-1 of *Torpedo californica;* however, it is somewhat below the observed molecular mass of the murine VAT-1 homolog protein (48 kDa) described in this paper (cf. Discussion).

ATPase Activity

The VAT-1 protein from *Torpedo californica* displays an ATPase activity [Linial and Levius, 1993a]; therefore, we assayed the murine VAT-1 homolog protein for this catalytic activity. The ATPase activity was determined by the release of [^{33}P]phosphate from [γ-^{33}P]ATP. Figure 7 shows the time curve of [^{33}P]phosphate release catalyzed by the purified VAT-1 homolog (closed circles). In the control reaction without VAT-1 homolog (open circles), ATP hydrolysis is strongly reduced. Addition of an excess of unlabeled ATP competes with the [γ-^{33}P]ATP, resulting also in a strong reduction of [^{33}P]phosphate release (squares). If we assume a linear reaction rate within the first 30 min, the calculated activity is 0.19 pmoles min^{-1}, and the specific activity is 480 pmoles min^{-1} mg^{-1} protein. These data suggest that the murine VAT-1 homolog, like the *Torpedo californica* VAT-1, displays an ATPase activity.

DISCUSSION

Here we present a purification procedure and biochemical data on a mammalian protein sharing sequence homology with the VAT-1 protein of the Pacific electric ray *Torpedo californica* [Linial et al., 1989]. The gene of the human VAT-1 homolog has been localized on chromosome locus 17q21 in the neighborhood of *BRCA1* [Friedman et al., 1995b]. The recently obtained complete genomic sequence of a 117 kb DNA fragment from 17q21 establishes the order of the genes encompassing *BRCA1*: centromere–*1FP35* (interferon-induced leucine zipper protein)–VAT-1 homolog–*Rho7* (GTP-binding protein)–*BRCA1–1A1-3B* (B-box protein pseu-

dogene)–telomere [Smith et al., 1996]. The analysis of the gene structure of the *VAT-1 homolog* resulted in the prediction of six exons. Further genes mapped by positional cloning to this chromosome locus include ten known human genes, four new human genes homologous to genes known in other species, and seven apparently novel genes [Friedman et al., 1995b]. It has been suggested that genes of this region other than *BRCA1* may also harbor critical somatic mutations as was shown for the plakoglobin gene [Aberle et al., 1995]. Furthermore, allelic imbalances in the region of *BRCA1* and a 400 kb deletion unit centromeric to the *BRCA1* gene were shown to be linked with ductal breast carcinoma and sporadic epithelial ovarian cancer, respectively [Munn et al., 1996; Tangir et al., 1996]. Due to its position in a region of chromosomal instability, the *VAT-1 homolog* gene might be a further candidate gene to harbor critical mutations in the neighborhood of *BRCA1* with possible implications for cancer. At present, no data are available on the number and nature of polymorphisms or mutations within the *VAT-1 homolog* gene in affected families or in the unaffected population. The possible role of the two described single-strand conformation polymorphisms is not known [Friedman et al., 1995b]. We have shown that the *VAT-1 homolog* is expressed in both a murine and a human breast cancer cell line. However, so far no data are available indicating a correlation between the abundance of the VAT-1 homolog protein and cancer.

The published sequences of the putative human gene (sequences U18009 and U25779) as well as the published analysis of the gene structure of the *VAT-1 homolog* (sequence L78833) suggest the molecular mass of the putative VAT-1 homolog protein to be approximately 30 kDa [Friedman et al., 1995b; Smith et al., 1996], which is in contrast to the molecular mass of the murine protein estimated to be 48 kDa. Reexamining the *VAT-1 homolog* gene structure using the sequence L78833, we suggest an upstream extension of exon 1, the expression of which would result in an mRNA with a size of approximately 2.8 kb and in a putative protein with a molecular mass of approximately 42 kDa. While the calculated size of the mRNA is in good agreement with the observed size (Fig. 6), the calculated molecular mass of the putative protein is below the observed apparent molecular mass of 48 kDa. The reason for this difference is not known, and further postulations are needed (e.g., extensive posttranslational modifications or a difference in the size between the human and murine gene).

By Southern blotting we obtained data demonstrating that *VAT-1 homolog* genes are common among vertebrates. We identified them in placental mammals (human, rat, guinea pig, rabbit, sheep, cow, *Putorius putorius*), marsupial mammals (*Macropus agilis*), birds (chicken, *Meleagris gallopavo, Poephila guttata*), turtles (*Chrysemys sp., Pseudemys scripta elegans*), frogs (*Rana temporaria*), and fishes *(Brachydanio rerio, Salmo gairdneri)* (not shown).

The data presented here suggest that the mammalian VAT-1 homolog, similar to the *Torpedo californica* VAT-1, displays an ATPase activity. By 2D-PAGE we could show that the isolated VAT-1 homolog exists in two isoforms with isoelectric points of approximately 5.8 (major isoform) and 5.7 (minor isoform) (Fig. 2C). Besides that, no further information on the mammalian protein is available so far.

Although the *Torpedo californica* VAT-1 protein binds calcium with low affinity [Levius and Linial, 1993] and may be involved in protein phosphorylation [Linial and Levius, 1993a], it has not yet been assigned unambiguously to a specific function. In this organism, the protein is specifically expressed in the electric lobe, while it is accumulated in nerve terminals of the electric organs, and copurifies with synaptic vesicles. Seventy percent of VAT-1 immunoreactivity is localized to the synaptic membrane, while 30% copurifies with larger membrane fragments. Within the synaptic vesicle membrane, VAT-1 forms oligomeric complexes [Linial, 1993] which appear to be dependent on the concentration of calcium [Linial et al., 1995]. Concerning the mammalian VAT-1 homolog, no comparable data are available. VAT-1 and VAT-1 homolog are more closely related to each other (53% identity) and distantly related to mammalian zeta-crystallins (26–27% identity) [Linial and Levius, 1993b]. Thus, VAT-1 and the VAT-1 homolog form a subgroup of the protein superfamily of medium-chain dehydrogenases/reductases [Persson et al., 1994]. Part of VAT-1 bears similarity with the enoyl reductase of the fatty acid synthase. This relationship may indicate an oxidoreductase activity to be coupled to the ATPase activity of both VAT-1 and VAT-1 homolog, although no experimental evidence is available so far. It remains a

		extended exon 1	
H. sapiens	106300	CCCTCCCGCT GGATCCCGCA GCCGCGGCTC TTCCCGACGC GTTCCGACTT CCCCAGCTGT	106359
EST05479	38	CCCTCCCGCT GGATCCCGCA GCCGCGGCTC TTCCCGACGC GTTCCGCCTT CCCCAGCTGT	97
		translation start	
H. sapiens	106360	GCACTCTCCA TCCAGCTGTG CGCTCTCGTC GGGAGTCCCA GCCATGTCCG ACGAGAGAGA	106419
EST05479	98	GCACTCTCCA TCCAGCTGTG NNGTCTCGTC GGGAGTCCCA GCCATGTCCG ACGAGAGANA	157
H. sapiens	106420	GGTAGCCGAG GCAGCGACCG GGGAAGACGC CTCTTCGCCG CCTCCGAAAA CCGAGGCAGC	106479
EST05479	158	GGTAGCCGAG GNAGCGACCG GGGAAGACG	186
H. sapiens	106480	GAGCGACCCC CAGCATCCCG CGGCCTCCGA AGGGGCCGCC GCCGCCGCCG CCTCGCCGCC	106539
H. sapiens	106540	ACTGCTGCGC TGCCTAGTGC TCACCGGCTT TGGAGGCTAC GACAAGGTGA AGCTGCAGAG	106599
		(exon 1)	
H. sapiens	106600	CCGGCCGGCA GCGGCCCCGG CCCCTGGGCC CGGCCAGCTG ACGCTGCGTC TGCGGGCCTG	106659
H. sapiens	106660	CGGGCTCAAC TTCGCAGACC TCATGGCTAG GCAGGGGCTG TACGACCGTC TCCCGCCTCT	106719
M. musculus	1	GACC TCATGGGCCG CCAAGGGCTG TACGACCGAC TACCGCCTCT	44
H. sapiens	106720	GCCTGTCACT CCGGGCATGG AGGGCGCGGG TGTTGTGATC GCAGTGGGCG AGGGAGTCAG	106779
M. musculus	45	GCCGGTCACC CCCGGCATGG AGGGCGCGGG CGTGGTAGTG GCAGTGGGCG AAGGCGTCGG	104
		exon 2	
H. sapiens	106780-89	CGACCGCAAG GCAGGAGACC GGGTGATGGT GTTGAACCGG TCAGGGATGT GGCAGGAAGA	109927-76
M. musculus	105	CGACCGTAAG GCAGGGGATC GGGTGATGGT GTTGAACCGG TCAGGGATGT GGCAGGAGGA	164
H. sapiens	109977	GGTGACTGTG CCCTCGGTCC AGACCTTCCT GATTCCTGAG GCCATGACCT TTGAGGAAGC	110036
M. musculus	165	AGTAACTGTG CCATCAGCCC AGACTTTCCT GATGCCTGAG GCCATGACCT TTGAAGAAGC	224
H. sapiens	110037	TGCTGCCTTG CTCGTCAATT ACATTACAGC CTACATGGTC CTCTTTGACT TCGGCAACCT	110096
M. musculus	225	TGCTGCCTTG CTGGTCAATT ATATCACCGC CTACATGGTT CTCTTTGACT TCGGCAACCT	284
		exon 3	
H. sapiens	110097-134	ACAGCCTGGC CACAGCGTCT TGGTACACAT GGCTGCAGGG GGTGTGGGTA TGGCTGCCGT	110520-41
M. musculus	285	GAGACCGGGC CACAGCGTCT TGGTACACAT GGCTGCAGGT GGCGTGGGCA TGGCAGCCCT	344
H. sapiens	110542	GCAGCTGTGC CGTACAGTGG AGAATGTGAC AGTGTTCGGA ACGGCCTCGG CCAGCAAGCA	110601
M. musculus	345	GCAGTTGTGC CGCACCGTGG AGAACGTGAC AGTGTTTGGA ACGGCCTCAG CCAGCAAGCA	404

(continued)

Figure 5.

H. sapiens	110602	**CGAGG**CA**CTG AAGGAGAA**TG **GGGTCACACA TC**CCATCGAC TATCACACGA CTGACTACGT	110661
M. musculus	405	**CGAGG**TG**CTG AAGGAGAA**CG **GGGTCACACA TC**	436
		exon 4	
H. sapiens	110662-90	GGATGAGATC AAGAAGATTT CCCCTAAAGC CTCTGGGTGG GTCAGATACT GCCAAGGGCT	110816-46
		exon 5	
	110847-85	ACAACCTCCT GAAACCCATG GGCAAAGTCG TCACCTATGG AATGGCCAAC CTGCTGACGG	112774-94
	112795	GCCCCAAACG GAACCTGATG GCCCTGGCCC GGACATGGTG GAATCAGTTC AGCGTGACAG	112854
	112855	CTCTGCAGCT GCTGCAGGCC AACCGGGCTG TGTGTGGCTT CCACCTGGGC TACCTGGATG	112914
	112915	GTGAGGTGGA GCTGGTCAGT GGTGTGGTGG CCCGCCTCCT GGCTCTGTAC AACCAGGGCC	112974
		exon 6	
	112975-3015	ACATCAAGCC CCACATTGAC TCAGTCTGGC CCTTCGAGAA GGTGGCTGAT GCCATGAAAC	113178-96
	113197	AGATGCAGGA GAAGAAGAAT GTGGGCAAGG TCCTCCTGGT TCCAGGGCCA GAGAAGCAGA	113256
		stop	
	113257	ACTAGGGCAA GTGGCTGTGA GACCCTAGAG ACCAGCGAAG GGAGAAGTTG	113306
		poly(A+)-signal	
	114677	.... TGCCTTTGTG TGTTTGTGTC AATAAAAAGC CAAACCCTGG GTCCTGCTTG	114726

Fig. 5. Alignment of VAT-1 homolog nucleotide sequences of the murine cDNA (EMBL/GenBank accession number X95562), of the putative exons of the human genomic DNA (EMBL/GenBank accession number L78833), and of the human expressed sequence tag EST05479 (GenBank accession number T07589). The positions of the putative exons in the human genomic sequence according to Smith et al. [1996] are designated (the first three nucleotides of every exon are underlined). The precise position of the transcription start site of the proposed extended exon 1 is not known. The putative translation start sites of the proposed extended exon 1 and of exon 1 according to Smith et al. [1996], the stop codon, and the polyadenylation signal are doubly underlined. Nucleotides identical in the murine and human or in EST05479 and the human VAT-1 homolog sequences are in **bold.** Note that the noncoding region of exon 6 of the human sequence between positions 113306 and 11677 has been omitted.

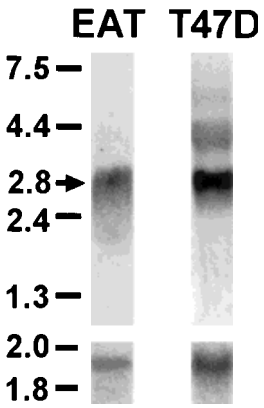

Fig. 6. Northern blot analysis of mRNA from murine (EAT) and human (T47D) breast cancer–derived cell lines. Blots were hybridized with a [^{32}P]phosphorous-labeled VAT-1 homolog probe (upper part) or a beta-actin probe (lower part). Numbers indicate the size of mRNA in kilobases. The arrow indicates the position of the VAT-1 homolog mRNA.

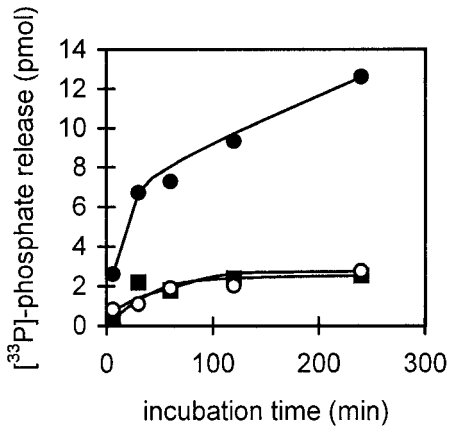

Fig. 7. ATPase activity of the murine VAT-1 homolog. Enzymatic release of [^{33}P]phosphate from [γ-^{33}P]ATP was measured in the presence of 0.4 µg VAT-1 homolog protein from fraction 49 (cf. Fig. 2B) (l), in the presence of 0.4 µg VAT-1 homolog and an excess of non-radioactive ATP (5 mM) (), and for control without VAT-1 homolog (○).

future task to elucidate the biochemical functions of the VAT-1 homolog protein and to determine whether its gene is affected by the chromosomal instability in the neighborhood of *BRCA1* in families with inherited predisposition to breast and ovarian cancer.

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