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A Novel Human Tocopherol-associated Protein

CLONING, *IN VITRO* EXPRESSION, AND CHARACTERIZATION*

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Vitamin E (α-tocopherol) is an essential dietary nutrient for humans and animals. The mechanisms involved in cellular regulation as well as in the preferential cellular and tissue accumulation of α-tocopherol are not yet well established. We previously reported (Stocker, A., Zimmer, S., Spycher, S. E., and Azzi, A. (1999) *IUBMB Life* 48, 49–55) the identification of a novel 46-kDa tocopherol-associated protein (TAP) in the cytosol of bovine liver. Here, we describe the identification, the molecular cloning into *Escherichia coli*, and the *in vitro* expression of the human homologue of bovine *TAP, hTAP*. This protein appears to belong to a family of hydrophobic ligand binding proteins, which have the CRAL (*cis*-retinal binding motif) sequence in common. By using a biotinylated α-tocopherol derivative and the IASys resonant mirror biosensor, the purified recombinant protein was shown to bind tocopherol at a specific binding site with K_d 4.6 $\times$ 10^{-7} M. Northern analyses showed that hTAP mRNA has a size of approximately 2800 base pairs and is ubiquitously expressed. The highest amounts of hTAP message are found in liver, brain, and prostate. In conclusion, hTAP has sequence homology to proteins containing the CRAL_TRIO structural motif. TAP binds to α-tocopherol and biotinylated tocopherol, suggesting the existence of a hydrophobic pocket, possibly analogous to that of SEC14.

Vitamin E is a physiological component of cellular membranes and lipoproteins. Since its discovery in 1922 by Evans and Bishop (1), researchers have confirmed the significance of α-tocopherol as the principal vitamin E homologue in human plasma (2). Roles in the maintenance of tissue integrity (3) in the prevention of atherosclerosis (4) and prostate cancer (5) as well as in the regulation of the immune response (6) have been proposed. Most of these effects have been attributed to two primary functions of α-tocopherol. One function of α-tocopherol is as a scavenger of reactive oxygen (7) and nitrogen species (8) able to break radical chain propagation reactions (9). The other function of α-tocopherol, more recently elucidated, is an additional role consisting of a structure-specific modulation of cellular functions such as adhesion (10), proliferation (11), and

apoptosis (12). Decreased levels of α-tocopherol can thus evoke unspecific damage to lipid, protein, and DNA caused by the unscavenged reactive oxygen and nitrogen species; they can, at the same time, induce defined cellular responses, which cannot be neutralized by α-tocopherol-related phenolic antioxidants (13, 14). The latter events can be related to α-tocopherol-induced protein kinase C inhibition (15), to the activation of the nuclear transcription factor κB (16), and to the modulation of the expression of specific genes, *e.g.* the gene of collagenase 1 (17), the gene of α-tropomyosin (18), and the gene for one of the scavenger receptors (19). These cellular events require specific recognition molecules that are able to bind tocopherol and feed this information further into the cell signaling pathways. Furthermore, due to its strong hydrophobicity and its primary location in the plasma membrane, α-tocopherol requires a carrier mechanism, which can regulate its cytosolic transport to other cellular sites. The mechanism by which α-tocopherol is redistributed within the cell and reaches mitochondria and the nucleus has not been studied.

Nutrition-related vitamin E deficiency in humans is a rare situation, although several lines of evidence support a relationship between low levels of α-tocopherol and human diseases, *e.g.* increased risk of atherosclerosis and prostate cancer. Recently, a group of patients having remarkably low levels of plasma α-tocopherol were studied in Tunisia (20, 21). In these patients, a genetic defect of an α-tocopherol-specific liver protein (α-TTP)[1] caused the disruption of tocopherol transfer from chylomicrons into plasma lipoproteins and a clinical syndrome, AVED (ataxia with isolated vitamin E deficiency). When these patients were given a high dose of α-tocopherol, the progression of the disease was reduced (22).

If a binding protein is needed for α-tocopherol transfer from chylomicrons via liver into plasma, it is important to ask if other proteins might be responsible for the distribution of α-tocopherol to intracellular compartments. Indeed a number of studies have been carried out in an attempt to isolate and characterize such proteins (23, 24).

Using radioactively labeled α-tocopherol as a tracer we have recently identified and briefly described a new α-tocopherol binding protein of molecular mass 46 kDa in the cytosol of bovine liver, which has been called α-tocopherol-associated protein (TAP) (25). In this paper, we present the identification,

[1] The abbreviations used are: α-TTP, α-tocopherol-transfer protein; bTAP/hTAP, bovine/human α-tocopherol-associated protein; CRAL, *cis*-retinal binding motif; SEC14, SEC14 cytosolic factor; His-hTAP, histidine-tagged human α-tocopherol-associated protein; IASys, interaction analysis system; HPLC, high performance liquid chromatography; RT-PCR, reverse transcription-polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; kb, kilobase(s).

molecular cloning, and the *in vitro* expression of the human homologue of TAP. The kinetic data of the interaction between recombinant TAP and a biotinylated tocopherol derivative are presented. In addition, the results of a structural analysis of its lipid binding domain and studies on the distribution of TAP in human tissues are reported.

EXPERIMENTAL PROCEDURES

Materials—Tritiated D-α-tocopherol (24.76 Ci/mmol) was synthesized by Amersham Pharmacia Biotech and further purified on a Nova-Pak C18 HPLC column from Millipore Corp. (Bedford, MA) using methanol/water (96/4, v/v) as the mobile phase and stored at −20 °C under nitrogen. The purity of D-α-[^{3}H]tocopherol was checked during the course of the studies by HPLC and/or by thin layer chromatography. The radioactivity was measured with a Packard liquid scintillation analyzer (Tri-Carb 2100 TR) using Irga-Safe Plus from Packard (Meriden, CT). All other analytical-grade chemicals were obtained from Sigma or Merck (Darmstadt, Germany).

Preparation of Bovine Liver Cytosol—Bovine liver was obtained from freshly slaughtered calves. The liver was perfused with a physiological NaCl solution, divided into portions, and frozen at −80 °C for future use. Thawed liver was homogenized using a Polytron homogenizer (Kinematica GmbH, Switzerland) with 2 volumes of homogenization buffer consisting of 0.01 M sodium phosphate, pH 7.4, 0.25 M sucrose, and 1 mM phenylmethylsulfonyl fluoride. All further purification steps were carried out at 4 °C. The homogenate was centrifuged at 5000 × g for 15 min to remove crude particles and whole cells. After ultracentrifugation of the supernatant at 140,000 × g for 90 min the obtained cytosol was stored in portions at −80 °C.

Gel Filtration—For gel filtration 80 ml of cytosol was mixed with 5 μCi of α-[^{3}H]tocopherol, incubated while stirring for 2 h at 4 °C, and fractionated on a 5- x 80-cm Sephacryl S-200 gel column (Pharmacia Fine Chemicals, Uppsala, Sweden) using a 0.01 M sodium phosphate buffer, pH 7.4 (buffer A). Fractions of 10 ml were collected, and the radioactivity of each fraction was measured.

Affinity Chromatography—The pooled tocopherol binding fractions from the gel filtration were incubated while stirring overnight at 4 °C with 2.5 μCi of α-[^{3}H]tocopherol. A 0.6- x 40-cm Affi-Gel Blue column (Bio-Rad) was equilibrated with buffer A. The incubation mixture was applied to the column, which was subsequently washed with 100 ml of the same buffer. Proteins were eluted with a linear NaCl gradient, consisting of 400 ml of buffer A and 400 ml of buffer A with 1.1 M NaCl and 2 mM EDTA. Fractions of 10 ml were collected and monitored for radioactivity.

Ion Exchange Chromatography—A Mono S HR 5/20 column (Amersham Pharmacia Biotech) was equilibrated with 0.02 M sodium phosphate, pH 6.5 (buffer B) at 4 °C. The pooled fractions from the first radioactive peak of the Affi-Gel Blue chromatography were concentrated and equilibrated with buffer B by ultrafiltration using Centriplus 10 concentrators (Amicon Inc., Beverly, MA) to 4 ml. After incubation for 1 h with 2.5 μCi of α-[^{3}H]tocopherol at 4 °C, the sample was applied to the column and the column was washed with 5 ml of the same buffer. For elution a linear NaCl gradient consisting of 12.5 ml of buffer B and 12.5 ml of buffer B with 1 M NaCl was run. Fractions of 1 ml were collected and monitored for radioactivity.

Photographic Cross-linking—Protein fractions containing radioactive tocopherol were irradiated for 30 s in a self-made device consisting of a 1-ml quartz cuvette, cooled to 4 °C, and placed at a 5-cm distance to a 150-watt xenon lamp.

Gel Electrophoresis, Isoelectric Focusing, and Fluorography—The purity of the α-[^{3}H]tocopherol-containing fractions was tested by SDS-PAGE (26), and gels were stained with Coomassie Brilliant Blue R-250. Native isoelectric focusing was performed on Servalyt Precotes 3–10 precast gels as described by the manufacturer (Serva, Heidelberg, Germany). Briefly, proteins were focused at 1500 V for 3500 volt-hours at 4 °C using 1 M NaOH as catholyte buffer and 1 M H$_3$PO$_4$ as anolyte buffer. The gels were fixed with 20% trichloroacetic acid and stained in Serva Violet 17. Fluorography was carried out by incubating the gels for 30 min with 1 M sodium salicylate and subsequent drying and exposure to Biomax mass spectrometry film (Eastman Kodak Co.).

Reversed-phase HPLC—For the final purification of TAP and also for the separation of peptide digests, obtained by cyanogen bromide cleavage, reversed-phase HPLC was performed. The samples were adjusted with distilled water to 50 μl and loaded onto a Nova-Pak C4 reversed-phase column (Millipore Corp.). A linear acetonitrile gradient (0–100%, 0.1% trifluoroacetic acid) at a flow of 0.5 ml/min was used for the separations. Elution of proteins or peptides was detected at 225 nm in

the UV channel and at the same time at 280-nm excitation and 320-nm emission in the fluorescent channel. Fractions of 0.5 ml were taken, and the samples were lyophilized in a Speedvac concentrator (Savant, Munich, Germany).

Cyanogen Bromide Cleavage—Mono S fractions containing the highest radioactivity were desalted and concentrated to 30 μl with Microcon 10 microconcentrators (Amicon Inc.) and mixed with 470 μl of 8 M urea, 0.4 M ammonium bicarbonate, pH 8.0. Reduction was achieved by 15-min incubation at 55 °C in the presence of 25 μl of 100 mM dithiothreitol. For carboxyamidation, 20 μl of 0.5 M iodoacetamide was added and the sample was kept for 15 min at room temperature in the dark. The sample was dissolved to 1 ml with distilled water and again concentrated to 30 μl. After adding 70 μl of 100% formic acid, the sample was cleaved with 3 μl of 5 M CNBr under nitrogen for 24 h in the dark. To remove formic acid the sample was exposed to a nitrogen flow for 30 min.

Amino Acid Sequencing of Peptide Fragments and Homology Search—The isolated peptides were sequenced by automated Edman degradation using a pulsed liquid-phase sequencer 477A on line with a phenylthiohydantoin analyzer (PE Applied Biosystems, Foster City, CA). The obtained amino acid sequences were analyzed with the EMBL/GenBank™ data bases. The BLAST algorithm was used to search for proteins homologous to bovine TAP.

Cloning of Human and Bovine TAP—The coding area of the cDNA for human *TAP* was amplified by RT-PCR using total RNA from human small colon as template. Oligonucleotide primers were synthesized based on the genomic *TAP* sequence with a sense oligonucleotide primer (5′-ATGAGCGGCAGAGTCGGCGA-3′) and an antisense oligonucleotide primer (5′-TTATTTCGGGGTGCCTGCCCCCA-3′) (Microsynth, Balgach, Switzerland). PCR was carried out by using the GeneAmp RNA PCR Kit (Perkin-Elmer) in a GeneAmp PCR System 9700 (PE Applied Biosystems). RT-PCR conditions were: reverse transcription for 15 min at 42 °C, denaturation for 5 min at 99 °C, and cooling for 5 min at 5 °C. Half of the resulting cDNA was subjected to 40 cycles of PCR under the following conditions: 30 s at 94 °C, 30 s at 68 °C, and 90 s at 72 °C. 1 ng of the obtained cDNA was used for reamplification in 30 cycles under the same conditions. The resulting PCR product was purified with the JETsorb gel extraction kit (Genomed, Bad Oeynhausen, Germany) and subcloned into the pGEM-T vector (Promega, Madison, WI). The construct was transformed into JM101 competent cells using electroporation with 1800 V, 25 microfarads, and 200 ohms in a Gene Pulser II electroporation system (Bio-Rad). Clones were sequenced using M13 forward and reverse oligonucleotide primers (Microsynth), and sequence analysis was done using the Wisconsin Sequence Analysis Package (version 9.1).

Bovine *TAP* was cloned using primers designed from the human sequence. Total RNA from bovine liver was purified using the RNeasy Mini Kit (Qiagen, Basel, Switzerland). RT-PCR and PCR were performed as described above with a sense oligonucleotide primer (5′-ATGAGCGGCAGAGTCGGCGA-3′) and an antisense oligonucleotide primer (5′-GTCTGGAAGCAGGACCTCCAC-3′) (Microsynth). PCR products were subcloned as described above.

Human RNA Dot Blot and Northern Blot Analysis of Human TAP—Full-length human *TAP* cDNA was labeled with [^{32}P]dATP using a random primed DNA labeling kit (Roche Molecular Biochemicals). For dot blot analysis, the probe was hybridized with a blot containing poly(A)$^+$ RNA from 50 different human tissues immobilized on a positively charged nylon membrane (CLONTECH Laboratories, Heidelberg, Germany). The hybridization was carried out as described in the user manual. For Northern blot analysis of human TAP, a multiple tissue blot (CLONTECH) was probed under high stringency conditions in accordance with the manufacturer's instructions.

Cloning of His-hTAP—For protein expression of human *TAP*, a construct containing a 6-histidine-tag (His) on the carboxyl-terminal site was designed. The antisense oligonucleotide primer for the PCR encoded the hexahistidine residue, the stop codon, and an *Eco*RI restriction site (5′-CCGGAATTCTCAGTGGTGGTGGTGGTGGTGTTTCGGGGTGCCTGCCCCCA-3′). The sense oligonucleotide primer contained an *Nde*I restriction site and the start codon (5′-ACAGGGAATTCCATATGAGCGGCAGAGTCGGCGA-3′). Cloned *hTAP* was used as template. The resulting PCR product was subcloned into the pGEM-T easy vector (Promega) and analyzed as described above. To be able to induce protein expression, the *His-hTAP* cDNA was subcloned into the expression vector pET 29b using the restriction sites for *Nde*I and *Eco*RI and then transformed into *Escherichia coli* BL21.

In Vitro Expression and Purification of Human His-hTAP—For protein overexpression the His-hTAP/pET 29b construct was transformed into *E. coli* BL21-CodonPlus(DE3)-RIL (Stratagene, La Jolla, CA). Cells

Human Tocopherol-associated Protein

were grown in a liquid culture to an A_{600} of 0.6–1.0, and the expression of *His-hTAP* was induced with 1 mM isopropylthiogalactopyranoside for 3 h. Cells were harvested by centrifugation at $5000 \times g$ for 5 min and resuspended in 32 ml of ice-cold binding buffer (5 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 7.9). For disruption of the cells, a French press with a pressure of 1000 p.s.i. was used. The lysate was centrifuged at $39,000 \times g$ for 20 min to remove debris. The supernatant fraction containing solubilized His-hTAP was used in affinity chromatography with His·Bind resin (Novagen). A 2.5-ml column was run with 0.5 ml/min, and fractions of 1 ml were collected. Purification of His-hTAP was achieved in general as described in the user manual using imidazole for protein elution. His-hTAP was eluted using 200 mM imidazole in elution buffer (200 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 7.9), and the collected fractions were neutralized using 1 ml of 80 mM Tris-HCl, pH 7.0, 20% glycerol. For the detection of His-hTAP protein, an anti-His$_6$ mouse monoclonal antibody (Roche Molecular Biochemicals) was used together with the enhanced chemiluminescence system (Amersham Pharmacia Biotech) in accordance to the manufacturers.

Determination of Binding Constants Using the IASys Biosensor—An IASys single channel resonant mirror biosensor (Fisons Applied Sensor Technology, Cambridge, UK) (27, 28) was used to measure the kinetic constants and affinity of His-hTAP to a biotinylated α-tocopherol derivative. The biotinylated α-tocopherol derivative was obtained by total synthesis, and its identity as 3,4-dihydro-2,5,7,8-tetramethyl-2-(tridecylamidopentyl)-19′-(biotinamid)-2H-1-benzopyran-6-ol was determined by ^{1}H NMR and mass spectrometry.[2]

Avidin (200-μg excess) was coupled to a sensing cuvette manufactured with a biotin layer, and biotinylated tocopherol (3-μg excess) was then coupled to the immobilized avidin. Binding of tocopherol was performed in phosphate-buffered saline containing 10% dimethyl sulfoxide. Phosphate-buffered saline buffer, pH 7.2, was used for all other binding reactions. The reaction vessel was stirred continuously with the aid of a propeller. Binding was measured at 2-s intervals, and the data readout from the biosensor was measured in units of arc-seconds. The reactions were routinely followed for at least 5 min. The k_{on} value for each binding experiment, as well as the kinetic constants, were calculated using the "Fast Fit" program (Fisons Applied Sensor Technology) (29).

Replacement of His-hTAP Bound [^{3}H]-α-Tocopherol with Aternate Ligands—Radioactive α-[^{3}H]tocopherol (7.9 μg) was dissolved in 100 μl of water by addition of 2-hydroxypropyl-β-cyclodextrin (144 mg). The unlabeled ligands were solubilized in water by 2-hydroxypropyl-β-cyclodextrin (144 mg in 100 μl) and adjusted to a final concentration of 2.5 mM. The concentrations of the dissolved unlabeled ligands were determined in water by UV spectrophotometry. The dissolved radioactive tocopherol (135 μl) was added to 100 μl of purified His-hTAP (10 mg/ml) in 10 mM Tris, pH 7.4, and incubated overnight at 4 °C. From this mixture, aliquots of 10 μl were added to 20 μl of the corresponding nonlabeled ligand and incubated overnight at 4 °C. To this mixture, 600 μl of binding buffer (5 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 7.9) containing 30 μl of His·Bind resin was added, and after shortly shaking the mixture the resin was centrifuged at $14,000 \times g$ for 1 min. The supernatant was carefully removed, and the resin was washed sequentially in 1 ml of binding buffer and then in 1 ml of binding buffer containing 2% BSA. The bound protein was eluted from the resin by adding 1 ml of elution buffer (1 M imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 7.9) containing 2% BSA. After centrifugation, the amount of α-[^{3}H]tocopherol bound to His-hTAP was determined in the supernatant by scintillation counting.

RESULTS

Purification of Bovine Tocopherol-associated Protein, TAP— The identification of the novel cytosolic tocopherol-binding protein was obtained by a modification of a previously shortly published protocol (25). The preparation of hepatic cytosol and the first purification step were performed in accordance to Kuhlenkamp *et al.* (30). Hepatic cytosol was incubated with α-[^{3}H]tocopherol and fractionated by size-exclusion chromatography on a Sephacryl S-200 column. A radioactive protein peak corresponding to a molecular mass range from 30 to 70 kDa was obtained (Fig. 1A). Further purification by Affi-Gel Blue affinity chromatography revealed two radioactive protein peaks (Fig. 1B). To prove that the radioactivity in both peaks
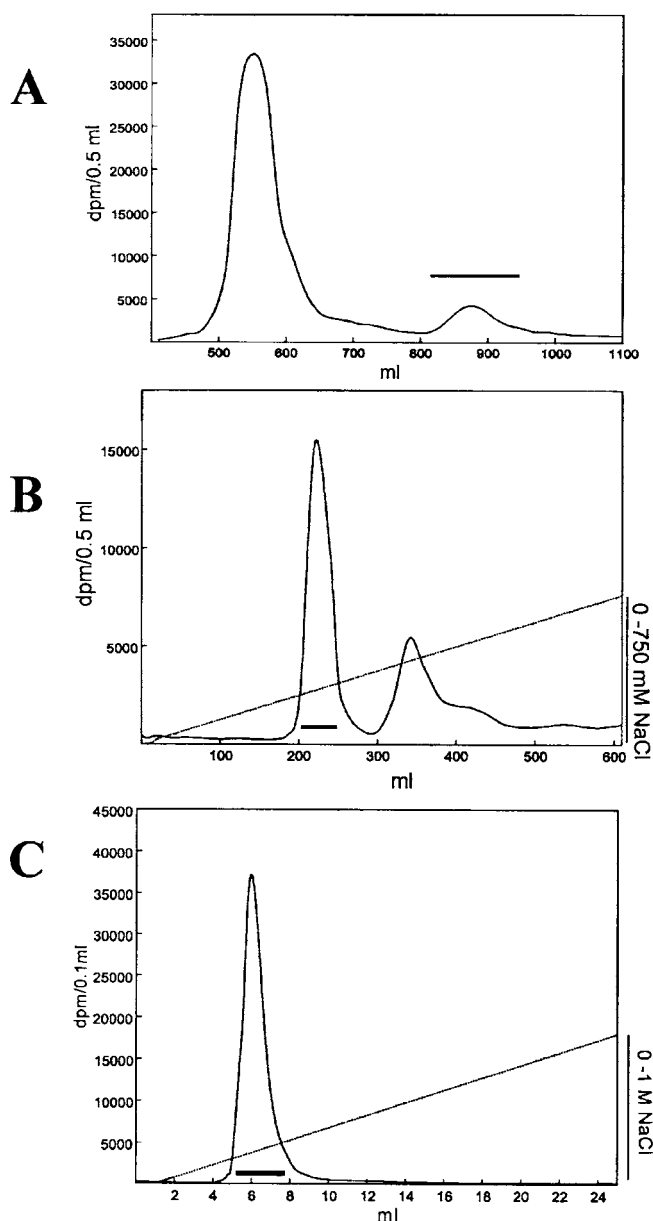
FIG. 1. **Elution of α-[^{3}H]tocopherol on Sephacryl S-200 gel filtration (A), Affi-Gel Blue affinity chromatography (B), and ion exchange chromatography (C).** The *horizontal bars* indicate pooled fractions used for further purification. *Broken lines* show salt gradients. See "Experimental Procedures" for details.

was associated with distinct proteins, native isoelectric focusing was performed. Fractions from peak 1 showed a major radioactive spot that corresponded to a protein with a pI of 8.1. The fractions of peak 2 revealed a double signal focusing around a pI of 6.5 (Fig. 2A). Fractions of both peaks were irradiated to covalently link the bound α-[^{3}H]tocopherol to the corresponding proteins. Subsequent fluorography revealed signals at 46 and at 31 kDa in fractions from peaks 1 and 2, respectively (Fig. 2B).

The α-tocopherol-transfer protein (α-TTP) from rat liver was shown to have a molecular mass of 31 kDa with reported isoforms having pIs of 5.3 and 5.4 (31). This led to the conclusion that the 31-kDa protein peak producing a double band at pI 6.5 represented the two isoforms of the corresponding bovine subtype of α-TTP. Thus, the additional protein contained in peak 1 had to be considered as a novel α-tocopherol-binding protein. Being the only function known of this novel protein, *i.e.*

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[2] A. Stocker and S. Zimmer, unpublished results.

A 1 2

pI

8.1 →

6.5 →

B 1 2

kDa

46 →

31 →
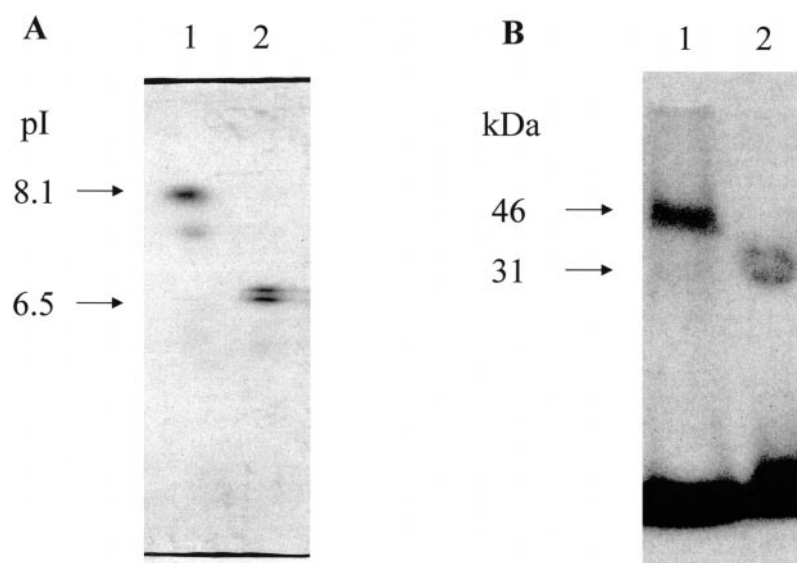
FIG. 2. **Fluorography of isoelectric focusing (*A*) and photographic cross-linking (*B*).** Radioactive signals in *lane 1* at pI of 8.1 and 46 kDa show bTAP. The signals in *lane 2* at pI 6.5 and 31 kDa show the hypothetical bovine tocopherol transfer protein.

that of associating α-tocopherol (used as a purification criterion), it was named tocopherol-associated protein (TAP). Further purification of bovine TAP was achieved by cation exchange chromatography on a Mono S column (Fig. 1*C*). SDS-PAGE analysis of the fraction with the highest radioactivity showed one predominant band at 46 kDa with minor contaminating proteins (Fig. 3*B*). A final purification step was performed by reversed-phase chromatography on a wide pore C4 column (Fig. 3*A*). SDS-PAGE analysis revealed one single protein band with an apparent molecular mass of 46 kDa (Fig. 3*B*). The estimation of the molecular mass of the purified native protein by gel filtration (data not shown) confirmed that bovine TAP was a monomeric protein with an apparent molecular mass of 46 kDa.

Cyanogen Bromide Cleavage and Peptide Identification—Because direct sequencing of the amino-terminal end by automated Edman degradation failed, a cyanogen bromide cleavage of bovine TAP was performed. This was followed by reversed-phase HPLC using a C4 column to separate the obtained peptide fragments. From the elution profile two major peaks at 23 and 26 min were fractionated and sequenced. Their Edman degradation analysis showed two sequences: EVLPSQRYNAHLVPEDGSL and FEENYPETLKXLFIVGAP, representing two peptides of 19 and 18 amino acids, respectively.

Identification of the Human TAP Gene and Sequence Homologies—The obtained peptide sequences of bovine TAP were used to search for known proteins in data banks using the BLAST sequence alignment program. Both bovine sequences matched with 84% and 83% identity with a hypothetical protein of 403 amino acids having a calculated molecular weight of 46,145. This hypothetical protein is coded by a human gene that is deposited at the EMBL/GenBank[TM] with accession number AL096881. Both methionine residues responsible for cyanogen bromide cleavage of the bovine protein were found to be conserved in the human sequence, indicating that this protein represents the human subtype of bovine TAP. The nucleotide sequence of human TAP derives from the *Homo sapiens* PAC clone DJ130H16, which has been sequenced and mapped by the Sanger Center chromosome 22 mapping group. The gene maps within chromosome 22 in region 22q12.1-ter and consists of 12 exons. A computational analysis indicated that human TAP is a homologue to a variety of functionally unrelated proteins (Fig. 4). It was recognized that these proteins share structurally conserved elements constituting a hydrophobic lipid binding pocket called CRAL_TRIO, the profile of which is deposited at the PFAM protein family data base (32).

Cloning of Bovine TAP—To confirm that the gene, identified through the data base analysis, represented the human homologue of *TAP*, bovine *TAP* was cloned using the primers coding for the human sequence. Using total RNA from bovine liver as template for RT-PCR, a 1.1-kb fragment was obtained showing the expected size according to the chosen primers. By cloning the 1.1-kb fragment into the pGEM-T vector and subsequently sequencing, the identity of the peptides obtained from cyanogen bromide cleavage of bovine TAP was confirmed. A comparison of the protein sequences of the bovine fragment of TAP with human TAP revealed a 95% homology (alignment not shown).

Cloning of Human TAP—To further study its body distribution, hTAP was cloned and its cDNA was used for *in vitro* expression and Northern analysis studies. RT-PCR was carried out using total RNA from human intestine and primers containing the 3'- or 5'-end sequence of human *TAP*. After reamplification the 1.2-kb cDNA fragment was cloned into the pGEM-T vector. Sequence analysis of the subcloned cDNA confirmed that the clone contained the entire open reading frame of human *TAP* with a 100% identity on the nucleotide sequence.

Tissue Distribution of Human TAP—To assess the presence and relative abundance of *hTAP* gene transcripts in human tissues, a commercially available Multiple Tissue Expression Array (CLONTECH) was used containing poly(A)$^+$ mRNAs from 50 different human tissues. Hybridization was carried out with α-[^{32}P]dATP-labeled cDNA of the human *TAP* clone. Expression levels of target mRNA were determined by analyzing the washed blot with a phosphor imager. The results show a broad tissue distribution of mRNA for *hTAP* with strong signals in liver, prostate, and brain tissues (Fig. 5*A*). To assess the relative expression levels of mRNA the strongest signal on the master blot was set to 100%, and all other signals were calculated as a percentage of it (Fig. 5*B*). Signals showing an expression level lower then 5% were neglected because of background variations. In contrast to the broad tissue distribution of *hTAP* in adult tissues, fetal tissues were found to have low expression levels in the range of the background. Northern analysis using a multiple tissue blot (CLONTECH) revealed one major mRNA transcript of approximately 2.8 kb and two minor transcripts of approximately 4.2 and 1.5 kb. The 2.8-kb transcript was found to be strongly expressed in liver and brain
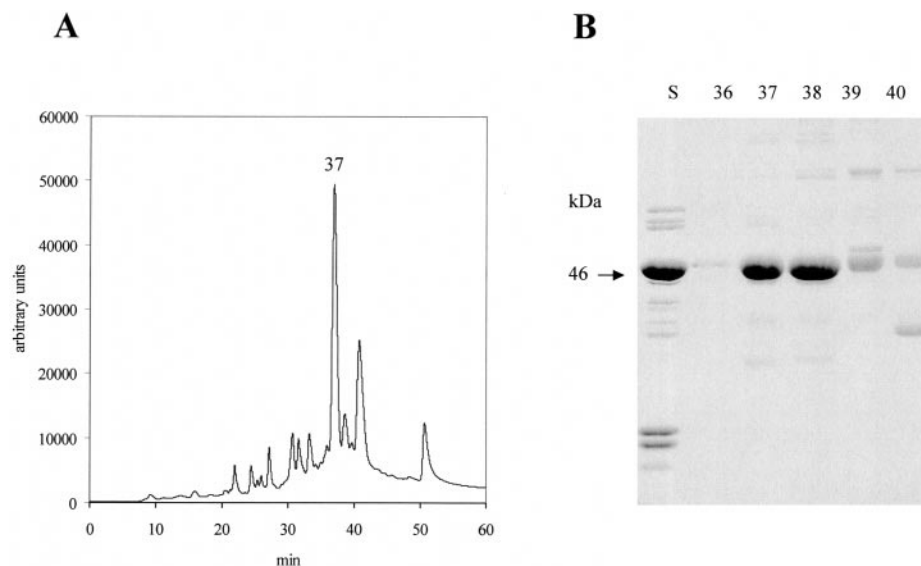
FIG. 3. **Reversed-phase HPLC of bTAP containing fractions from ion exchange chromatography.** *A*, protein was eluted on a C4 reversed-phase column with a linear acetonitrile gradient (0–100%, 0.1% trifluoroacetic acid). *B*, SDS-PAGE of purified bTAP (Coomassie staining). *Lane S* indicates the fraction from Mono S purification; *lanes 36*, *37*, *38*, and *39* represent fractions from the purification by reversed-phase chromatography.

FIG. 4. **Alignment of the CRAL_TRIO-domain from human TAP with homologous proteins.** Human TAP (amino acids 63–246; EMBL/GenBank® accession number AL096881) has been aligned with human cellular retinal-binding protein (J04213), human tyrosine phosphatase (M83738), squid retinal-binding protein (S68871), 45-kDa secretory protein from rat olfactory epithelium (AJ132352), yeast phosphatidylinositol transfer protein (X15483), and human α-tocopherol transfer protein (D49488). Gaps (.) have been inserted to demonstrate maximum sequence similarity. Residues identical or showing conserved substitution with those of human TAP are indicated with *shaded letters*. Conserved amino acid substitutions are defined as pairs of residues belonging to one of the following groups: S/T/P/A/G; N/D/E/Q; H/R/K; M/I/L/V; and F/Y/W.

and weakly expressed in kidney (Fig. 6). The existence of a human 2.8-kb mRNA transcript (AL096881) encoding for a hypothetical protein with homology to *Rattus norvegicus* 45-kDa secretory protein (AJ132352) has been proposed recently. Based on these results and the dot blot analysis described above, we conclude that the major mRNA transcript of *hTAP* has 2818 base pairs and is widely expressed in human tissues, with the highest levels being found in liver, brain, and prostate.

Cloning of His-hTAP, in Vitro Expression and Purification—To permit a convenient protein isolation of hTAP, we designed a construct containing a His-tag with six carboxyl-terminal histidine residues. PCR was carried out using an antisense oligonucleotide primer containing the sequence of the His-tag, a stop codon, and an *Eco*RI restriction site for ligation into the expression vector pET 29b. The sense oligonucleotide primer contained an *Nde*I restriction site next to the start codon. The probe was subcloned into the expression vector pET

29b under the control of the T7*lac* promoter, and the sequence of the construct was confirmed through sequence analysis. The plasmid was transformed into the BL21-CodonPlus(DE3)-RIL cell line, which contains an additional plasmid coding for *argU*, *ileY*, and *leuW* tRNA genes to avoid limitation of certain tRNA species (33). Overexpression of the recombinant His-hTAP protein was induced with 1 mM isopropylthiogalactopyranoside for 3 h, and protein expression was confirmed by Western blot analysis using an anti-His$_6$ antibody (Fig. 7*B*). After cell disruption with a French press, the recombinant His-hTAP was located in the soluble fraction. Purification of His-hTAP was achieved by affinity chromatography using His·Bind resin charged with Ni^{2+} ions by stepwise increasing imidazole concentrations (Fig. 7*A*).

His-hTAP Binding to Biotinylated α-Tocopherol Derivative Using the IASys Biosensor—The interaction of His-hTAP with the immobilized biotinylated α-tocopherol derivative was stud-
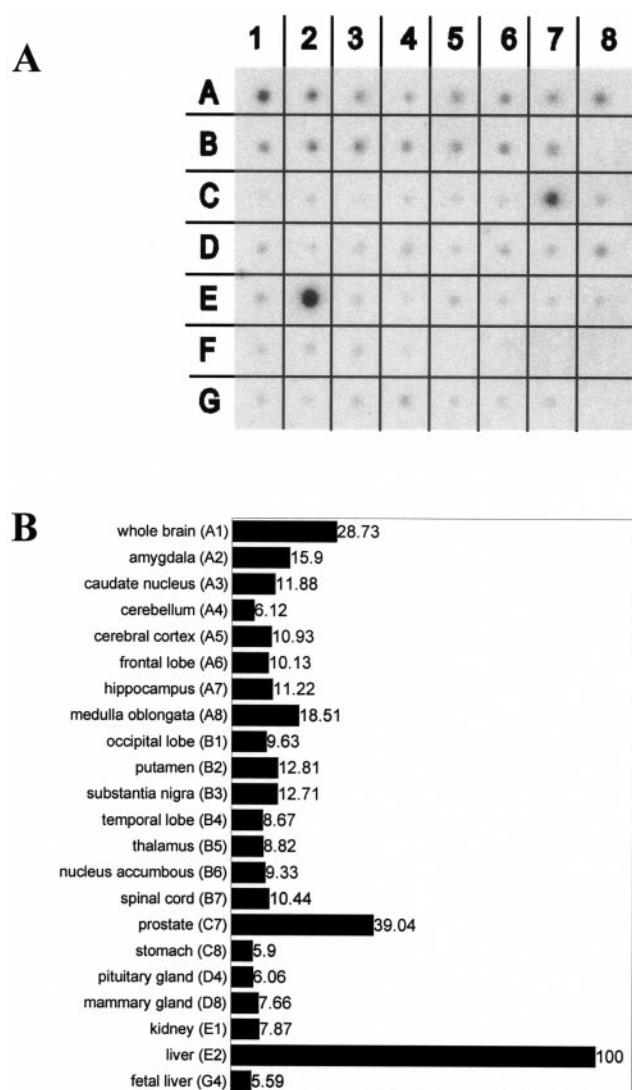
FIG. 5. **Multiple tissue expression analysis of *hTAP.*** *A*, autoradiodiagram of human RNA master blot (CLONTECH) hybridized with a ^{32}P-labeled cDNA of hTAP. *B*, dot-to-dot variations are expressed as percentage of maximal intensity relative to the liver tissue.
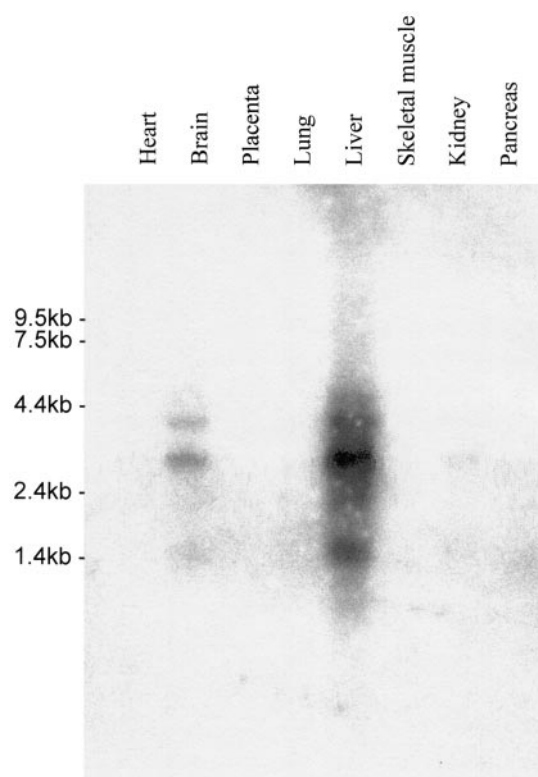

FIG. 6. **Northern analysis of hTAP.** The size distribution of the *hTAP* transcript was analyzed using a human multiple tissue Northern blot (CLONTECH) hybridized with a ^{32}P-labeled cDNA of hTAP.
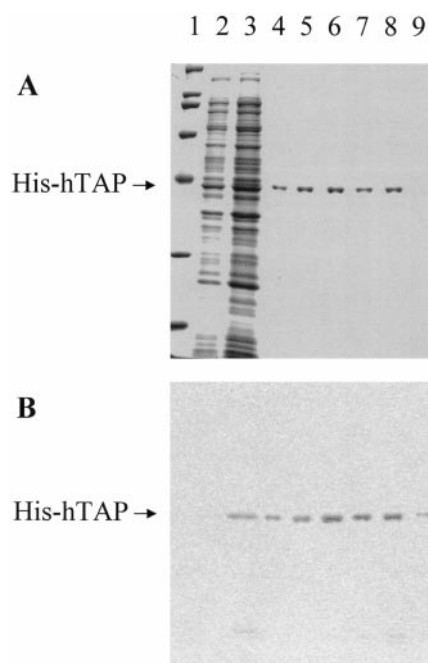

FIG. 7. **Analysis of purification of His-hTAP.** The purification of recombinant His-hTAP was followed by SDS-PAGE (*A*) and Western blotting (*B*). *Lane 1* shows the molecular weight standard, *lanes 2* and *3* show the *E. coli* supernatants uninduced and after induction. In the *residual lanes* the elution of pure His-hTAP on Ni-NTA-Sepharose using 200 mM imidazole is depicted.

ied with the IASys resonant mirror biosensor. In these experiments the biotin label of the derivative was immobilized by binding to avidin coupled to the biotin surface of a cuvette, and the binding at different His-hTAP concentrations was monitored over a minimum 5-min period. As shown in Fig. 8 the derivative consists of a biotin label being attached by an amide bond to the tail of the synthetic straight-chain *R*-α-tocopherol derivative. The plots of the response observed following the addition of the indicated concentrations of His-hTAP and BSA show that His-hTAP binds significantly to the tocopherol derivative, whereas no significant binding was observed with BSA (Fig. 9*A*). Comparing the plots at different concentrations the binding of His-hTAP was found to be concentration-dependent in the range of 100 nM to 5 μM. When the binding data for the interaction of His-hTAP with immobilized tocopherol were fitted to the double exponential curve, a linear relationship was observed between k_{on} and ligand concentration according to the equation $k_{on} = k_{diss} + k_{ass}$ [ligand] (Fig. 9*B*). Using this method, the k_{ass} ($1.759 \pm 0.113 \times 10^3$ M^{-1} s^{-1}) and k_{diss} ($8.08 \pm 2.46 \times 10^{-4}$ s^{-1}) values for the interaction are given by the slope and *y* intercept of the plot, respectively. Values of dissociation constants (K_D) were calculated from the relationship K_D

$= k_{diss}/k_{ass}$. For the binding of TAP to the tocopherol derivative, the K_D value was calculated to be 4.6×10^{-7} M.

Replacement by Various Ligands for His-hTAP-bound α-[^{3}H]Tocopherol—To assess the binding specificity of His-hTAP for various forms of tocopherol and hydrophobic ligands,
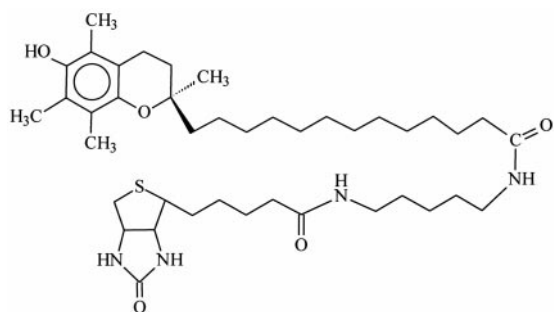
3,4-dihydro-2,5,7,8-tetramethyl-2-(tridecylamidopenty-19'-(biotinamid)-2H-1-benzopyran-6-ol

FIG. 8. **Biotinylated α-tocopherol derivative.** The structure of total synthetic biotinylated α-tocopherol, as confirmed by ^{1}H NMR and mass spectrometry; the proposed systematic name is given underneath.

replacement of α-[^{3}H]tocopherol by a 64-fold excess of each alternative ligand was determined. The results of Table I represent the average of duplicate experiments ± S.E. Tocopherols were the most efficient species in replacing His-hTAP-bound α-[^{3}H]tocopherol followed by biotinylated α-tocopherol derivative. 3-*sn*-Phosphatidylcholine appeared to be without effect.

DISCUSSION

Strategy to Reveal the Existence of a New α-Tocopherol-Binding Protein—The present study describes the finding, cloning, and *in vitro* expression of the human gene encoding the tocopherol-associated protein (*hTAP*). Initial investigations on the cellular distribution of vitamin E have led to the identification of a novel cytosolic protein (bTAP) from bovine liver that binds radioactively labeled α-tocopherol. Peptide mapping of bTAP revealed two peptide sequences matching with high homology to a human gene encoding for a 403-amino acid protein of unknown function. To obtain conclusive evidence for the hypothesis that this protein was identical with the human subtype of bTAP a 1.1-kb fragment of the bovine gene was amplified by RT-PCR using primers deriving from the human gene and bovine RNA as template. The high degree (95%) of sequence homology of the amplicon gave unequivocal evidence that this gene represents the human subtype of *bTAP*.

Tocopherol Binding by His-hTAP—α-Tocopherol appears to be specifically selected in the human body and exerts specific functions uniquely related to its structure. The former event is performed by the liver α-TTP; the latter are α-tocopherol exclusive actions at the level of cell signal transduction that result in α-tocopherol-dependent gene expression. How does the regulation of signal transduction and gene expression by α-tocopherol take place? Is there a need for a cellular protein recognizing α-tocopherol? To establish if hTAP may play such a role, the interaction between the protein and tocopherol was investigated. The technique employed simulates the *in vivo* situation where a cytosolic protein (hTAP) accesses a hydrophobic surface (the membrane) that contains α-tocopherol. A biotinylated α-tocopherol derivative was immobilized and used as a ligand for the binding measurements performed using an IASys-resonant mirror system and recombinant His-hTAP. The biotinylated α-tocopherol has been shown (cf. Table I) to bind with hTAP, as indicated by the competition experiments with hTAP-bound radioactive α-tocopherol, although with a lower affinity. The obtained data show a dose-dependent saturable binding of His-hTAP to the biotinylated tocopherol, and at the same time they have excluded the possibility of nonspecific interactions as shown by the absence of binding of BSA. The data show a dissociation constant of 4.6×10^{-7} M. This
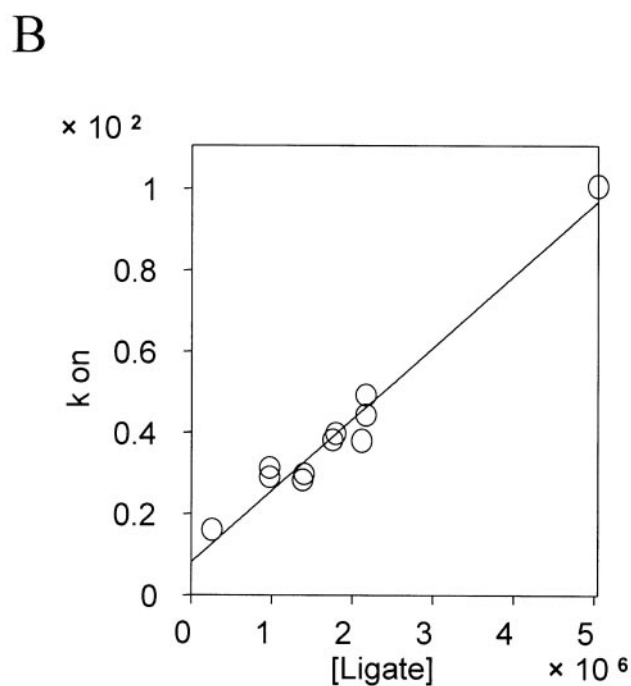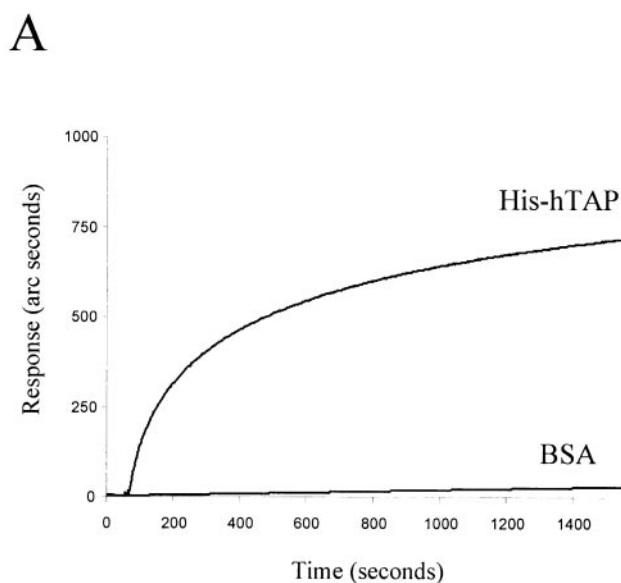

FIG. 9. **Interaction analysis of soluble His-hTAP with the immobilized biotinylated α-tocopherol derivative.** *A*, comparison of the interaction of 100 μg of His-hTAP and 100 μg of BSA with immobilized, biotinylated α-tocopherol derivative via avidin, coupled to the biotin surface of a cuvette. The measurements were made by an IASys resonant mirror biosensor. A saturating response of 800 arc-seconds was observed for His-hTAP, equivalent to 1.5 ng/mm^2, according to the information supplied by the manufacturer for planar cuvette surfaces. *B*, determination of binding constants for the interaction of His-hTAP with immobilized biotinylated α-tocopherol from three separate experiments, using the IASys resonant mirror biosensor. For each concentration of His-hTAP, the rate constants (k_{on}) were determined by the linearization procedure using the Fast Fit program. The plot of k_{on} *versus* His-hTAP concentrations gave a straight line with a slope representing k_{ass} and the intercept with the *y* axis equivalent to k_{diss}. The calculated correlation coefficient for this data is 0.982.

suggests that the recombinant His-hTAP is expressed with a functional lipid-binding domain and that it binds to biotinylated tocopherol well within physiological concentrations. However, the efficiency of cold α-tocopherol in competing with the

TABLE I
Replacement of His-hTAP bound α-[³H]tocopherol by various ligands

Replacement of α-[³H]tocopherol bound to His-hTAP was determined in the presence of 64-fold excess of the indicated ligands as described under "Experimental Procedures." The replacement factors have been calculated from the mean values ± S.E. taking the value of 3-*sn*-phosphatidylcholine as 1.

Ligand	dpm	Mean ± S.E.	Replacement
RRR-α-tocopherol[a]	2342 2080	2211 ± 185	3.82 ± 0.32
RRR-β-tocopherol	1682 1570	1626 ± 79	5.19 ± 0.25
RRR-γ-tocopherol	1592 1485	1539 ± 76	5.48 ± 0.27
RRR-δ-tocopherol	2066 1989	2028 ± 54	4.15 ± 0.11
Biotinylated α-tocopherol	6849 6575	6712 ± 194	1.26 ± 0.04
3-*sn*-Phosphatidylcholine	8225 8646	8436 ± 298	1.00 ± 0.04

[a] RRR, indicates the stereospecificity of the carbon atoms 2, 4′, and 8′.

hot hTAP-bound one is higher than that of the biotinylated form. This indicates that the dissociation constant obtained with this method is underestimated.

These findings are also compatible with an *in vivo* role of hTAP in tocopherol-mediated intracellular functions. The specificity of α-tocopherol binding to hTAP has been studied by competition experiments between radioactive α-tocopherol bound to hTAP and a number of hydrophobic molecules dissolved in the aqueous medium by β-cyclodextrin. The use of a radioactive assay, the only one available at the present moment, has limited the analysis to the question of tocopherol specificity in comparison with other ligands. Further studies on the relative affinity of the different tocopherols require the development of a more sensitive assay. However, it was already possible to show that more α-tocopherol remains bound to the His-hTAP when biotinylated tocopherol is used as a competitor. All other tocopherols were equally effective at the concentrations used (1.7 mM). A titration of the release of bound α-tocopherol at lower concentrations of other tocopherols would be needed to establish their relative affinities, but the assay precision does not permit this information to be obtained.

Analogy with CRAL Proteins—The structural analysis of *hTAP* indicates that it is a member of the widespread SEC14-like protein family. All members of this family have a common conserved lipid-binding domain called CRAL_TRIO. The structural determinants of the CRAL_TRIO domain have been identified by Sha *et al.* (34) by resolving the crystal structure of the phosphatidylinositol transfer protein from yeast (SEC14). The *SEC14* gene product has been shown to play an essential role in yeast Golgi function. The presence of an SEC14-like lipid-binding domain in hTAP indicates that this region may be involved in hTAP membrane localization. In contrast to *SEC14* and *α-TTP*, the *hTAP* gene product contains a unique 157-amino acid carboxyl-terminal extension. The strong overall sequence homology of *hTAP* with the 45-kDa secretory protein from rat (AJ132352), which also contains this extension, indicates that both proteins are closely related. The 45-kDa rat secretory protein is present in cells of the olfactory epithelium, the apical region of the trachea, the surface layer of the ciliated bronchial epithelium, and the epidermis (35). Opposite to this, *hTAP* mRNA is ubiquitous and strongly expressed exclusively in adult tissues.

The possibility that *hTAP* represents an additional α-tocopherol transfer protein cannot be excluded. However, in contrast to α-TTP, which is present essentially only in the liver (36), hTAP mRNA is ubiquitous and present in liver, in the prostate, and in brain in larger amounts. α-TTP lacks the carboxyl terminus domain, which suggests there is a different functional role for hTAP. Affinity data of α-TTP that suggest a recognition of the different tocopherols and tocotrienols cannot yet be obtained for TAP, although the latter can distinguish between tocopherols and other hydrophobic ligands as well as biotinylated tocopherol.

Possible Function of hTAP—The presence of hTAP expression in almost all tissues, although in different amounts, is indicative of an important general cellular function of this protein. Its lack in fetus liver may suggest also an embryogenetic role.

The search for an hTAP function may be guided by results obtained with analogous proteins. SEC14 has been suggested to play a role in phospholipid exchange (37, 38). The GTP binding motive, found to be present together with GTP binding in TAP,[3] is similar to what has been described for the rat secretory protein (39). A GTP binding/GTPase activity may be important to confer to hTAP regulation properties in its α-tocopherol redistribution function. Proteins having SEC14-like domains can interact with ras[21] and through this binding be able to suppress ras[21] activity (40).

Conclusions—In conclusion, intracellular proteins with the ability to bind and redistribute α-tocopherol as well as α-tocopherol receptor molecules appear to be needed to fully understand the mechanism of action of this molecule. It is still too early to state if hTAP is one or the other, but in either case hTAP may be an important molecule (or a member of a family of molecules) capable of binding α-tocopherol and responsible for important intracellular events.

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GENOMICS PROTEOMICS AND BIOINFORMATICS:

A Novel Human Tocopherol-associated Protein: CLONING, IN VITRO EXPRESSION, AND CHARACTERIZATION

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