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Original Research Article

Identification of a Novel Cytosolic Tocopherol-Binding Protein: Structure, Specificity, and Tissue Distribution

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Summary

α-Tocopherol plays an important role as a lipid-soluble antioxidant. It is present in all major mammalian cell types and shows tissue-specific distribution. This suggests the presence of specific proteins involved in intracellular distribution or metabolism of α-tocopherol. A diminution of tocopherol plasma concentrations contributes to the development of diseases such as vitamin E deficiency (AVED), atherosclerosis, and prostate cancer. Further evidence has been obtained for the existence of sites in cellular metabolism and signal transduction where α-tocopherol potentially plays a regulatory role. A signal transduction modulation specific for α-tocopherol has been described in several model systems. Using radioactively labeled α-tocopherol as tracer, we have isolated a new α-tocopherol-associated protein (TAP) from bovine liver. This protein has a molecular mass of 46 kDa and an isoelectric point of 8.1. From its partial amino acid sequence, a human gene has been identified with high homology to the newly described protein. Sequence analysis has established that the new TAP has structural motifs suggesting its belonging to a family of hydrophobic ligand-binding proteins (RALBP, CRALBP, α-TTP, SEC 14, PTN 9, RSEC 45). Human TAP has been cloned into *Escherichia coli*, and its tissue-specific expression has been assessed by Northern blot analysis.

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Keywords Atherosclerosis; AVED; cloning; gene expression; prostate cancer; α-tocopherol; α-tocopherol-associated protein (TAP); tocopherol-binding protein.

INTRODUCTION

The protective effects of vitamin E (D-α-tocopherol) against propagation of free radical damage of biological membranes are well documented for both in vitro and in vivo models (*1, 2*). The transport of absorbed α-tocopherol by low-density lipoproteins in the plasma compartment and the characterization of a liver-specific α-tocopherol transfer protein have been described (*3*). However, the mechanism of tissue uptake, storage, and degradation of α-tocopherol in mammalian cells is not fully understood. Tissue-specific distribution of various vitamin E forms—tocotrienols and tocopherols—in mammals suggests that they have unique roles in cellular functions and may indicate a specific regulation at the level of single tissues (*4*). The presence of tocopherol-associated proteins involved in intracellular transport or metabolism may fulfill the regulatory role required by such an uneven distribution. Disorders in tocopherol transport and diminution of its plasma concentrations contribute to the development of diseases such as, vitamin E deficiency (AVED)[2] and atherosclerosis, respectively (*5, 6*). Besides the presence of the α-tocopherol transfer protein in liver (*7*), and in small amounts, in other tissues (*8*), further evidence has been obtained for the existence of potential sites in cellular metabolism and signal transduction where α-tocopherol plays a regulatory role. In particular a signal transduction modulation specific for α-tocopherol has been described in several model systems (*9–11*).

In this study we describe for the first time the identification and characterization of a novel cytosolic tocopherol-associated protein (TAP) containing structural motifs that suggest it belongs to a class of hydrophobic ligand-binding proteins (RALB, CRALB, α-TTP, SEC 14, PTN 9, RSEC 45). The function of this protein is not yet established but it may be involved in cellular transport of α-tocopherol and α-tocopherol-dependent cell signaling.

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[2]Abbreviations: AVED, ataxia with vitamin E deficiency; RT-PCR, reverse transcriptase–polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TAP, α-tocopherol-associated protein; TTP, α-tocopherol-transfer protein; TLC, thin-layer chromatography.

EXPERIMENTAL PROCEDURES

Materials

Tritiated D-α-tocopherol (24.76 Ci/mmol) was purchased from Amersham International (Bucks., U.K.). Sephacryl S-200, Mono S HR 5/20 column, and pI marker proteins (pI 3.5–9.3) were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Affi-Gel Blue gel (100–200 mesh) and broad range molecular standards for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and gel filtration were obtained from Bio-Rad Laboratories (Hercules, CA). For ultrafiltration, Microcon- and Centricon-10 concentrators from Amicon Inc. (Beverly, MA) were used. Servalyt Precotes 3–10 precast gels for isoelectric focusing were obtained from Serva (Heidelberg, Germany). For fluorography, Biomax MS film from Eastman Kodak Co. (Rochester, NY) was used. Human RNA Master Blot was purchased from Clontech Laboratories (Heidelberg, Germany). All other chemicals were obtained from Sigma Co. (St. Louis, MO) or Merck (Darmstadt, Germany) unless otherwise stated.

Methods

Tritiated D-α-tocopherol was further purified on a Nova-Pak C_{18} HPLC column from Millipore Corp. (Bedford, MA), with methanol/water (96/4, v/v) used as the mobile phase, and then 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 by thin-layer chromatography (TLC) (*12*). The radioactivity was measured with a Packard liquid scintillation analyzer (Tri-Carb 2100 TR) and using Irga-Safe Plus from Canberra Packard (Meriden, CT).

To establish that the radioactivity bound to TAP was from α-tocopherol, we extracted the tritiated tocopherol with $CHCl_3$. The extract was separated by TLC and the zone containing tocopherol was analyzed by scintillation counting.

Preparation of Bovine Liver Cytosol. Bovine liver was obtained from freshly slaughtered calves. The liver was perfused with 10 l of 0.9% (w/v) NaCl divided into 200-g portions and frozen at −80 °C for future use. For the preparation of cytosol, a 200-g portion was thawed and cut into small pieces in homogenization buffer (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 liver was homogenized to a 33% (w/v) homogenate with a Polytron (Kinematica GmbH, Switzerland) for 5 min. The homogenate was centrifuged at 5000 *g* for 15 min. The supernatant was then centrifuged at 140 000 *g* for 90 min and the subsequent supernatant obtained was stored at −80 °C.

Purification of TAP. For gel filtration 80 ml of supernatant was mixed with 5 μCi of [^{3}H]α-tocopherol, incubated while stirring for 2 h, and applied to a 5 × 80 cm Sephacryl S-200 column with 0.01 M sodium phosphate, pH 7.4 (buffer A), as previously described (*13*). Fractions (10 ml) were collected, and the radioactivity of each fraction was measured by scintillation counting. The fractions corresponding to proteins with a molecular mass of 30–70 kDa were pooled (150 ml/run) and then incubated with 2.5 μCi of [^{3}H]α-tocopherol overnight (15 h). This mixture was next applied to a 0.6 × 40 cm Affi-Gel Blue column in buffer A. The column was washed with 100 ml of buffer A, and the proteins were eluted with a linear salt gradient (0–0.75 M NaCl). Pooled fractions of the first tocopherol-binding peak from Affi-Gel Blue chromatography were equilibrated in 0.02 M sodium phosphate, pH 6.5, by ultrafiltration and then incubated for 1 h with 2.5 μCi of [^{3}H]α-tocopherol. Cation-exchange chromatography was carried out on a Mono S column equilibrated in 0.02 M sodium phosphate, pH 6.5. Proteins were eluted with a linear salt gradient (0–1 M NaCl).

CNBr-Cleavage and Sequencing of Peptide Fragments. The Mono S-eluted fractions containing the most radioactivity were concentrated to a final volume of 30 μl and mixed with a solution of 470 μl of 8 M urea and 0.4 M ammonium bicarbonate, pH 8.0. The mixture was heated for 15 min at 55 °C in the presence of 25 μl of 100 mM dithiothreitol. After the mixture had cooled to room temperature, 20 μl of 0.5 M iodoacetamide was added and the mixture was incubated for 15 min in the dark. After dilution to 1 ml with distilled water, the sample was concentrated to 30 μl final volume. Then 70 μl of 100% formic acid followed by 3 μl of 5 M CNBr was added and the sample was overlaid with nitrogen and kept in the dark for 24 h. Formic acid was removed from the sample by evaporation under a nitrogen flow for 30 min. The volume of the mixture was adjusted with distilled water to 50 μl and its components were separated on a C_4 reversed-phase column eluted with a linear acetonitrile gradient (0–100%) containing 0.1% trifluoroacetic acid and a flow rate of 0.5 ml/min. Detection of the peptides was monitored by absorbance at 225 nm in the ultraviolet channel and by 280 nm excitation and 320 nm emission in the fluorescence channel. Lyophilized samples were subjected to automated Edman degradation on a gas-phase sequencer on line with a phenytoin analyzer. The obtained amino acid sequences were compared with Gen Bank/EMBL databases.

Electrophoresis. The SDS-PAGE was performed as previously described (*14*), using 15% slab gels and staining with colloidal Coomassie Brilliant Blue R-250 (*15*). Photo-crosslinking was carried out in a self-made device by using 1-ml quartz cuvettes at 4 °C and irradiating tocopherol-containing protein fractions at a distance of 5 cm with a 150 W xenon lamp for 10, 30, 60, and 120 s. Native isoelectric focusing was carried out with precast gels and a catholyte buffer of 1 M NaOH and an anolyte buffer of 1 M H_3PO_4. During focusing the temperature was kept at 4 °C, and the sample applicator strips were removed after 45 min. Focusing was finished after 3500 Vh with maximum power supply settings at 1500 V, 6 mA, and 0.8 W/cm. The gels were fixed with 20% trichloroacetic acid and stained in Serva Violet 17. Before fluorography, the gels were incubated 30 min with 1 M sodium salicylate; after drying, they were exposed at −80 °C.

Cloning and Sequencing of cDNA Fragments. The coding area of the cDNA for human TAP was amplified by reverse transcriptase–polymerase chain reaction (RT-PCR). Total

RNA from human intestine was used as template and two synthetic oligonucleotide were obtained from Microsynth (Balgach, Switzerland). The sequence of the 5′ oligonucleotide primer was 5′-ATGAGCGGCAGAGTCGGCGA-3′, that of the 3′ oligonucleotide primer was 5′-TTATTTCGGGGTGCCTGCCCCCA-3′.

Full-length human TAP cDNA was cloned into the pGEM-T vector from Promega (Madison, WI). Plasmid DNA sequencing with M13 forward and reverse primer was carried out by Microsynth. Sequence analysis was done by using the Wisconsin Sequence Analysis Package (version 9.1) from GCG (Genetics Computer Group, Madison, Wisconsin).

Human RNA Master Blot. Human TAP cDNA was labeled with a random primed DNA labeling kit from Boehringer (Mannheim, Germany). Poly A^+ RNAs from 50 different human tissues were tested with a dot-blot obtained from Clontech. The hybridization was carried out as described in the users manual. The results were analyzed using a GS-250 Molecular Imager from Bio-Rad Laboratories.

RESULTS

Purification, CNBr-Cleavage, and Sequencing of Peptide Fragments of Bovine TAP

Using radioactively labeled [^{3}H]α-tocopherol as tracer, we identified a novel TAP from bovine liver. Gel filtration of hepatic cytosol preincubated with [^{3}H]α-tocopherol yielded a radioactive protein peak in the 30- to 70-kDa fractions (Fig. 1). Further purification of the pooled 30- to 70-kDa fractions by Affi-Gel Blue affinity chromatography revealed two radioactive protein peaks. The molecular mass of the proteins carrying radioactive tocopherol was determined by photo-cross-linking experiments. The first radioactive protein peak contained a predominant band at 46 kDa in SDS-PAGE, which migrated to pH 8.1 in native isoelectric focusing (Fig. 2). The second peak contained a protein of 31 kDa migrating as two spots at pH 6.5, probably representing the bovine subtypes of hepatic tocopherol transfer protein (TTP) (*16*). Final purification of the pooled 46-kDa fractions by Mono S revealed one major radioactive protein peak that had a molecular mass of 46 kDa in SDS-PAGE (Fig. 3). The radioactivity of the protein peak was shown to be partially displaceable by α-tocopherol in a previously described gel filtration method (*13*). Attempts to assess the N-terminal amino acid sequence of TAP failed presumably because the molecule was blocked at the N-terminus. The purified protein was cleaved by CNBr treatment. The resulting peptide fragments were separated by reversed phase HPLC, and two peptide fragments were sequenced by automated Edman degradation.

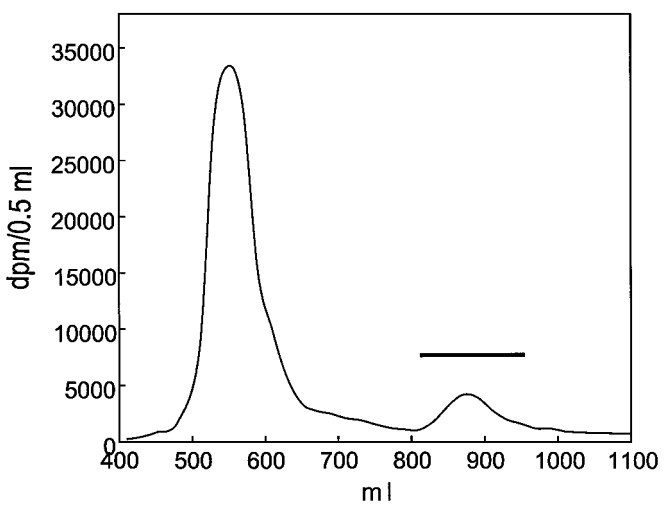

Figure 1. Sephacryl S-200 gel filtration of bovine liver cytosol. Eluted radioactivity was monitored. The horizontal bar indicates the pooled fractions that were used for further purification. See *Experimental Procedures* for details.

Identification and Cloning of Human TAP Gene

Comparing the sequences of the bovine peptide fragments with Genbank/EMBL DNA databases revealed a human gene (Accesssion no. G3213000). The gene maps to within chromosome 22 in region 22q12.1-ter. The sequence of this region derives from *Homo sapiens* PAC clone DJ130H16, which has been sequenced and mapped by the Sanger Center chromosome 22 mapping group (*17*). Using total RNA from human intestine, we amplified a 1.2-kb cDNA by RT-PCR (*18*). The resulting band was cut from a gel, reamplified, and subcloned into the pGEM-T vector. Sequence analysis of the subcloned cDNA yielded an open reading frame encoding 403 amino acids. Comparison with G32113000 revealed 100% identity for the portion between the two primer binding sites.

A computer-assisted analysis of protein sequences revealed that the primary structure of human TAP was homologous with several proteins. Identity of the 63–246 sequence was 70% with 45-kDa secretory protein from rat olfactory epithelium (E1371392), 42% with the squid retinal-binding protein (P49193), 26% with the human cellular retinal-binding protein (P12271), 30% with the yeast phosphatidylinositol-transfer protein (P24280), 32% with the human tyrosine phosphatase (P43378), and 29% with the human α-tocopherol transfer protein (P49638). All of these proteins contain a similar structural domain, the CRAL-TRIO, which forms a hydrophobic pocket responsible for ligand binding. The profile of this domain is deposited at the Pfam protein families database (*19*). By using the CRAL-TRIO domain of TAP (aa 63–246) for alignment, and including conservative replacements in the calculation, the homologies to the other members of the family were raised significantly. Fig. 4 shows the results of the alignment of the CRAL-TRIO domain of TAP. In conclusion, our sequence analysis establishes that the gene G3213000, which represents human TAP, has structural motifs suggesting its membership in a family of hydrophobic ligand-binding proteins.

Distribution of TAP in Human Tissues

Subcloned cDNA encoding for TAP was labeled with [α-^{32}P]dATP and used for Northern analysis. The labeled probe

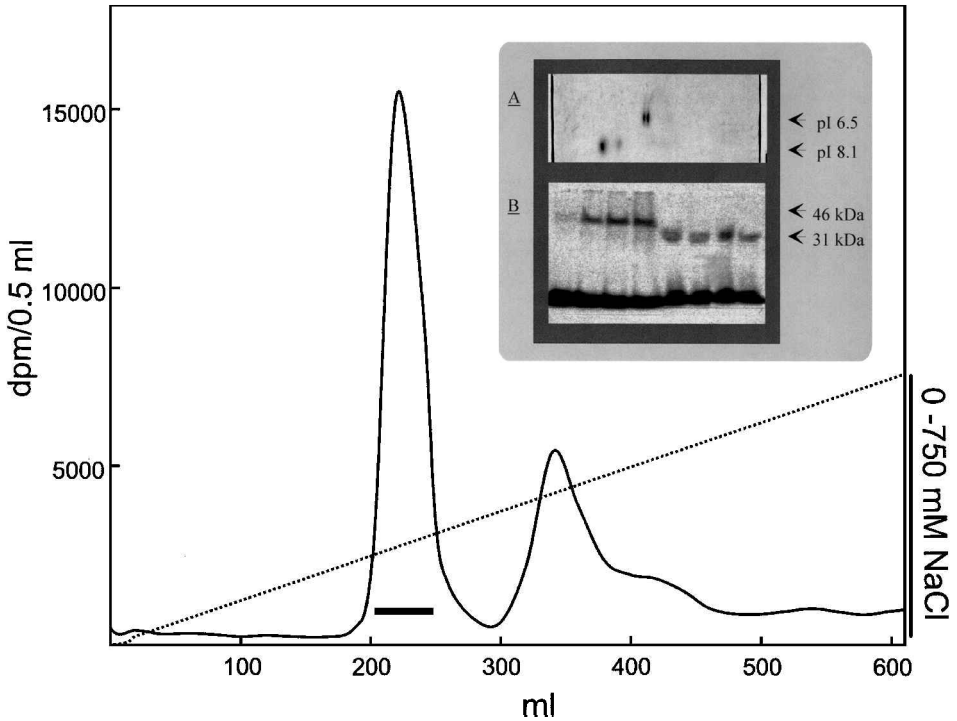

Figure 2. Affi-Gel Blue affinity chromatography of the 30- to 70-kDa fraction of bovine liver cytosol. Protein was eluted with a salt gradient (0–750 mM NaCl, broken line). Elution of radioactivity was monitored (solid line). The bar indicates the pooled fractions that were used for further purification. See text for details. Inset: Fluorograms of (*A*) native isoelectric focusing and (*B*) cross-linking experiments. Radioactive signals at pI 8.1 and 46 kDa show bovine TAP; those at pI 6.5 and 31 kDa show hypothetical bovine tocopherol transfer protein (TTP).

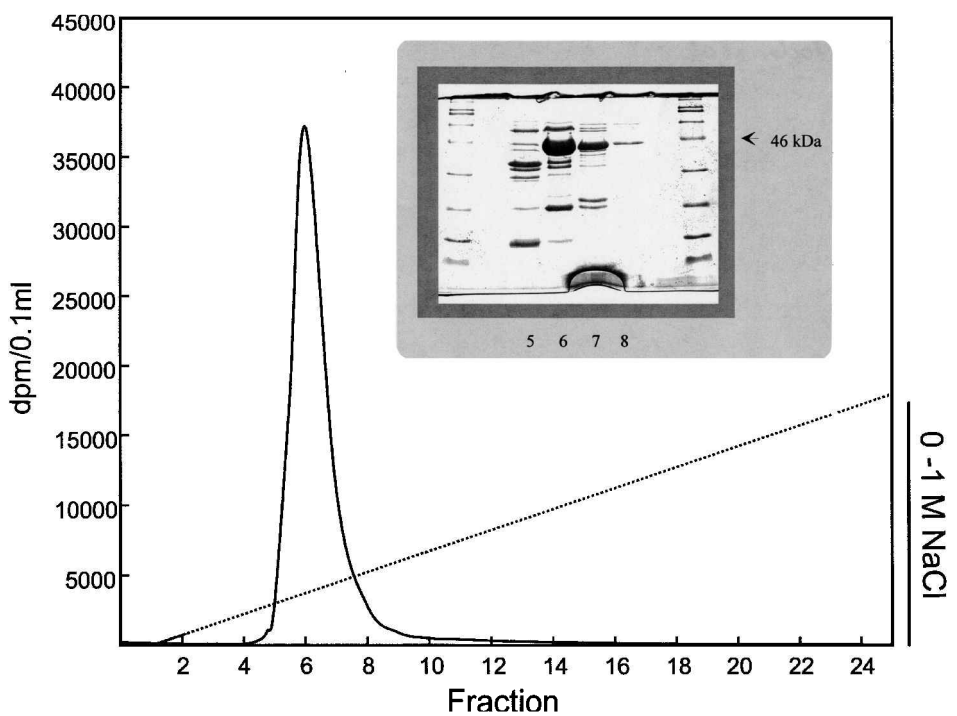

Figure 3. Ion-exchange chromatography of the TAP containing fraction from Affi-Gel Blue chromatography. Protein was eluted with a salt gradient (0–1 M NaCl, broken line). Elution of radioactivity was monitored (solid line). See text for details. Inset: SDS-PAGE of enriched bovine TAP (Coomassie staining). Outside lanes are molecular standards (from top to bottom, in kDa: 200, 116, 97, 66, 45, 31, 21.5, 14.2, 6.5), fractions 5, 6, 7 and 8 are shown in the middle lanes.

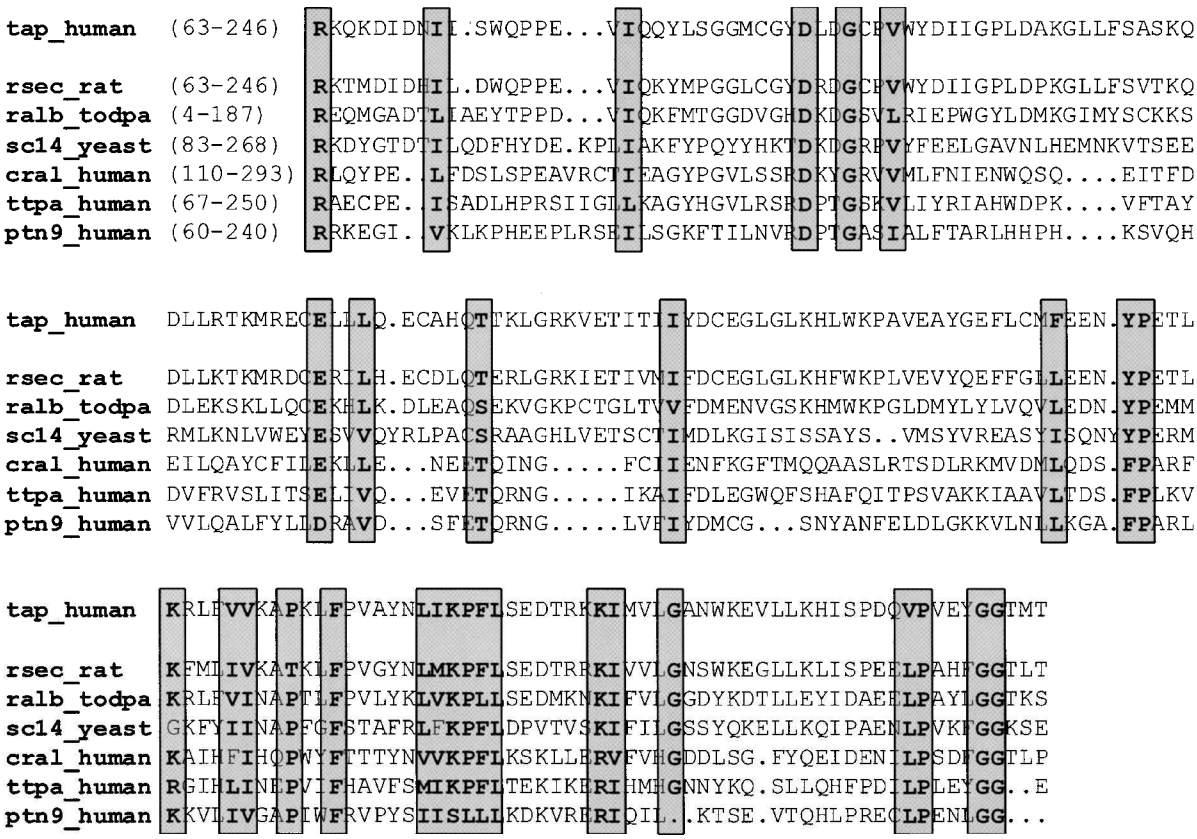

Figure 4. Alignment of the CRAL-TRIO domain from human TAP with homologous proteins. Human TAP has been aligned with human cellular retinal-binding protein (CRAL_HUMAN), human tyrosine phosphatase (PNT9_HUMAN), squid retinal-binding protein (RALB_TODPA), 45-kDa secretory protein from rat olfactory epithelium (RSEC_RAT), yeast phosphatidylinositol-transfer protein (SC14_YEAST), and human α-tocopherol transfer protein (TTPA_HUMAN). Gaps are marked with dots inserted to demonstrate maximum sequence similarity. Residues, identical or showing conserved substitutions with those of human TAP, are shown in bold letters and are enclosed by solid lines. Conserved amino acid substitutions are defined as pairs of residues belonging to one of the following groups: S, T, P, A, and G; N, D, E, and Q; H, R, and K; M, I, L, and V; and F, Y, and W.

was hybridized to a RNA master blot containing poly A^+ RNAs from 50 different human tissues immobilized on a positively charged nylon membrane (*20*). Relative expression levels of target mRNA were determined by analyzing the washed blot with a phosphorimager. The results show that the highest level of mRNA for TAP is expressed in human liver (Fig. 5).

The signals from all other tissues were calculated as a percentage of the liver signal (= 100%). TAP is widely expressed in human tissues, with highest levels in adult liver (but not in fetal liver), prostate, and neural tissues. Our results indicate that human TAP may have a central function in cellular regulation.

DISCUSSION

TAP is a novel α-tocopherol-binding cellular protein. Evidence that the protein binds α-tocopherol comes from the purification method itself, by which only those proteins that tightly bind tocopherol are isolated. The present paper reports on the isolation of this protein from bovine liver cytosol, as well as the identification and cloning of its human homologue.

Computer searches of protein and nucleotide databases with two sequence fragments of bovine TAP led to the identification of a Genbank entry for the human gene of TAP, which is localized at the 22q12.1-qter region of chromosome 22.

Comparison of the human TAP primary structure with that of other known proteins revealed a high degree of sequence identity with a previously identified 45-kDa secretory protein from rat olfactory epithelium. A less extended, but still notable, degree of sequence identity was also observed with a class of proteins having in common the capability to recognize hydrophobic ligands. The secretory protein, as well as the other members of this group, contains a structural motif designated as the CRAL-TRIO domain. This domain consists of a string motif that forms a hydrophobic pocket and presumably is responsible for the recognition of the corresponding ligand. Besides two retinal-binding

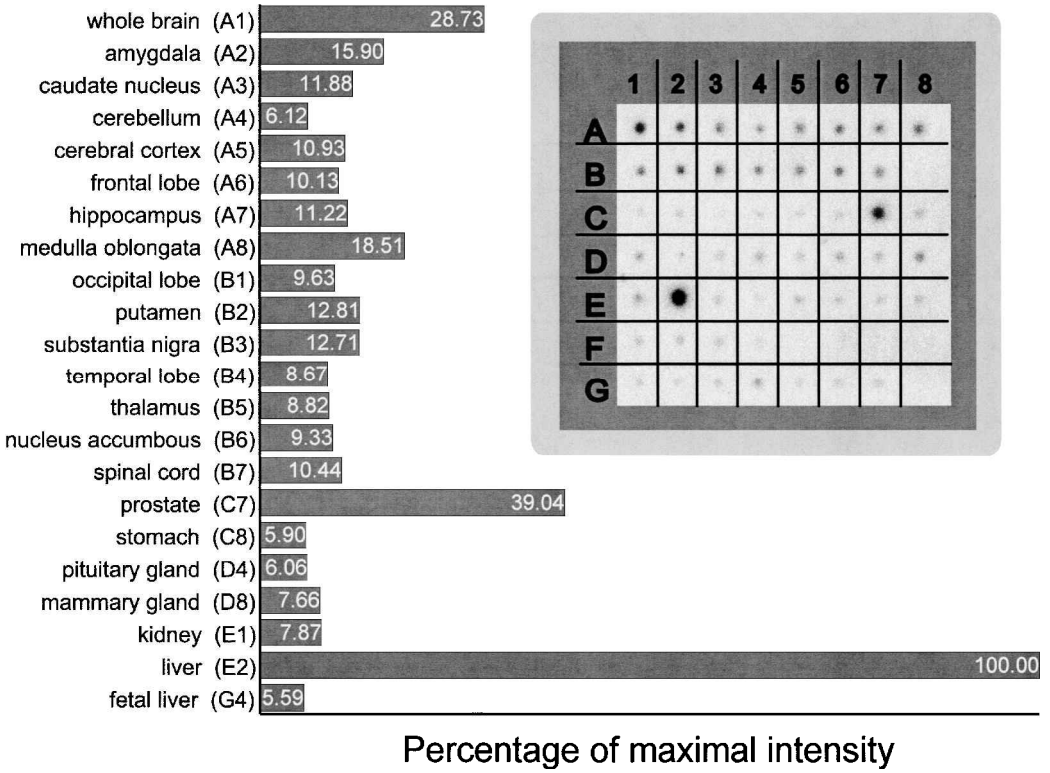

Figure 5. Autoradiogram of human RNA master blot hybridized to human TAP. Dot-to-dot variations are expressed as percentage of maximal intensity relative to the liver tissue. Spots that are not named in the legend had < 5% of maximal intensity.

proteins (RALB and CRALB), the phosphatidylinositol-transfer protein SEC 14, and the tyrosine phosphatase PTN 9, the most prominent member of this group is α-TTP. α-TTP seems to be expressed almost exclusively in liver, where it is responsible for the discrimination of α-tocopherol and its transport into the plasma (*21*). This specific localization makes α-TTP an improbable candidate for mediating α-tocopherol tissue and intracellular distribution (*22*). However, although lipophilic compounds may be expected to traverse biological membranes by simple diffusion, in fact, they may require membrane receptors or transporters, or both [for example, the 4 subtypes of PGE membrane receptors (*23*) or the free fatty acids (*24*)].

A recently identified 15-kDa tocopherol-binding protein in the cytosol of various animal tissues has been suggested to be a specific intracellular carrier of α-tocopherol (*25*). The structure and function of this protein along with the possible relationship with the one described here are still a matter of speculation.

Northern blot analyses provided clear evidence that this protein is widely distributed in human tissues. Our results also show that highest concentrations of TAP are found in liver, prostate, and neural tissues. Both the rather unusual tissue distribution of TAP and its ability to recognize α-tocopherol in its native state support the idea that human TAP is involved in intracellular tocopherol-related functions. The physiological function of TAP is being investigated and will be the subject of a forthcoming report.

As mentioned above, discrimination and incorporation of α-tocopherol into nascent lipoprotein particles appears to be determined by the α-TTP in the liver (*26*). Recently, patients with vitamin E deficiency (AVED) have been described who exhibit neurologic abnormalities and vitamin E deficiency but no abnormality in lipoprotein metabolism (*27*). The molecular basis of this pathology has been found in a genetically defective α-TTP (*5*). Further evidence of a correlation between low plasma concentrations of tocopherol and disease comes from epidemiological studies (*28, 29*) and the intervention studies (*6, 30*), which suggest that supplementation with α-tocopherol substantially reduces the risk of cardiovascular disease.

The identification of a new protein related with α-tocopherol cellular functions and the availability of its cDNA clone isolated in the present study should help in clarifying the structure of this protein and its physiological function. Ultimately this discovery should lead to a better understanding of the molecular basis of tocopherol-mediated intracellular events.

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