Chapter 9
The Tocopherol Transfer Protein: Regulator of Vitamin E Status

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Keywords Tocopherol · Transfer protein · Phosphoinositide · Oxidative stress

Key Points

- TTP is the only known specific regulator of α-tocopherol status in vertebrates.
- Genetic defects in the gene encoding TTP cause vitamin E deficiency in humans.
- Vectorial transport of vitamin across cells by TTP is driven by opposing concentration gradients of tocopherol and PI(4,5)P_2 between organelles.
- Expression of TTP in the CNS protects vulnerable neurons from oxidative stress.
- TTP expression and activity are regulated to maintain vitamin E homeostasis.

Introduction

Many molecules that are essential for the growth and survival of cells and organisms are virtually or poorly insoluble in aqueous solvents. The list of such ligands is long and includes lipophilic molecules that are de novo synthesized within cells (e.g., phospholipids, fatty acids, cholesterol, sex hormones), as well as essential hydrophobic compounds that are derived from the diet (e.g., the fat-soluble vitamins A, D, and E). The poor solubility of such hydrophobic molecules in aqueous solution poses significant thermodynamic and kinetic barriers on their movement in the aqueous milieu of cells and

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tissues. Two general mechanisms evolved to achieve regulated transport of such lipids through the cytosol: (1) transport within the bilayers of lipid vesicles, as seen in the trafficking of newly synthesized phospholipids and sterols from the endoplasmic reticulum to other intracellular organelles [1–4], and (2) by lipid binding and transfer proteins. These proteins bind their lipid ligands (retinoids, some sterols, tocopherol, squalene, etc.) with high affinity and specificity and have been shown to accelerate the movement of those lipids between lipid membranes in vitro. Several distinct structural motifs have evolved to function as specific lipid binding domains and occur in lipid transfer proteins such as the fatty acid binding proteins (FABPs), StAR-related lipid-transfer (START) domain proteins, oxysterol binding proteins (OSBP) and related proteins (ORPs), and phosphatidylinositol transfer proteins (PITPs). Of specific relevance to this chapter is a large family of proteins that contain the CRAL-TRIO lipid binding domain which includes the vitamin E binding protein TTP (SMART-SM00516; PFAM-PF00650). The defining protein of this family is the yeast phospholipid transfer protein Sec14, containing a phospholipid binding cavity with a floor made of four parallel and two antiparallel beta-strands. Several α-helices cover the β-sheet, and the binding site is gated by a flexible hinged helix ("the lid"). The Sec14 domain is found in 6024 proteins across all eukaryotic phyla, with as many as 49 identified and predicted Sec14 domain-containing proteins in humans. In addition to Sec14, the CRAL-TRIO family [5–7] includes the tocopherol transfer protein (discussed in detail below), neurofibromin [8], the yeast Sfh proteins [9], supernatant protein factor [10, 11], the human Sec14-like proteins (e.g., Sec14L4), Dbl-like activators of Rho GTPases [12–14], the tyrosine phosphatase MEG2 [15], and clavesin [16].

Tocopherol Binding Proteins It is generally believed that tocopherol binding proteins serve to "solubilize" the hydrophobic vitamin, thereby facilitating its transport to intracellular sites. Indeed, incubation of crude cytosol fractions with radioactively labeled α-tocopherol followed by chromatographic separation revealed the presence of tocopherol binding proteins in a number of tissues (e.g., [17–21]). Since in cells the hydrophobic tocopherol resides predominantly in membranes, specific binding proteins may also be expected to interact with lipid bilayers. Experimentally, this criterion has been applied in the form of "tocopherol transfer assays" that measure each protein's ability to facilitate the transfer of a labeled tocopherol between lipid vesicles (e.g., [22, 23]).

It is important to note that conclusive identification of a protein's "real" physiological ligand using in vitro binding assays should be interpreted with caution. Unlike water-soluble protein-ligand interactions, many lipophilic ligands will exhibit a measurable affinity to most hydrophobic binding pockets within protein folds (e.g., cholesterol binds to CRAL-TRIO proteins' binding pockets, although no evidence exists that this behavior has any physiological relevance). The threshold for non-specific binding needs to be evaluated directly and carefully [24, 25].

TTP The best characterized tocopherol binding and transfer protein is the α-tocopherol transfer protein (TTP), that belongs to the CRAL-TRIO protein family [26, 27], and is the focus of this chapter. Inoue and colleagues reported the identification, purification, and cloning of a soluble, 33 kDa protein that bound tocopherol with high affinity from rat liver cytosol [22, 23] and, later, its cloning from a human liver cDNA library [28]. The gene encoding TTP is highly conserved among species (94% homology between the rat, mouse, and human proteins). Two in vitro biochemical hallmarks were assigned as "signature" activities of TTP: (1) high affinity binding of tocopherol and (2) facilitation of tocopherol transfer between lipid membranes [20, 29, 30]. The ligand binding preference of this protein is: α-*RRR*-tocopherol>> β- tocopherol >> γ- tocopherol, δ tocopherol, essentially identical to the biological potency of the different tocols in bioassays [30]. Thus, it is generally accepted that TTP is the major physiological mechanism responsible for the selective retention of α-*RRR*- tocopherol in vivo and, therefore, a key mediator of vitamin E action [31]. While expression of TTP is highest in the liver, it is becoming apparent that the protein is also expressed in the brain and placenta and that expression in these tissues is of high physiological importance [32–35].

The role of TTP and tocopherol in human health is underscored by the fact that heritable mutations in the TTP gene are the only known cause for the autosomal recessive disorder ataxia with isolated vitamin E deficiency (AVED; [36]). AVED patients present progressive neurodegeneration and low (or undetectable) serum levels of α-tocopherol. Multiple mutations in TTP were identified in AVED patients that impair the protein's function. We have recently shown that clinical severity of the disease is correlated with defects in TTP function in vitro and in cultured cells [37–40]. The role of TTP in vitamin E status and human health was underscored by observations made in mouse models in which expression of the *ttpA* gene has been disrupted [41–43]. While the homozygous TTP$^{-/-}$ mice are normal in many respects, their levels of circulating tocopherol are extremely low, and they manifest three major pathologies that underscore the critical roles of α-tocopherol in health. In support of epidemiological correlations between vitamin E intake and cardiovascular disease, TTP$^{-/-}$ mice are more susceptible to formation of atherosclerotic lesions [41]. Additionally, female TTP$^{-/-}$ mice are infertile ([43]), in accordance with the original identification of vitamin E as a "fertility factor" (see below). As TTP$^{-/-}$ mice age, they display cerebellar ataxia symptoms, reminiscent of those associated with AVED in human patients ([42]; see below).

Heart-TTP This ~14 kDa polypeptide was demonstrated to bind labeled α-tocopherol in homogenates prepared from rat, rabbit, and bovine hearts [44–46], rat liver [45, 47], and human placenta [48, 49]. The protein was later said to be "present in all tissues" [50], but since the protein was never sequenced, it is impossible to determine whether the biochemical activity demonstrated in different tissues originates from the same biochemical entity. Since thorough biochemical characterization was described only for the form purified from the heart, we focus our discussion on this isoform and refer to it as "heart-TTP." Since its identification >20 years ago, heart-TTP received surprisingly limited experimental attention. Current knowledge of this protein can be summarized as follows: (1) The protein was purified to homogeneity using conventional chromatography approaches, yet has never been sequenced nor cloned. (2) Purified heart-TTP displays rapid and saturable binding to [^{3}H]-α-tocopherol. (3) The purified protein displays pronounced substrate specificity for α-tocopherol, showing no binding for oleic acid, neither to the γ or δ vitamers of tocopherol. (4) Binding of [^{3}H]-α-tocopherol is tight (Kd = 3 nM) and stoichiometric (0.9 mole α-tocopherol per mole protein). (5) The protein is monomeric in solution. (6) Heart-TTP facilitates the in vitro transfer of α-tocopherol between lipid vesicles.

Tocopherol-Associated Protein (TAP)/Supernatant Protein Factor (SPF) Stocker et al. [51] isolated and later cloned [52] a 45 kDa protein from bovine liver that bound radiolabeled α-tocopherol and termed it "tocopherol-associated protein" (TAP). It was later noted that this protein is identical to a squalene binding protein that functions in the de novo cholesterol biosynthetic pathway, previously purified and characterized by Bloch's group [53–55]. Moreover, competitive ligand binding measurements revealed that this protein's affinity for α-tocopherol is at the non-specific range (>600 nM; [24]), shedding doubt on the relevance of this protein to vitamin E biology [24, 56, 57]. Thus, although SPF is not a bona fide tocopherol binding protein, its documented impact on cell proliferation and behavior [58–60] renders it a fascinating subject of study on the roles that sterol binding proteins play in cell biology. It is important to note that recent changes in nomenclature now refer to this protein as Sec14L2.

Three-Dimensional Structure of TTP

Published three-dimensional structures of the human [61, 62] and mouse [63] TTP isoforms reveal a compact protein, with a deeply buried hydrophobic ligand binding pocket, and a charged surface that renders the protein highly soluble (Fig. 9.1a). Comparison of the apo- and holo-structures reveals a

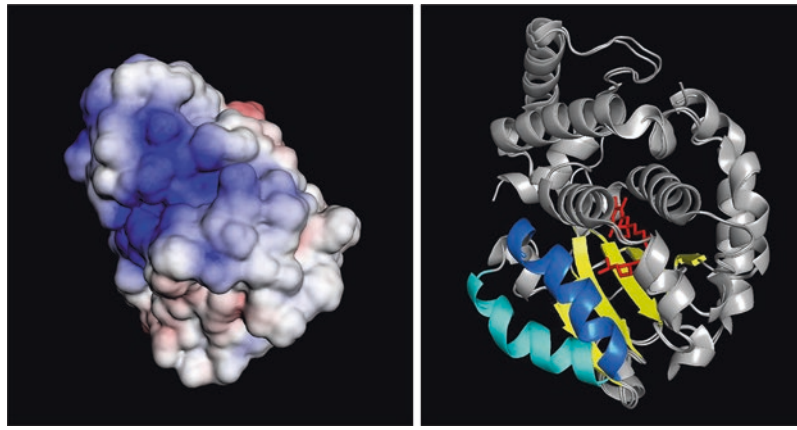

Fig. 9.1 Three-dimensional structure of TTP. **Left panel**: Depiction of the protein's surface electrostatic potential. PyMOL was used for visualization, and the electrostatic surface was calculated using the PyMOL plugin APBS via the PDB2PQR Server (http://nbcr-222.ucsd.edu/pdb2pqr_2.1.1/). The PARSE force field was used, and PROPKA to assign protonation states at pH 7. The solvent accessible surface was visualized using 3DMol and colored from −5 kT/e (dark blue) to +5 kT/e (dark red). The deep blue patch is largely a result of residues R59, R68, K190, R192, K217, and R221. **Right panel**: Backbone aligned x-ray crystal structures of TTP in the open ligand-free form (PDB 1OIZ) and closed ligand-bound form (PDB 1OIP). The mobile helical gate comprising residues 201–213 is colored dark blue in the closed form and aquamarine in the open form. Tocopherol bound to 1OIP is shown in red

significant conformational change that occurs upon ligand binding. The major movement of tertiary structure occurs in a helical "lid" (residues 201–213) that covers the entrance to the binding site, a structural feature conserved among all members of the CRAL-TRIO protein family. The flexibility of the helical lid is illustrated by measurements of the distance between the alpha-carbons of F213 and K177 in TTP which is ~16.0 Å in the ligand-free conformation and shortens to 6.5 Å in the tocopherol-bound conformation [61]. Similar ligand-induced conformational shifts are seen in the yeast phospholipid transfer protein Sec14 [64]. The currently prevailing hypothesis regarding ligand transfer by CRAL-TRIO proteins proposes that the amphipathic lid provides a molecular mediator for membrane insertion and ligand exchange [27, 64, 65]. Such mobile structural units appear to be a common feature of ligand-induced conformational selection in other proteins and enzymes as well [66–68].

The biochemical function of TTP, transferring the lipophilic α-tocopherol between intracellular membranes, necessitates extraction of the ligand from one bilayer and depositing it in another. For this activity the protein must, at least transiently, interact with lipid bilayers. While structural determinations of the protein were based on soluble preparations, and did not include membranes, calculations on the free energy changes on protein insertion into a hydrophobic phase have revealed that indeed, TTP utilizes specific residues to interact with membranes, as shown in Fig. 9.2. Interactions with a membrane occur by insertion of TTP's hydrophobic residues into the outer leaflet of the bilayer. Residues F169, F165, I202, and M209 are particularly important, as they provide the side chains that insert into the bilayer. Accordingly, substitution of either F165 or F169 with aspartic acid all but abolishes membrane binding of TTP and abrogates its activity in facilitating vitamin E transport in cells [65].

Recent findings provide important mechanistic insights into how the lid mediates membrane association and ligand retrieval/release by TTP [63, 69]. The key appears to involve an additional ligand, PI(4,5)P_2, which binds to a patch of basic residues on TTP's surface. Binding of PI(4,5)P_2 to TTP induces a conformational change whereby the lid "opens," allowing for the release of the bound vitamin E and its deposition into the "target" membrane. The interior hydrophobic surface of the lid may actually insert into the bilayer at this step as suggested in calculations of the free energy of insertion of these protein features into a hydrophobic phase [65, 70]. This ligand exchange mechanism appears

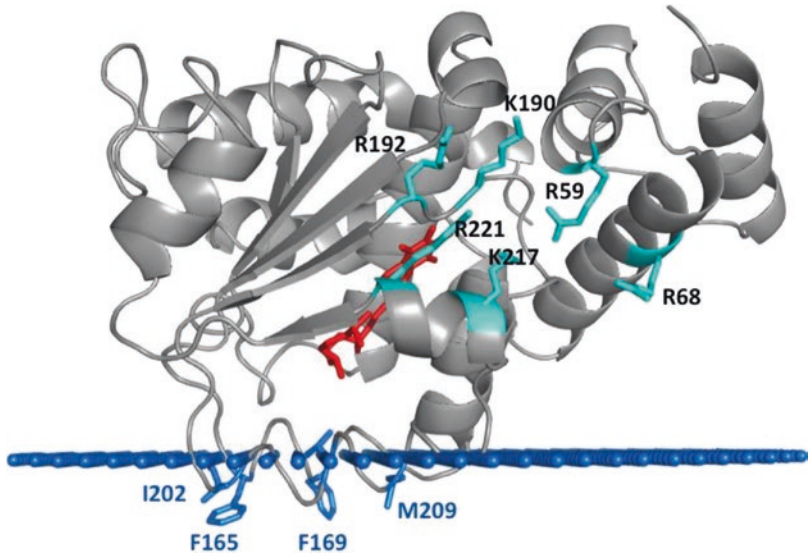

Fig. 9.2 Calculated orientation of human tocopherol transfer protein (PDB 1OIP) with respect to a hydrophobic phase of a phospholipid bilayer. Image is downloaded from the Orientation of Proteins in Membranes database at http://opm.phar.umich.edu. The plane of blue spheres marks the approximate surface of the acyl chain methylene carbons moving away from the glycerol moiety. Residues F165, F169, I202, and M209 in blue are shown deeply inserted into the interior of the bilayer. Tocopherol is shown in red. Residues in aquamarine are key basic amino acids making up the basic patch as seen in Fig. 9.1 (left)

Table 9.1 Ligand and membrane binding affinities of wild-type TTP and K217A

Protein	K_d for PM vesicles (μM)	PM B_{max} (μRIU)	PM + 4% $PI(4,5)P_2$ B_{max} (μRIU)
TTP(wild-type)	1.3 ± 0.26	270 (100%)	470 (175%)
TTP(K217A)	0.43 ± 0.09	570 (100%)	320 (56%)

(i) *PM* plasma membrane lipids (44% DOPC 20% DOPE, 19% cholesterol, 10% sphingomyelin, 5% DOPS, 2% DOPA). When 4% $PI(4,5)P_2$ was included, PC content was reduced by the same amount
(ii) SPR protein injections were made at concentrations of protein near the K_d values for the respective proteins: wtTTP = 1.3 μM; K217A = 0.5 μM. μRIU = micro-refractive index units

to be conserved from yeast to man, as it has been observed/suggested in the homologous CRAL-TRIO proteins Sec14 [27] and CRALBP [71].

A key feature of the dynamic ligand-induced conformational switching in TTP appears to be mediated by Lys217. Substitution of this residue eliminated TTP binding to $PI(4,5)P_2$, abolished TTP-mediated tocopherol secretion in cultured hepatocytes, and disrupted the protein's intracellular localization to a pattern where the protein constitutively resides near the plasma membrane [63]. These findings are supported by in vitro studies in which we determined the affinity of TTP proteins to model plasma membranes (Table 9.1). We found that TTP(K217A) bound NBD-tocopherol with similar affinity as the wild-type protein, indicating that the substitution did not lead to gross structural perturbations [65]. Using surface plasmon resonance (SPR), we determined the ability of the proteins to bind to tethered vesicles mimicking plasma membrane lipid composition. We found that the K217A protein bound to membranes with slightly higher affinity than the wild-type TTP. However, when the experiments included membranes containing $PI(4,5)P_2$, the K217A mutant protein showed profoundly weakened affinity to the membranes, as compared to wild-type TTP. Preliminary SPR studies also suggest that the presence of tocopherol weakens TTP's affinity to $PI(4,5)P_2$-containing membranes.

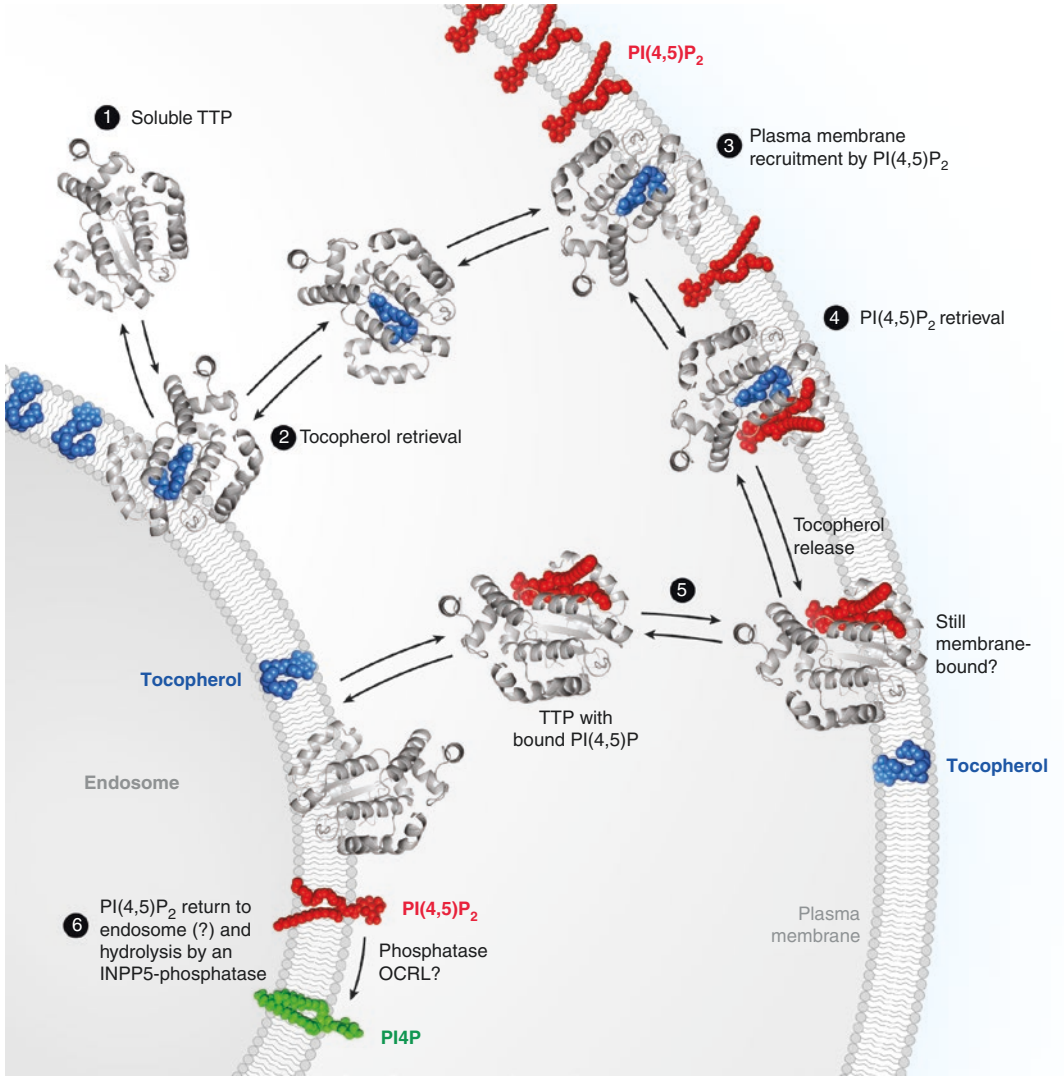

Fig. 9.3 Tocopherol transport across cells. See text for details

These data show that TTP's affinity to lipid bilayers is increased by the presence of $PI(4,5)P_2$ and that Lys217 is critical for this discriminatory binding behavior. Thus, the K217A mutant seems to have lost the ability to complete the ligand exchange reaction; it therefore retains appreciable affinity for tocopherol even when bound to $PI(4,5)P_2$. The mutant protein therefore remains bound to both ligands and "stuck" along the membrane in cells (i.e., cannot complete step 4 in the model shown in Fig. 9.3).

Mechanisms of TTP-Mediated Tocopherol Transfer In Vivo

In cells, the non-vesicular movement of small molecules by transfer proteins requires elements of temporal and spatial control. The protein-ligand complex should form where the two partners are at concentrations that drive favorable thermodynamics of binding. Furthermore, once the protein-ligand

complex is formed, directional transfer of the ligand cargo to a new location requires a favorable binding event elsewhere in the cell. Finally, to achieve net vectoral transport of the ligand, directionality of the process must be maintained. We propose a model for the mechanism by which TTP transports vitamin E across cells that relies on two principle features: the exchange of tocopherol and PI(4,5)P_2 (see above) and concentration gradients of the two ligands between different intracellular locations. This model, depicted in Fig. 9.3, is based on experimental findings [63, 69, 72] and on a similar phenomenon, namely, the transport of sterols and phosphoinositides, by members of the OSBP family [73–76].

According to our model, TTP picks up α-tocopherol from the site of the vitamin's arrival in cells, the endocytic compartment [39]. Interaction between TTP and endosomal vesicles is driven by the high concentrations of tocopherol at this site and possibly by the high curvature of these membranes [77, 78] and is mediated by a hydrophobic face of the protein that offers two key phenylalanine residues for submersion into the hydrophobic membrane (residues F169, F165; see Fig. 9.2 and [65, 79]). The binding of tocopherol shifts the conformation of the helical lid (including residues 198–221) to close on the binding site, thereby weakening the protein's affinity for the bilayer. The protein then dissociates to the cytosol as a soluble TTP-tocopherol complex. The TTP-tocopherol complex then partitions to the plasma membrane (3) where concentrations of PI(4,5)P_2 are high [80]. Although TTP does not contain a known binding motif specific for phosphoinositides, it contains a basic patch on the surface (comprising Arg59, Arg68, Lys190, Arg192, Lys217, Arg221) [63, 72] that offers a favorable environment for electrostatic and H-bonding interactions with the phosphorylated inositol head group of PI(4,5)P_2 [63].

Although the detailed sequence of the ligand exchange event is not yet clear, the end result is the deposition of tocopherol into the plasma membrane bilayer and abstraction of PI(4,5)P_2 by TTP from the membrane (5). The TTP-PI(4,5)P_2 complex then departs to the cytosol and returns to the endosomal compartment (6) where the PI(4,5)P_2 is dropped off and is hydrolyzed to PI(4)P by an endosomal resident phosphatase such as OCRL [81–84]. Alternatively, TTP might specifically promote the hydrolysis of bound PI(4,5)P_2 by presenting it to a plasma membrane-resident phosphatase such as SHIP2 [85, 86]. Directionality of the transport process is governed by the concentration gradients of PI(4,5)P_2 (high in plasma membrane and low in endosomes) and an opposite concentration gradient of tocopherol (high in the endosomal compartment, low in plasma membrane) [72].

TTP and Neurological Function

The primary manifestations of vitamin E deficiency in laboratory animals are neuropathologies, similar to those presented by human AVED patients [36, 87, 88], underscoring the critical roles that α-tocopherol plays in the CNS. We refer the reader to a detailed review on this topic that was recently published elsewhere [89, 90] and only briefly discuss here a few points.

TTP is expressed at significant levels in glia (astrocytes), the cells that provide metabolic support for neurons. In these cells, TTP regulates supply of α-tocopherol to neighboring neurons, in a manner highly reminiscent of the protein function in hepatocytes, as depicted in Fig. 9.4.

Specifically, TTP mediates the trans-cellular transport of the vitamin from vesicles of the endocytic compartment to a transporter at the astrocyte plasma membrane, from which the vitamin is exported by a transporter to an acceptor apolipoprotein for delivery to its final destination. The two tissues utilize slightly different membrane transporters (ABCA1 in hepatocytes vs. ABCG4 in the CNS), different lipoprotein acceptors (ApoA-based in hepatocytes vs. ApoE-based in the CNS), and different destinations (systemic circulation in hepatocytes vs. cerebrospinal fluid in the CNS) yet the overall process seems to share most functional steps and players. Importantly, in both tissues expression of the *TTPA* gene is stimulated under conditions of oxidative stress.

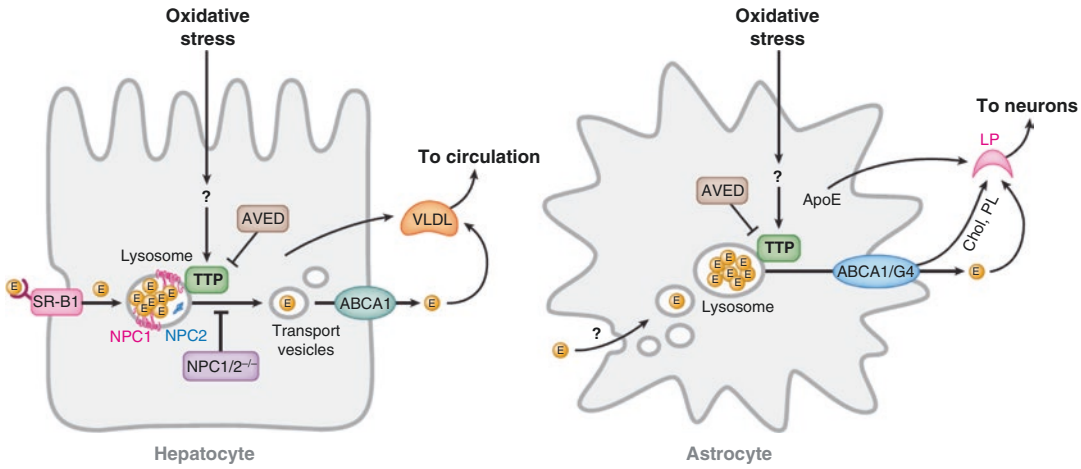

Fig. 9.4 Mechanisms of TTP-facilitated tocopherol transport in hepatocytes and astrocytes. (Drawn after Ulatowski, 2013 [91])

Expression of TTP in the liver ensures supply of diet-derived tocopherol to the circulation and, in turn, to all extra-hepatic tissues. What, then, is the evolutionary need that selected for "redundant" expression of the protein in the CNS? The unique sensitivity of the CNS to oxidative damage might be a key answer to this question. Thus, TTP appears to serve two protective functions in the CNS: to provide and "safeguard" a separate pool of vitamin E that sustains neurological function and to regulate vitamin E transport to neurons in a manner independent from body-wide fluctuations. It is possible, and likely, that similar reasons dictated expression of the protein in the female reproductive system.

TTP and Fertility

Almost a century ago, vitamin E was identified as a plant component that is critical for female fertility in laboratory animals (hence the name tocopherol, *Gr.* to bear offspring). Specifically, vitamin E was shown to be critical to successful pregnancy in rodents; without it, pregnant females would resorb the developing embryo into the uterine wall and pregnancy would be terminated [92]. Considerations of vitamin E actions often rely in part on this biological activity (the gestation-resorption assay) [93, 94]. Since vitamin E deficiency is not common in humans, deeper understanding of this phenomenon came only after the advent of the TTP-null mice. Shortly after generating this genetic model, Arai and colleagues reported that TTP-null crosses were not fertile. They further found that male fertility was not affected, that pregnancies could be rescued by supplementation with α-tocopherol or a synthetic lipophilic antioxidant, and that infertility was an outcome of placental defect [43]. In a later report, the same group demonstrated that transfer of fertilized TTP-null eggs to wild-type females subverted the fertility defect. Taken together, these finding demonstrated that adequate α-tocopherol delivery via TTP is critical for placental function, but not for embryonic development per se [95]. Thus, it appears that the situation in the reproductive system is analogous to that in the CNS: TTP's critical function is in supplying the oxidative stress-susceptible embryo with the antioxidant, and defects in TTP lead to functional failure. A question that begs urgent research is whether these findings have relevance to female fertility in humans. It is worth noting in

this regard that TTP is expressed in human placental trophoblasts [34, 96] and that expression of the protein increases upon induction of oxidative stress in cultured human trophoblasts [97]. These observations strongly suggest that a similar scenario exists. The urgency of research into this question is underscored by the extremely high prevalence of unexplained miscarriages in women [98], the abundance of single nucleotide polymorphisms in the *TTPA* gene [99], and the total inattention at present to vitamin E status in women of child-bearing age.

Vitamin E Homeostasis: Dynamic Regulation of TTP

For a long time, vitamin E status was assumed to be a passive reflection of dietary intake ("we are what we eat"). Recent evidence, however, demonstrates that TTP's function is not merely to transport the vitamin among tissues and compartments, but also to **regulate** tissue levels of vitamin E in response to changing physiological conditions. Regulation of vitamin E status via changes in TTP actions is achieved through a number of mechanisms:

- *Transcriptional regulation of TTP.* Expression of the gene encoding TTP is not constant. Rather, TTP mRNA levels were shown to be regulated by a number of transcriptional modulators [99] including hormones (e.g., TNFα), nuclear receptors (e.g., PPAR), second messengers (cAMP), and hypoxia mediators (DFX) [99]. Although the physiological or pathological conditions under which these stimuli affect TTP expression in vivo are yet to be described, the data clearly demonstrate that TTP expression, and in turn vitamin E levels and distribution, is dynamically regulated. Especially intriguing are the findings showing that TTP expression is markedly enhanced under conditions where vitamin E is in demand, i.e., under elevated oxidative stress or when the need for increased trafficking rises, i.e., upon elevated levels of α-tocopherol.
- *Post-translational regulation of TTP.* A more immediate and transient regulation of TTP happen at the post-translational level. Thus, it was shown that tocopherol increases the lifetime of the TTP protein by protection of the protein from proteasomal degradation [100]. Moreover, our recent findings show that the TTP protein is transiently phosphorylated on tyrosine residue(s) and that this modification has profound effect on the protein's activity. The data show that TTP is phosphorylated on tyrosine residue(s) (Fig. 9.5a), that the phosphorylation is stimulated by serum-derived growth factors (Fig. 9.5b), that substitution of Tyr269 to phenylalanine markedly reduces phosphorylation on TTP (Fig. 9.5c), and that the signature biological activity of TTP, namely, facilitation of tocopherol secretion from cultured hepatocytes, is abolished by the Tyr269Phe mutation as well as by treatment with Iressa, a selective inhibitor of the epidermal growth factor receptor (EGFR; Fig. 9.5d). Taken together, these data strongly support the notion that TTP-mediated tocopherol transport is regulated by classical mitogenic signaling cascades.

Current Gaps in Our Understanding of TTP and Vitamin E Despite a wealth of information derived from decades of vitamin E research, a number of important questions remain unanswered and await detailed focused attention from experimental scientists:

- Is vitamin E status associated with infertility in women, and what is the impact of TTP defects in this phenomenon?
- How is TTP activity regulated in normal and pathological states?
- What is the function of TTP in yet unexplored sites of expression (e.g., lung, kidney)?
- What are the medical consequences of subclinical vitamin E deficiency and polymorphisms in the *TTPA* gene?

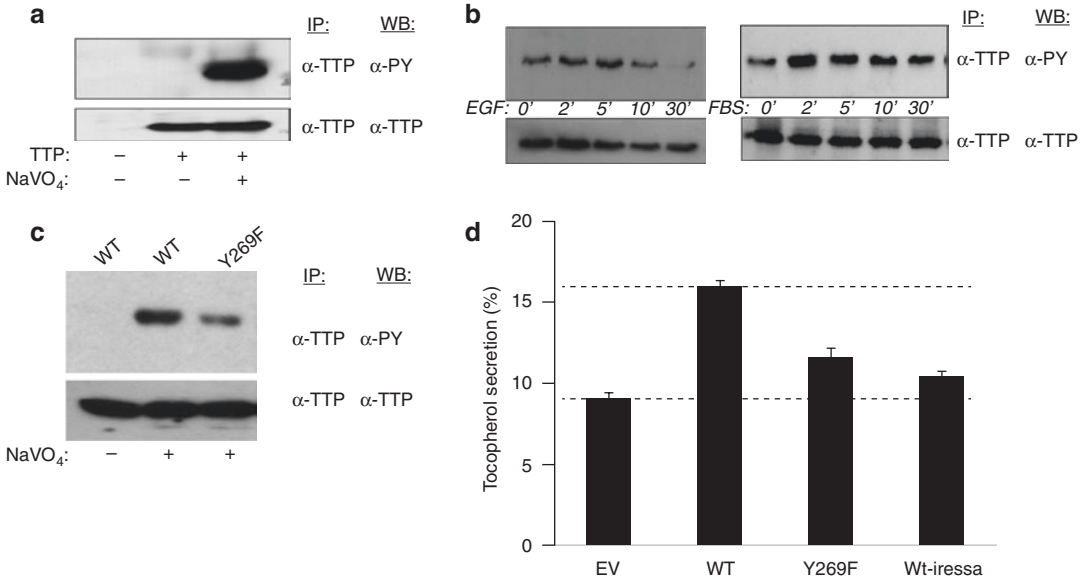

Fig. 9.5 Regulated phosphorylation of tyrosine residue(s) in TTP. All experiments were done in cultured HepG2 hepatocytes. (**a**) **TTP is tyrosine-phosphorylated.** TTP was immunoprecipitated using anti-TTP antibodies in lysates from cells transfected with TTP (or mock)-encoding plasmids. Tyrosine phosphorylation was visualized by immunoblotting with an anti-PY antibody (clone 4G10), in the presence or absence of the phosphatase inhibitor sodium vanadate. (**b**) **Tyrosine phosphorylation of TTP is stimulated by growth factors.** TTP-transfected cells were serum-starved for 20 h and then stimulated for the indicated time with epidermal growth factor (EGF; 50 ng/ml) or bovine serum (FBS; 10%) prior to analysis as in panel A. (**c**) **Tyr269 is a likely phosphorylation site in TTP.** Cells were transfected with the indicated construct prior to analysis as in panel A. (**d**) **Tyrosine phosphorylation of TTP regulates tocopherol secretion in cultured hepatocytes.** Cells were transfected as indicated, and secretion of loaded [^{14}C]-α-tocopherol was measured as in (Qian *Biochemistry*, **2006**, *45*: 8236). Where indicated, the EGFR inhibitor Iressa (Gefitinib; 5 μM) was added to the culture media during the secretion phase of the experiment

Conclusion

Since the earliest discovery of fatty acid [101, 102] and phospholipid [103, 104] binding proteins, function has been deduced from in vitro intermembrane transfer assays. We are now growing to appreciate that the function of lipid transfer proteins is more nuanced than ligand binding and random membrane encounters. How is directional ligand transport achieved and controlled? Many transfer proteins recognize specific membrane lipid compositions, often guided by organelle- and membrane-specific phosphatidylinositol phosphates [105] and by protein-protein interactions at membrane contact sites [2, 74, 75].

In vertebrates, the tocopherol transfer protein is responsible for the selective retention of dietary α-tocopherol during passage of the vitamin through the liver and for its secretion into plasma lipoproteins. Kono et al. [63, 69] have described the possible first steps in TTP-mediated tocopherol transport as specific recognition of plasma membrane PI(4,5)P_2 by the TTP-tocopherol complex that is followed by ligand exchange of phospholipid for the bound tocopherol. Still, aspects of this delivery cycle remain to be described. Is the PI(4,5)P_2 actually extracted from the plasma membrane in vivo? Which phosphatase is responsible for turnover of the extracted PI(4,5)P_2 and where does this hydrolysis occur? Answers to these questions will likely require tools for altering PIP metabolism [106–109], as well as fluorescent and bio-conjugated forms of phosphoinositides [110] to enable tracking and identification of potential protein binding partners.

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