Vitamin E and Phosphoinositides Regulate the Intracellular Localization of the Hepatic α-Tocopherol Transfer Protein*

Stacey Chung‡, Mikel Ghelfi‡, Jeffrey Atkinson§, Robert Parker¶, Jinghui Qian§, Cathleen Carlin‖, and Danny Manor‡‡†

From the Departments of *Nutrition, **Pharmacology, and †Microbiology and Molecular Biology, School of Medicine, Case Western Reserve University, Cleveland, Ohio 44106, the §Department of Chemistry, Brock University, St. Catharines, Ontario L2S 3A1, Canada, and the ¶Division of Nutritional Sciences, Cornell University, Ithaca, New York 14853

α-Tocopherol (vitamin E) is an essential nutrient for all vertebrates. From the eight naturally occurring members of the vitamin E family, α-tocopherol is the most biologically active species and is selectively retained in tissues. The hepatic α-tocopherol transfer protein (TTP) preferentially selects dietary α-tocopherol and facilitates its transport through the hepatocyte and its secretion to the circulation. In doing so, TTP regulates body-wide levels of α-tocopherol. The mechanisms by which TTP facilitates α-tocopherol trafficking in hepatocytes are poorly understood. We found that the intracellular localization of TTP in hepatocytes is dynamic and responds to the presence of α-tocopherol. In the absence of the vitamin, TTP is localized to perinuclear vesicles that harbor CD71, transferrin, and Rab8, markers of the recycling endosomes. Upon treatment with α-tocopherol, TTP- and α-tocopherol-containing vesicles translocate to the plasma membrane, prior to secretion of the vitamin to the exterior of the cells. The change in TTP localization is specific to α-tocopherol and is time- and dose-dependent. The aberrant intracellular localization patterns of lipid binding-defective TTP mutants highlight the importance of protein-lipid interaction in the transport of α-tocopherol. These findings provide the basis for a proposed mechanistic model that describes TTP-facilitated trafficking of α-tocopherol through hepatocytes.

Vitamin E is a plant-derived lipid that was discovered as a dietary component vital for female fertility in rodents (1) and for neuronal health in humans (2). Eight vitamin E forms are synthesized by plants, differing in the degree of methylation of the chromanol ring and the saturation of the isoprenoid side chain (3). Of the eight naturally occurring forms of vitamin E, α-tocopherol exhibits the most potent biological activity in preventing deficiency-induced reproductive failure in rodents (4, 5). α-Tocopherol’s efficacy in scavenging free radicals (i.e. functioning as an antioxidant) is thought to underlie its critical roles in health (6–8), but additional redox-independent actions have been recently proposed (9).

The major regulator of vitamin E status is the α-tocopherol transfer protein (TTP),2 and mutations in the TTPA gene cause heritable vitamin E deficiency. In humans, mutations in the TTPA gene cause systemic vitamin E deficiency accompanied by neurological compromise, primarily spinocerebellar ataxia (10–17). Patients afflicted with this disorder (ataxia with vitamin E deficiency; OMIM 277460) present with debilitating neurodegeneration, the severity of which depends on the specific mutation’s effect on TTP’s biochemical activity (18, 19). Life-long vitamin E supplementation can delay or reverse disease progression, especially when patients are treated in earlier stages (20, 21). Disruption of the TtpA gene in mice recapitulated the human ataxia with vitamin E deficiency disorder (22). TTP is expressed at high levels in parenchymal cells of the liver (23, 24), but low level expression has been described also in the cerebellum (25) and the placenta (26–28). In vitro, TTP displays selective high affinity binding to α-tocopherol and facilitation of α-tocopherol transfer between lipid vesicles (29–31). In hepatocytes, TTP was shown to have a punctate perinuclear localization and significant distribution overlap with lysosomal markers (32, 33). The function of hepatic TTP is to facilitate secretion of the ingested vitamin from the hepatocytes, prior to its association with lipoproteins and delivery to non-hepatic tissues (34–36).

The distinct substrate specificity of TTP, together with the selective hepatic catabolism of all other forms of vitamin E (37), explains the remarkable isomer discrimination observed in vivo favoring α-tocopherol (38). Moreover, expression levels of TTP are a key determinant of circulating α-tocopherol levels (22, 36). Importantly, the transcriptional response of the TTPA gene to oxidative stress (39, 40) and the profound effect of phosphoinositides on TTP activity (41) suggest that TTP regulates α-tocopherol biology in a dynamic and homeostatically controlled fashion. Despite these insights, the molecular mechanisms by which TTP facilitates α-tocopherol trafficking are still poorly understood. Here, we report our findings on the regulation of TTP’s intracellular localization and the mechanisms by which the protein facilitates secretion of α-tocopherol from hepatocytes.

2 The abbreviations used are: TTP, α-tocopherol transfer protein; NBD-α-tocopherol, (R)-2,5,7,8-tetramethylchroman-2-(9-[(7-nitrobenz(1,2,3)oxadiazol-4-ylamino)nonyl] chroman-6-ol; IPP-CAXA, membrane targeted 5'-phosphatase domain of synaptoplakin 1; RFP, red fluorescent protein; PH, pleckstrin homology, PI(3,4)P₂, phosphatidylinositol 3,4-bisphosphate; PI(4,5)P₂, phosphatidylinositol 4,5-bisphosphate.
**Results**

**Uptake of \( \alpha \)-Tocopherol in Cultured Hepatocytes**—To examine the uptake of \( \alpha \)-tocopherol into hepatocytes with high spatial resolution, we utilized NBD-\( \alpha \)-tocopherol, a fluorescent analog that we have previously shown to be a reliable reporter of the vitamin’s location in cells and when bound to TTP (19, 30, 31, 33, 42–46). To examine the time course of vitamin E uptake, cells were incubated with the serum:NBD-\( \alpha \)-tocopherol complex at 4 °C, and the unbound vitamin was washed with serum-free media and uptake initiated by switching the cells back to 37 °C. The intracellular distribution of NBD-\( \alpha \)-tocopherol was monitored over time in live cells using confocal fluorescence microscopy. As shown in Fig. 1, NBD-\( \alpha \)-tocopherol was transported in a time-dependent manner to the cell interior, where it was observed in distinct peri-nuclear punctae after 30 min. At that point, NBD-\( \alpha \)-tocopherol co-localized with LysoTracker, a fluorescent reporter that selectively marks lysosomes, as we previously observed in fixed cells (Fig. 1A) (33, 46). These observations support our previous findings that \( \alpha \)-tocopherol is taken up through endocytosis (33) and extend these findings to live cells. Interestingly, we found that very shortly (<5 min) after uptake, NBD-\( \alpha \)-tocopherol also co-localizes to large (0.50–2 \( \mu \)m) round structures in the cell periphery, where it remained for the duration of the uptake process (Fig. 1B). The strong co-staining of these structures with Nile Red, a marker of neutral lipids, strongly suggests that they are lipid droplets (47). These findings indicate that lipid droplets are intimately involved with the uptake and trafficking of \( \alpha \)-tocopherol in hepatocytes (see under “Discussion”).

**Effect of \( \alpha \)-Tocopherol on the Intracellular Localization of TTP**—To study the role of TTP in vitamin E transport, we investigated whether the presence of \( \alpha \)-tocopherol affects the protein’s intracellular localization pattern. Because TTP is not endogenously expressed in McARH7777 cells, we transfected the cells with the human TTPA as we have done in the past (19, 33). Cells were grown in vitamin E-deficient media and transiently transfected with a plasmid encoding TTP prior to visualization by confocal fluorescence microscopy. We found the intracellular distribution of TTP was dramatically influenced by \( \alpha \)-tocopherol; in the absence of the vitamin, the protein exists in distinct peri-nuclear punctae that are marked by LAMP1, an established resident of the late endocytic/lysosomal compartment (19, 33). However, upon addition of \( \alpha \)-tocopherol, the protein re-distributes to a diffuse pattern throughout the cytosol (Fig. 2A). To examine the specificity of this phenomenon, and its relevance to vitamin E physiology, we tested two mis-sense mutants of TTP that cause heritable vitamin E deficiency in humans (48), and the protein’s biochemical activities in vitro are very similar to those of the wild-type protein (18). We found that the intracellular localization of the protein and its response to \( \alpha \)-tocopherol treatment are indistinguishable from the wild-type protein (Fig. 2C). These findings with TTP mutants show that the \( \alpha \)-tocopherol-induced shift in TTP’s intracellular localization is key to the protein’s biological activity, and it reflects its ligand-specific binding activity. We observed a similar response to \( \alpha \)-tocopherol in live cells that express RFP-tagged TTP but not in cells that express the RFP tag alone (Fig. 2D). Quantification of TTP’s intracellular local-
The intracellular localization of TTP is regulated by vitamin E. Because established hepatocyte cell lines do not endogenously express TTP, we utilized transient transfection to express the human TTPA gene in McARH7777 cells. A–C, indicated TTP variant was transiently transfected (in the pCDNA3.1 vector) into vitamin E-depleted McARH7777 cells. Where indicated, serum-complexed α-tocopherol (35 μM) was added to the cultures for 24 h prior to fixing, immunostaining, and imaging by confocal fluorescence microscopy. D, RFP-tagged TTP-expressing (in the pTagRFP plasmid; left) or RFP-expressing (right) plasmids were transiently transfected and treated as in A, and protein localization was determined by live-cell fluorescence microscopy. E, kinetics of α-tocopherol-induced shift in TTP’s localization. Fraction of TTP was determined at different times after addition of 35 μM serum-complexed α-tocopherol as in D, and fraction of the cells in which the protein displayed a punctate pattern was quantified by visual inspection of fluorescence micrographs (total of 300 – 400 cells in each of four images per condition). Shown are averages and standard deviations in one representative of three independent experiments. F, dose-response of α-tocopherol-induced shift in TTP’s localization. Fraction of cells in which the protein displayed a punctate pattern was quantified 24 h after treatment with the indicated concentration of serum-complexed α-tocopherol (or cholesterol) as in E. Asterisks indicate statistically significant difference from control (p < 0.05), determined by Student’s t test.
small GTPase Rab8 (Fig. 3B), both of which mark the recycling endosomes as follows: small vesicles that originate from the endocytic pathway and provide a rapid route for recycling of internalized, activated cell-surface receptors and regulated by Rab GTPases (49). Importantly, not only TTP but also α-tocopherol resides in recycling endosomes, as seen by the colocalization of NBD-α-tocopherol, TTP, and transferrin (Fig. 3C). The addition of α-tocopherol caused a marked redistribution of TTP but did not affect the distribution of Lamp1, Rab8, or CD71 (Figs. 2A and 3A and B). Taken together, these findings indicate that TTP-facilitated transport of vitamin E within hepatocytes utilizes recycling endosomes that replenish the plasma membrane with selected proteins and cargo (50–53).

**Dynamic Changes in Intracellular Localization of α-Tocopherol and TTP**—The observations that the intracellular localization of TTP is affected by α-tocopherol raise the possibility that TTP actively participates in the process of transporting the vitamin to its site of secretion at the plasma membrane following endocytosis of α-tocopherol. To examine the kinetics of this process in greater detail, we employed a “pulse-chase” approach that allows us to observe the time-dependent localization of both TTP and NBD-α-tocopherol following uptake. Vitamin E-depleted cells were first incubated with a low concentration (10 μM) of serum-complexed NBD-α-tocopherol at 4 °C. Under this condition, the hydrophobic vitamin adheres to the surface of the cells but cannot be internalized, thus providing a means to initiate synchronized uptake of α-tocopherol by switching back to normal temperature (33). After washing unbound tocopherol, the cells were returned to 37 °C and imaged with confocal fluorescence microscopy. We observed that the levels of NBD-α-tocopherol rapidly decreased within cells that express TTP, reflecting the TTP-facilitated secretion of the vitamin; intracellular NBD fluorescence was reduced to ~40% of its initial levels within 90 min (cells outlined in white in Fig. 4, A and B). In contrast, intracel-

**FIGURE 3. TTP localizes to the recycling endosomes.** McARH7777 cells stably expressing pTRE2-TTP and induced with doxycycline for 48 h were fixed and immunostained with antibodies directed against TTP (red), the transferrin receptor (A; green), or Rab8 (B; green). Where indicated, serum-complexed α-tocopherol was added to 35 μM for 48 h prior to fixing, immunostaining, and visualization under the confocal fluorescence microscope. C, McARH7777 cells were incubated with Alexa Fluor 633-labeled transferrin (blue) and NBD-tocopherol (green) at 4 °C for 1 h prior to incubation at 37 °C for 30 min, fixing and visualization with confocal fluorescence microscopy. White arrows indicate sites of co-localization. Data are representative of three independent experiments. Scale bars, 5 μm.
FIGURE 4. Time-dependent changes in the intracellular localization of NBD-α-tocopherol and TTP. McARH7777 cells were transiently transfected with pTagRFP-TTP. Forty eight hours after transfection, cells were pulse-loaded with 10 μM serum-complexed NBD-α-tocopherol, and live confocal images of NBD-α-tocopherol (green) or TTP (red) were taken at the indicated times. A, representative images showing cells that do (white outline) or do not (yellow outline) express TTP. Scale bars, 5 μm. B, quantification of intracellular NBD-α-tocopherol fluorescence over time in cells that do (white bars) or do not (black bars) express TTP. Shown are averages and standard errors of NBD-α-tocopherol fluorescence quantified in confocal images of ~50 cells of each group from three independent experiments. Asterisks denote statistical significance with \( p < 0.01 \), calculated by Student’s \( t \) test. Fluorescence intensities were normalized to the values of non-TTP-expressing cells. C, time-dependent changes in the intracellular localization of TTP. Localization of TTP in perinuclear (black bars) or peripheral (white bars) cellular locations during secretion of NBD-α-tocopherol was manually determined in three images (20–50 cells) of each condition. Asterisks denote statistically significant differences determined by Student’s \( t \) test (\( p < 0.01 \)). Data are representative of three independent experiments.
Regulation of the Tocopherol Transfer Protein

Lular content of tocopherol changed very little in hepatocytes that did not express TTP (cells outlined in yellow in Fig. 4, A and B). Importantly, the intracellular localization of TTP itself also changed markedly during this time period: initially, most of the protein (>70%) was observed in endosomes (Fig. 4C). After 90 min, however, >60% of the TTP-containing vesicles relocated to the cell periphery, adjacent to the plasma membrane (white arrows in Fig. 4A; Fig. 4, A and C). We note that the change in TTP localization from the cell interior to the periphery occurs shortly after α-tocopherol treatment (30–60 min), although the localization shift after sustained high level treatment with α-tocopherol takes many hours (Fig. 2). We attribute this variation to the different experimental protocols employed; when α-tocopherol is added at high concentrations and for prolonged periods, the observed localization pattern of TTP reflects its presence in multiple cellular locations (endosomes, plasma membrane, and intermediate trafficking locations), exhibiting an “average” diffuse cytosolic pattern (Fig. 2). However, when the vitamin is delivered in a short synchronized “pulse,” the localization pattern of TTP reflects its time-dependent location at various stages of the transport process. Taken together, these observations reveal that TTP is co-transported together with α-tocopherol as the vitamin travels from the hepatocyte’s endocytic compartment to the plasma membrane.

Role of Protein-Lipid Interactions in TTP-facilitated α-Tocopherol Transport—The extreme lipophilic nature of α-tocopherol restricts its localization to either lipid membranes or droplets (Fig. 1) or to a protein’s hydrophobic binding pocket (54). It is therefore not surprising that the biological actions of TTP require direct interactions between the protein and lipid bilayers (31). Kono et al. (41) recently demonstrated that a positively charged patch in TTP’s surface binds phosphatidylinositols, especially PI(4,5)P₂ and PI(3,4)P₂, and that binding of these lipids modulated TTP’s tocopherol transfer activity. In addition, hydrophobic residues in TTP’s A8 helix were shown to mediate the protein’s association with lipid bilayers and to be critical for TTP-facilitated α-tocopherol secretion in cultured hepatocytes (43). We therefore examined how these protein-lipid interactions affect TTP’s intracellular localization and activity. To examine the role of phosphoinositides, we utilized the membrane-targeted inositol 5′-phosphatase domain of synaptotagmin 1 (IPP-CAAX, where AA is aliphatic amino acid), previously shown to efficiently deplete the plasma membrane of PI(4,5)P₂ (55–58). The suitability of this reagent is demonstrated in Fig. 5A; in control cells, the pleckstrin homology (PH) domain of PLCδ binds PI(4,5)P₂ at the plasma membrane, and thus it selectively highlights this region in the cell (Fig. 5A). However, in cells that express IPP-CAAX, the PH domain redistributes throughout the cytosol, reflecting depletion of the plasma membrane pools of PI(4,5)P₂ (Fig. 5Aa) (55–58). Importantly, expression of IPP-CAAX abrogated TTP activity; in the presence of the inositol phosphatase, TTP-facilitated secretion of NBD-α-tocopherol was completely abolished (Fig. 5B). Thus, the presence of 5-phosphoinositides at the plasma membrane is essential for TTP-mediated transport of α-tocopherol. Moreover, expression of IPP-CAAX had a dramatic impact on the intracellular localization of TTP, even in the absence of vitamin E; the protein’s intracellular localization pattern changed from the vesicular compartment described above to a diffuse cytosolic pattern (Fig. 5C). These findings indicate that high levels of 5′-phosphoinositides at the plasma membrane play a key role in trafficking of the TTP-positive vesicles, likely by providing directionality to the transport process, from the cell interior to the PI(4,5)P₂-rich plasma membrane (see under “Discussion”). Indeed, membrane pools of phosphatidylinositols are known to play a key role in directing recycling endosomes toward the plasma membrane (59).

Next, we examined how mutations that impair the interactions of TTP with membrane lipids affect the protein’s intracellular localization. When bound to TTP, PI(4,5)P₂ was shown to directly interact with a positively charged “patch” on the protein’s surface, which includes Lys²¹⁷. Moreover, the TTP(K217A) substitution disrupted phosphoinositide binding to TTP and concomitantly abolished α-tocopherol transport by the protein (41). We found that the intracellular localization of the TTP(K217A) mutant was drastically different from that of the wild-type protein; the mutant protein was localized in a “band” along the cell’s plasma membrane, and this pattern was not altered by the presence of α-tocopherol (Fig. 6B). These observations confirm that the ability of TTP to interact with phosphoinositides is critical for the protein’s function and suggest that Lys²¹⁷ is essential for coupling the binding of phosphoinositide to TTP with the release of the protein-bound α-tocopherol to the membrane and the subsequent dissociation of the protein from the membrane (see under “Discussion”). We also examined the role of TTP’s hydrophobic surface (helix A8) in the protein’s intracellular localization pattern. We found that the TTP(F169A) and TTP(F169D) mutants were dispersed throughout the cytosol and that this pattern was not affected by treatment with α-tocopherol (Fig. 6, C and D). These findings indicate that distinct lipid-protein interactions affect TTP’s intracellular localization and function in different ways; the hydrophobic stretch around Phe¹⁶⁹ is required for association of the protein with all membranes, whereas the positive “patch” around Lys²¹⁷ mediates the selective association with the phosphoinositide-enriched plasma membrane that is required for releasing α-tocopherol from the protein binding pocket. Although these interactions are independent of each other, they are both critical for TTP’s function.

Discussion

Despite the realization of vitamin E’s important role in human health nearly a century ago (60), the distribution patterns and concentrations of vitamin E in tissues were generally assumed to be a passive reflection of dietary intake. However, recent findings suggest that this is not the case. It is now clear that hepatic TTP regulates the selective secretion of ingested α-tocopherol from hepatocytes to circulating lipoproteins that then deliver the vitamin to extra-hepatic tissues. In doing so, hepatic TTP functions as a critical “gatekeeper” that determines body-wide levels and distribution of the vitamin. Thus, circulating levels of α-tocopherol are dependent on the TTP gene dose (26), and heritable mutations in the TPPA gene cause familial vitamin E deficiency (12, 24, 36), the clinical severity of which correlates with the degree of biochemical compromise to TTP function (19). Moreover, transcription of the TPPA gene is
subject to regulation by physiological states and some hormones (40). Further homeostatic control is provided by the fact that TTP’s stability in cells is modulated by ubiquitination which, in turn, is regulated by α-tocopherol (61). Finally, TTP is also expressed in the brain (25, 62) and the placenta (28, 39, 63). It is likely that in these locations, TTP provides an additional layer of regulation that maintains local α-tocopherol sufficiency in the most sensitive tissues, i.e. the central nervous system and the developing embryo. These observations provide strong support to the notion that TTP provides homeostatic control of α-tocopherol levels in vivo in the face of fluctuating intake and alterations in physiological demands.

A number of studies are aimed to understand how vitamin E trafficking is regulated in hepatocytes and to elucidate TTP’s roles in that process. It is well established that secretion of α-tocopherol from hepatocytes is sensitive to disruption of lysosomal integrity (33, 34, 46, 64, 65), thereby implicating vesicular transport. Arai and co-workers (34) have shown that TTP-facilitated secretion of α-tocopherol from hepatocytes relies on a novel transport pathway that is independent of Golgi function.
and therefore uncoupled from the hepatic assembly and export of nascent lipoproteins. The intracellular localization of TTP was reported to be cytosolic in some reports (32) and associated with vesicles of endocytic origin in others (19, 33). We show that during secretion from hepatocytes, both α-tocopherol and TTP reside in vesicles marked by the small GTPase Rab8, transferrin, and the transferrin receptor CD71 (Fig. 3). These data indicate that α-tocopherol is trafficked through the recycling endosomes: vesicles that replenish the plasma membrane with recycled receptors and some endocytic cargo and are controlled by Rab8 and its downstream effectors (51, 53). To our surprise we found that in live cells, α-tocopherol is also located in lipid droplets, marked by the neutral lipid stain Nile red (Fig. 1). To our knowledge, a trafficking route that delivers newly endocytosed lipids directly to lipid droplets has not been described in the literature. However, a number of reports documented physical and functional interactions between lipid droplets and endosomes. Thus, endosomal Rab GTPases were shown to associate with lipid droplets in a GTP-dependent fashion and to regulate the exchange of markers between the two compartments (66). Interestingly, Rab8 was shown to be a critical regulator of lipid droplet fusion and growth (67, 68). Thus, it appears that endosome-lipid droplet interactions may be another example of the growing repertoire of dynamic exchange between cell organelles (69).

A major finding reported here is the dynamic change in TTP location during α-tocopherol secretion, and the importance of protein-lipid interactions in this process. Thus, although in vitamin E-depleted cells TTP resides in the perinuclear punctae described above, addition of α-tocopherol caused a marked dose-dependent change in the protein’s intracellular localization. We observed this under two distinct sets of experimental conditions as follows: 1) to observe a “single turnover” of the transport process, we treated cells with a brief low-dose pulse of α-tocopherol. This allowed us to monitor the directional trafficking of the protein- and vitamin-containing vesicles toward the cell’s periphery (Fig. 4). This transition likely reflects the directional transport of TTP- and α-tocopherol-containing vesicles to sites of secretion at the cell’s plasma membrane, presumably where an ABC-type transporter resides (33, 70–72). 2) When the cells are exposed to a sustained (many hours) high dose treatment with α-tocopherol, TTP is dispersed from the punctae to a uniform cytosolic pattern with only a few visible vesicular structures (Figs. 2 and 3). This distribution likely reflects a composite localization of TTP as the protein undergoes multiple cycles of α-tocopherol transport.

An important determinant of α-tocopherol trafficking is TTP’s association with membrane resident lipids. Previous studies have shown that association of TTP with lipid bilayers involves an interaction between a hydrophobic surface in TTP (composed of the side chains of Phe<sup>165</sup>, Phe<sup>169</sup>, Ile<sup>202</sup>, Val<sup>206</sup>, and Met<sup>209</sup>) and the lipid bilayer. Thus, the TTP(F169A) mutant is unable to associate with lipid bilayers and concomitantly does not facilitate α-tocopherol transport (43). Here, we extend these findings and show that the TTP(F169A) mutant displays a diffuse cytosolic distribution that is not affected by α-tocopherol (Fig. 6). Recently, Kono <em>et al.</em> (41) have shown that interactions with phosphoinositides are also critical for TTP function. A positive patch on TTP’s surface (composed of the side chains of Arg<sup>59</sup>, Arg<sup>192</sup>, and Lys<sup>217</sup>) mediates the binding of TTP to PI(3,4)P<sub>2</sub> or PI(4,5)P<sub>2</sub> in the membrane. Importantly, mutations of residues that line this surface region, such as the TTP(K217A) mutant, are defective for the homologous Sec proteins, binding of phosphoinositides
induces an allosteric conformational change that opens an amphipathic helix at the entrance to the ligand (tocopherol) binding pocket, thus initiating ligand-phosphoinositide exchange (73, 74). Indeed, Kono et al. (41) observed direct exchange of PI(4,5)P2 for tocopherol upon incubation of TTP with lipid vesicles in vitro. The constitutive localization of TTP(K217A) at the plasma membrane indicates that Lys217 communicates the conformational change induced by PI(4,5)P2 binding to the protein’s tocopherol binding pocket. Thus, we propose that in the TTP(K217A), the two events are uncoupled, and hence, the protein remains bound to both tocopherol and the plasma membrane. Confirmation of this model awaits further structural and biophysical studies.

It has been established that in multiple cell types, PI(4,5)P2 is selectively enriched in the plasma membrane, levels of these lipids in intracellular organelles are significantly lower, and this concentration gradient drives directional trafficking (59, 75). Our findings that enzymatic depletion of plasma membrane phosphoinositides perturbs TTP’s function and localization pattern (Fig. 5) suggest that this concept applies also to α-tocopherol trafficking in hepatocytes. Thus, our working model, depicted in Fig. 7, purports that the PI(4,5)P2 gradient “drives” the TTP-mediated α-tocopherol transport toward the plasma membrane, whereas the inverse gradient of α-tocopherol (low in the plasma membrane, high at the endocytic compartment) directs the return portion of the transport cycle.

Experimental Procedures

Cell Culture—McARH7777 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% horse serum (Gibco) and 10% fetal bovine serum (Denville Scientific) at 37 °C and 5% CO2. Where noted, cells were depleted from vitamin E by sequential passaging (>10 passages) in DMEM supplemented with 20% defined fetal bovine serum (Hyclone) determined to be α-tocopherol-free by GC-MS or by the manufacturer’s certificate of analysis (e.g. Hyclone, lot no. AAM211101). Transient transfections were carried out using polyethyleneimine (76). α-Tocopherol or NBD-α-tocopherol were delivered to cells after complexing to serum lipoproteins as described previously (33). Briefly, the vitamin was dried under vacuum and incubated with serum at 37 °C for 1 h, followed by rotary mixing overnight at 4 °C. The next day, DMEM was added to achieve a final serum concentration of 20% (v/v). Concentration of NBD-α-tocopherol was determined spectrophotometrically, using an extinction coefficient ε466 nm = 22,000 M⁻¹ cm⁻¹ (30, 42).

α-Tocopherol Treatment—We employed two separate protocols for treating vitamin E-depleted cells with α-tocopherol or NBD-α-tocopherol. 1) Sustained high dose treatment was used (Figs. 2, 3, 5C, and 6). Serum-complexed α-tocopherol was added to culture media to a final concentration of 0–35 μM for the indicated times (>12 h). This protocol was used to visualize the steady-state distribution of TTP through continuous cycling of vitamin E transport. 2) Synchronized pulse-chase treatment was used (Figs. 1 and 4). Cells were pre-chilled on ice for 1 h, and serum-complexed NBD-α-tocopherol was added to the media to a final concentration of 10 μM. After 1 h, the cells were washed with cold serum-free media and returned to 37 °C. This procedure allowed us
to examine the time-dependent single turnover transport of the vitamin through the cell.

**Immunofluorescence**—McARH7777 cells were seeded onto collagen-coated Ibidi μ-slides or dishes. For live-cell imaging, cells were stained with 100 nm LysoTracker DND-99 Red (Life Technologies, Inc.) or 100 nm Nile Red (Life Technologies, Inc.). For immunostaining, cells were seeded on collagen-coated coverslips, fixed with 3.7% paraformaldehyde for 15 min, and permeabilized with 0.2% Triton in 2% goat serum for 1 h at room temperature. Antibodies used include anti-Lamp 1 (1:100; Enzo Life Sciences), anti-Rab8 (1:25; BD Biosciences), anti-transferrin receptor (CD71; 1:150; Pharmingen), and anti-TTP (1:1500 (40)). Fluorescence of intracellular NBD-α-tocopherol was quantified by ImageJ software (77). Cells were outlined using the freehand trace tool, and the raw integrated density was measured, averaged, and compared between groups. All imaging experiments were carried out on a Zeiss LSM 510 Meta confocal microscope (Neurosciences Imaging Center, Case Western Reserve University) using a ×100 oil objective and argon (488 nm) or HeNe (543 nm) lasers.

**Analytical Determinations**—α-Tocopherol concentrations were measured by GC-MS on a Hewlett-Packard 6890 gas chromatograph coupled to a Hewlett-Packard 5892 mass selective detector operated in selected ion mode as described previously (78).

**Statistical Analyses**—Statistical significance of data were determined using the Student’s t test. p values < 0.05 were taken as the threshold for significance. Data were analyzed and graphed using Igor Pro software (WaveMetrics, Inc.).

**Reagents**—NBD-α-tocopherol, an analog in which the fluorescent NBD moiety is attached to the vitamin’s phytyl side chain, was synthesized as described earlier (30, 42). d-α-Tocopherol (99% pure) was purchased from Cole Parmer. Polyethyleneimine was purchased from Polysciences, Inc. Collagen from rat tail tendon was purchased from Roche Applied Science. The GFP-TTP and RFP-TTP constructs were generated by cloning the human TTP reading frame into the pEGFP-C2 plasmid into McARH7777 cells, followed by selection in hygromycin (200 μg/ml) for 3 weeks. The GFP-tagged PH domain of PLCβ was a gift from Tobias Meyer (Addgene plasmid 21179) (79). The membrane-targeted 5’-phosphatase domain of synaptojanin 1 (IPP-CAX) was a generous gift from Volker Hauke at the Leibniz-Instituts für Molekulare Pharmakologie in Berlin, Germany. All other reagents were of highest purity available.

**Author Contributions**—S. C. conducted the experiments, analyzed the results, and wrote first draft of the paper. M. G. synthesized the fluoroscent analog used in experiments. J. A. designed the analog, supervised the synthesis, and edited the manuscript. R. P. analyzed lipids by GC-MS and edited the manuscript. J. Q. conducted the transferrin experiment (Fig. 3C). C. C. provided ideas for experiments, discussed the data, and edited the manuscript. D. M. conceived the idea for the project, supervised the experimental work, and wrote the paper.

**Acknowledgments**—We are grateful to Volker Hauke at the Leibniz-Instituts für Molekulare Pharmakologie in Berlin, Germany, for the generous gift of the IPP-CAX construct, to Oshikazu Imanishi from the Case Western Reserve University Department of Pharmacology for valuable reagents, and to Maryanne Pendergast from the Case Western Reserve University Neuroscience Imaging Facility for expert microscopy support. Artwork was provided by Fiona Martin.

**References**

1. Evans, H. M., and Bishop, K. S. (1922) On the existence of a hitherto unrecognized dietary factor essential for reproduction. *Science* **56**, 650–651.

2. Ulatowski, L. M., and Manor, D. (2015) Vitamin E and neurodegeneration. *Neurobiol. Dis.* **48**, 74–83.

3. DellaPenna, D., and Pogson, B. J. (2006) Vitamin synthesis in plants: tocopherols and carotenoids. *Annu. Rev. Plant Biol.* **57**, 711–736.

4. Hosomi, A., Arita, M., Sato, Y., Kiyose, C., Ueda, T., Igarashi, O., Arai, H., and Inoue, K. (1997) Affinity for α-tocopherol transfer protein as a determinant of the biological activities of vitamin E analogs. *FEBS Lett.* **409**, 105–108.

5. Leth, T., and Sondergaard, H. (1977) Biological activity of vitamin E compounds and natural materials by the resorption-gestation test, and chemical determination of the vitamin E activity in foods and feeds. *J. Nutr.* **107**, 2236–2243.

6. Ingold, K. U., Webb, A. C., Witter, D., Burton, G. W., Metcalfe, T. A., and Muller, D. P. (1987) Vitamin E remains the major lipid-soluble, chain-breaking antioxidant in human plasma even in individuals suffering severe vitamin E deficiency. *Arch. Biochem. Biophys.* **259**, 224–225.

7. Burton, G. W. (1994) Vitamin E: molecular and biological function. *Proc. Nutr. Soc.* **53**, 251–262.

8. Traber, M. G., and Atkinson, J. (2007) Vitamin E, antioxidant and nothing more. *Free Radic. Biol. Med.* **43**, 4–15.

9. Azzi, A. (2007) Molecular mechanism of α-tocopherol action. *Free Radic. Biol. Med.* **43**, 16–21.

10. Ouahchi, K., Arita, M., Kayden, H., Henri, F., Ben Hamida, M., Sokol, R., Arai, H., Inoue, K., Mandel, J. L., and Koenig, M. (1995) Ataxia with isolated vitamin E deficiency is caused by mutations in the α-tocopherol transfer protein. *Nat. Genet.* **9**, 141–145.

11. Gotoda, T., Arita, M., Arai, H., Inoue, K., Yokota, T., Fuku, Y., Yazaki, Y., and Yamada, N. (1995) Adult-onset spinocerebellar dysfunction caused by a mutation in the gene for the α-tocopherol transfer protein. *N. Engl. J. Med.* **333**, 1313–1318.

12. Cavalier, L., Ouahchi, K., Kayden, H. J., Di Donato, S., Reutenauer, L., Mandel, J. L., and Koenig, M. (1998) Ataxia with isolated vitamin E deficiency: heterogeneity of mutations and phenotypic variability in a large number of families. *Am. J. Hum. Genet.* **62**, 301–310.

13. Sokol, R. J. (1988) Vitamin E deficiency and neurologic disease. *Annu. Rev. Nutr.* **8**, 351–373.

14. Traber, M. G., Sokol, R. J., Kohlschutter, A., Yokota, T., Muller, D. P., Dufour, R., and Kayden, H. J. (1993) Impaired discrimination between stereoisomers of α-tocopherol in patients with familial isolated vitamin E deficiency. *J. Lipid Res.* **34**, 201–210.

15. Yokota, T., Shiojiri, T., Gotoda, T., and Arai, H. (1996) Retinitis pigmentosa and ataxia caused by a mutation in the gene for the α-tocopherol transfer protein. *N. Engl. J. Med.* **335**, 1770–1771.

16. Yokota, T., Shiojiri, T., Gotoda, T., Arita, M., Arai, H., Ohga, T., Kanda, T., Suzuki, J., Imai, T., Matsumoto, H., Harino, S., Kiyosawa, M., Mizusawa, H., and Inoue, K. (1997) Friedreich-like ataxia with retinitis pigmentosa caused by the Hs101Gln mutation of the α-tocopherol transfer protein gene. *Am. J. Hum. Genet.* **61**, 845–852.

17. Yokota, T., Uchihara, T., Kumagai, J., Shiojiri, T., Pang, J. J., Arita, M., Arai, H., Hayashi, M., Kiyosawa, M., Okeda, R., and Mizusawa, H. (2000) Post-mortem study of ataxia with retinitis pigmentosa by mutation of the α-tocopherol transfer protein gene. *J. Neurol. Neurosurg. Psychiatry* **68**, 521–525.
Regulation of the Tocopherol Transfer Protein

18. Morley, S., Panagabko, C., Shineman, D., Mani, B., Stocker, A., Atkinson, J., and Manor, D. (2004) Molecular determinants of heritable vitamin E deficiency. Biochemistry 43, 4143–4149
19. Qian, J., Atkinson, J., and Manor, D. (2006) Biochemical consequences of heritable mutations in the α-tocopherol transfer protein. Biochemistry 45, 8236–8242
20. Gabsi, S., Goudier-Khouja, N., Belai, S., Fki, M., Kefi, M., Turki, I., Ben Hamida, M., Kayden, H., Mebazaa, R., and Rentati, F. (2001) Effect of vitamin E supplementation in patients with ataxia with vitamin E deficiency. Eur. J. Neurol. 8, 477–481
21. Schuelle, M., Mayatepek, E., Inter, M., Becker, M., Pfeiffer, E., Speer, A., Hübner, C., and Finchk, B. (1999) Treatment of ataxia in isolated vitamin E deficiency caused by α-tocopherol transfer protein deficiency. J. Pediatr. 134, 240–244
22. Yokota, T., Igarkashi, K., Uchihara, T., Jishage, K., Tomita, H., Inaba, A., Li, Y., Arita, M., Suzuki, H., Mizusawa, H., and Inoue, A. (2001) Delayed-onset ataxia in mice lacking α-tocopherol transfer protein: model for neuronal degeneration caused by chronic oxidative stress. Proc. Natl. Acad. Sci. U.S.A. 98, 15185–15190
23. Sato, Y., Hagiwara, K., Arai, H., and Inoue, K. (2013) Purification and characterization of the α-tocopherol transfer protein from rat liver. FEBS Lett. 588, 41–45
24. Traber, M. G., and Arai, H. (1999) Molecular mechanisms of vitamin E transport. Annu. Rev. Nutr. 19, 343–355
25. Hosomi, A., Goto, K., Kondo, H., Iwatsubo, T., Yokota, T., Ogawa, M., Arita, Aoki, J., Arai, H., and Inoue, K. (1998) Localization of α-tocopherol transfer protein in rat brain. Neurosci. Lett. 256, 159–162
26. Jishage, K., Arita, M., Igarashi, K., Iwata, T., Watanabe, M., Ogawa, M., Ueda, O., Kamada, N., Inoue, K., Arai, H., Suzuki, H., and Nakis, A., Jeschke, U., and Rotzoll, D. E. (2012) Oxidative stress stimulates α-TTP-PIPs interaction underlies familial vitamin E deficiency. Science 340, 1106–1110
27. Manor, D., and Morley, S. (2008) Mechanisms of ligand transfer by the hepatic tocopherol transfer protein: model for neuronal transport. Biochem. Med. 14, 3721–3736
28. Zhang, W. X., Thakur, V., Lomize, A., Pogozhieva, I., Panagabko, C., Cecchini, M., Baptista, M., Morley, S., Manor, D., and Atkinson, J. (2011) The contribution of surface residues to membrane binding and ligand transfer by the α-tocopherol transfer protein (α-TTP). J. Mol. Biol. 405, 972–988
29. Zhang, W. X., Frahm, G., Morley, S., Manor, D., and Atkinson, J. (2009) Effect of bilayer phospholipid composition and curvature on ligand transfer by the α-tocopherol transfer protein. Lipids 44, 631–641
30. Morley, S., Thakur, V., Danielpour, D., Parker, R., Arai, H., Inoue, K., Barnholtz-Sloan, J., Klein, E., and Manor, D. (2010) The tocopherol transfer protein promotes prostate cancer cell to vitamin E. J. Biol. Chem. 285, 35578–35589
31. Ulatowski, L., Parker, R., Davidson, C., Yanjanin, N., Kelley, T., Corey, D., Atkinson, J., Porter, F., Arai, H., Wallkley, S. U., and Mannor, D. (2011) Altered vitamin E status in Niemann-Pick type C disease. J. Lipid Res. 52, 1400–1410
32. Greenspan, P., Mayer, E. P., and Fowler, S. D. (1985) Nile red: a selective fluorescent stain for intracellular lipid droplets. J. Cell Biol. 100, 965–973
33. Rentati, A., Deng, H. X., Hung, W. Y., Nayer, M., Ahmed, M. S., He, X., Tim, R., Stumpf, D. A., Siddique, T., and Ahmed. (1996) Human α-tocopherol transfer protein: gene structure and mutations in familial vitamin E deficiency. Ann. Neurol. 39, 295–300
34. Stenmark, H. (2009) Rab GTPases as coordinators of vesicle traffic. Nat. Rev. Mol. Cell Biol. 10, 513–525
35. Kanerva, K., Uronen, R. L., Blom, T., Li, S., Bittman, R., Lappalainen, P., Peränen, J., Raposo, G., and Ikonen, E. (2013) LDL cholesterol recycles to the plasma membrane via a Rab8b-Msyosin5b-dependent membrane transport route. Dev. Cell 27, 249–262
36. Sharma, M., Giridharan, S. S., Rahajeng, J., Naslavsky, N., and Caplan, S. (2009) MICAL-L1 links EHD1 to tubular recycling endosomes and regulates receptor recycling. Mol. Biol. Cell 20, 5181–5194
37. Huber, L. A., Pimplikar, S., Parton, R. G., Virta, H., Zerial, M., and Simons, K. (1993) Rab8, a small GTPase involved in vesicular traffic between the TGN and the basolateral plasma membrane. J. Cell Biol. 123, 35–45
38. Nagabhushana, A., Chalasani, M. L., Jain, N., Radha, V., Rangaraj, N., Balasubramanian, D., and Swarup, G. (2010) Regulation of endocytic trafficking of transferrin receptor by optineurin and its impairment by a glaucoma-associated mutant. BMC Cell Biol. 11, 4
39. Atkinson, J., Epand, R. F., and Epand, R. M. (2008) Tocopherols and tocotrienols in membranes: a critical review. Free Radic. Biol. Med. 44, 739–764
40. Milosevic, J., Sorensen, J. B., Lang, T., Krauss, M., Nagy, G., Haucke, V., Jahn, R., and Neher, E. (2005) Plasma mememal phosphatidylinositol-4,5-bisphosphate level regulates the releasable vesicle pool size in chromaffin cells. J. Neurosci. 25, 2557–2565
41. Nakatsu, B., Baskin, J. M., Chung, J., Tanner, L. B., Shui, G., Lee, S. Y., Pirruccello, M., Hao, M., Ingolia, N. T., Wenk, M. R., and De Camilli, P. (2012) PtdIns4P synthesis by PH4KIIα at the plasma membrane and its impact on plasma membrane identity. J. Cell Biol. 199, 1003–1016
42. Krauss, M., Kinuta, M., Wenk, M. R., De Camilli, P., Takei, K., and Haucke, V. (2003) ARf6 stimulates clathrin/AP-2 recruitment to synaptic membranes by activating phosphatidylinositol-4,5-phosphate kinase type I. J. Cell Biol. 162, 113–124
43. Posor, Y., Eichhorn-Gruenig, M., Puchkov, D., Schöneberg, J., Ullrich, A.,
Lampe, A., Müller, R., Zarbakhsh, S., Gulluni, F., Hirsch, E., Krauss, M., Schultz, C., Schnoranzer, J., Noé, F., and Haucke, V. (2013) Spatiotemporal control of endocytosis by phosphatidylinositol-3,4-bisphosphate. *Nature* **499**, 233–237.

Di Paolo, G., and De Camilli, P. (2006) Phosphoinositides in cell regulation and membrane dynamics. *Nature* **443**, 651–657.

Niki, E., and Traber, M. G. (2012) A history of vitamin E. *Ann. Nutr. Metab.* **61**, 207–212.

Thakur, V., Morley, S., and Manor, D. (2010) Hepatic α-tocopherol transfer protein: ligand-induced protection from proteasomal degradation. *Biochemistry* **49**, 9339–9344.

Copp, R. P., Wisniewski, T., Hentati, F., Larnaout, A., Ben Hamida, M., and Kayden, H. J. (1999) Localization of α-tocopherol transfer protein in the brains of patients with ataxia with vitamin E deficiency and other oxidative stress related neurodegenerative disorders. *Brain Res.* **822**, 80–87.

Kaempf-Rotzoll, D. E., Igarashi, K., Aoki, J., Jishage, K., Suzuki, H., Tamai, H., Linderkamp, O., and Arai, H. (2002) α-Tocopherol transfer protein is specifically localized at the implantation site of pregnant mouse uterus. *Biol. Reprod.* **67**, 599–604.

Bjørneboe, A., Bjørneboe, G. E., Hagen, B. F., Nossen, J. O., and Drevon, C. A. (1987) Secretion of α-tocopherol from cultured rat hepatocytes. *Biochim. Biophys. Acta* **922**, 199–205.

Yévenes, L. F., Klein, A., Castro, J. F., Marin, T., Leal, N., Leighton, F., Alvarez, A. R., and Zanlungo, S. (2012) Lysosomal vitamin E accumulation in Niemann-Pick type C disease. *Biochim. Biophys. Acta* **1822**, 150–160.

Liu, P., Barta, R., Zehmer, J. K., Ying, Y. S., Zhu, M., Serrero, G., and Anderson, R. G. (2007) Rab-regulated interaction of early endosomes with lipid droplets. *Biochim. Biophys. Acta* **1773**, 784–793.

Gao, Q., and Goodman, J. M. (2015) The lipid droplet-a well-connected organelle. *Front. Cell Dev. Biol.* **3**, 49.

Regulation of the Tocopherol Transfer Protein

Olam, J. F., Vaughan, A. M., and Stocker, R. (2001) ATP-binding cassette transporter A1 mediates cellular secretion of α-tocopherol. *J. Biol. Chem.* **276**, 39898–39902.

Oliveir, M., Bott, G. R., Frisal, E., Nowick, M., Plengpanich, W., Desmarchelier, C., Roi, S., Quinn, C. M., Gelissen, I., Jessup, W., Van Eck, M., Guérin, M., Le Goff, W., and Reboul, E. (2014) ABCG1 is involved in vitamin E efflux. *Biochim. Biophys. Acta* **1841**, 1741–1751.

Shichiri, M., Takanazawa, Y., Rotzoll, D. E., Yoshida, Y., Kokubu, T., Ueda, K., Tamai, H., and Arai, H. (2010) ATP-binding cassette transporter A1 is involved in hepatic α-tocopherol secretion. *J. Nutr. Biochem.* **21**, 451–456.

Sha, B., Phillips, S. E., Bankaitis, V. A., and Luo, M. (1998) Crystal structure of the *Saccharomyces cerevisiae* phosphatidylinositol-transfer protein. *Nature* **391**, 506–510.

Bankaitis, V. A., Mousley, C. J., and Schaaf, G. (2010) The Sec14 superfamily and mechanisms for crosstalk between lipid metabolism and lipid signaling. *Trends Biochem. Sci.* **35**, 150–160.

Vicinanza, M., D’Angelo, G., Di Campli, A., and De Matteis, M. A. (2008) Phosphoinositides as regulators of membrane trafficking in health and disease. *Cell. Mol. Life Sci.* **65**, 2833–2841.

Thomas, M., Lu, J. J., Ge, Q., Zhang, C., Chen, J., and Klblanov, A. M. (2005) Full deacylation of polyethyleneimine dramatically boosts its gene delivery efficiency and specificity to mouse lung. *Proc. Natl. Acad. Sci. U.S.A.* **102**, 5679–5684.

Schneider, C. A., Rasband, W. S., and Eliceiri, K. W. (2012) NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods* **9**, 671–675.

Sontag, T. J., and Parker, R. S. (2007) Influence of major structural features of tocopherols and tocotrienols on their α-oxidation by tocopherol-α-hydroxylase. *J. Lipid Res.* **48**, 1090–1098.

Stauffer, T. P., Ahs, S., and Meyer, T. (1998) Receptor-induced transient reduction in plasma membrane PtdIns(4,5)P2 concentration monitored in living cells. *Curr. Biol.* **8**, 343–346.