THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 291, NO. 33, pp. 17028–17039, August 12, 2016

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

Received for publication, April 22, 2016, and in revised form, June 7, 2016 Published, JBC Papers in Press, June 15, 2016, DOI 10.1074/jbc.M116.734210

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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). Lifelong 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.

* This work was supported by National Institutes of Health Grant DK067494 (to D. M.). The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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[2] The abbreviations used are: TTP, α-tocopherol transfer protein; NBD-α-tocopherol, (*R*)-2,5,7,8-tetramethylchroman-2-[9-(7-nitrobenz[1,2,5]oxadiazol-4-ylamino)nonyl] chroman-6-ol; IPP-C*AAX*, membrane targeted 5′-phosphatase domain of synaptojanin 1; RFP, red fluorescent protein; PH, pleckstrin homology; PI(3,4)P_2, phosphatidylinositol 3,4-bisphosphate; PI(4,5)P_2, phosphatidylinositol 4,5-bisphosphate.

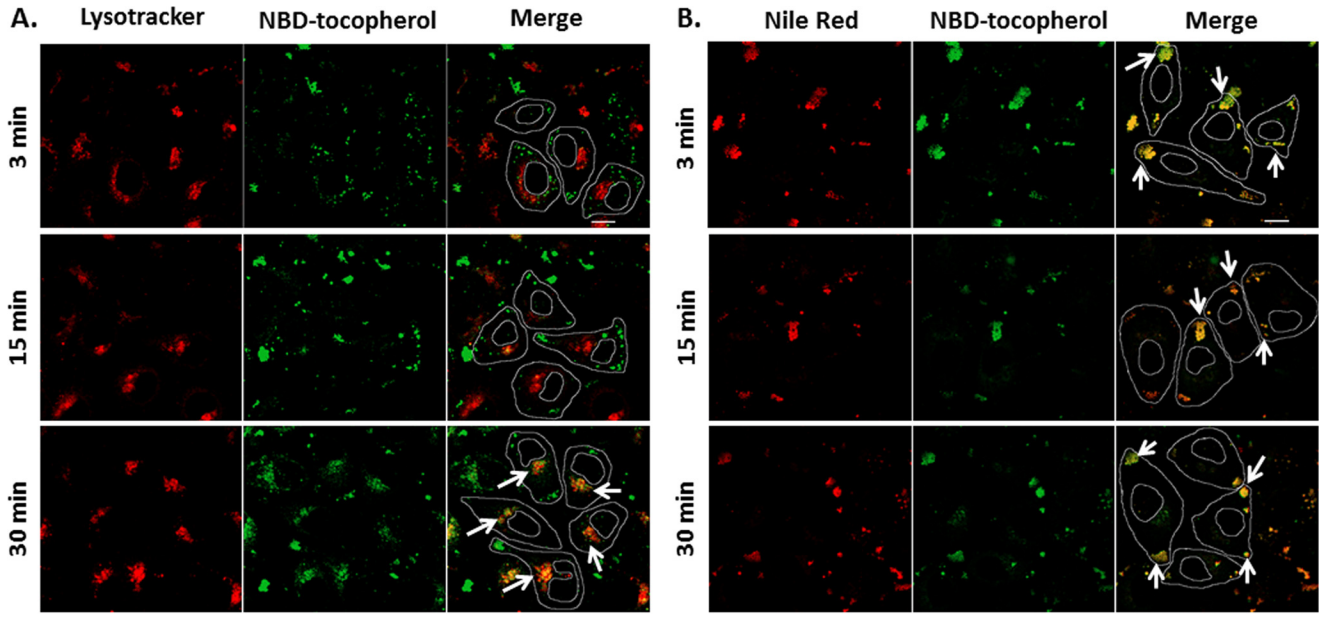

FIGURE 1. **Localization of NBD-α-tocopherol during uptake into hepatocytes.** McARH7777 cells were incubated with serum-complexed NBD-α-tocopherol at 4 °C for 1 h prior to washing and incubation at 37 °C for the indicated times. Live cells were co-stained with 100 nM LysoTracker Red (*A*) or 100 nM Nile Red (*B*) and visualized by confocal fluorescence microscopy. NBD-α-tocopherol is shown in *green*, and LysoTracker and Nile red are shown in *red*. *Arrows* indicate sites of co-localization. *Scale bar,* 10 μm.

Results

Uptake of α-Tocopherol in Cultured Hepatocytes—To examine the uptake of α-tocopherol into hepatocytes with high spatial resolution, we utilized NBD-α-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-α-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-α-tocopherol was monitored over time in live cells using confocal fluorescence microscopy. As shown in Fig. 1, NBD-α-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-α-tocopherol co-localized with LysoTracker, a fluorescent reporter that selectively marks lysosomes, as we previously observed in fixed cells (Fig. 1*A*) (33, 46). These observations support our previous findings that α-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-α-tocopherol also co-localizes to large (0.50–2 μm) round structures in the cell periphery, where it remained for the duration of the uptake process (Fig. 1*B*). 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 α-tocopherol in hepatocytes (see under "Discussion").

Effect of α-Tocopherol on the Intracellular Localization of TTP—To study the role of TTP in vitamin E transport, we investigated whether the presence of α-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 α-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 α-tocopherol, the protein re-distributes to a diffuse pattern throughout the cytosol (Fig. 2*A*). 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. The TTP(R221W) variant is associated with a severe early-onset disease (12). *In vitro*, the TTP(R221W) mutant displays weak binding affinity to α-tocopherol, impaired ability to catalyze inter-membrane transport of the vitamin (18), and a profound defect in facilitating secretion of α-tocopherol from hepatocytes (19). We observed that the intracellular localization pattern of TTP(R221W) mutant is constitutively punctate and is not affected by treatment with α-tocopherol (Fig. 2*B*). The TTP(R192H) mutant, however, is associated with a mild late-onset form of 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 α-tocopherol treatment are indistinguishable from the wild-type protein (Fig. 2*C*). These findings with TTP mutants show that the α-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 α-tocopherol in live cells that express RFP-tagged TTP but not in cells that express the RFP tag alone (Fig. 2*D*). Quantification of TTP's intracellular local-

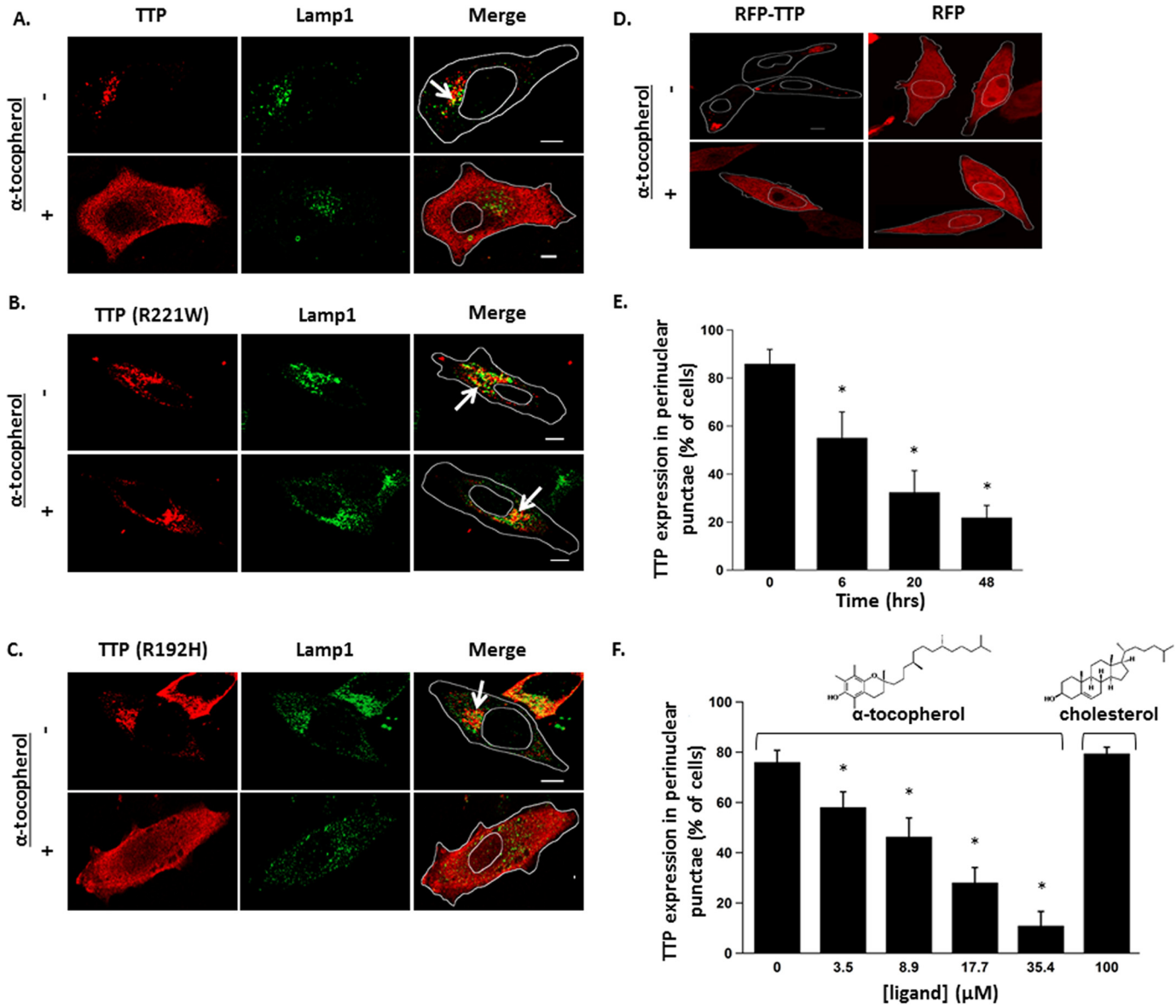

FIGURE 2. **Intracellular localization of TTP is regulated by vitamin E.** Because established hepatocyte cell lines do not endogenously express TTP, we utilized transient transfection to express the human *TTPA* gene in McARH7777 cells. *A–C,* indicated TTP variant was transiently transfected (in the 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. Localization 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.

ization response to α-tocopherol revealed that TTP was in distinct punctae in ~90% of the cells before vitamin E treatment, but 24 h after the addition of α-tocopherol TTP was punctate in only less than 30% of the cells (Fig. 2*E*). Moreover, the change in TTP's distribution was markedly dose-responsive, with a half-maximal α-tocopherol effect achieved at a physiological concentration range (~9 μM; Fig. 2*F*). Importantly, the change in intracellular localization of TTP was highly ligand-specific; addition of cholesterol, which shares some physicochemical properties with α-tocopherol but does not bind TTP (42), did not affect TTP's distribution pattern, even at a high concentration (Fig. 2*F*). Taken together, these observations show that the intracellular localization of TTP changes dramatically in response to α-tocopherol and strongly suggest that the protein's shift to a different intracellular location underlies TTP's action as a facilitator of α-tocopherol trafficking.

To better understand the molecular mechanisms of vitamin E transport, we employed confocal immunofluorescence microscopy to identify the vesicular compartment where TTP resides. In addition to the overlap with the lysosomal marker Lamp1 (Fig. 2*A*), TTP-positive vesicles showed strong spatial overlap with the transferrin receptor (CD71; Fig. 3*A*) and the

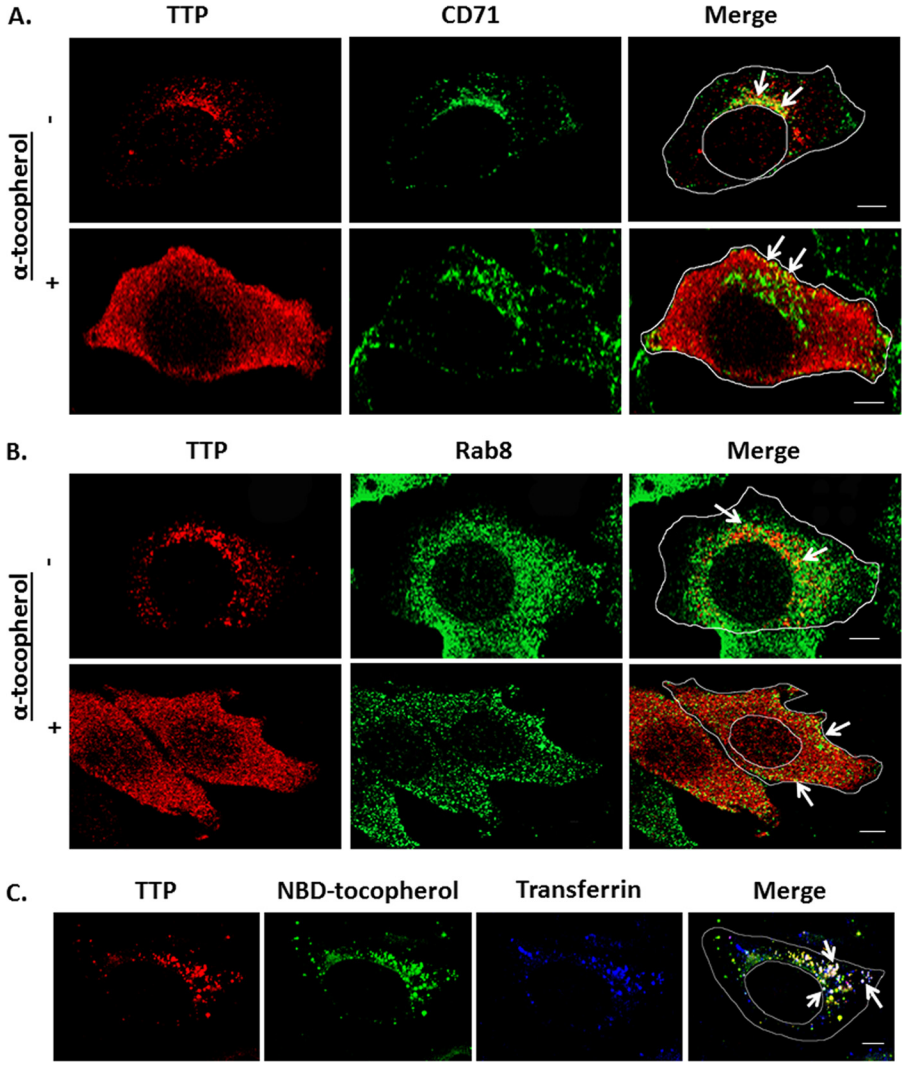

FIGURE 3. **TTP localizes to the recycling endosomes.** McARH7777 cells stably expressing pTRE2-TTP and induced with doxycycline for 48 h were fixed and immunostained with antibodies directed against TTP (*red*), the transferrin receptor (*A*; *green*), or Rab8 (*B*; *green*). Where indicated, serum-complexed 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.

small GTPase Rab8 (Fig. 3*B*), 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 co-localization of NBD-α-tocopherol, TTP, and transferrin (Fig. 3*C*). The addition of α-tocopherol caused a marked re-distribution of TTP but did not affect the distribution of Lamp1, Rab8, or CD71 (Figs. 2*A* and 3, *A* 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 un-bound tocopherol, the cells were returned to 37 °C and imaged with confocal fluorescence microscopy over time. As expected, 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-

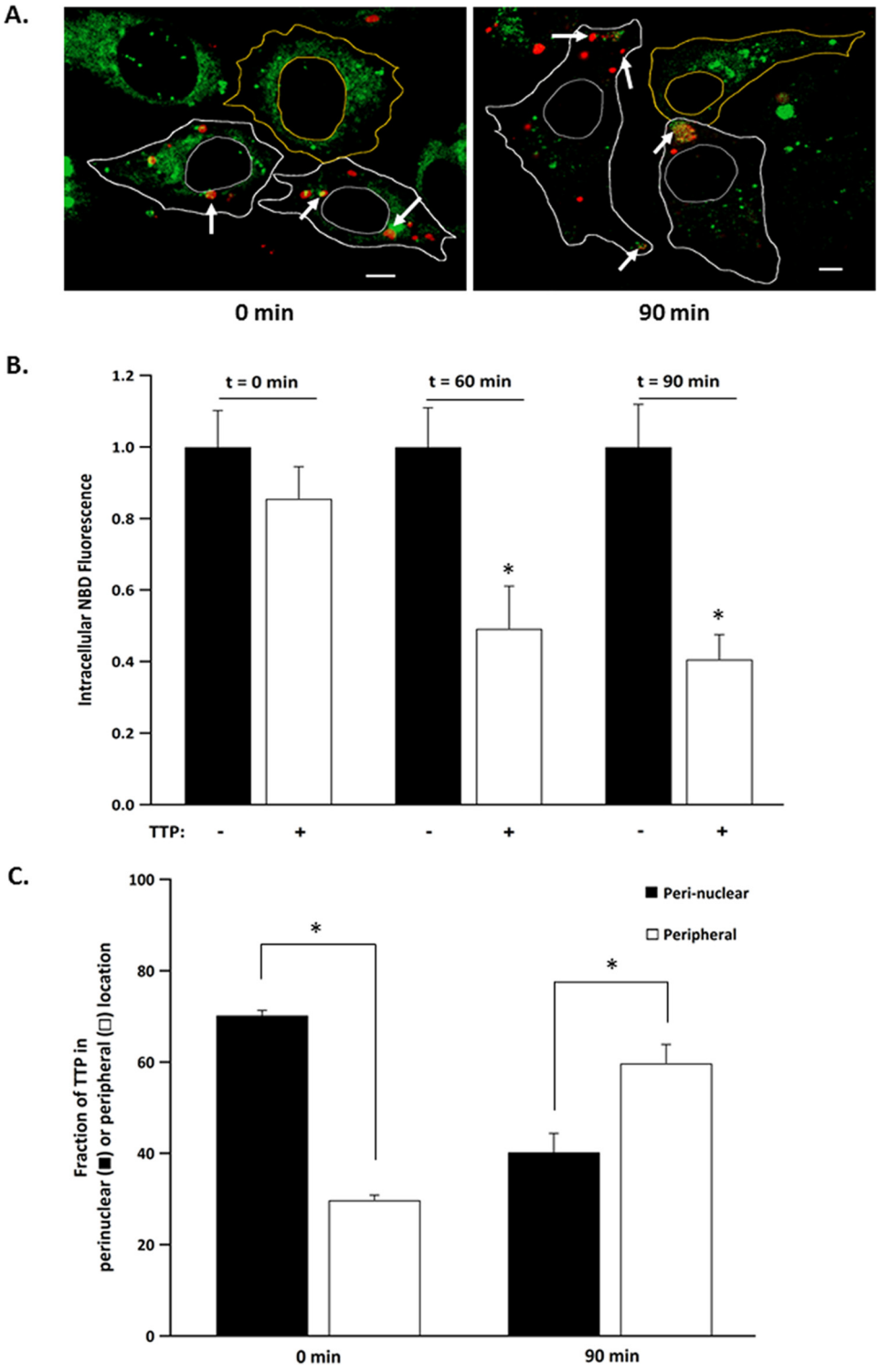

FIGURE 4. **Time-dependent changes in the intracellular localization of NBD-α-tocopherol and TTP.** McARH7777 cells were transiently transfected with pTagRFP-TTP. Forty eight hours after transfection, cells were pulse-loaded with 10 μM serum-complexed NBD-α-tocopherol, and live confocal images of NBD-α-tocopherol (*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.

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. 4*C*). After 90 min, however, >60% of the TTP-containing vesicles relocated to the cell periphery, adjacent to the plasma membrane (*white arrows* in Fig. 4*A*; 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 $PI(4,5)P_2$ and $PI(3,4)P_2$, 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 synaptojanin 1 (IPP-C*AAX*, where *AA* is aliphatic amino acid), previously shown to efficiently deplete the plasma membrane of $PI(4,5)P_2$ (55–58). The suitability of this reagent is demonstrated in Fig. 5*A*; in control cells, the pleckstrin homology (PH) domain of PLCδ binds $PI(4,5)P_2$ at the plasma membrane, and thus it selectively highlights this region in the cell (Fig. 5*A*). However, in cells that express IPP-C*AAX*, the PH domain redistributes throughout the cytosol, reflecting depletion of the plasma membrane pools of $PI(4,5)P_2$ (Fig. 5*A*a) (55–58). Importantly, expression of IPP-C*AAX* abrogated TTP activity; in the presence of the inositol phosphatase, TTP-facilitated secretion of NBD-α-tocopherol was completely abolished (Fig. 5*B*). Thus, the presence of 5-phosphoinositides at the plasma membrane is essential for TTP-mediated transport of α-tocopherol. Moreover, expression of IPP-C*AAX* 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. 5*C*). 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 $PI(4,5)P_2$-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, $PI(4,5)P_2$ was shown to directly interact with a positively charged "patch" on the protein's surface, which includes Lys^{217}. 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. 6*B*). These observations confirm that the ability of TTP to interact with phosphoinositides is critical for the protein's function and suggest that Lys^{217} 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 Phe^{169} is required for association of the protein with all membranes, whereas the positive "patch" around Lys^{217} 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 *TTPA* 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 *TTPA* gene is

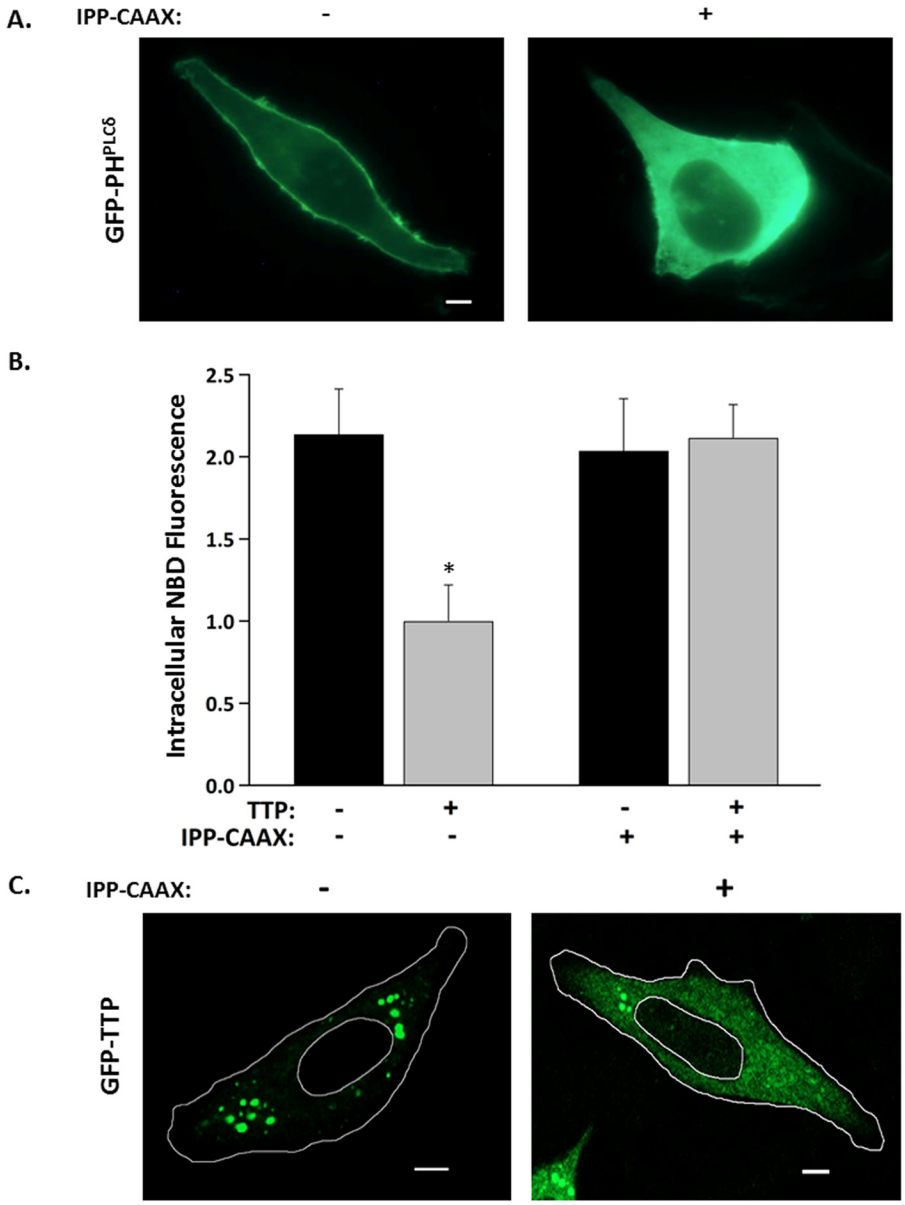

FIGURE 5. **Depletion of plasma membrane PI(4,5)P_2 inhibits TTP function.** McARH7777 cells stably expressing pTagRFP-TTP were grown without vitamin E as in Fig. 2. Where indicated, cells were also transiently transfected with membrane-targeted inositol 5′-phosphatase domain of synaptojanin 1 encoded in the pcDNA3-HA vector (IPP-C*AAX* (55–58)). Cells were then pulse-loaded with 10 μM serum-complexed NBD-α-tocopherol as described under "Experimental Procedures" and imaged by fluorescence microscopy. *A,* depletion of plasma membrane PI(4,5)P_2 by IPP-C*AAX*. Cells were co-transfected with a construct encoding pEGFP-tagged PH domain of PLCδ. *Left panel,* localization of the PH domain is restricted to the cells' plasma membrane (79). *Right panel,* upon expression IPP-C*AAX*, the PH domain is no longer at the plasma membrane, indicating depletion of membrane PI(4,5)P_2 (55–58). *Scale bar,* 5 μm. *B,* TTP-mediated secretion of α-tocopherol is dependent upon plasma membrane PI(4,5)P_2. Secretion of NBD-α-tocopherol in TTP-expressing cells was quantified as in Fig. 4. Where indicated, cells were also transfected with pCDNA3-encoded IPP-C*AAX*. *Asterisk* denotes statistical significance with $p < 0.01$, calculated by Student's *t* test. *C,* depletion of plasma membrane PI(4,5)P_2 causes mislocalization of TTP. McARH7777 cells were transiently co-transfected with plasmids encoding GFP-tagged TTP and IPP-C*AAX* (or empty vector) prior to live cell confocal fluorescence imaging as in Fig. 2*D*. *Scale bar*, 5 μm. Figure is representative of three independent experiments.

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

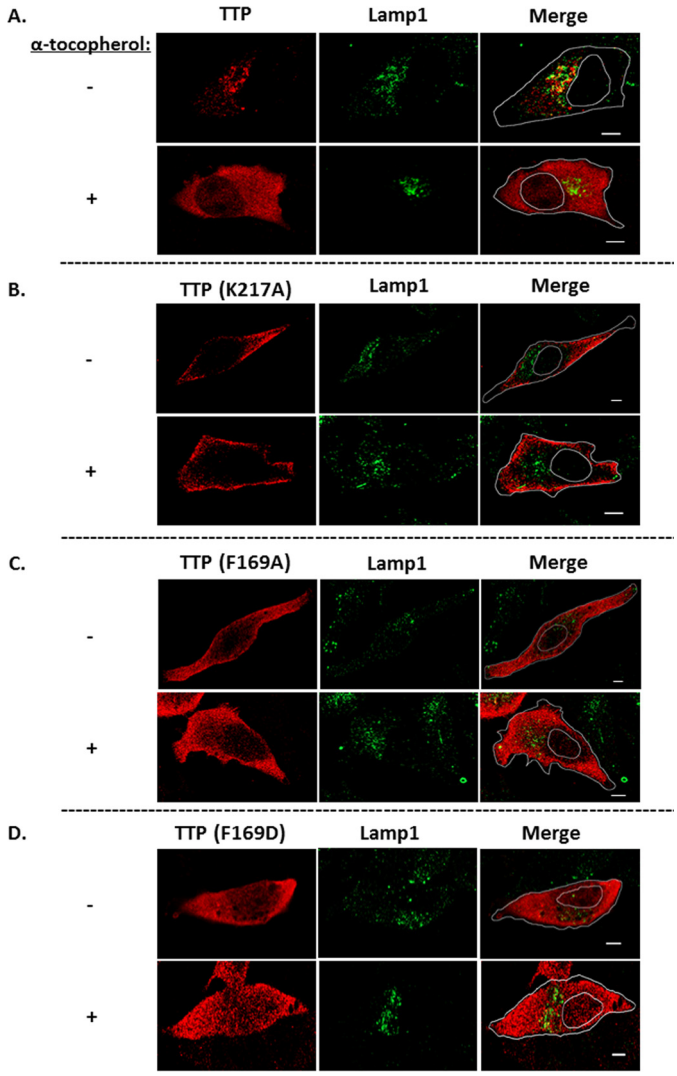

FIGURE 6. **Roles of protein-lipid interactions on the intracellular localization of TTP: substitutions of Phe^{169} and Lys^{217} affect TTP localization.** McARH7777 cells were transiently transfected with the indicated variant of TTP and treated with serum-complexed 35 μM α-tocopherol as in Fig. 2. Cells were fixed and immunostained using anti-TTP (*red*) and anti-Lamp1 (*green*) antibodies, prior to visualization with confocal fluorescence microscopy. *Scale bar,* 5 μm.

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 (*i.e.* not through the endoplasmic reticulum) 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^{165}, Phe^{169}, Ile^{202}, Val^{206}, and Met^{209}) 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 *et al.* (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^{59}, Arg^{68}, Lys^{190}, Arg^{192}, Lys^{217}, and Arg^{221}) mediates the binding of TTP to PI(3,4)P_2 or PI(4,5)P_2 in the membrane. Importantly, mutations of residues that line this surface region, such as the TTP(K217A) mutant, are defective in phosphoinositide binding and unable to facilitate tocopherol trafficking (41). We show that the TTP(K217A) displays a unique intracellular distribution; the protein is concentrated in a thin band along the plasma membrane, and this pattern is not affected by α-tocopherol (Fig. 6). These findings indicate that other residues in TTP's positive surface patch (*i.e.* Arg^{59}, Arg^{192}, and Arg^{221}) are sufficient for mediating TTP's association with the plasma membrane, but Lys^{217} is necessary for subsequent steps in which α-tocopherol is "unloaded" from the protein's binding pocket. According to the "bulldozer model" for the homologous Sec proteins, binding of phosphoinositides

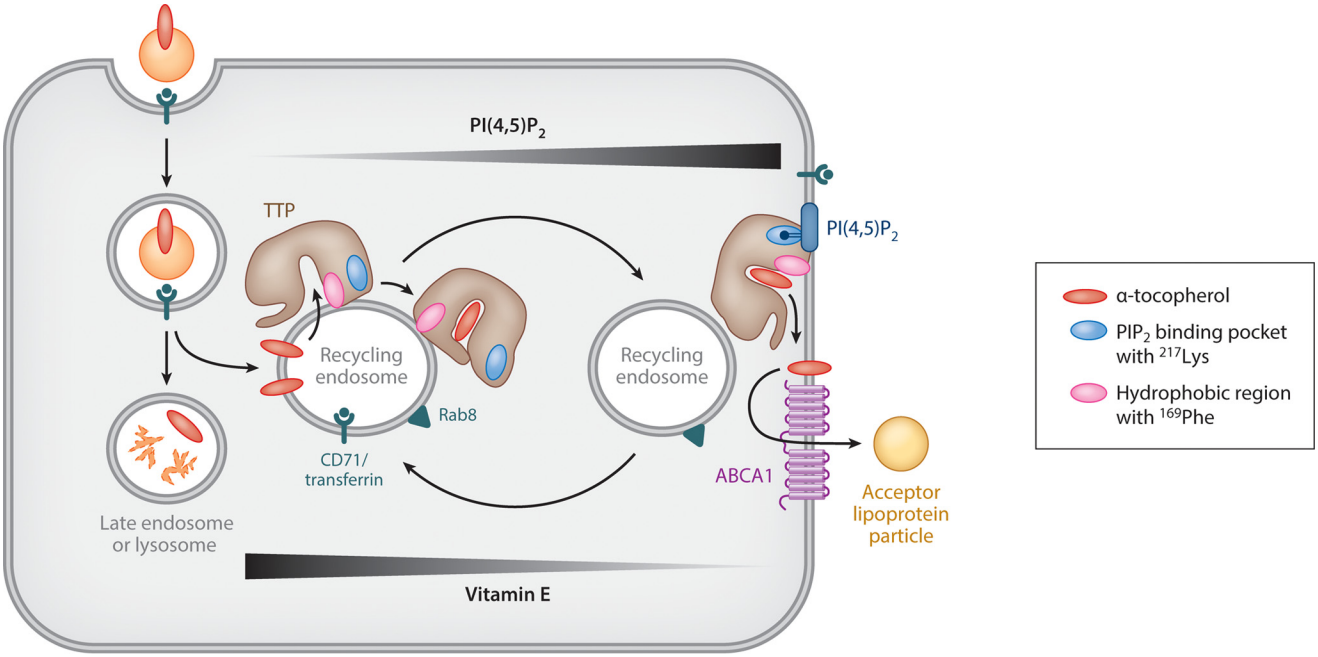

FIGURE 7. **Proposed model of TTP-facilitated vitamin E trafficking in hepatocytes.** TTP initially associates with endocytic vesicles through the hydrophobic surface that contains Phe^{169}. After uptake through receptor-mediated endocytosis, recycling endosomes that contain the transferrin receptor, Rab8, TTP and vitamin E then travel to the plasma membrane, where the vesicles "dock" at $PI(4,5)P_2$-rich domains. Binding of $PI(4,5)P_2$ to TTP's positively charged "patch" that includes Lys^{217} causes a conformational change in TTP, leading to release of the protein-bound vitamin E to the plasma membrane. Subsequently, TTP cycles back to vitamin E-containing organelles to repeat this cycle of regulated vitamin E secretion. Directionality of the transport process is provided by the opposite concentration gradients of $PI(4,5)P_2$ (high at the plasma membrane) and α-tocopherol (high at the endocytic compartment). After release to the plasma membrane, vitamin E exits through an ABC-type transporter and associates with lipoprotein particles that deliver it to extra-hepatic tissues. Adapted from Ref. 35 with the author's permission.

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 $PI(4,5)P_2$ for tocopherol upon incubation of TTP with lipid vesicles *in vitro*. The constitutive localization of TTP(K217A) at the plasma membrane indicates that Lys^{217} communicates the conformational change induced by $PI(4,5)P_2$ 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, $PI(4,5)P_2$ 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 $PI(4,5)P_2$ 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% CO_2. 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 $\epsilon_{466\ nm} = 22{,}000\ M^{-1}\ cm^{-1}$ (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, 5*C*, 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-Lamp1 (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 $\times$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 5872 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 and pTagRFP-N plasmids, respectively. Stable cell lines were generated by co-transfection of these constructs together with the pcDNA3.1/hygro(+) plasmid into McARH7777 cells, followed by selection in hygromycin (200 μg/ml) for 3 weeks. The GFP-tagged PH domain of PLCδ was a gift from Tobias Meyer (Addgene plasmid 21179) (79). The membrane-targeted 5′-phosphatase domain of synaptojanin 1 (IPP-C*AAX*) 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 fluorescent analog used in experiments. J. A. designed the analog, supervised the syntheses, and edited the manuscript. R. P. analyzed lipids by GC-MS and edited the manuscript. J. Q. conducted the transferrin experiment (Fig. 3*C*). 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-CAAX 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.

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