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Hepatic α-Tocopherol Transfer Protein: Ligand-Induced Protection from Proteasomal Degradation[†]

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ABSTRACT: There are eight naturally occurring forms of the dietary antioxidant vitamin E. Of these, only α-tocopherol is retained at high levels in vertebrate plasma and tissues. This selectivity is achieved in part by the action of the hepatic α-tocopherol transfer protein (TTP), which facilitates the selective incorporation of dietary α-tocopherol into circulating lipoproteins. We examined the effects of vitamin E on TTP expression in cultured hepatocytes. Treatment with vitamin E precipitated a time- and dose-dependent increase in the steady-state levels of TTP. This stabilization was caused by α-tocopherol-induced attenuation of the ubiquitination of TTP and its subsequent degradation by the proteasome. In vitro, vitamin E protected TTP from proteolytic degradation by trypsin, suggesting ligand-induced changes in protein conformation. Cell fractionation studies showed that TTP is distributed between the cytosolic and membranous organelle fraction, and that tocopherol induced the translocation of some TTP from the cytosol to the organelle fraction. Furthermore, vitamin E markedly attenuated the degradation of organelle-bound TTP. These findings suggest that vitamin E imparts a distinct conformation on TTP that is associated with localization to a specific cellular compartment, where the protein is less susceptible to proteasomal degradation.

α-Tocopherol is a lipophilic, plant-derived neutral lipid that functions as a chain breaking antioxidant in biological membranes (*1*). The effective radical trapping ability of vitamin E is thought to underlie the strict biological requirement for this nutrient and its protective actions against a number of human pathologies. While plants synthesize eight forms of vitamin E (α-, *β*-, *γ*-, and *δ*-tocopherols and -tocotrienols), animals retain primarily α-tocopherol as their lipid-phase antioxidant (*2*).

The tissue distribution of α-tocopherol shares many steps with the transport of other neutral lipids. After ingestion, the vitamin is taken up by enterocytes and packaged in chylomicra that deliver the vitamin to the liver. Delivery from the liver to peripheral tissues is mediated by circulating low- and high-density lipoprotein particles (*3*, *4*). The only known transport mechanism that is specific to α-tocopherol occurs in the liver, where the α-tocopherol transfer protein (TTP)[1] selectively binds this vitamin and facilitates its secretion from hepatocytes (*4*, *5*). Heritable mutations that perturb TTP function in humans are the only known cause for ataxia with vitamin E deficiency (AVED, OMIM 600415), a familial disorder characterized by vitamin E deficiency and progressive loss of movement coordination (*6*, *7*). Mice in which expression of the *ttpA* gene is disrupted are vitamin E deficient and display neurological symptoms similar to those observed in AVED patients (*8*, *9*). Progression of the AVED pathology can sometimes be prevented by high-dose vitamin E supplementation in both $Ttp^{-/-}$ mice and human patients, further underscoring the relationship among TTP, α-tocopherol levels, and CNS health. To date, TTP is the only known protein that specifically regulates body-wide levels of α-tocopherol.

The molecular mechanisms by which TTP influences trafficking of vitamin E in the liver have received significant attention (*10*−*14*). The protein is expressed in parenchymal cells of the liver and is believed to bind newly arrived α-tocopherol in the endocytic compartment. Through a poorly understood process, TTP then facilitates the transfer of α-tocopherol to transport vesicles that deliver the vitamin to its site of secretion in the hepatocyte plasma membrane (*12*, *13*). Despite the central roles that TTP plays in maintaining vitamin E adequacy, very little is known regarding the mechanisms that regulate the expression and biochemical activity of the protein. We report here our findings with regard to the factors that govern the stability of TTP in cultured cells and its regulation by vitamin E.

MATERIALS AND METHODS

Reagents. (*RRR*)-α-Tocopherol was purchased from Cole-Palmer. Cycloheximide and chloroquine were purchased from Sigma Aldrich. Doxycycline and MG132 were obtained from Calbiochem. *Clasto*-lactacystin *β*-lactone, ubiquitine aldehyde, and *N*-ethylmalemide (NEM) were purchased from Boston Biochemicals. Except where noted, TTP was visualized on Western blots using monoclonal antibodies directed against the protein's amino-terminal hemagglutinin tag (HA.11, Covance Inc.; see ref *12*). Anti-ubiquitin and anti-actin antibodies were purchased from Biomol Inc. and Cell Signaling Inc., respectively. The 12D7 anti-TTP antibody was a generous gift of H. Arai (University of Tokyo, Tokyo, Japan). Immortalized human

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[1]Abbreviations: EDTA, ethylenediaminetetraacetate; FBS, fetal bovine serum; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PBS, phosphate-buffered saline; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide electrophoresis; TTP, tocopherol transfer protein.

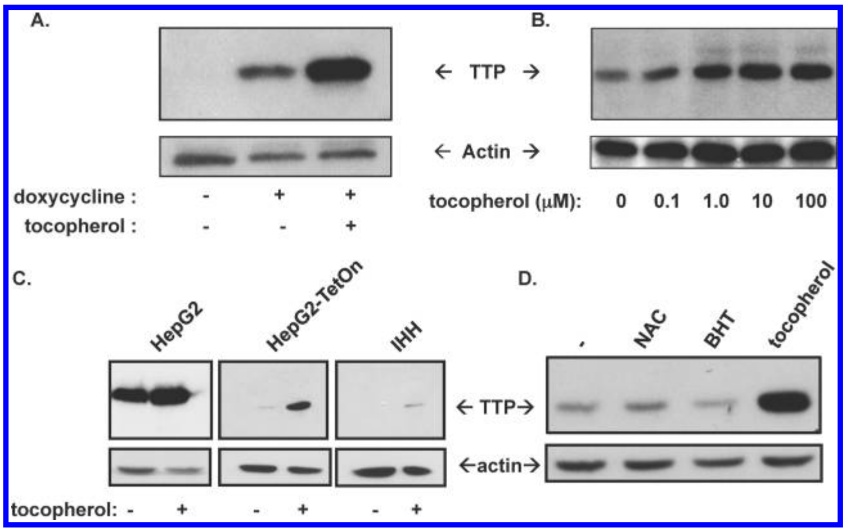

FIGURE 1: Treatment with vitamin E increases TTP expression levels. (A) Expression of TTP was induced in HepG2-TetOn-TTP cells with doxycycline. Where indicated, 100 μM α-tocopherol was included in the culture medium for 48 h. TTP was visualized in whole cell lysates using anti-HA immunoblotting. (B) Dose response of the stabilizing effect of α-tocopherol, with the conditions described for panel A. (C) Cultured HepG2 cells were transiently transfected with a pCDNA3.1 construct encoding the TTP open reading frame and treated as described above. Forty-eight hours post-transfection, expression of TTP was examined in whole cell lysates by immunoblotting using anti-TTP 12D7 antibodies. (D) Immortalized human hepatocytes were treated with α-tocopherol as described above, and the level of expression of endogenous TTP was examined in whole cell lysates as in panel A.

hepatocytes (*15*) were a generous gift of R. Ray (Saint Louis University, Saint Louis, MO).

Cell Culture. HepG2-TetOn-TTP cells stably expressing HA-tagged TTP under the control of the inducible TetOn promoter were described previously (*12*). Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (Atlanta Biologicals) in the presence of G418 (Gibco) and hygromycin (Invitrogen) at 100 μg/mL each. TTP expression was induced via addition of 1 μg/mL doxycycline to the growth medium 48 h prior to cell lysis. α-Tocopherol was added to the culture medium (final concentration of 100 μM, unless specified otherwise) from ethanolic stocks 48 h prior to cell lysis. Equal volumes of ethanol were added to control cultures. Proteolysis inhibitors were added from DMSO stocks to a concentration of 100 μM (chloroquine) or 10 μM (MG132 and *Clasto*-lactacystin β-lactone) for the indicated durations. Immortalized human hepatocytes (IHH) were cultured in DMEM supplemented with 10% defined fetal bovine serum containing <0.1 μM α-tocopherol (HyClone). All other methods were described previously (*12*, *16*). Unless otherwise indicated, cell lysates were prepared by incubation for 10 min on ice in 20 mM HEPES (pH 7.4), 1 mM EDTA, 150 mM NaCl, 1% Nonident-P40 (NP-40), 20 mM NaF, 20 mM β-glycerol phosphate, 1 mM sodium vanadate, 10 μg/mL leupeptin, and 10 μg/mL aprotinin, followed by microcentrifugation at 21000*g*.

Ubiquitination Assays. Cells were treated with the proteasome inhibitor MG132 for 5−6 h prior to termination of the experiment. Cells were lysed in 20 mM HEPES (pH 7.4), 150 mM NaCl, 1% NP-40, 20 mM NaF, 20 mM β-glycerol phosphate, 1 mM EDTA, 1.0 mM phenylmethanesulfonyl fluoride, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 1 mM sodium vanadate, 1 μM ubiquitin aldehyde, and 1 mM *N*-ethylmaleamide. TTP was immunoprecipitated using Sepharose beads conjugated to anti-HA antibodies (Covance Inc.). Precipitates were resolved via SDS−PAGE. After transfer, the membranes were autoclaved for 30 min., and ubiquitination of TTP was detected by Western blotting using anti-ubiquitin antibodies.

In Vitro Proteolysis. Recombinant TTP was purified from overexpressing *Escherichia coli* as previously described (*16*). TTP [12.5 μM protein in 20 mM Tris (pH 8.0) and 150 mM NaCl] was incubated with 100 μM (*RRR*)-α-tocopherol (or ethanol control) in silicon-coated tubes for 3 h on ice. Trypsin (7 μM) was added to the reaction mixture and incubated for the indicated duration at 37 °C. The reaction was terminated when the mixture was boiled in Laemmeli buffer, after which samples were resolved via SDS−PAGE and visualized by Coomassie staining. Signal intensity was measured by densitometry using Scion image (developed at the National Institutes of Health and available at http://rsb.info.nih.gov/nih-image/).

Cell Fractionation. Cells were trypsinized and washed three times by centrifugation at 6800*g* in cold PBS. Pellets were resuspended in 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.2 mM phenylmethanesulfonyl fluoride, 10 μg/mL leupeptin, and 10 μg/mL aprotinin and lysed by five freeze−thaw cycles. Centrifugation at 24000*g* for 1 h at 4 °C was used to separate the cytosol (supernatant) and the organelle (pellet) fractions.

RESULTS

While characterizing the expression of ectopically expressed TTP in cultured HepG2 cells, we observed that the steady-state expression levels of the protein increased by 2−3-fold when vitamin E was included in the culture medium (Figure 1A). The increase in the level of TTP expression was concentration-dependent over a physiological range of vitamin concentrations [0.1−100 μM (Figure 1B)]. A vitamin E-induced increase in the level of expression of TTP was also evident in HepG2 cells transiently expressing TTP, as well as in immortalized human hepatocytes (IHH) that endogenously express the protein (Figure 1C). Importantly, two synthetic antioxidants, *N*-acetylcysteine (NAC) and butylated hydroxytoluene (BHT), had no effect on the levels of expression of the TTP protein under conditions where α-tocopherol caused a marked increase (Figure 1D). Taken together, these observations indicate that vitamin E specifically upregulates the steady-state expression levels of TTP.

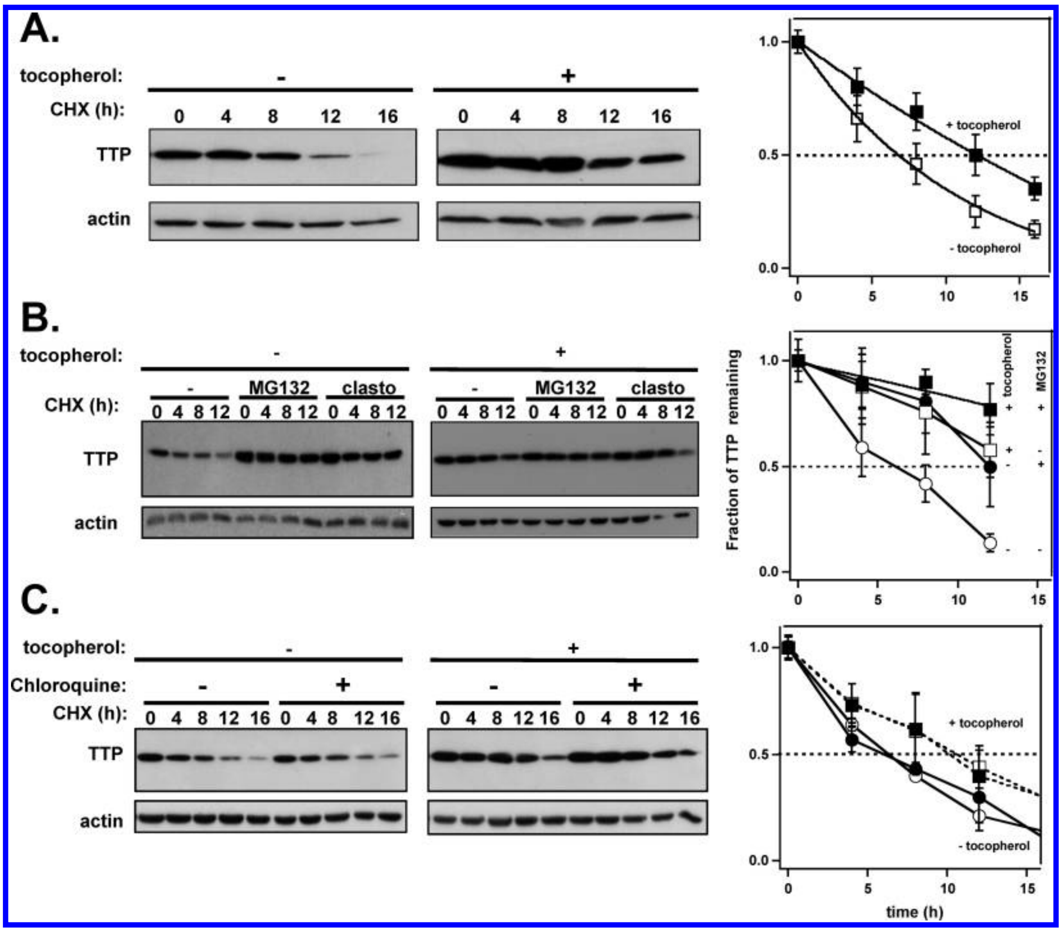

FIGURE 2: α-Tocopherol protects TTP from proteasomal degradation. Expression levels of TTP were determined by quantitative densitometric image analysis. (A) HepG2-TetOn-TTP cells were cultured and treated as described in the legend of Figure 1A. Forty-eight hours after induction, cycloheximide (CHX, 100 μg/mL) was added for the indicated duration prior to lysis and immunoblotting as described in the legend of Figure 1. (B and C) The proteasomal inhibitors MG132, *Clasto*-lactacystin β-lactone, and chloroquine were added to concentrations of 10 μM, 10 μM, and 100 μg/mL, respectively, for 4 h prior to cell lysis. Shown are averages and standard deviations of three independent experiments.

Because α-tocopherol increases the levels of expression of ectopically expressed TTP, where an extrinsic promoter drives gene expression, we considered the possibility that the ligand exerts its effect on a post-translational level. Thus, we measured the effect of the vitamin on the turnover rate of TTP. Cells were incubated with the protein synthesis inhibitor cycloheximide, and the levels of TTP were measured at various times. In the absence of α-tocopherol, levels of TTP declined with a half-life ($t_{1/2}$) of 6 h (Figure 2A). Treatment with α-tocopherol slowed the degradation of TTP by 2-fold [$t_{1/2}$ = 12 h (Figure 2A)]. These results indicate that α-tocopherol modulates TTP expression at the level of protein stability, rather than at the level of transcription or translation. In support of this notion, quantitative real-time RT-PCR revealed no α-tocopherol-induced changes in the levels of TTP mRNA in either HepG2-TeTOn-TTP or IHH cells (data not shown).

The specific pathway(s) through which proteins are degraded can be identified with the aid of specific pharmacologic inhibitors. Treatment with MG132 or *Clasto*-lactacystin β-lactone, each an inhibitor of the proteasomal degradation pathway (*17*, *18*), markedly attenuated the degradation of TTP (Figure 2B). Inhibition of lysosomal proteolysis with chloroquine, on the other hand, did not affect the rate of TTP degradation (Figure 2C). Taken together, these findings indicate that TTP is subject to degradation by the proteasome and that binding of α-tocopherol protects the protein from this fate, thereby elevating its steady-state levels.

Proteasomal degradation of proteins involves changes in protein ubiquitination. We therefore examined whether TTP is ubiquitinated and whether this modification is altered by inclusion of α-tocopherol in the cell culture medium. TTP was immunoprecipitated from α-tocopherol-treated (or control) HepG2-TetOn-TTP cells, and the extent of its modification was assessed by anti-ubiquitin immunoblotting. As shown in Figure 3A, a ubiquitinated protein band was evident at ~40 kDa, in addition to several additional bands at higher molecular masses. These bands represent ubiquitinated forms of TTP because they were visible only when the expression of TTP was induced with doxycycline (compare the left- and right-most lanes of Figure 3A). Importantly, treatment of the cells with α-tocopherol all but eliminated TTP ubiquitination (compare the middle- and right-most lanes of Figure 3A). These findings reveal that α-tocopherol attenuates the post-translational ubiquitination of TTP. The effect of α-tocopherol is specific to TTP, because treatment with vitamin E did not affect the global protein ubiquitination pattern observed in anti-ubiquitin blots of total cell extracts (Figure 3B). We conclude that the degradation of TTP is regulated by post-translational ubiquitination and that α-tocopherol increases TTP levels by attenuating the rates of ubiquitination and degradation of the protein.

A likely molecular mechanism that underlies the effect of α-tocopherol on the ubiquitination and degradation of TTP involves a ligand-induced conformational change that affects the

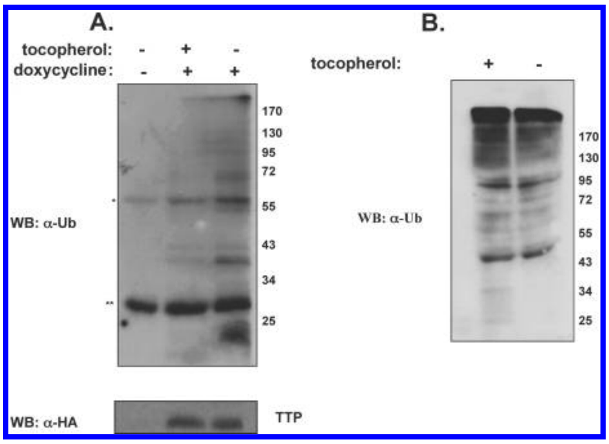

FIGURE 3: α-Tocopherol attenuates the ubiquitination of TTP. (A) Expression of TTP was induced for 48 h in HepG2-TetOn cells in the absence or presence of 100 μM α-tocopherol. MG132 was added to a concentration of 10 μM 6 h prior to cell lysis. TTP was immunoprecipitated using Sepharose-conjugated anti-HA antibodies, and the precipitate was resolved by SDS−PAGE and immunoblotted with anti-HA and anti-ubiquitin antibodies. (B) Lysates were prepared from tocopherol-treated (or control) cells, and immunoblotted using anti-ubiquitin antibodies. Representative of three independent experiments. Asterisks denote heavy and light antibody chains.

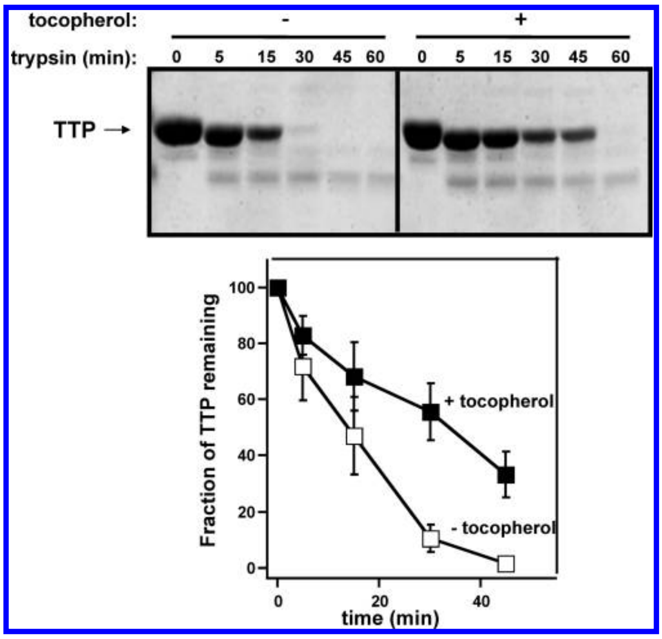

FIGURE 4: α-Tocopherol protects TTP from tryptic digestion in vitro. Purified, recombinant TTP (12 μM) was preincubated with 100 μM α-tocopherol (or ethanol control) for 3 h prior to the addition of trypsin (7 μM). The reaction mixture was incubated at 37 °C for the indicated durations prior to SDS−PAGE and Coomassie staining. The bottom panel shows the quantification of the time-dependent tryptic proteolysis of TTP. Shown are averages and standard deviations of four independent experiments.

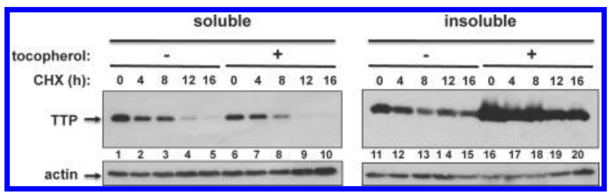

FIGURE 5: Intracellular distribution of TTP. The cell culture and treatments were as described in the legend of Figure 2. Cells were lysed by a freeze-thawing and centrifuged to separate the cytosolic (supernatant) and organelle (pellet) fractions. Representative of three independent experiments.

accessibility of surface residues to components of the ubiquitination machinery. Indeed, comparison of the three-dimensional structures of holo- and apo-TTP reveals that the protein undergoes a significant reorganization upon ligand binding. Specifically, a surface-exposed amphiphatic helix (residues 198−221) functions as a "lid" that closes the binding pocket when it is occupied by α-tocopherol (*19*, *20*). To examine how the ligand-induced conformational changes affect the surface topology of TTP, we monitored the susceptibility of purified TTP to proteolytic degradation by trypsin in vitro. As shown in Figure 4, incubation of purified, recombinant TTP with trypsin led to efficient degradation of the protein, with a half-life of approximately 15 min. However, when the protein was precomplexed with α-tocopherol, proteolysis was significantly inhibited. These data indicate that ligand binding by TTP induces a significant conformational change in the protein that may sterically hinder the accessibility of some surface residues.

To address the physiological consequences of the ligand-induced conformational changes in TTP, we examined whether treatment with α-tocopherol affects the protein's intracellular localization pattern. Originally, TTP was considered to be a soluble protein, because it was purified from high-speed supernatant of liver homogenate (*21*). However, recent immunofluorescence microscopy studies have shown that TTP is associated with vesicles of the endocytic pathway, specifically late endosomes and lysosomes (*10*, *12*, *13*). These observations raise the possibility that TTP is loosely associated with endocytic vesicles, such that disruption of the cell releases the protein to the cytosol. We therefore used a gentle freeze−thaw lysis protocol and separated the soluble and organelle-associated fractions by centrifugation. We used this method to determine the localization of TTP in the presence and absence vitamin E treatment and the rates by which the protein is degraded in the different cellular fractions. As shown in Figure 5, the majority of TTP (> 60%) is in the organelle-associated fraction of the cell. Protein turnover measurements using cycloheximide revealed that cytosolic TTP was degraded with a half-life of ~8 h, similar to the rate we observed in total cell lysates (Figures 1 and 2). Treatment with vitamin E caused a slight (~10%) but consistent decrease in the levels of cytosolic TTP (in Figure 5, right panel, compare lanes 1 and 6), suggesting that some TTP translocates away from this compartment. Conversely, treatment with α-tocopherol resulted in a significant increase in the levels of organelle-bound TTP (in Figure 5, right panel, compare lanes 11 and 16). Vitamin E did not affect the turnover rate of TTP in the cytosol. The degradation rate of membrane-associated TTP, on the other hand, was significantly retarded in the presence of the vitamin ($t_{1/2} \gg 16$ h), such that the levels of TTP in this pool were dramatically increased (Figure 5, right panel). Taken together, these findings show that (1) TTP is distributed between the cytosol and membranous organelles, (2) treatment with vitamin E triggers the translocation of a some cytosolic TTP to the membrane-associated pool, and (3) treatment with vitamin E slows the degradation of organelle-associated TTP, leading to increased levels of the protein in this compartment.

DISCUSSION

Multiple lines of evidence indicate that the hepatic α-tocopherol transfer protein is the main regulator of α-tocopherol status in rodents and humans. First, mutations in the *TTPA* gene are the only known cause of familial ataxia with vitamin E deficiency, observed in humans with an otherwise normal lipid profile (AVED) (*6*, *22*). Second, targeted disruption of the *TtpA* gene in mice results in vitamin E deficiency, accompanied by neurological symptoms analogous to those displayed by AVED patients (*8*). Importantly, a linear correlation exists between the steady-state plasma levels of α-tocopherol and *TtpA* gene dosage in the $TTP^{-/-}$, $TTP^{+/-}$, and $TTP^{+/+}$ genotypes (*9*). Finally, close biochemical examination revealed that of all related lipid-binding proteins from the CRAL_TRIO family, only TTP binds α-tocopherol with high affinity and selectivity (*23*). It is therefore generally accepted that TTP function in the liver is the key, if not the only, specific biochemical determinant of α-tocopherol levels in humans and rodents (*3*, *4*).

TTP is believed to regulate body-wide levels of α-tocopherol by directing the trafficking of the dietary vitamin within the hepatocyte. Newly acquired vitamin E enters hepatic cells by endocytosis of lipoprotein complexes, and α-tocopherol is routed by TTP to export vesicles that deliver it to the plasma membrane (*11*, *12*). α-Tocopherol is then secreted from the plasma membrane via an ATP-binding cassette transporter (*12*, *24*) prior to association with lipoproteins and delivery to peripheral tissues (*25*−*27*). Intracellular localization studies provided important clues about the molecular mechanisms of action of TTP. Although hepatic TTP was originally purified from liver cytosol (*21*), a number of observations suggest that the protein is associated with cellular organelles. Arai and colleagues observed that upon treatment with the lysosomotropic agent chloroquine, TTP's diffuse distribution pattern changed to a punctate appearance that overlaps that of LBPA, a lipid resident of late endosomes (*10*). Qian et al. reported that TTP was constitutively associated with EEA1- and LAMP1-positive vesicles, i.e., endosomes and lysosomes (*12*, *13*). These observations suggest that TTP may associate with its ligand at the surface of endocytic vesicles, the vitamin's "port of entry" into hepatocytes. However, as fluorescence microscopy did not demonstrate a vitamin E-induced change in TTP's localization pattern, the nature of subsequent steps remained enigmatic.

Here, we employed a gentle detergent-free lysis protocol followed by fractionation and immunoblotting to determine the compartment in which TTP resides. We discovered that >60% of TTP is associated with intracellular organelles. Importantly, treatment of the cells with vitamin E caused a small but significant change in the localization of TTP; ∼10% of the protein shifted from the cytosolic to the organelle-associated pool. We postulate that TTP binds α-tocopherol on the surface of endocytic vesicles and dissociates to the cytosol prior to delivering the vitamin to preformed transport vesicles that carry it to the plasma membrane. The failure to detect ligand-induced changes in the localization of TTP by immunofluorescence microscopy may have arisen from sensitivity constraints that prevented the detection of small changes.

The critical role that TTP plays in maintaining vitamin E status raises the possibility that antioxidant homeostasis in the face of oxidative stress may be regulated by changes in the protein's expression levels or activity. This notion prompted a number of animal studies that yielded confusing and sometimes conflicting results. Kim et al. (*28*) reported that increased vitamin E intake lowered the levels of TTP mRNA and protein in rat liver. Conversely, Shaw et al. (*29*) reported that TTP protein levels did not respond to dietary supplementation but decreased in response to vitamin E depletion. When comparing the gene expression profile of vitamin E-deficient versus supplemented rats, Barella et al. (*30*) noted that the TTP transcript did not change. Fechner et al. (*31*) reported that a supplementation−fasting protocol in vitamin E-depleted rats led to a dramatic increase in the level of TTP mRNA. Copp and colleagues reported that the levels of TTP are increased in brain sections of humans afflicted with oxidative stress-related diseases such as AVED, Alzheimer's disease, and Down's syndrome (*32*). Recently, Bella et al. observed that TTP protein levels increased in mice supplemented with vitamin E but did not change in response to smoke-induced oxidative stress (*33*). To examine the mechanisms that regulate the levels of TTP, we utilized cultured hepatocytes that express TTP endogenously (IHH cells) or ectopically (HepG2 cells). We observed that, in both cell types, treatment with vitamin E increased the level of TTP protein by 2−3-fold but did not alter the level of TTP mRNA. We found further that vitamin E stabilizes the protein by attenuating the rates at which TTP is ubiquitinated and degraded by the proteasome. As other antioxidants did not affect the expression level of TTP, α-tocopherol appears to affect the stability of TTP by inducing a specific conformation rather than doing so via indirect effects on cellular oxidative stress or redox status. This notion is further supported by the findings that α-tocopherol changed TTP's sensitivity to proteolysis by trypsin in vitro.

The most striking conformational change that accompanies ligand binding in TTP is a translational movement of residues 198−221 that cover the occupied ligand binding pocket (*20*). The inter-relationship among ligand-induced conformational changes, ubiquitination pattern, and intracellular localization of TTP remains to be clarified.

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