Biochemical Consequences of Heritable Mutations in the α-Tocopherol Transfer Protein†

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ABSTRACT: Tocopherol transfer protein (TTP) regulates vitamin E status by facilitating the secretion of tocopherol from liver to circulating lipoproteins. Heritable mutations in the *ttpA* gene, encoding for TTP, result in ataxia with vitamin E deficiency (AVED) syndrome, typified by low vitamin E levels and a plethora of neurological disorders. The molecular mechanisms by which TTP facilitates tocopherol secretion are presently unknown. We recently showed that vitamin E is taken up by hepatocytes through an endocytic process and that, shortly following uptake, the vitamin is found primarily in lysosomes. We showed further that TTP is localized to late endocytic vesicles and that it facilitates the intracellular trafficking of tocopherol from lysosomes to the plasma membrane. To gain insight into the molecular mechanisms that underlie TTP actions, we studied the physiological impact of three naturally occurring heritable mutations in the *ttpA* gene (the R59W, R221W, and A120T substitutions). We found that these mutations impair the ability of TTP to facilitate the secretion of vitamin E from cells. Furthermore, the degree of impairment corresponded to the severity of the AVED pathology associated with each mutation. In cells that express mutated TTP proteins, vitamin E did not traffic to the plasma membrane and remained "trapped" in lysosomes. In addition, we observed that substitution mutations that cause the AVED syndrome impart a marked instability on the TTP protein. These observations suggest that the physiological role of TTP is anchored in its ability to direct vitamin E trafficking from the endocytic compartment to transport vesicles that deliver the vitamin to the site of secretion at the plasma membrane.

The term vitamin E refers to a family of neutral plant lipids composed of a chromanol ring and a phytyl side chain. Members of the vitamin E family differ by the methylation pattern of their chromanol ring (α- vs *β*- vs *γ*- vs *δ*-), the degree of saturation of their phytyl tail (tocopherols vs tocotrienols), and the stereochemistry of three chiral centers in the molecule (*R* vs *S* stereoisomers). Vitamin E molecules demonstrate potent radical trapping (i.e., antioxidant) activity both in vitro and in vivo (*1*, *2*), and it is generally accepted that this scavenging activity is at the basis for the physiological requirement for vitamin E. In support of this notion, adequate vitamin E intake was reported to offer protection from a number of pathologies associated with elevated oxidative stress, such as cardiovascular disease, cancer, and inflammation (*3*−*5*).

In vivo, vitamin E status is regulated by two principal processes that take place in the liver. First, vitamers other than α-tocopherol (e.g., the *β*-, *γ*-, and *δ*-tocopherols) are degraded to water-soluble catabolites by the *ω*-hydroxylase cytochrome P450 CYP4F2 (*6*). Second, α-tocopherol is selectively retained by the hepatic tocopherol transfer protein (TTP),[1] which facilitates the secretion of this form from hepatic cells to circulating lipoproteins (*7*−*9*).

The critical role of TTP in regulating vitamin E status is demonstrated by the observation that, in humans, heritable mutations in the *ttpA* gene result in very low plasma tocopherol levels and a neurological syndrome termed ataxia with vitamin E deficiency (AVED; *10*, *11*). These mutations can be divided into two general categories, those that are associated with a milder, later onset neuropathy (e.g., the A120T, H101Q, and R192H substitutions) and those associated with a severe, early onset pathology (e.g., R221W, R59W, and E141K) (*8*, *12*). $TTP^{-/-}$ mice exhibit low plasma levels of tocopherol (*13*, *14*), are infertile (*15*), and display neurological symptoms similar to those presented by human AVED patients (*16*). To date, two activities have been attributed to TTP. The protein catalyzes the transfer of tocopherol between donor and acceptor membranes in vitro (*17*−*20*), and it facilitates the secretion of vitamin E from cultured hepatocytes (*7*, *9*, *21*). Whether these two activities are connected, as well as the molecular mechanisms that underlie them, remains unknown.

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[1] Abbreviations: TTP, α-tocopherol transfer protein; AVED, ataxia with vitamin E deficiency; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; LBPA, lysobisphosphatidic acid; LAMP1, lysosomal-associated membrane protein 1; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; ABC-A1, ATP-binding cassette transporter A1; HA, hemagluttin epitope; DMEM, Dulbecco/Vogt-modified Eagle's essential medium; FBS, fetal bovine serum; Chx, cycloheximide.

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Using a fluorescent analogue of vitamin E, NBD-tocopherol, we recently showed that, following uptake by endocytosis, vitamin E accumulates in endosomes and lysosomes, where TTP is localized (*9*, *21*). Furthermore, we demonstrated that TTP facilitates the intracellular transport of the vitamin from lysosomes to the plasma membrane (*9*). Here, to better understand the mechanisms that underlie intracellular trafficking of vitamin E and the role of TTP in this process, we investigated the impact of a number of heritable TTP mutations on the intracellular transport of vitamin E.

MATERIALS AND METHODS

Molecular Constructs and Stable Cell Lines. The HA-tagged TTP was amplified from the pTRE2 vector (*9*) while adding the *Cla*I and *Xba*I restriction sites at the 5′ and 3′, respectively, and the resultant PCR product was ligated into pCDNA3.1-Hygro (Invitrogen). This construct was used as template for site-directed mutagenesis (QuickChange; Invitrogen) to generate the TTP constructs harboring each of the AVED substitution mutations A120T, R221W, R59W. All constructs were verified by sequencing at the Cornell BioResource facility. The TTP constructs (or "empty" vector as a control) were transfected into HepG2/C3A cells using Fugene6 (Roche), and stable clones were selected by culturing in DMEM supplemented with 10% FBS and 200 μg/mL hygromycin. After clonal selection and propagation, cells were lysed in 20 mM HEPES (pH 7.4), 1 mM EDTA, 150 mM NaCl, 1% NP40, 20 mM sodium fluoride, 20 mM β-glycerophosphate, 1 mM sodium vanadate, and 200 μM PMSF. TTP expression was assessed by anti-HA immunoblotting (HA.11 antibody; Covance Inc.). The anti-HA and anti-TTP antibodies recognize all TTP variants with essentially identical sensitivity (data not shown).

Tocopherol Secretion. The preparation of tocopherol−serum complexes and the tocopherol secretion assay were performed as described previously (*9*). Briefly, HepG2-TTP cells were cultured in 24-well plates and incubated with media containing [^{14}C]-*RRR*-α-tocopherol (Amersham Biosciences Corp.) as the serum complex for 36 h. The cells were then washed extensively in DMEM/10% FBS and incubated in DMEM. At the indicated times, the media were collected, the cells were lysed, and the radioactivity in the media and cell lysates was measured by scintillation counting. Under these experimental conditions, HepG2 synthesize and secrete the lipoprotein ApoA, which serves as the lipid acceptor in the media (*9*). Tocopherol secretion was calculated as follows:

$$\text{tocopherol secretion (\%)} = \frac{\text{CPM}_{\text{media}}}{\text{CPM}_{\text{cells}} + \text{CPM}_{\text{media}}} \times 100$$

Confocal Microscopy. The intracellular localization of TTP was determined using anti-HA antibodies (Covance Inc.) as described earlier (*9*). Intracellular trafficking of vitamin E was monitored using a fluorescent analogue of vitamin E, NBD-tocopherol, which we recently described (*9*, *22*, *23*). Confocal images were collected on a Leica TCS-T2 microscope at the Cornell BioResource facility.

TTP Stability. To assess the impact of AVED mutations on TTP stability, we measured the rate of protein degradation in the presence of a protein synthesis inhibitor. Specifically, cells were treated with cycloheximide (100 μg/mL) for the indicated duration, and the TTP level was measured in cell lysates by immunoblotting. Where indicated, cells were cotreated with the proteasomal inhibitor MG132 (5 μM). Western blots were scanned, and intensities of the TTP band were quantitated using Scion-Image software.

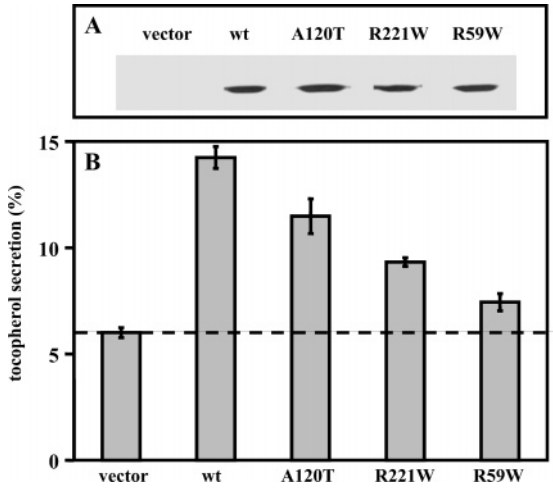

FIGURE 1: Facilitation of vitamin E secretion by TTP. The indicated cell lines were generated as described in Materials and Methods, and clones with similar TTP expression levels were selected for functional studies. (A) Expression levels of TTP in the different clones were evaluated by anti-HA western blotting. (B) Tocopherol secretion activities in the HepG2-TTP stable cell lines. Cells were loaded with [^{14}C]-*RRR*-α-tocopherol, and the appearance of radioactivity in the media was assayed after 24 h as described in Materials and Methods. Shown are averages and standard deviations of quadruplicate plates, representative of five independent experiments.

RESULTS

Tocopherol Secretion Activity of TTP Variants. To date, only one physiological activity was ascribed to TTP, namely, the facilitation of tocopherol secretion from parenchymal cells of the liver (*7*, *9*, *21*). We therefore hypothesized that hepatocytes that overexpress mutant TTP proteins will be compromised with respect to vitamin E secretion. To test this notion, we stably expressed three such mutant TTP constructs in the human hepatoma cell line HepG2: the TTP-(A120T) variant, shown to give rise to the late-onset, mild form of AVED, and the TTP(R221W) and TTP(R59W) mutants, each associated with the severe, early-onset form of the disease (*12*). We then examined the ability of these cell lines to secrete vitamin E to the media using an established protocol that employs radiolabeled tocopherol (*9*). We chose to focus on clones that express the different TTP variants to the same level, as determined by immunoblotting (Figure 1A). We observed that overexpression of the wild-type TTP was accompanied by a ca. 2.5-fold increase in tocopherol secretion during a 24 h period as compared to mock-transfected cells (Figure 1B), in agreement with previous observations (*7*, *9*). Clones that stably express either of the three AVED mutants exhibited significant reduction in their ability to secrete tocopherol. Specifically, the secretion activity exhibited by cells expressing the A120T, R221W, and R59W variants of TTP was reduced to 67%, 40%, and 17% of the activity exhibited by the wild-type protein, respectively (Figure 1B). To confirm that the

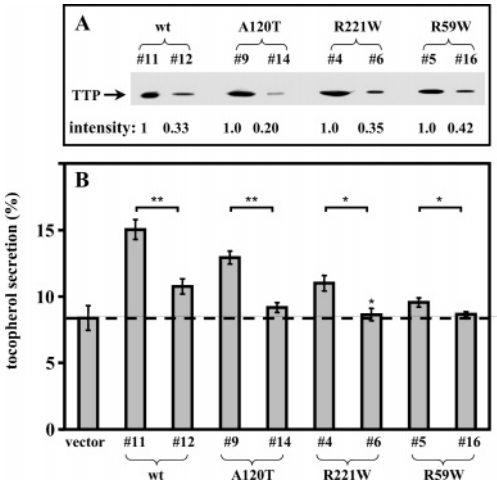

FIGURE 2: Dependence of tocopherol secretion on TTP expression levels. For each TTP variant, two stable transfectants were selected, each expressing the protein to different level. (A) Expression levels of TTP in the different clones were evaluated by anti-HA western blotting, and band intensities were quantified by computerized densitometry. (B) Tocopherol secretion was evaluated in each stable transfectant as described in Figure 1. Shown are averages and standard deviation of quadruplicate plates, representative of four independent experiments. Statistical significance: asterisks denote *P* value of less than 0.05 (**) or less than 0.5 (*) in a standard Student *t* test.

observed impact of AVED mutations on tocopherol secretion is indeed the outcome of compromised TTP activity rather than clonal variation, we compared the TOH secretion activity of cell lines that express different levels of each TTP variant. As seen in Figure 2, for each TTP mutant, stimulation of tocopherol secretion correlated well with the expression level of the protein as assayed by immunoblotting. For example, TTP(A120) clone 14 expresses the protein 5-fold less than TTP(A120) clone 9 and exhibited similar reduction in tocopherol secretion (Figure 2). We observed a similar dose−response relationship among all cell lines. Taken together, these data confirm that mutations in the *ttpA* gene identified in human AVED patients indeed cause impairment in TTP's biochemical activity. Furthermore, the degree of functional impairment among the various TTP alleles directly correlates to the severity of the clinical symptoms displayed by human carriers of these mutations. Thus, the order of functional impairment imparted by AVED mutations is R59W > R221W > A120T, paralleling the severity of clinical phenotype observed in patients bearing these mutations (*12*).

Facilitations of Intracellular Trafficking of Vitamin E by TTP Variants. We have recently characterized a novel fluorescent analogue of vitamin E, C9-NBD-tocopherol, and showed that it is a valuable tool for studying TTP function in vitro and in vivo (*9*, *22*, *23*). To gain insights into the physiological impacts of heritable mutations in TTP, we compared the mutant and wild-type proteins with respect to their effect on the intracellular trafficking of NBD-tocopherol using the stable cell lines described above. We observed that cells that express either the wild-type version of TTP or its R221W variant accumulated NBD-tocopherol in lysosomes with similar kinetics (data not shown), in agreement with previous observations that TTP does not participate in the vitamin E uptake (*9*). However, cells that express the mutant TTP(R221W) protein are remarkably different with regard to the movement of NBD-tocopherol from the lysosomes to the plasma membrane, as shown in Figure 3. In cells that express wild-type TTP, NBD-tocopherol exits from the lysosomal compartment to the plasma membrane within 2 h after loading, and >90% of the vitamin is found near the plasma membrane after 3 h (Figure 3B). In cells that overexpress the TTP(R221W), on the other hand, the major fraction of NBD-tocopherol remains in endocytic vesicles during the time period of the experiment. Kinetic analyses demonstrate that the capacity of the mutant to facilitate intracellular tocopherol transport is diminished by >4-fold as compared to that of the wild-type protein (Figure 3B).

Taken together, these results show that the tocopherol secretion activity of TTP is closely correlated to its ability

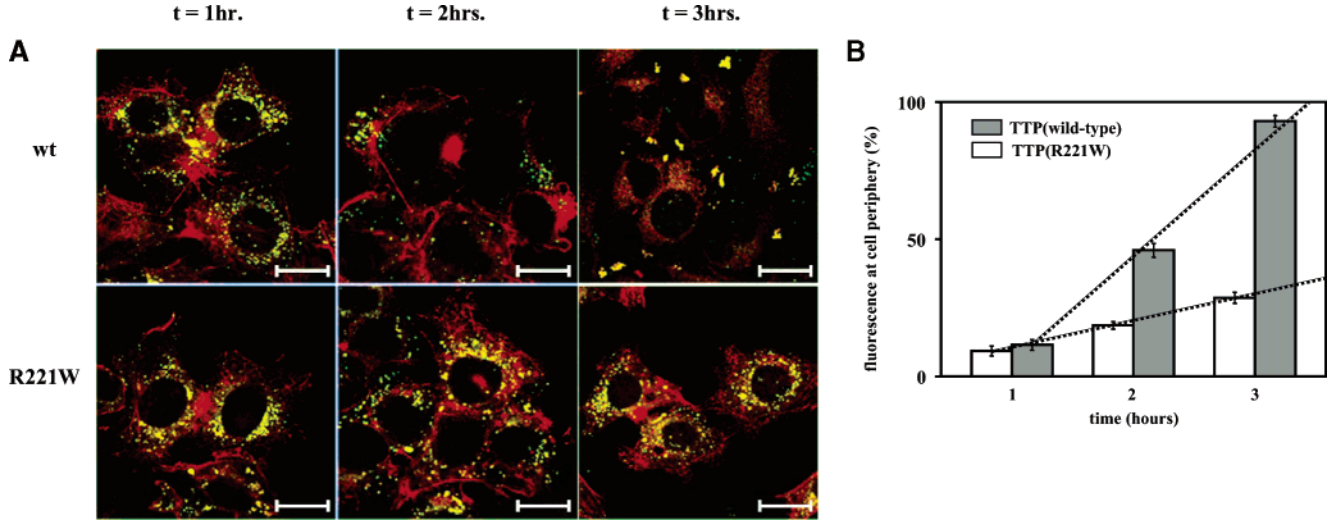

FIGURE 3: Intracellular trafficking of NBD-tocopherol in HepG2-TTP cells. (A) HepG2 cells stably expressing wild-type TTP or the TTP-(R221W) mutant were cultured on microscope slides and incubated with NBD-tocopherol (as serum complexes) for 1 h at 4 °C. After washing, the cells were incubated in regular media (DMEM/10% FBS) at 37 °C for indicated time before processing for fluorescence microscopy. Color code: red, actin (visualized with Texas Red-conjugated phalloidin); green, NBD-tocopherol. (B) Quantitation of NBD-tocopherol transport. For each cell, the intensity of NBD-tocopherol fluorescence was measured at the cell periphery (i.e., within 2 μm of the plasma membrane) and expressed as percent of the total NBD-tocopherol fluorescence in that cell. Each data set represents average and standard deviations obtained from 40 cells. Bar = 12 μm.

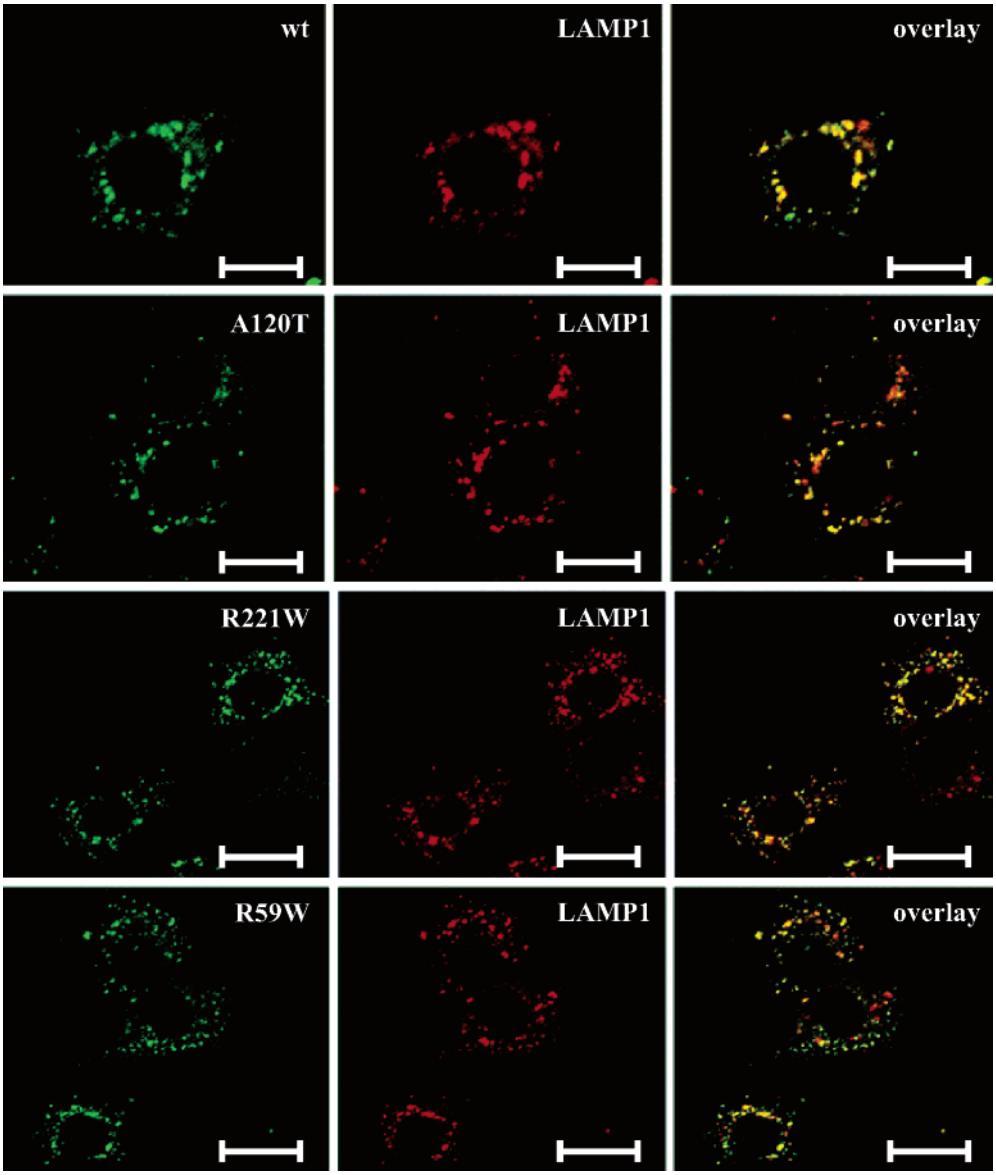

FIGURE 4: Intracellular localization of wild-type and mutant TTP proteins. HepG2 cells that stably express the indicated TTP construct were cultured on microscope chamber slides and prepared for microscopy as detailed in Materials and Methods. TTP was visualized by staining with anti-HA antibody (green), and the late endosomes and lysosomes were visualized with anti-LAMP-1 antibodies (red). Bar = 12 μm.

to facilitate intracellular transport of the vitamin, i.e., that, in hepatocytes, TTP catalyzes the rate-limiting step in tocopherol secretion. Moreover, the data indicate that trafficking of vitamin E from lysosomes to the plasma membrane is the biochemical step that is compromised by heritable mutations in the *ttpA* gene.

Intracellular Localization of AVED Mutants. Others and we have previously shown that TTP colocalizes with molecular markers of the endocytic pathway (*9*, *21*) and that pharmacologic inhibitors of lysosome function abolish TTP-mediated secretion of vitamin E (*9*, *21*, *24*). We therefore set out to examine the effects of AVED on the cellular localization of TTP. To test this hypothesis, we studied the intracellular localization of three TTP mutants that give rise to the mild [TTP(A120T)] and severe [(TTP(R221W), TTP-(R59W)] forms of AVED. We observed that the localization pattern of the mutant TTP constructs shows extensive overlap with the lysosomal marker LAMP1, similar to the pattern exhibited by the wild-type protein, indicating that the mutated proteins are properly localized (Figure 4). The data indicate that the defects in the physiological activity of the mutant TTP variants arise from perturbations to other biochemical properties of the proteins.

Stability of Mutant TTP Proteins. Another property that could be affected by heritable mutations in TTP is protein stability. To directly evaluate the effects of AVED substitutions on the turnover rate of TTP, we treated cells that stably express the different mutants with the protein synthesis inhibitor cycloheximide and examined the rate of TTP degradation by immunoblotting.

We observed that the wild-type TTP protein degrades over time, with an apparent half-life of approximately 16 h. Treatment of the cells with vitamin E (50 μM *RRR*-α-tocopherol) did not alter the turnover rate of TTP (Figure 5A). When the cells were treated with MG132, an inhibitor of the proteasomal degradation pathway, the degradation of TTP was drastically slowed, with less than 8% of the protein disappearing over 16 h (Figure 5A). These observations

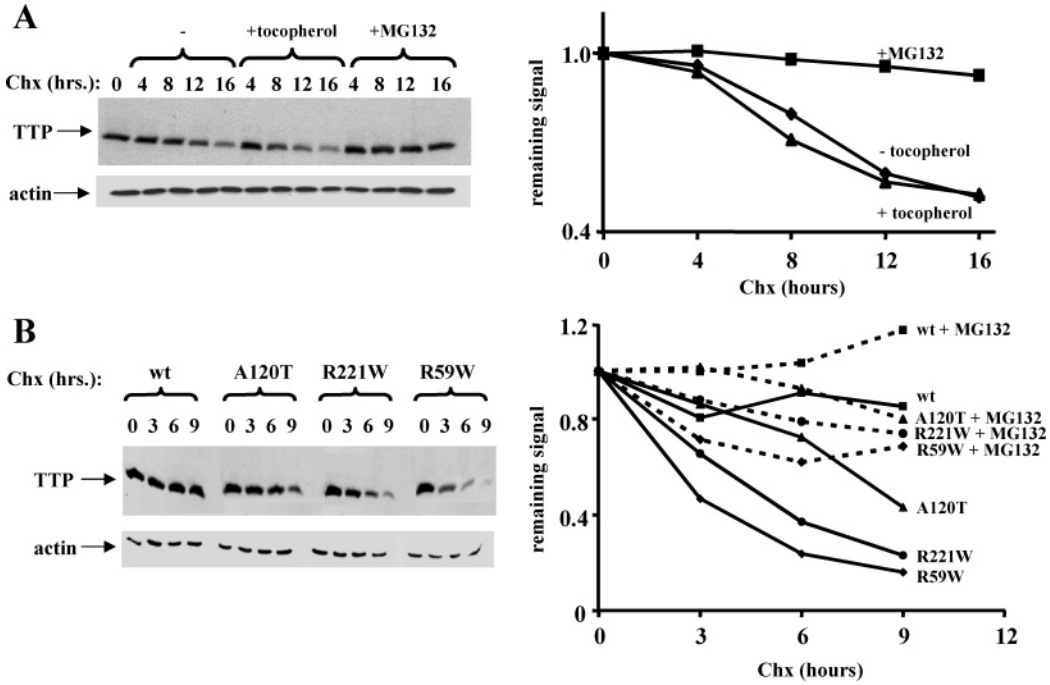

FIGURE 5: Stability of TTP proteins in vivo. (A) HepG2 cells were seeded in triplicate six-well plates and treated with cycloheximide (Chx, 100 μg/mL) for the indicated duration. Where indicated, serum-complexed tocopherol (final concentration 50 μM) was included in the culture media 20 h prior to cycloheximide addition. MG132 (5 μM) was added where indicated to inhibit the proteasomal pathway. TTP levels were evaluated in cell lysates by anti-HA immunoblotting. (B) Intensities of TTP bands in each condition were quantified by computerized densitometry. Shown are averages and standard deviations of four independent experiments.

indicate that the stability of TTP is regulated by the proteasomal pathway, possibly involving ubiquitination of the protein by a protein−ubiquitin ligase that destines TTP for degradation by the proteasome (*25*). In support of this notion, treatment of the cells with the lysosomotropic agent chloroquine did not affect the turnover rate of TTP, thus ruling out the lysosome-mediated protein degradation pathway (data not shown). TTP proteins that harbor AVED mutations degraded much faster than the wild-type protein in cultured hepatocytes. Specifically, the half-life values of TTP(A120T), TTP(R221W), and TTP(R59W) were 8.3, 4.6, and 2.8 h, respectively (Figure 5B). Inhibition of proteasomal degradation by treatment with MG132 caused a marked stabilization of TTP (Figure 5B). Moreover, protein stability appears to be directly related to the clinical phenotype associated with each mutation, with the shortest lifetime exhibited by the variant that leads to the most severe form of the disease (R59W). Similar results were obtained when we examined the steady-state expression levels of the different TTP variants. We transiently transfected the different constructs into HepG2 cells and measured the TTP levels after 48 h by immunoblotting. The relative expression level of the wild-type, A120T, R221W, and R59W protein was 1.0, 0.78, 0.5, and 0.12, respectively (data not shown). We conclude that mutations in the *ttpA* gene that cause vitamin E deficiency decrease the stability of the gene product.

DISCUSSION

Genetic linkage analyses indicated that the molecular defects that cause heritable vitamin E deficiency arise from mutations in the *ttpA* gene (*10*, *12*, *26*−*28*). In support of this notion, $TTP^{-/-}$ mice have very low vitamin E levels and present the clinical symptoms associated with AVED in humans (*13*−*16*). A critical insight into the role of TTP in regulating vitamin E status was obtained when Arai and colleagues reported that overexpression of TTP in a rat hepatocyte cell line was accompanied by enhanced secretion of vitamin E to the culture media (*7*). These observations led to the currently accepted model in which dietary vitamin E is transported in chylomicra particles from intestinal cells to the liver, where TTP regulates its secretion to plasma lipoproteins (*8*, *29*, *30*). However, the molecular mechanisms by which TTP stimulates tocopherol secretion from hepatocytes are still poorly understood. Deciphering the exact biochemical functions of TTP and its regulatory modes will therefore greatly enhance our understanding of vitamin E biology in both health and disease.

We recently reported that TTP expression does not influence the kinetics or the extent of vitamin E uptake in HepG2 cells (*9*). Furthermore, we have shown that tocopherol enters the hepatocyte through the endocytic pathway, involving the scavenger receptor SR-BI, and that, shortly after uptake, the vitamin accumulates in late endocytic vesicles, where TTP resides (*9*, *21*). In agreement with these observations, lysosomotrophic agents were shown to abolish TTP-mediated tocopherol efflux (*9*, *21*). Since the lysosomal localization of TTP is constitutive, we propose that following lipoprotein degradation in the lysosome, TTP facilitates the delivery of tocopherol to transport vesicles that then exit the cell through the plasma membrane. There is considerable evidence that the ATP-driven transporter ABCA1 participates in this final secretory step (*9*, *31*).

The availability of detailed information regarding the nature and impact of heritable mutations in TTP is important on several levels. First, functional characterization of TTP variants will shed light on the only known human pathology that is directly related to abnormal vitamin E status. Second,

mutated TTP constructs can serve as invaluable tools that can be utilized for deciphering the physiological reactions that are catalyzed by the protein. Lastly, mutant versions of TTP offer a unique opportunity for understanding the structure−function relationship in this protein and the mechanistic bases for its physiological actions. Previous reports established that some AVED mutations negatively impact on the ability of TTP to facilitate intermembrane tocopherol transfer in vitro (*20*, *27*). However, how these mutations affect TTP function in a physiological setting is presently unknown. Thus, we investigated the functional outcomes of three naturally occurring substitution mutations in TTP, each of which is known to cause the neuropathological AVED syndrome in human patients. The R221W and R59W substitutions give rise to the severe, early-onset form of the disease, while the A120T mutation is associated with a milder, late-onset phenotype (*12*). Using hepatocyte cell lines that stably express the different TTP alleles, we demonstrate that these mutants are compromised in their ability to stimulate the secretion of vitamin E from hepatocytes (Figures 1 and 2) and that the extent of impairment in TTP activity correlates with the severity of the clinical phenotype associated with each mutation. We further show that this defect stems specifically from inability of the mutated proteins to trigger the intracellular translocalization of vitamin E: while expression of wild-type TTP protein induces dramatic translocation of vitamin E from the lysosomes to the plasma membrane, this process is markedly inhibited in cells that express mutated TTP (Figure 3).

It was previously reported that TTP expression is regulated by vitamin E status in rat liver (*32*), by gestational phase in murine uterus (*15*), and by developmental stage in neonatal rat liver (*33*). The molecular mechanisms that underlie these changes in TTP levels are presently unknown. The data shown here demonstrate that TTP degrades with a half-life of approximately 16 h and that degradation is mediated by the proteasomal pathway (Figure 5). These observations raise the possibility that TTP function may be regulated by posttranslational mechanisms, in which the steady-state expression level of the protein is changed in response to some cellular stimuli. Identification of the factors that may impact such regulatory mode is of particular interest. While we found that treatment of the cells with vitamin E does not affect TTP stability, it is possible that modulation of TTP turnover involves other factors that are absent from the immortalized hepatocyte cell line used in this study. Importantly, we find that three naturally occurring substitution mutations in TTP (A120T, R221W, and R59W) result in a marked increase in the rate of the degradation of the protein by the proteasome (Figure 5), suggesting that these genetic defects may decrease steady-state levels of the protein.

Our data demonstrate a number of important points: First, TTP is indeed critical in mediating tocopherol secretion from hepatocytes, as postulated in the past (*34*−*36*). Second, TTP functions to facilitate the transport of vitamin E from lysosomes to a yet undetermined point of exit at the plasma membrane. Third, mutations that cause vitamin E deficiency in humans interfere with TTP-facilitated transport of vitamin E between these cellular compartments. Lastly, these mutations significantly shorten the half-life of the *ttpA* gene product in cells. Future studies will utilize these TTP variants to understand the molecular mechanisms by which TTP influences the intracellular transport of vitamin E and the modes by which this activity is regulated in vivo.

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