Molecular Basis of the Acceleration of the GDP-GTP Exchange of Human Ras Homolog Enriched in Brain by Human Translationally Controlled Tumor Protein*§

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Ras homolog enriched in brain (Rheb), a small GTPase, positively regulates the mTORC1 pathway. The GDP–GTP exchange of Rheb has been suggested to be facilitated by translationally controlled tumor protein (TCTP). Here we demonstrate that human TCTP (hTCTP) interacts with human Rheb (hRheb) and accelerates its GDP release in vitro and that hTCTP activates the mTORC1 pathway in vivo. To investigate the underlying mechanism, we built structure models of GDP- and GTP-bound hRheb in complexes with hTCTP and performed molecular dynamics simulations of the models, which predict key residues involved in the interactions and region of hRheb undergoing conformational change during the GDP-GTP exchange. These results are verified with site-directed mutagenesis and in vitro biochemical and in vivo cell biological analyses. Furthermore, a crystal structure of the E12V mutant hTCTP, which lacks the guanine nucleotide exchange factor activity, shows that the deficiency appears to be caused by loss of a salt-bridging interaction with Lys-45 of hRheb. These data collectively provide insights into the molecular mechanisms of how TCTP interacts with hRheb and activates the mTORC1 pathway.

The mammalian target of rapamycin (mTOR)§1 belongs to the phosphoinositide 3-kinase-related kinase family. It is a central regulator of cell growth and cell proliferation by integrating signals, including growth factors, nutrients, and energy status, and has been implicated to play important roles in cancer cell metabolism (1). mTOR functions in the context of two distinct multiprotein complexes. The mTORC1 complex is sensitive to rapamycin. It regulates transcription, protein translation, ribosomal biogenesis, and autophagy, and thus affects fundamental cell processes, including cell growth and cell cycle progression (1). The complex comprises mTOR, raptor, and mLST8 (2, 3), and its major substrates include ribosomal S6 kinase 1 (S6K1) (4), eukaryote initiation factor 4E-binding protein 1 (4E-BP1) (4), and the newly identified serum- and glucocorticoid-induced protein kinase 1 (SGK1) (5). 4E-BP1 and S6K1 are involved in initiation of protein translation and protein synthesis, respectively (1). Activation of SGK1 by mTOR results in phosphorylation and cytoplasmic mislocalization of p27 and may promote G1 progression (5). The mTORC2 complex is rapamycin-insensitive and contains mTOR, rictor, and mLST8 (6). It phosphorylates Akt on Ser-473 and regulates cytoskeleton (7, 8).

A small GTPase, Ras homolog enriched in brain (Rheb), is a critical regulator of mTORC1 (9). However, the underlying mechanism is unclear. Bai et al. showed that Rheb can activate mTOR through binding to FKBP38, an endogenous inhibitor of mTOR, in a GTP-dependent manner to prevent the interaction of FKBP38 with mTOR (10). However, this result has been doubted by Wang et al. (11).

Like other small GTPases, Rheb cycles between the active GTP-bound and inactive GDP-bound forms, and regulation of Rheb is mediated by regulators, including GTPase-activating protein and guanine nucleotide exchange factor (GEF). One identified GTPase-activating protein for Rheb is tuberous sclerosis complex (TSC) formed by tumor suppressor proteins TSC1 and TSC2 (12–14). By binding to Rheb and stimulating its GTPase activity, the TSC1-TSC2 complex reduces the level of GTP-bound Rheb and thus inhibits the mTOR/S6K1/4E-BP1/SGK1 pathway (5, 12–14). Consistently, mutations on either TSC1 or TSC2 gene lead to high mTOR activity, which is associated with tuberous sclerosis syndrome, an autosomal-dominant genetic disorder that manifests largely as benign tumors in brain, heart, skin, and kidney and can cause severe complications, including mental retardation, seizure, and autism (15).
The long searched for GEF for Rheb was proposed to betranslationally controlled tumor protein (TCTP) based ongenetic data (16), but this has become controversial (11, 17),providing a strong motivation for our studies. As implicated inits name, TCTP is regulated at both translational and posttranslational levels in response to a wide range of extracellularsignals and conditions (18) and exerts diverse functions invarious cell processes such as cell cycle progression, cell growth,microtubule stabilization, and apoptosis (reviewed in Ref. 19).In Arabidopsis thaliana, TCTP has been shown to be animportant regulator of growth, but whether and/or how the TORactivity is affected by plant TCTP was not studied (20). TCTP homozygous knock-out in mouse is embryonic-lethal, and themutant embryos are smaller than the wild-type embryos andhave reduced number of cells in epiblast (21). In Drosophila,down-regulation of TCTP results in phenotypes, including reduced cell size, cell number, and organ size (16), similar to those of Drosophila Rheb (dRheb) mutant (22). Genetic epistasis experiments suggested that Drosophila TCTP (dTCTP) is epistatic to dRheb but acts upstream of S6K (16). Although TCTP was discovered about two decades ago, its GEF-likeactivity was not realized until recently. TCTP structurally resembles a GEF, namely Mss4 (23). Indeed, in vitro experimentshowed that dTCTP binds to nucleotide-free dRheb and specifically stimulates the GDP-GTP exchange of dRheb (16). Moreover, co-immunoprecipitate experiments showed that dTCTP interacts with dRheb, and reducing the TCTP level inDrosophila S2 cells results in lowered GTP-bound dRheb, further suggesting that Rheb is a downstream target of TCTP in vivo (16). However, the GEF activity of TCTP on Rheb and the role of TCTP in S6K regulation have been doubted in two recent papers. Rehmann et al. showed that the GEF activity of human TCTP (hTCTP) on human Rheb (hRheb) could not be detected using an in vitro real-time fluorescence-based in-solution assay (17). Wang et al. reported that overexpression of hTCTP had no significant effect on the phosphorylation level of S6, a substrate of S6K, in stressed HEK293 cells (11). In addition, TCTP knockdown by siRNA did not affect the phosphorylation level of S6K (17) or S6 (11).

In this work, we demonstrated with in vitro biochemicalexperiment that hTCTP can accelerate the GDP release of hRheb, and with in vivo cell biological experiments that overexpression of hTCTP can result in enhanced phosphorylation of S6K, whereas knockdown of hTCTP has an opposite effect, supporting the notion that hTCTP is a GEF of hRheb and a regulator of the mTORC1 pathway. To investigate the molecularmechanism of the GEF activity of hTCTP on hRheb, we carried out homology modeling studies of the hRheb-hTCTP complexes based on the crystal structures of GDP- and GTP-bound hRheb (24) and hTCTP using the crystal structure of the Rab8-Mss4 complex as a template (25), and further employed molecular dynamics (MD) simulation to the modeled complexes. Our modeling and simulation results predicted the key residues that are involved in the hTCTP-hRheb interaction and the important region that undergoes conformational change during the GDP-GTP exchange of hRheb and reached a different conclusion than that by Rehmann et al. (17). These results were validated by using mutagenesis studies, in vitro biochemicaland in vivo cell biological analyses. We also determined thecrystal structure of the E12V mutant hTCTP, which lacks theGEF activity and cannot activate the mTORC1 pathway. Thecomputational, structural, biochemical, and cell biologicaldata together provide insights into the molecular mechanism ofthe GDP-GTP exchange of hRheb accelerated by hTCTP.

EXPERIMENTAL PROCEDURES

Cloning, Expression, and Purification—The hTCTP gene was amplified by PCR from the cDNA of human HEK293T cells with the sense primer 5'-ATGATTATCTACCGGAGCTC-3' and the antisense primer 5'-ACATTTTTCCATTTCAAC-3', and this gene fragment was further amplified with the sense primer 5'-AACATATGATTATCTACCGGAGCTC-3' (with the Ndel restriction site underlined) and the antisense primer 5'-AACCTCGAGACATTTTTCCATTTCAAC-3' (with the Xhol restriction site underlined) to incorporate the restriction sites. The gene fragment was inserted into the Ndel and Xhol restriction sites of the pET-22b (+)-His expression vector, and the plasmid was used as a template to generate an E12V mutant hTCTP with QuickChange® site-directed mutagenesis kit (Stratagene). The plasmid encoding the mutant was transformed into Escherichia coli BL21(DE3) strain (Novagen). When the culture of the transformed cells reached an A600 of 0.6–0.8, protein expression was induced by 0.1 mM isopropyl 1-thio-β-D-galactopyranoside at 20 °C for 18 h. The cells were lysed by sonication in the lysis buffer (20 mM Tris-HCl, pH 7.4, 500 mM NaCl, 10% glycerol, and 2 mM dithiothreitol). Proteinpurification was carried out by affinity chromatography using anickel-nitrilotriacetic acid column (Qiagen) with the lysis buffer supplemented with 20 mM and 200 mM imidazole serving aswashing buffer and elution buffer, respectively. The elution fractions containing the target protein were further purified bygel filtration using a Superdex G-75 HiLoad 16/60 column (Amersham Biosciences) in buffer A (10 mM Tris-HCl, pH 7.4,100 mM NaCl, 10% glycerol, and 2 mM dithiothreitol). The tar-get protein fractions were pooled and concentrated to ~30 mg/ml for crystallization. The hTCTP gene fragment was alsoamplified with the sense primer 5'-AGGATCCATGATTCATGCATCGGAGCTC-3' (with the BamH restriction site underlined) and the antisense primer 5'-ACTCGAGATACTTTTCTACCGGAACGTTACTTTTCTACCGGAGCTC-3' (with the Xhol restriction site underlined) and cloned into the BamH and Xhol restriction sites of the pEGX-4T vector, and the site-directed mutagenesis was performed to construct various GST-hTCTP mutants. The GST-tagged wild-type and mutant hTCTP proteins were expressed in E. coli BL21(DE3) cells as described above. The cells were lysed by sonication in phosphate-buffered saline buffer, and the protein was purified with glutathione-Sepharose beads (Amersham Biosciences). All of the purification processes were carried out at 4 °C (except for gel filtration at 16 °C) to reduce potential proteolysis and denaturation of the target protein. Thequality of the purified protein was assessed by SDS-PAGE andCoomassie Blue staining. His-tagged hRheb was expressed andpurified as described previously (24).

In Vitro GST Pulldown Assay—To investigate the effects ofmutations of the key residues involved in the hRheb-hTCTPinteraction, we performed in vitro protein-protein binding
Structure Model of the hRheb-hTCTP Complex

assay. Previous studies have shown that the purified hRheb was bound with GDP and Mg^{2+} (24). To remove the bound GDP and Mg^{2+}, 0.5 ml of the purified protein was incubated with 10-fold volume of buffer A supplemented with 10 mM EDTA and subsequently ultracentrifuged to 0.5 ml. Such process was repeated five times. GTP loading of hRheb was achieved by incubating the nucleotide-free hRheb with 25 mM GTP and 10 mM MgCl_2. For experiments presented in Fig. 1A, ~150 μg of the wild-type GST-hTCTP protein was immobilized onto the glutathione-Sepharose beads and then incubated with 300 μg of the nucleotide-free or nucleotide-bound hRheb protein in binding buffer (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10% glycerol, and 10 mM EDTA for nucleotide-free hRheb or 10 mM MgCl_2 for nucleotide-bound hRheb). The bound proteins were finally eluted by elution buffer (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10% glycerol, and 10 mM EDTA for nucleotide-free hRheb or 10 mM MgCl_2 for nucleotide-bound hRheb). The bound proteins were finally eluted by elution buffer (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10% glycerol, and 10 mM EDTA) at 4 °C for 2 h. The mixture was then washed ten times with chilled washing buffer (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10% glycerol, and 10 mM EDTA) at 4 °C for 2 h. The mixture was then washed ten times with chilled washing buffer supplemented with 0.5% Tween 20. The eluted proteins were resolved by 12% SDS-PAGE, transferred to polyvinylidene difluoride, and then blotted with anti-His antibodies (Tiangen). The GST protein served as a negative control.

In vitro GDP Release Assay—In vitro GDP release assay was performed as described by Hsu et al. with minor modifications (16). Briefly, 1 μM nucleotide-free His-hRheb was incubated with 1 μM [3H]GDP and 1.5 μM wild-type or mutant GST-hTCTP in buffer B (50 mM HEPES, pH 7.6, 100 mM NaCl, 2.5 mM Mg^{2+}, and 1 mM dithiothreitol) at 25 °C for 60 min. The GDP/GTP exchange reaction of hRheb was initiated by addition of excess GTP (~100 μM). At different time points 5 μl of the mixture was loaded to nitrocellulose membrane. After the membrane was dried, it was washed four times with 1 ml of ice-cold buffer B. The amount of the radiolabeled GDP bound to hRheb was quantified by scintillation counting. To determine the dose effect of hTCTP on hRheb, the GDP/GTP exchange assay was carried out at different concentration (0–1.5 μM) of the wild-type GST-hTCTP at 15 min after initiation of the reaction.

Cell Culture, Transfection, and Immunoblotting—For in vivo functional assay, the wild-type and mutant hTCTP and human S6K were cloned to the Xhol and BamHI restriction sites of the pHA-N3 vector (modified from the pGFP-N3) with the primers 5′-CCGCTCGAGATGATTATCTACCGGGACCTC-3′ (with the Xhol site underlined) and 5′-CCGGATCCCTTAAAGATT- TTTGCTATTAAAAACAT-3′ (with the BamHI site underlined). HEK293T cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 0.1 mg/ml streptomycin. For co-transfection of the plasmids, 5 × 10⁵ HEK293T cells were seeded in 6-well plate and 16 h later were transfected with 2 μg of the pHA-S6K plasmid and 2 μg of the pHA-hTCTP plasmid or the empty vector with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Forty hours after transfection, the cells were treated by replacing the medium with n-phosphate-buffered saline to remove the amino acids and serum. The cells were then assayed at 45, 60, 75, and 90 min after the treatment for analysis of the wild-type hTCTP, and at 75 min for analysis of the mutant hTCTP.

For co-transfection of the plasmids and siRNA for hTCTP, 2.5 × 10⁵ HEK293T cells per well were seeded in 6-well plates and 12 h later were transfected with 2 μg of the pHA-S6K plasmid and 100 pmol of hTCTP siRNA or control siRNA with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Sixty hours after transfection, the cells were assayed at 0, 10, 20, and 30 min after removal of amino acids. The oligoribonucleotide sequences of hTCTP siRNA are 5′-AGGGAACUUGAGAAGAGTT-3′ (sense) and 5′-CUGUCUUCAAGUGUUCCUTT-3′ (antisense) (GenePharma). For co-transfection of the plasmids and siRNA for hRheb, 2.5 × 10⁵ HEK293T cells per well in 6-well plates were transfected with 1.2 μg of the pHA-S6K plasmid, 1.2 μg of the pHA-hTCTP plasmid or the empty vector, and 60 pmol of hRheb siRNA or control siRNA with Lipofectamine 2000. Sixty hours after transfection, the cells were depleted with amino acids and assayed at 75 min post treatment. The oligoribonucleotide sequences of hRheb siRNA-1 were 5′-GAAGAGCUCUGAU- UGGATT-3′ (sense) and 5′-UCCAUAGCUCCUUCUU- CCTT-3′ (antisense), and those of hRheb siRNA-2 were 5′-UCAGUGAAGUUUGAGUUUUTT-3′ (sense) and 5′-AAACACGAAACUAACAGUGAGGGAGTT-3′ (antisense) (GenePharma). The sequences of control siRNA for all the RNA interference experiments are UUCUCUGAAGCUGAAGCUGTUTT (sense) and AGCUUGACACGUCCUUAGATT (antisense).

The antibodies used were for phosphoryl-p70 S6K (Thr-389, Cell Signaling), hRheb (Cell Signaling), Actin (Biyotime), HA (Biyotime), and hTCTP (Abcam), and the signal was detected by the ECL Advanced Western Blotting Detection Kit (Amer sham Biosciences).

Homology Modeling and Molecular Dynamics Simulation—The initial models of the hRheb-hTCTP complexes were built by superimposing the GDP-bound hRheb (PDB code 1XTQ) or GTP-bound hRheb (PDB code 1XTS) onto Rab8, and hTCTP (PDB code 1YZ1) onto Mss4, respectively, in the crystal structure of the Rab8-Mss4 complex (PDB code 2FU5) using the program VMD (version 1.8.4) (26). The models were subjected to molecular mechanics refinement using software package Groningen Machine for Chemical Simulations (GROMACS) 3.3 (27) with the GROMOS 53a6 force field (28). Conformations of the homology models of the complexes and the crystal structures of hRheb-GDP, hRheb-GTP, and hTCTP were sampled by molecular dynamics simulations. All starting structures were first subjected to energy minimization and subsequently placed in the center of a box that extended a minimum of 10 Å from the protein surface and solvated with simple point charge water molecules (29). In addition to the proteins and nucleotides, the system contains 16,131 water molecules, 36 Na⁺ ions, and 29 Cl⁻ ions with a final salt concentration of 100 mM NaCl to neutralize charges in the system and to emulate physiological conditions. A short equilibration run of 100 ps was then performed with the protein atoms restrained. The production runs were 15 ns long. Electrostatic interactions were evaluated using...
the Particle-Mesh Ewald method (30, 31) with a real space cut-off of 1.0 nm. Van der Waals interactions were modeled by a Lennard-Jones potential with a 1.4 nm cut-off. All of the bonds were constrained for the protein using the LINCS algorithm (32). During the simulations, temperature was maintained at 300 K, and the system pressure was coupled using the Berendsen method (33). Simulations of each set-up were repeated three times with different random initial velocities. The average structure was calculated from the last 10-ns time period of the 15-ns trajectory (path of the movements of the simulated model) using snapshots at intervals of 10 ps. Overall root mean square deviation (r.m.s.d.) values of the proteins were calculated using the backbone atoms only. r.m.s.d. values of the switch I region and the bound nucleotide were calculated using all atoms except hydrogen. The figures were prepared using PyMOL and VMD molecular visualization programs (26).

**Crystallographic Data Collection and Structure Determination**—Crystallization was carried out at 4 °C using the hanging drop, vapor-diffusion method. Crystals of the E12V mutant hTCTP were grown in drops containing equal volumes of the protein solution (10 mg/ml) and the reservoir solution (0.1 M Tris-HCl, pH 8.2, and 22% polyethylene glycol 6000). Diffraction data were collected from a flash-cooled crystal at beamline NW12 of Photon Factory, Japan, and processed using the program HKL2000 (34). Statistics of the diffraction data are summarized in Table 1.

The structure of the E12V mutant hTCTP was solved with the molecular replacement method implemented in the program Phaser of the CCP4 suite (35) using the structure of the wild-type hTCTP (PDB code 1YZ1) as the search model. The initial structure refinement was carried out with the program CNS (36) following the standard protocols, and the final structure refinement was performed with the maximum likelihood algorithm implemented in the program REFMAC5 (37). A free R-factor monitor calculated with 5% of randomly chosen reflections and a bulk solvent correction were applied throughout the refinement. There are four monomers in the asymmetric unit, which were refined independently. Model building was performed with the program COOT (38) and guided by SIGMAA-weighted 2Fo−Fc and Fo−Fc maps. A summary of the structure refinement statistics is given in Table 1.

**RESULTS AND DISCUSSION**

**hTCTP Can Activate the mTORC1 Pathway**—Previously, Hsu et al. showed that dTCTP can facilitate the GDP-GTP exchange of dRheb and regulate the TOR pathway (16). However, two research groups recently reported that hTCTP has no GEF activity toward hRheb and suggested that hTCTP is not involved in the mTORC1 pathway (11, 17). To resolve this issue, we first repeated the in vitro GST pulldown assay and GDP release experiments by Hsu et al. (16). Our results show that hTCTP is able to bind nucleotide-free hRheb weakly and to bind GDP- and GTP-bound hRheb very weakly (Fig. 1A) and that hTCTP can accelerate the GDP release of hRheb (Fig. 1B), which are consistent with those reported by Hsu et al. (16). To investigate whether hTCTP is able to activate hRheb and thus the mTORC1 pathway in vivo in stressed cells, we examined the effects of overexpression and down-regulation of hTCTP on S6K activation in amino acid-depleted HEK293T cells, which is a different protocol from that by Rehmann et al. in which the S6K activity was maintained at a basic level or induced by insulin (17). As shown in Fig. 1C, the mTORC1 pathway was activated when the cells were depleted of amino acids. However, in the cells overexpressing hTCTP, the mTORC1 pathway remained activated with apparently higher phosphorylation levels of S6K after removal of amino acids. Moreover, the elevated phosphorylation level of S6K was sustained for ~75 min after the amino acid depletion treatment and then decreased to a low level similar to that in the control cells at 90 min. These results clearly indicate that hTCTP prolongs activation of the mTORC1 pathway. Because Glu-127 of dTCTP has been shown to be critical for its GEF activity toward dRheb (16), a corresponding E12V mutant of hRheb was also analyzed, and we show that this mutant lacks the ability to prolong S6K phosphorylation (see details later). We further examined the effect of down-regulation of hTCTP on the phosphorylation of S6K. As shown in Fig. 1D, hTCTP siRNA significantly decreased the level of phosphorylated S6K in amino acid-depleted HEK293T cells. The discrepancies between our results and those obtained by the two other groups (11, 17) may be due to the differences of the assay systems. We studied the effect of hTCTP in cells depleted of amino acids without serum starvation overnight, whereas Wang et al. carried out the experiments with cells starved with serum overnight and further depleted of amino acids for 90 min with or without insulin treatment (11) and Rehmann et al. examined the effect of hTCTP in mammalian cells with serum starvation overnight with or without supplementation of insulin (17). Based on our in vitro and in vivo results and analyses of the previously reported data, we conclude that hTCTP does harbor GEF activity on hRheb and can regulate the mTORC1 pathway. Furthermore, we examined the effect of knockdown of hRheb by RNA interference on the activation of S6K by hTCTP overexpression. As shown in Fig. 1E, at 75 min after the treatment, hRheb knockdown by either of the two Rheb siRNAs utilized in this study substantially inhibited S6K activation even when hTCTP was overexpressed (Fig. 1E), indicating that hTCTP is an upstream activator of hRheb and the activation of S6K by hTCTP is dependent on hRheb.

**Homology Models of the hRheb-hTCTP Complexes Predict the Key Residues Involved in the Interaction**—The previous results by Hsu et al. (16) and our biochemical and cell biological data have demonstrated that hTCTP has a weak GEF activity toward hRheb. To understand the molecular basis of the interaction between hTCTP and hRheb, we attempted to prepare the hRheb-hTCTP complex and perform structural study of the complex. However, various experiments so far have failed to obtain a stable hRheb-hTCTP complex for crystallization presumably due to the weak interaction between hRheb and hTCTP. Alternatively, structure-based homology modeling is a powerful method to predict transient protein-protein interaction and to provide relatively accurate interaction information (39). Recently the crystal structure of the Rab8-Mss4 complex has revealed the mechanism of facilitation of the nucleotide exchange of Rab8 by Mss4 (25). Small GTPases are structurally similar with moderate sequence homology across the superfamily. Comparison of the amino acid sequences of hRheb and
**Structure Model of the hRheb-hTCTP Complex**

**FIGURE 1. hTCTP plays a regulatory role in the mTORC1 pathway through its GEF activity toward hRheb.** A, in vitro binding assay of GST-fused hTCTP with nucleotide-free or nucleotide-bound hRheb. The pulldown His-hRheb was detected by Western blot, and the inputs of HRheb and GST-hTCTP were analyzed by Coomassie Blue staining. B, in vitro GDP release assay of hRheb in the absence or presence of hTCTP. All experiments were performed in triplicate, and the error bars represent the standard deviations. The GEF activity of hTCTP is expressed as the percentage of the remaining[^3H]GDP to the initial[^3H]GDP bound to hRheb after the exchange reaction taking place for 15 min. C, effect of hTCTP overexpression on S6K phosphorylation in amino acid-depleted HEK293T cells. Approximately 5 x 10⁵ HEK293T cells were transfected with 2 μg of the pHA-S6K plasmid and 2 μg of the pHA-hTCTP plasmid or the empty vector. Forty hours after transfection, the cells were starved by replacing the medium with Dulbecco's phosphate-buffered saline to remove the serum and amino acids. The phosphorylation level of S6K was analyzed at indicated time points post deprivation treatment. Actin served as a loading control. D, effect of TCTP down-regulation on S6K phosphorylation. HEK293T cells were co-transfected with 100 pmol of control siRNA or TCTP siRNA along with 2 μg of pHA-S6K. The phosphorylation level of S6K was analyzed at indicated time points after removal of the amino acids. E, hRheb dependence of S6K phosphorylation in hTCTP-overexpressing cells. HEK293T cells were co-transfected with 60 pmol of control siRNA, hRheb siRNA-1, or hRheb siRNA-2 along with 1.2 μg of pHA-S6K and 1.2 μg of pHA-hTCTP or the vector control. The phosphorylation level of S6K was analyzed at 75 min after removal of the amino acids.

Rab8 with the BLAST program from NCBI (blast.ncbi.nlm.nih.gov/Blast.cgi) shows that hRheb shares a sequence identity of 29% and a sequence similarity of 52% with Rab8, and the structure of hRheb resembles that of Rab8 with an r.m.s.d. of 4.3 Å for 98 Ca atoms. Although hTCTP and Mss4 do not share evident sequence homology, the two proteins are structurally similar with an r.m.s.d. of 2.9 Å for ~80 Ca atoms of the core region (23). Thus, we constructed three-dimensional homology models of the hRheb-GDP-hTCTP and hRheb-GTP-hTCTP complexes based on the crystal structure of the Rab8-Mss4 complex (Fig. 2A, see “Experimental Procedures” for details of the model building). The modeled complexes resemble each other except the switch I region of hRheb (Fig. 2B). In the work by Rehmann et al., a model of the hTCTP-Rab8 complex was constructed by superposing hTCTP with the Mss4/Rab8 structure and using Rab8 as a model for hRheb (17). In our model, the crystal structure of hRheb is used rather than that of Rab8 in which the switch I region forms a loop instead of an α-helix, and thus the steric clash between the insertion of hTCTP and hRheb predicted by Rehmann et al. is not observed.

In our model, at the protein-protein interface strand β2 of hRheb (the nomenclature of the secondary structures of hRheb is after that of Yu et al. (24)) interacts with strand β7 of hTCTP (the nomenclature of the secondary structures of hTCTP is shown in supplemental Fig. S1) to form an intermolecular anti-parallel β-sheet as observed in the Rab8-Mss4 complex (25) (Fig. 2C). The main chains of residues Glu-40R (residues of hRheb will be designated by a superscripted suffix R hereafter), Asn-41R and Thr-42R on β2 of hRheb interact with those of Glu-80T, Thr-81T, and Ser-82T on β7 of hTCTP. The main chain of Thr-44R forms hydrogen bonds with the side chain of Gln-79T; the side chains of Thr-42R and Glu-40R form a hydrogen bond with the side chains of Glu-80T and Thr-81T, respectively. Moreover, the β1–β2 loop (residues 9–12) and the TCTP2 signature motif (residues 138–140) of hTCTP also make interactions with β2 of hRheb (Fig. 2D). Specifically, residue Glu-138T shares a similar physicochemical property as the equivalent residue Asp-96 of Mss4 and forms a salt bridge with Lys-45R (equivalent to Arg-48 of Rab8), which is also conserved in the Rab8-Mss4 complex. Residues Glu-12T and Met-140T are also involved in the interactions which, however, differ from their counterparts in the Rab8-Mss4 complex. Glu-12T forms a salt bridge with Lys-45R, whereas the equivalent residue Arg-29 of Mss4 is not involved in interaction with Rab8. Met-140T makes hydrophobic contacts with Ile-24R, Val-32R, and Phe-43R, whereas the equivalent residue Glu-98 of Mss4 forms a salt bridge with Lys-46 of Rab8 (corresponding
to Phe-43R). These distinct interactions may account for the specificities of these GEFs for their respective targets.

**MD Simulations of the Modeled Complexes Suggest a Major Conformational Change of Switch I of hRheb in the GDP-GTP Exchange**—Various structural studies of small GTPases have shown that switch I, switch II, and/or the P-loop are essential for nucleotide binding and usually undergo significant conformational changes in the GDP-GTP exchange (40, 41). In the Rab8-Mss4 complex, switch I of the unliganded Rab8 interacts directly with Mss4 and displays a conformation substantially different from that of the homolog Sec4, suggesting that Mss4 binding may induce a conformational change of switch I of Rab8 (25). Analysis of the hRheb-hTCTP models predicts that switch I of hRheb assumes different conformations when bound with GDP and GTP, even though it does not participate in interaction with hTCTP, whereas both switch II and the P-loop adopt similar conformations and have no interaction with hTCTP in both complexes. To identify the mobile region(s) of the hRheb-hTCTP complexes and sample more conformational spaces, we carried out MD simulations of the

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**FIGURE 2.** Homology models of the hRheb-hTCTP complexes. A, overall structure of the modeled hRheb-GDP-hTCTP complex. hRheb is colored in cyan and hTCTP in yellow. The secondary structural elements are labeled, and the bound GDP is shown with a ball-and-stick model. Switch I, switch II, and the P-loop of hRheb and the TCTP2 region of hTCTP are colored in blue, green, pink, and purple, respectively. Strand β2 of hRheb forms an intermolecular β-sheet with strand β7 of hTCTP. B, comparison of the GDP- and GTP-bound hRheb (cyan and violet, respectively) in the structure models of the hRheb-hTCTP complexes showing the conformational differences of the switch I region of hRheb. C, comparison of the structure model of the hRheb-GDP-hTCTP complex and the crystal structure of the Rab8-Mss4 complex. hRheb is colored in cyan and hTCTP in yellow. Rab8 and Mss4 are colored in green and red, respectively. D, comparison of part of the interaction interface in the hRheb-GDP-hTCTP complex and the Rab8-Mss4 complex. The key residues that are predicted to be involved in the interactions are shown with side chains. The inter-molecular salt bridges are indicated with dashed lines. The color coding of the proteins is the same as in Fig. 1C.
modeled complexes as the MD simulation method has been increasingly successful in characterization of protein-protein interaction (42). We performed 15-ns simulations of the modeled complexes and the crystal structures of hRheb-GDP, hRheb-GTP, and hTCTP, respectively. The overall r.m.s.d. values of the backbone atoms of the complexes fluctuate around 2.5 Å, which is about the average r.m.s.d. between the simulated and experimental structures (43).

Analysis of the MD simulation trajectory of the modeled hRheb-GDP-hTCTP complex shows that switch I of hRheb displays a large movement (Fig. 3A). Comparison of the initial and average structures indicates that switch I of hRheb displaces ~4 Å from its starting position toward hTCTP (Fig. 3B). In the average structure, switch I of hRheb interacts with hTCTP mainly through three residues: Tyr-35R, Asp-36R, and Ile-39R (Fig. 3C). Tyr-35R and Ile-39R form a hydrophobic patch and point toward a hydrophobic cluster of hTCTP consisting of Phe-83T, Tyr-91T, and Pro-142T. Additionally, Asp-36R of hRheb forms a salt bridge with Lys-90T of hTCTP. The movement of switch I is accompanied by conformational and positional changes of the bound nucleotide. Comparison of the initial and average structures shows that GDP exhibits a displacement of ~6 Å from its starting position (Fig. 3A). In the initial model, GDP is bound in the canonical mode: the nucleoside moiety of GDP is sandwiched by 310 helix and the switch I-containing loop, and the diphosphoryl group is stabilized by the P-loop. In the average structure, displacement of switch I weakens the constraints on the nucleoside, leading to the movement of the nucleoside with the tip of the base pointing away from the binding pocket (Fig. 3B). The diphosphoryl group maintains a similar position which appears to be constrained by the stable P-loop.

In contrast, simulation of the modeled hRheb-GTP-hTCTP complex shows that both switch I and the bound GTP are stable, and Tyr-35R covers the top of the nucleotide and forms a hydrogen bond with the γ-phosphate, which is also seen in the crystal structure (24). Similarly, simulations of the GDP- and GTP-bound hRheb

FIGURE 3. MD simulations of the structure models of the hRheb-hTCTP complexes and the crystal structures of hRheb. A, r.m.s.d. values of switch I (residues Asp-33 through Asn-41) of hRheb (top panel) and the bound GDP in the hRheb-GDP-hTCTP complex exhibit significant deviations. B, conformational differences of switch I in the crystal structure of hRheb-GDP (gray) and the average structures of the simulated hRheb-GDP alone (cyan) or in complex with hTCTP (green). The other regions of hRheb are stable in the simulations. For clarity, only the crystal structure of hRheb-GDP (gray) is shown with the whole structure. The average structures were calculated from the MD trajectories of the last 10 ns. The bound nucleotides are shown with ball-and-stick models. C, the interactions between switch I of hRheb and hTCTP in the simulated hRheb-GDP-hTCTP complex. A potential salt bridge between Asp-36R of switch I and Lys-90T of hTCTP is indicated with a dashed line. Several residues at the interaction interface are shown for reference.
structures show that, in the GDP-bound hRheb, switch I exhibits some conformational change at the beginning of the simulation but converges to a stable conformation along the simulation and GDP exhibits a displacement of ∼5 Å; whereas in the GTP-bound hRheb, both switch I and GTP maintain stable positions (Fig. 3A). Analyses of the GTP- and GDP-bound hRheb structures provide a possible explanation for these results. In the hRheb-GDP structure, switch I of hRheb has little contacts with the nucleotide, and thus both switch I and GDP have relatively high flexibility; whereas in the GTP-hRheb structure, GTP binds tightly to switch I via four hydrogen bonds, which restrict the flexibility of switch I and GTP (supplemental Fig. S2) (24). During all of the simulations, switch II and the P-loop undergo little conformational changes (data not shown), which are consistent with the observation that these regions do not show obvious conformational differences in the GDP- and GTP-hRheb structures (24). The simulation results, together with the previous crystal structure results, suggest that switch I of hRheb has a great flexibility and undergoes a significant conformational change upon GDP-GTP exchange. In the presence of hTCTP, switch I of hRheb moves toward hTCTP and decreased contacts between switch I and GDP result in increased dynamics of switch I and GDP, suggesting that hTCTP binds to the switch I region of hRheb and opens the nucleotide-binding site to facilitate GDP dissociation. This is consistent with the notion that GEFs function by disturbing the nucleotide-binding site of small GTPases through induction of conformational changes of the switch regions and/or the P-loop (44).

Validation of the Key Residues in the hRheb-hTCTP Interaction—Analyses of the hRheb-hTCTP models and the MD simulations have allowed us to predict the key residues participating in the hRheb-hTCTP interaction and the region undergoing significant conformational change during the GDP-GTP exchange.

FIGURE 4. Validation of the key residues in the hRheb-hTCTP interaction. Binding assay of wild-type and mutant hRheb with the GST-fused wild-type hTCTP (A) and that of wild-type hRheb with the GST-fused wild-type and mutant hTCTP (B). The GST pulldown results are shown in the upper panel, and equal loading of hRheb and GST-fused hTCTP proteins are in the middle and lower panels, respectively. The apparent molecular weight of the K45D mutant hRheb is slightly larger than that of the other hRheb proteins for an unknown reason. GST served as a negative control. C, in vitro GDP release assay of hRheb accelerated by wild-type and mutant hTCTP. D, in vivo functional analysis of the capability of wild-type and mutant hTCTP to activate the mTORC1 pathway. The phosphorylation level of S6K was examined at 75 after removal of the amino acids. Actin served as a loading control.
Structure Model of the hRheb·hTCTP Complex

exchange of hRheb. Alignments of the available Rheb sequences from 24 species and the corresponding TCTP sequences show that the involved residues Tyr-35R, Glu-12T, and Glu-138T are strictly conserved and residues Phe-43R, Lys-45R, and Met-140T are highly conserved (supplemental Fig. S1). Besides, residues Asp-36R and Lys-90T are conserved in mammals and birds. To further investigate the functional roles of the residues at the interaction interface, we performed site-directed mutagenesis studies and in vitro GST pull-down assays. We examined Lys-45R of switch I of hRheb and Glu-12T and Glu-138T of hTCTP2, which are predicted to form two salt-bridging interactions (Fig. 2D). The results of the hRheb-GDP·hTCTP complex model predict that the switch I region of hRheb interacts with hTCTP via extensive hydrophobic interactions. In particular, Tyr-43R and Ile-39R of hRheb make hydrophobic contacts with several residues of hTCTP, including Phe-83T, Tyr-91T, and Pro-142T. Mutation of Tyr-35R to Ala impairs these hydrophobic contacts and therefore diminishes the binding of hRheb with hTCTP (Fig. 4A). In the simulated hRheb-GDP·hTCTP complex, Asp-36R of hRheb forms a salt bridge with Lys-90T of hTCTP. Consistently, the K90E mutant hTCTP exhibits impaired binding with hRheb due to loss of the salt bridge (Fig. 4B).

We further examined the abilities of the hTCTP mutants to accelerate the GDP release of hRheb in vitro and to regulate the mTORC1 pathway in vivo. Our results show that the activities of the hTCTP mutants are correlated well with their binding abilities with hRheb in general. The E12V, K90E, and E138A mutants of hTCTP exhibit significantly diminished capabilities to stimulate GDP dissociation of hRheb (Fig. 4C). The R5A mutant seems to have a slightly enhanced GEF activity compared with the wild-type protein, but the difference appears to be statistically not significant. Because the Y35A and K45D mutants of hRheb showed impaired binding with hTCTP, their abilities to respond to hTCTP in the GDP release assay were also examined. The velocities of GDP release by the mutants alone were similar to that of the wild-type hRheb (supplemental Fig. S3). However, neither mutant showed enhanced GDP release in the presence of hTCTP (Fig. 4C). The in vivo activities of those mutants were further analyzed. Consistently, the mutations (E12V, K90E, and E138A) that impair the GEF activity of hTCTP in vitro also impede its ability to activate the mTORC1 pathway in vivo (Fig. 4D). Quantitative analysis of results in Fig. 4D shows that the level of the phosphorylated S6K in cells transfected with the E138A mutant is ∼50% of that in cells with the wild-type hTCTP. In particular, phosphorylation of S6K was not observed in the cells overexpressing the E12V and K90E mutants of hTCTP. These results indicate that the key residues of hTCTP involved in the interaction with hRheb are important for its GEF activity toward hRheb and its regulatory function in the mTORC1 pathway, supporting the notion that hTCTP regulates the mTORC1 pathway via its interaction with hRheb.

Table 1: Statistics of x-ray diffraction data and structure refinement

| Diffraction Data Statistics | Resolution (Å) | Space group | a (Å) | b (Å) | c (Å) | Mosaicity | Total reflections | Unique reflections | Redundancy |
|-----------------------------|----------------|-------------|------|------|------|----------|------------------|-------------------|------------|
| 50.0-2.60 (2.69-2.60)¹      | P2₁2₁2₁        | 78.2        | 81.5 | 139.9| 1.4   | 110,151   | 27,481           | 4.0 (4.7)        |
| 97.5 (96.7)                 |                |             |      |      |       | 13.6 (2.9)          |
| 9.6 (46.7)                  |                |             |      |      |       | 7.6           |

¹ The numbers in parentheses refer to the highest resolution shell.

Refinement and Structure Model Statistics

| No. of reflections (Rmerge (%)) | 27,481 |
|-------------------------------|--------|
| Working set                   | 26,115 |
| Free R set                    | 1.366  |
| R-factor                      | 0.237  |
| Free R-factor¹                | 0.280  |
| Subunits/ASU                  | 4      |
| Total protein atoms           | 4,792  |
| Total solvent atoms           | 38     |
| Averaged B-factor (Å²)        | 55.8   |
| Protein atoms                 | 55.9   |
| Solvent atoms                 | 50.7   |
| r.m.s.d. bond lengths (Å)     | 0.090  |
| r.m.s. bond angles (°)        | 0.89   |
| Ramachandran plot (%)         | 92.4   |
| Most favored                  |        |
| Allowed                       | 7.6    |
| Luzzati atomic positional error (Å) | 0.43 |

Table 1 statistics of x-ray diffraction data and structure refinement

The previous biochemical data have shown that mutation of Glu-12T of dTCTP to Val abolishes its GEF activity toward dRheb (16). Our in vitro biochemical and in vivo cell biological assays also demonstrate that the E12V mutant of hTCTP has undetectable binding to hRheb and an abolished GEF activity to hRheb (Fig. 4, A and C) and can no longer activate the mTORC1 pathway (Fig. 4D). Sequence analysis shows that Glu-12 of TCTP is strictly conserved in all species (supplemental Fig. S1). These results indicate that Glu-12T of hTCTP is a key residue involved in the interaction with hRheb. To understand the molecular basis of the effect caused by the E12V mutation of hTCTP, we determined the crystal structure of the E12V mutant of hTCTP at 2.6-Å resolution (Table 1). The space group of the E12V mutant belongs to P2₁2₁2₁, which is different from that of the wild-type hTCTP (P2₁) (PDB code 1YZ1). The overall structure of the mutant is very similar to that of the wild-type protein with an overall r.m.s.d. of 1.2 Å for all atoms, indicating that the E12V mutation does not cause obvious conformational change of the protein (Fig. 5A). The previous structural analysis of the wild-type hTCTP by Thaw et al. suggests that Glu-12T, Leu-78T, and Glu-138T of hTCTP may form a potential small GTPase-binding groove (23). In the structure models of the hRheb·hTCTP complexes, both Glu-12T and Glu-138T of hTCTP form salt-bridging interactions with Lys-45R of hRheb. The importance of these two residues is further supported by both in vitro and in vivo assay results. However, Leu-78T of hTCTP is not involved in direct interaction with hRheb. Detailed structural comparison of the wild-type and the E12V mutant hTCTP in the putative GTPase-binding groove region shows that both Leu-78T and Glu-12T of hTCTP are key residues involved in the interaction with hRheb. This result is consistent with the in vitro data showing that mutation of Glu-12T of dTCTP to Val abolishes its GEF activity toward dRheb.
and Glu-138<sup>T</sup> maintain similar side-chain conformations, whereas Arg-5<sup>T</sup> adopts different side-chain conformations. In the wild-type hTCTP structure, the side chain of Arg-5<sup>T</sup> points toward and may form a potential electrostatic interaction with the side chain of Glu-12<sup>T</sup> (~4 Å); while in the E12V mutant hTCTP, the side chain of Arg-5<sup>T</sup> points away from the side chain of Val-12<sup>T</sup> apparently due to the change of the side chain (Fig. 5B). As the potential electrostatic interaction between Arg-5<sup>T</sup> and Glu-12<sup>T</sup> could weaken the interaction between Glu-12<sup>T</sup> and Lys-45<sup>R</sup> by neutralizing the negative charge of Glu-12<sup>T</sup>, we predict that mutation of Arg-5<sup>T</sup> to Ala would enhance the salt-bridging interaction between Glu-12<sup>T</sup> and Lys-45<sup>R</sup> and thus the hRheb-hTCTP interaction. As expected, the R5A mutant hTCTP shows an increased binding ability with hRheb (Fig. 4B) and displays a GEF activity comparable to if not stronger than that of wild-type hTCTP (Fig. 4C), further supporting the importance of the interaction between Glu-12<sup>T</sup> of hTCTP and Lys-45<sup>R</sup> of hRheb. The R5A mutant hTCTP did not exhibit an increased activating ability for the mTORC1 pathway probably due to the very low expression level of the mutant in the cells (Fig. 4D). These results not only validate our homology models but also support the notion that Glu-12<sup>T</sup> is essential for hTCTP function due to its critical role in the interaction with hRheb.

It is noteworthy that there are four hTCTP molecules in the asymmetric unit that form two homodimers, and there is an intermolecular disulfide bond between Cys-172<sup>T</sup> of adjacent monomers (Fig. 5A). Considering that murine TCTPs are prone to interaction with each other through a C-terminal region of residues 126–172 (45), the disulfide bond seen in the mutant hTCTP structure may account for the tendency of TCTP to dimerize or oligomerize. Further investigation is needed to find out whether dimerization of TCTP and formation of the disulfide bond have any roles in the modulation of TCTP function.

In summary, our biochemical, cell biological, modeling, and structural data together demonstrate that hTCTP positively regulates the mTORC1 pathway via acceleration of the GDP-GTP exchange of hRheb. Our results not only resolve the argument about the involvement of hTCTP in the mTORC1 pathway but also provide insights into the molecular mechanism of the biological function of hTCTP in the mTORC1 pathway.

**Protein Data Bank Accession Code**—The structure of the E12V mutant hTCTP has been deposited with the RCSB Protein Data Bank under accession code 3EBM.
Structure Model of the hRheb-hTCTP Complex

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