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Identification of TBC7 having TBC domain as a novel binding protein to TSC1–TSC2 complex

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Abstract

TBC7, a TBC (Tre-2/Bub2/Cdc16) 1 domain protein, was identified as a novel binding protein to the TSC1–TSC2 tumor suppressor complex by peptide mass fingerprinting analysis of the proteins immunoprecipitated with FLAG-epitope tagged TSC1 and TSC2 from the transfected mammalian cells. The *in vivo* and *in vitro* association of TBC7 and the TSC1–TSC2 complex was confirmed by the co-immunoprecipitation and pull-down analysis, respectively, and TBC7 was revealed to bind to the C-terminal half region of TSC1, which is distinct from the binding site with TSC2. The immunofluorescence microscopy and subcellular fractionation showed that TBC7 co-localizes with the tumor suppressor complex in the endomembrane. Overexpression of TBC7 enhanced ubiquitination of TSC1 and increased phosphorylation of S6 protein by S6 kinase, that is located in the mTOR-signaling pathway. These results indicate TBC7 could take a part in the negative regulation of the tumor suppressor complex through facilitating the downregulation of TSC1.

Keywords: TSC1; TSC2; TBC7; Rheb; mTOR; Protein phosphorylation; Ubiquitination

Tuberous sclerosis complex (TSC) is an autosomal dominant disorder characterized by hamartoma formation in various organs caused by mutation in either the *TSC1* or *TSC2* tumor suppressor gene [1]. The *TSC1* and *TSC2* gene products, also known as hamartin and tuberin, respectively, form a stable dimer to function as a GTPase activating protein (GAP) toward Rheb, a small GTPase [2]. Through the stimulation of GTP hydrolysis of Rheb, the TSC1–TSC2 complex negatively regulates the mammalian target of rapamycin (mTOR), an evolutionarily conserved serine/threonine protein kinase that phosphorylates the translational regulators such as S6 kinase and 4E-BP1 to control the protein synthesis. Importantly, the TSC1–TSC2 complex receives inputs not only from the phosphatidylinositol 3-kinase-Akt signaling pathway but also from those including Erk-p90 ribosomal S6 kinase and LKB1-AMPK resulting in the phosphorylation of TSC2 [3]. The phosphorylation reactions of TSC2 by Akt and Erk dissociate TSC1 from TSC2, whereas the modification reaction by AMPK enhances its GAP activity. On the other hand, the TSC1–TSC2 complex is retained in the endomembrane to regulate Rheb and its downstream mTOR pathway, because Rheb localizes in the membrane in a farnesylation-dependent manner [4]. The conflicting results, however, have been reported concerning the subcellular localization of TSC1 and TSC2. For instance, these two proteins are fractionated exclusively in the membrane fraction [5]; they distribute both in the cytosol and membrane fractions [6]; and TSC1 is restricted only in the membrane fraction while localization of TSC2 in these fractions is regulated by the Akt-mediated phosphorylation [7]. Moreover, there are several proteins that associate with either TSC1 or TSC2 [8]. For example, TSC1 and TSC2 have been shown to be associated with RB1CC1/FIP200 and HERC1 ubiquitin ligase, respectively, to be downregulated through the ubiquitin-proteasomal pathway [9–11]. Thus,

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the activity of the TSC1–TSC2 complex is regulated by multiple mechanisms.

In this study, we demonstrate that TBC7, a protein containing *TBC* (Tre-2/Bub2/Cdc16) 1 domain [12], is a novel binding protein to the TSC1–TSC2 complex, and that the introduction of TBC7 enhances the mTOR signaling pathway through the interaction with TSC1 and its downregulation.

Materials and methods

Antibodies. The anti-FLAG (M2), anti-myc (9E10), and anti-α-tubulin antibodies were purchased from Sigma; the anti-HA antibodies (12CA5 and 3F10) were from Roche; the anti-TSC1, anti-S6 ribosomal protein, and anti-phospho-S6 ribosomal protein (pS235/236) antibodies were from Cell Signaling Technology; the anti-GST antibody was from Upstate Biotechnology; the anti-TSC2 (C-20) and anti-calnexin (H-70) antibodies, and normal rabbit immunoglobulin G were from Santa Cruz Biotechnology. The horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit antibodies were obtained from Jackson ImmunoResearch Laboratories and Bio-Rad, respectively. The Alexa Fluor 488-labeled anti-rat and Alexa Fluor 546-labeled anti-mouse antibodies were obtained from Invitrogen. The rabbit polyclonal anti-peptide antiserum recognizing TBC7, T98, was produced by Immuno-Biological Laboratories by immunizing rabbits with the synthetic peptide VSDATPQAEVYLRMYQ LES (amino acids 98–116) coupled with thyroglobulin.

cDNAs and siRNAs. The cDNA encoding human TBC7 (Accession No. NM_016495, GeneCopoeia) was amplified by PCR with following primers: 5′-GCAGTGGAATTCATGACTGAGGACTCTCAGAGAA-3′ and 5′-GCAGTGCTCTCGAGTCAGCTTGAATGGACCGGG-3′, where underlined sequences indicate the restriction enzyme sites added. The product was cloned into pcDNA3 with HA- or myc-epitope tag. The GST-TBC7 fusion protein vector was generated by cloning the PCR product into pGEX. FLAG-tagged TSC1 and FLAG-tagged TSC2 in pcDNA3 were kindly provided by Dr. David J. Kwiatkowski (Brigham and Women's Hospital, Boston). Myc-tagged TSC1 wild type (amino acids 1–1164) and its deletion mutants, N (amino acids 1–510), C (amino acids 511–1164), C-1 (amino acids 511–880), C-2 (amino acids 881–1164), and C-C (amino acids 730–996), were generated in pcDNA3. The expression vector of HA-tagged ubiquitin in pCGN was kindly provided by Dr. Mitsuyoshi Nakao (Kumamoto University). Small interfering RNA (siRNA) for targeting the coding sequence of human TSC1 was designed as described [13]. Scramble control siRNA was purchased from iGENE Therapeutics.

Cell culture and transfection. HEK293, HeLa, and HeLa S cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma) with 10% fetal bovine serum (Gibco BRL), and NIH3T3 cells were maintained in DMEM with 10% bovine serum (Gibco BRL) at 37 °C in a 5% CO_2 incubator. HEK293 cells were transfected with expression vectors by lipofection using lipofectAMINE (Invitrogen), and NIH3T3 and HeLa S cells were transfected by nucleofection using Nucleofector 3 (AMAXA Biosystems) according to the manufacturers' protocols. Unless otherwise indicated, 1 μg of each plasmid was employed for 10^7 cells. For the siRNA studies, HEK293 cells were transfected with 240 pmol of either TSC1 or control siRNA by nucleofection. The cells were then cultured for one to two days before the experiments.

Immunoprecipitation. The following procedures were carried out at 0–4 °C. The cells were washed with Dulbecco's phosphate-buffered saline (D-PBS) and lysed with Buffer A (20 mM Tris–HCl at pH 7.5, 120 mM NaCl, 1 mM EDTA, 20 mM β-glycerophosphate, 5 mM EGTA, 0.3% CHAPS, 20 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 4 μg/ml leupeptin, and 1 mM dithiothreitol). After centrifugation at 15,000*g* for 25 min, the supernatant was incubated with 15 μl of Protein G-Sepharose (GE Healthcare) coupled with each antibody for 2 h, and the immunoprecipitate was washed three times with Buffer A.

Mass spectrometry. FLAG-TSC1 and -TSC2 immunoprecipitates were eluted from the resin with Buffer A containing 200 μg/ml FLAG peptide (Sigma), and the proteins were separated by SDS–PAGE and visualized by silver staining. Mass spectrometric analysis was carried out essentially as described [14].

In vitro GST pull-down assay. GST-TBC7 and GST proteins expressed in *Escherichia coli* were purified as described [15]. HEK293 cell lysate in Buffer A was incubated for 2 h at 4 °C with glutathione–Sepharose immobilized with 38, 150, and 600 pmol of either GST-TBC7 or GST. The resin was then washed three times with Buffer A.

Immunoblotting. The samples were separated by SDS–PAGE, and the proteins were transferred to a polyvinylidene difluoride membrane and subjected to immunoblotting using each primary antibody. After incubation with the HRP-conjugated secondary antibodies, detection of the proteins was carried out by the enhanced chemiluminescence reaction. When endogenous TBC7 was detected, HRP-conjugated Protein A (Bio-Rad) was employed instead of the HRP-conjugated secondary antibodies.

Results and discussions

Identification of TBC7 as a novel binding protein to the TSC1–TSC2 complex

The TSC1 and TSC2 proteins with FLAG-epitope tag were immunoprecipitated from the transfected HEK293 cells, and the recovered proteins were visualized by silver staining after SDS–PAGE (Fig. 1A). In addition to the FLAG-epitope tagged proteins, the protein bands of approximate molecular mass of 70 and 30 kDa were detected. The former was found in the control immunoprecipitate, and thus the 30-kDa protein (p30) was further studied. Peptide mass fingerprinting analysis using mass spectrometry identified the protein as TBC7 [*TBC* (Tre-2/Bub2/Cdc16) 1 domain family member *7*, Accession No. NP_057579]. Human TBC7 consists of 293 amino acids with a putative TBC domain composed of approximately 200 amino acid residues (Supplemental Fig. 1A). The TBC domain is conserved among eukaryotes, and the human genome is predicted to encode at least 50 proteins containing this domain [12,16]. Several TBC proteins, such as GAP-CenA, RN-Tre, and RabGAP-5, have been shown to have a GAP activity for the Rab small GTPase family [17–19]. In addition, it has been reported that three 'fingerprint' sequence motifs, RxxxW, IxxDxxR, and YxQ, in the N-terminal region of the TBC domain are highly preserved among the members of the Rab-GAP family [20], and that the arginine residue in the second motif is essential for the GAP activity [21]. The database search revealed that TBC7 orthologs exist in *Homo sapiens*, *Mus musculus*, *Xenopus laevis*, and *Drosophila melanogaster*, but not in *Caenorhabditis elegans* or two yeasts (Supplemental Fig. 1B). Furthermore, the TBC domains of human TBC7 and its orthologs only have the first 'fingerprint' motif but lack other two motif sequences (Supplemental Fig. 1B). It is thus unclear whether TBC7 has a GAP activity, and accordingly the function of TBC7 has not been elucidated. Northern blot analysis revealed that a single *TBC7* mRNA of approximately 1.1 kb is highly expressed in heart, and slightly in kidney, liver, and placenta among human tissues (Supplemental Fig. 2). On the other hand, *TSC1* mRNA was widely expressed among human tissues in consistency with

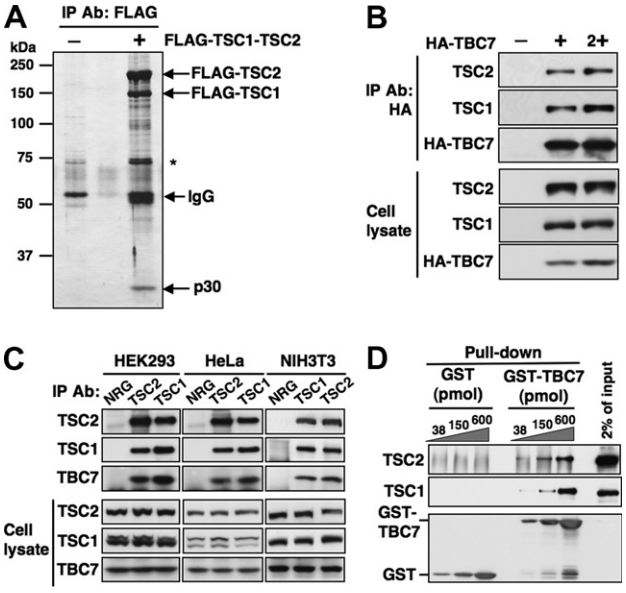

Fig. 1. Association of TBC7 with the TSC1–TSC2 complex. (A) Identification of a 30-kDa protein as a TSC1–TSC2 complex associated protein. The lysates from HEK293 cells transfected with the FLAG-TSC1 and -TSC2 vectors or empty vector were subjected to immunoprecipitation with the anti-FLAG antibody, and the proteins eluted from resin were visualized by silver staining after SDS–PAGE. FLAG-TSC1, FLAG-TSC2, IgG, and a 30-kDa protein (p30) are indicated by arrows. Asterisk shows a protein of 70 kDa. The positions of the size standards are indicated in kDa. (B) Association of epitope-tagged TBC7 with the endogenous TSC1–TSC2 complex. HEK293 cells were transfected with HA-TBC7 or the empty vector. Where indicated as 2+, the twice as much as amounts of the HA-TBC7 expression vector was employed. Immunoprecipitates with the anti-HA antibody (12CA5) were analyzed by immunoblotting with the indicated antibodies. (C) Association of endogenous TBC7 and the TSC1–TSC2 complex. The cell lysates and immunoprecipitates with the anti-TSC1 or -TSC2 antibody from the cell lines were analyzed by immunoblotting with the indicated antibodies. Normal rabbit immunoglobulin G (NRG) was used as a control. (D) Association of TBC7 with the TSC1–TSC2 complex *in vitro*. The cell lysates from HEK293 cells were incubated with glutathione–Sepharose coupled with either GST or GST-TBC7, and the proteins were analyzed by immunoblotting with the indicated antibodies.

the previous report [22]. These results indicate that TBC7 and the tumor suppressor protein are expressed differently among the tissues, although the profiles are not exclusive to each other.

TBC7 associates with the TSC1–TSC2 complex in vivo and in vitro

The epitope-tagged TBC7 was expressed in HEK293 cells and its association with the endogenous TSC1 and TSC2 was examined (Fig. 1B). Both of the endogenous tumor suppressor proteins were detected in the HA-TBC7 immunoprecipitate but not in the control immunoprecipitate. The amounts of co-precipitated TSC1 and TSC2 increased in parallel with the expression level of HA-TBC7. Immunoblotting using a polyclonal antibody against TBC7 indicated that TBC7 is detected in the immunoprecipitates by anti-TSC1 and anti-TSC2 antibodies prepared from three different cell lines, HEK293, HeLa, and NIH3T3 cells, but not in those by normal rabbit immunoglobulin G (Fig. 1C). Furthermore, the *in vitro* pull-down analysis showed that both of TSC1 and TSC2 obtained from HEK293 cells bound to the bacterial GST-TBC7 in a dose-dependent manner, but not to the control protein (Fig. 1D). On the other hand, phosphorylation of TSC2 by Akt/PKB and Erk leads to the dissociation of the TSC1–TSC2 complex [3]. The interaction of TBC7 with the TSC1–TSC2 complex was, however, not altered by the growth factor stimuli such as insulin, epidermal growth factor, and serum (data not shown).

TBC7 binds to TSC1 through the coiled-coil domain of TSC1

When the three proteins were co-expressed, myc-TSC1 and FLAG-TSC2 were efficiently immunoprecipitated with HA-TBC7 (Fig. 2A). The association of myc-TSC1 with HA-TBC7 was detected efficiently in the cells even without FLAG-TSC2 as in the cells these three proteins were co-expressed. In contrast, the association of FLAG-TSC2 to HA-TBC7 was little in the cells without transfection of myc-TSC1. These results raise the possibility that TBC7 associates with TSC1 rather than TSC2. A small amount of FLAG-TSC2 recovered with HA-TBC7 in the absence of myc-TSC1 may be associated with the endogenous TSC1 in the immunoprecipitate. Indeed, siRNA-mediated knockdown of TSC1 led to the decrease in the amount of endogenous TBC7 recovered in the TSC2 immunoprecipitate (Fig. 2B). Therefore, TBC7 was concluded to associate directly with TSC1. To determine the binding region in TSC1 with TBC7, a series of the TSC1 deletion mutants were generated (Fig. 2C). TSC1 has potential transmembrane and coiled-coil domains at the N- and C-terminal regions, respectively [8]. As shown in Fig. 2D, TSC2 bound to both of the N- and C-terminal fragments of TSC1 in cultured cells in consistency with the previous report [23]. On the other hand, TBC7 was not detected in the immunoprecipitate of the N-terminal fragment (N) but in those of the C-terminal fragment (C) and the coiled-coil domain (C-C). Furthermore, the C-terminal half of the coiled-coil domain (C-2) was sufficient for the binding of TBC7 but unsatisfactory for TSC2, whereas the N-terminal half of the domain (C-1) did not associate with either TBC7 or TSC2. Taken together, TBC7 binds to the C-terminal half of the coiled-coil domain in TSC1, which is distinct from the TSC2 binding region.

Co-localization of TBC7 and the TSC1–TSC2 complex

The intracellular localization of TBC7 was examined by immunostaining HA-TBC7 in NIH3T3 cells co-expressed with myc-TSC1 and FLAG-TSC2. Immunofluorescence microscopy revealed the co-localization of HA-TBC7 with myc-TSC1 in the cytoplasm (Supplemental Fig. 3A). The staining of myc-TSC1 had a punctate appearance

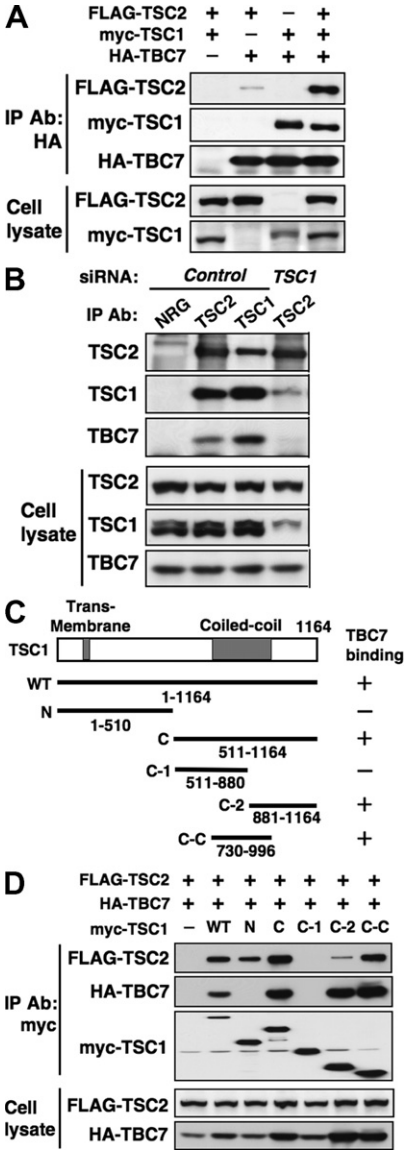

Fig. 2. Association of TBC7 with TSC1. (A) Association between TBC7 and the TSC1–TSC2 complex. HEK293 cells were transfected with different combinations of myc-TSC1, FLAG-TSC2, HA-TBC7, and the empty vectors as indicated. The cell lysates and immunoprecipitates with the anti-HA antibody were analyzed by immunoblotting with the indicated antibodies. (B) Effect of TSC1 knockdown. HEK293 cells were transfected with either TSC1 or control siRNA. The cell lysates and immunoprecipitates with the anti-TSC1 or -TSC2 antibody were analyzed by immunoblotting with the indicated antibodies. NRG was used as a control. (C) Deletion mutants of TSC1. The wild type (WT) of TSC1 having potential transmembrane and coiled-coil domains and the deletion mutants were employed for the binding analysis. Numbers indicate amino acids. The results shown in (D) are summarized on the right. (D) Association between TBC7 and TSC1 mutants. HEK293 cells were transfected with FLAG-TSC2, HA-TBC7, and each of myc-TSC1 constructs or the empty vector. The cell lysates and immunoprecipitates with the anti-myc antibody were analyzed by immunoblotting with the indicated antibodies.

consistent with the previous study reporting that TSC1 is located in the cytoplasmic vesicles [5], and HA-TBC7 also showed the punctate pattern in the cytoplasm. FLAG-TSC2 showed the staining pattern similar to these two proteins in the cells (data not shown). The subcellular localization of TBC7 was further analyzed comparing with that of TSC1 and TSC2 in HEK293 cells after separation into S100 and P100 fractions employing α-tubulin and calnexin as the markers of cytosolic and membrane proteins, respectively [24]. Under the conditions employed, both of the tumor suppressor proteins recovered predominantly in the P100 fraction and were present in the S100 fraction, whereas TBC7 was detected exclusively in the P100 fraction (Supplemental Fig. 3B). These results indicate that TBC7 localizes in the cytoplasmic vesicles of the endomembrane in association with the TSC1–TSC2 complex.

TBC7 and TSC-mTOR-signaling pathway

The role of TBC7 was investigated in the association with the TSC1–TSC2 complex that suppresses the mTOR-signaling pathway by attenuating the Rheb activity [2]. Namely, the phosphorylation of ribosomal protein S6 through activation of S6 kinase in downstream of mTOR was examined in HeLa S cells overexpressing TBC7. As shown in Fig. 3A, overexpression of HA-TBC7 significantly increased phosphorylation of S6 protein at Ser235/236. These results imply that the overexpression of TBC7 enhances the mTOR pathway, and it is attractive to assume that TBC7 eliminates the negative regulation of mTOR by the TSC1–TSC2 complex. It has been shown that RB1CC1/FIP200 interacts with the middle region of TSC1 including the coiled-coil domain

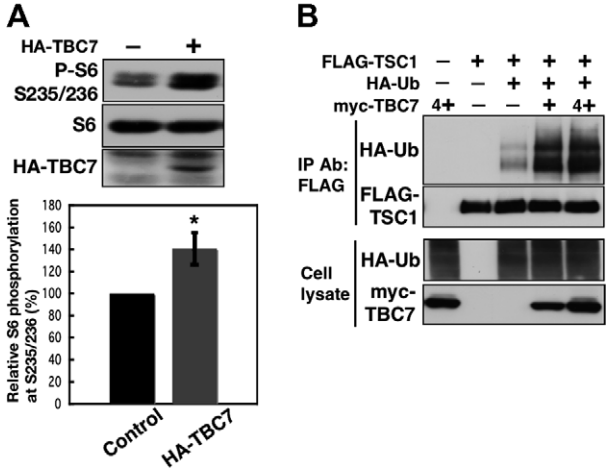

Fig. 3. TBC7 in the mTOR signaling pathway. (A) TBC7 and S6 phosphorylation. HeLa S cells were transfected with HA-TBC7 or empty vector, and the cell lysates were analyzed by immunoblotting with the indicated antibodies. Upper half shows the representative result. Lower half shows the relative S6 phosphorylation quantitated by densitometry using NIH image (version 1.63). The OD of each S6 phosphorylation blot was individually normalized to that of the S6 protein blot. The bars represent relative means ± S.D. of data from four independent experiments. $^{*}P < 0.05$. (B) Ubiquitination of TSC1. HEK293 cells were transfected with different combinations of FLAG-TSC1, HA-ubiquitin, myc-TBC7, and the empty vectors as indicated. Where indicated as 4+, four times as much as amounts of the HA-TBC7 expression vector was employed. The cell lysates and immunoprecipitates with the anti-FLAG antibody were analyzed by immunoblotting with the indicated antibodies.

and induces the ubiquitin-mediated degradation of TSC1 resulting in the reduction of the TSC1–TSC2 complex [9,10]. We thus postulated that TBC7 is involved in the ubiquitination of TSC1, as TBC7 also interacts with TSC1 in its coiled-coil domain as described above. To test this hypothesis, myc-TBC7 was co-expressed with FLAG-TSC1 and HA-ubiquitin in HEK293 cells, and FLAG-TSC1 was immunoprecipitated to assess the effect of TBC7 on the ubiquitination of TSC1. As expected, overexpression of myc-TBC7 accelerated TSC1 ubiquitination (Fig. 3B). TBC7 may enhance the ubiquitination of TSC1 independently or cooperate with RB1CC1/ FIP200 to downregulate the tumor suppressor protein. Recently, it has been shown that TSC2 is released from the cytoplasmic vesicles by the Akt-dependent phosphorylation, whereas TSC1 associates with hypophosphorylated TSC2 in the cytoplasmic vesicles where the complex exerts the GAP activity toward Rheb [7]. Thus, in addition to the regulation of TSC2 by the phosphorylation reaction, it seems to be possible that TSC1 is downregulated by TBC7 in the cytoplasmic vesicles via the induction of its ubiquitination to prevent the complex formation with TSC2, and that the Rheb activity is consequently blocked. On the other hand, it has been reported that the TSC complex immunoprecipitated from cultured cells by the anti-TSC2 antibody works as a GAP not only for Rheb but also for Rab5 [25]. The immunoprecipitate may include the endogenous TBC7 having the TBC domain shared among the Rab-GAP family. We could not detect, however, the significant association of Rab5 with TBC7 (data not shown) consistent with the TBC7 sequence lacking the two 'fingerprint' motif sequences common among the Rab-GAP family (Supplemental Fig. 1B). Even though TBC7 does not work as GAP for the Rab family, the results do not necessarily rule out the role of TBC7 in the regulation mechanisms other than TSC–mTOR pathway. Further studies are required to clarify the detail roles of TBC7 in the signaling pathways.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.07.011.

References

[1] J.P. Cheadle, M.P. Reeve, J.R. Sampson, D.J. Kwiatkowski, Molecular genetic advances in tuberous sclerosis, Hum. Genet. 107 (2000) 97–114.
[2] K. Inoki, H. Ouyang, Y. Li, K.L. Guan, Signaling by target of rapamycin proteins in cell growth control, Microbiol. Mol. Biol. Rev. 69 (2005) 79–100.
[3] D.J. Kwiatkowski, B.D. Manning, Tuberous sclerosis: a GAP at the crossroads of multiple signaling pathways, Hum. Mol. Genet. 14 (2005) R251–R258.
[4] K. Takahashi, M. Nakagawa, S.G. Young, S. Yamanaka, Differential membrane localization of ERas and Rheb, two Ras-related proteins involved in the phosphatidylinositol 3-kinase/mTOR pathway, J. Biol. Chem. 280 (2005) 32768–32774.
[5] T.L. Plank, R.S. Yeung, E.P. Henske, Hamartin, the product of the tuberous sclerosis 1 (TSC1) gene, interacts with tuberin and appears to be localized to cytoplasmic vesicles, Cancer Res. 58 (1998) 4766–4770.
[6] Y. Yamamoto, K.A. Jones, B.C. Mak, A. Muehlenbachs, R.S. Yeung, Multicompartmental distribution of the tuberous sclerosis gene products, hamartin and tuberin, Arch. Biochem. Biophys. 404 (2002) 210–217.
[7] S.L. Cai, A.R. Tee, J.D. Short, J.M. Bergeron, J. Kim, J. Shen, R. Guo, C.L. Johnson, K. Kiguchi, C.L. Walker, Activity of TSC2 is inhibited by AKT-mediated phosphorylation and membrane partitioning, J. Cell Biol. 173 (2006) 279–289.
[8] M. Rosner, A. Freilinger, M. Hengstschlager, Proteins interacting with the tuberous sclerosis gene products, Amino Acids 27 (2004) 119–128.
[9] B. Gan, Z.K. Melkoumian, X. Wu, K.L. Guan, J.L. Guan, Identification of FIP200 interaction with the TSC1–TSC2 complex and its role in regulation of cell size control, J. Cell Biol. 170 (2005) 379–389.
[10] T. Chano, M. Saji, H. Inoue, K. Minami, T. Kobayashi, O. Hino, H. Okabe, Neuromuscular abundance of RB1CC1 contributes to the non-proliferating enlarged cell phenotype through both RB1 maintenance and TSC1 degradation, Int. J. Mol. Med. 18 (2006) 425–432.
[11] H. Chong-Kopera, K. Inoki, Y. Li, T. Zhu, F.R. Garcia-Gonzalo, J.L. Rosa, K.L. Guan, TSC1 stabilizes TSC2 by inhibiting the interaction between TSC2 and the HERC1 ubiquitin ligase, J. Biol. Chem. 281 (2006) 8313–8316.
[12] P.M. Richardson, L.I. Zon, Molecular cloning of a cDNA with a novel domain present in the tre-2 oncogene and the yeast cell cycle regulators BUB2 and cdc16, Oncogene 11 (1995) 1139–1148.
[13] T. Nobukuni, M. Joaquin, M. Roccio, S.G. Dann, S.Y. Kim, P. Gulati, M.P. Byfield, J.M. Backer, F. Natt, J.L. Bos, F.J. Zwartkruis, G. Thomas, Amino acids mediate mTOR/raptor signaling through activation of class 3 phosphatidylinositol 3OH-kinase, Proc. Natl. Acad. Sci. USA 102 (2005) 14238–14243.
[14] N. Oshiro, R. Takahashi, K.I. Yoshino, K. Tanimura, A. Nakashima, S. Eguchi, T. Miyamoto, K. Hara, K. Takehana, J. Avruch, U. Kikkawa, K. Yonezawa, The proline-rich Akt substrate of 40 kDa (PRAS40) is a physiological substrate of mammalian target of rapamycin complex 1, J. Biol. Chem. 282 (2007) 20329–20339.
[15] K. Hara, Y. Maruki, X. Long, K. Yoshino, N. Oshiro, S. Hidayat, C. Tokunaga, J. Avruch, K. Yonezawa, Raptor, a binding partner of target of rapamycin (TOR), mediates TOR action, Cell 110 (2002) 177–189.
[16] A. Bernards, GAPs galore! A survey of putative Ras superfamily GTPase activating proteins in man and Drosophila, Biochim. Biophys. Acta 1603 (2003) 47–82.
[17] M.H. Cuif, F. Possmayer, H. Zander, N. Bordes, F. Jollivet, A. Couedel-Courteille, I. Janoueix-Lerosey, G. Langsley, M. Bornens, B. Goud, Characterization of GAPCenA, a GTPase activating protein for Rab6, part of which associates with the centrosome, EMBO J. 18 (1999) 1772–1782.
[18] L. Lanzetti, V. Rybin, M.G. Malabarba, S. Christoforidis, G. Scita, M. Zerial, P.P. Di Fiore, The Eps8 protein coordinates EGF receptor signalling through Rac and trafficking through Rab5, Nature 408 (2000) 374–377.
[19] A.K. Haas, E. Fuchs, R. Kopajtich, F.A. Barr, A GTPase-activating protein controls Rab5 function in endocytic trafficking, Nat. Cell Biol. 7 (2005) 887–893.
[20] A. Rak, R. Fedorov, K. Alexandrov, S. Albert, R.S. Goody, D. Gallwitz, A.J. Scheidig, Crystal structure of the GAP domain of

Gyp1p: first insights into interaction with Ypt/Rab proteins, EMBO J. 19 (2000) 5105–5113.

[21] S. Albert, E. Will, D. Gallwitz, Identification of the catalytic domains and their functionally critical arginine residues of two yeast GTPase-activating proteins specific for Ypt/Rab transport GTPases, EMBO J. 18 (1999) 5216–5225.

[22] M. van Slegtenhorst, R. de Hoogt, C. Hermans, M. Nellist, B. Janssen, S. Verhoef, D. Lindhout, A. van den Ouweland, D. Halley, J. Young, M. Burley, S. Jeremiah, K. Woodward, J. Nahmias, M. Fox, R. Ekong, J. Osborne, J. Wolfe, S. Povey, R.G. Snell, J.P. Cheadle, A.C. Jones, M. Tachataki, D. Ravine, J.R. Sampson, M.P. Reeve, P. Richardson, F. Wilmer, C. Munro, T.L. Hawkins, T. Sepp, J.B. Ali, S. Ward, A.J. Green, J.R. Yates, J. Kwiatkowska, E.P. Henske, M.P. Short, J.H. Haines, S. Jozwiak, D.J. Kwiatkowski, Identification of the tuberous sclerosis gene TSC1 on chromosome 9q34, Science 277 (1997) 805–808.

[23] A. Astrinidis, W. Senapedis, E.P. Henske, Hamartin, the tuberous sclerosis complex 1 gene product, interacts with polo-like kinase 1 in a phosphorylation-dependent manner, Hum. Mol. Genet. 15 (2006) 287–297.

[24] V. David, F. Hochstenbach, S. Rajagopalan, M.B. Brenner, Interaction with newly synthesized and retained proteins in the endoplasmic reticulum suggests a chaperone function for human integral membrane protein IP90 (calnexin), J. Biol. Chem. 268 (1993) 9585–9592.

[25] G.H. Xiao, F. Shoarinejad, F. Jin, E.A. Golemis, R.S. Yeung, The tuberous sclerosis 2 gene product, tuberin, functions as a Rab5 GTPase activating protein (GAP) in modulating endocytosis, J. Biol. Chem. 272 (1997) 6097–6100.