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TBC domain family, member 15 is a novel mammalian Rab GTPase-activating protein with substrate preference for Rab7

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Abstract

Ypt/Rabs are Ras-related GTPases that function as key regulators of intracellular vesicular trafficking. Their slow intrinsic rates of GTP hydrolysis are catalyzed by GTPase-activating proteins (GAPs). Ypt/Rab-GAPs constitute a family of proteins that contain a TBC (Tre-2/Bub2/Cdc16) domain. Only three of the 51 family members predicted in the human genome are confirmed Ypt/Rab-GAPs. Here, we report the identification and characterization of a novel mammalian Ypt/Rab-GAP, TBC domain family, member 15 (TBC1D15). TBC1D15 is ubiquitously expressed and localized predominantly to the cytosol. The TBC domain of TBC1D15 exhibits relatively high homology with that of Gyp7p, a yeast Ypt/Rab-GAP. Furthermore, TBC1D15 stimulates the intrinsic GTPase activity of Rab7, and to a lesser extent Rab11, but is essentially inactive towards Rab4 or Rab6. These data increase the number of mammalian TBC domain family members with demonstrated Rab-GAP activity to four, and suggest that TBC1D15 may be involved in Rab7-mediated late endosomal trafficking.

Keywords: Ypt/Rab; Rab7; GTPase-activating protein; TBC domain; Gyp7p

Ypt/Rab proteins encompass a large family of ras-related small GTPases that play central roles in coordinating intracellular membrane trafficking pathways in eukaryotic cells. In *Saccharomyces cerevisiae*, there are 11 Ypt/Rabs [1], while at least 60 different family members are predicted in the human genome [2]. Members of the Ypt/Rab family localize to distinct membranes where they have been shown to control events such as transport vesicle motility, and tethering to the appropriate target compartment prior to SNARE-mediated vesicle fusion [3–5]. Ypt/Rabs cycle between inactive or GDP-bound, and active or GTP-bound states. Interconversion between these states is catalyzed by guanine nucleotide exchange factors that promote the exchange of GDP for GTP, and GTPase-activating proteins (GAPs), that stimulate the slow intrinsic rate of GTP hydrolysis by Ypt/Rabs. In the GTP-bound state, Ypt/Rabs associate directly with a diverse and specific set of effector molecules [3–5]. Ypt/Rab effectors include the exocyst subunit Sec15 [6,7], which is involved in vesicle tethering to the plasma membrane, as well as molecular motor proteins belonging to the myosin and kinesin families [8–10]. Rab-GAP catalyzed GTP hydrolysis is believed to result in disassembly of effector complexes and recycling of Ypt/Rabs between membranes.

Rab-GAPs comprise a superfamily of proteins that share a TBC (Tre-2/Bub2/Cdc16) domain. The TBC domain has been shown to be sufficient for the catalytic activity and substrate specificity of two yeast Rab-GAPs, Gyp1p, and Gyp7p [11]. TBC domains contain conserved sequence motifs [12], as well as an invariant arginine residue that is essential for GAP activity [11]. Like the Ras- and Rho-GAPs, the Rab-GAPs are believed to employ

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an 'arginine-finger' catalytic mechanism involving the invariant arginine in the TBC domain [11,13]. In *S. cerevisiae*, there are 10 Rab-GAP-related proteins (denoted as Gyp proteins), 8 of which (Gyp1p-8p) have been confirmed to possess Rab-GAP catalytic activity in vitro [14–19]. Some of the yeast Rab-GAPs characterized to date stimulate the GTPase activities of a number of different Ypt/Rabs and posses overlapping substrate specificities. For example, Gyp1p stimulates the intrinsic GTPase activity of Sec4p, Ypt1p, Ypt7p, and Ypt51p [11,15]. In contrast, Gyp5p, Gyp6p, and Gyp7p exhibit clear substrate preferences for Ypt1p, Ypt6p, and Ypt7p, respectively [14,18,19]. The in vivo activities of the yeast Rab-GAPs have been more difficult to define, largely because their mutants lack observable phenotypes. However, since Gyp1p localizes to the Golgi and functions specifically as a negative regulator of Ypt1p in intact cells, the in vivo substrate specificities of the Rab-GAPs may be more restricted than observed in vitro [20].

Rab-GAPs are poorly characterized in higher eukaryotes. Although the human genome predicts 51 TBC domain-containing proteins [21], only three of them have been demonstrated to possess Rab-GAP activity. Among the mammalian Rab-GAPs, GAPCenA localizes to centrosomes as well as the cytosol, and exhibits a substrate preference for Rab6, while also stimulating the GTPase activity of Rab4 and Rab2 in vitro [22]. Two mammalian TBC domain family members, RN-Tre and PRC17, have been identified that stimulate Rab5 GTPase activity and demonstrate transforming activity, possibly by regulating the endocytosis of growth factor receptors [23–25]. Three non-TBC domain family members, Rab3 GAP [26], tuberous sclerosis complex 2 [27], and the p85α subunit of phosphatidylinositol 3′-kinase [28], have also been shown to possess Rab-GAP activity. Here, we report the identification and characterization of a fourth mammalian TBC domain family, member 15 (TBC1D15), that displays a marked substrate preference for the Rab7 GTPase.

Materials and methods

Construction of cDNA clones. The cDNA clone encoding TBC1D15 was identified in a yeast two-hybrid screen for factors that interact with the cytoplasmic domain (amino acids Met1-Lys94) of murine vesicle-associated membrane protein-2 (VAMP2) in 3T3-L1 adipocytes as described previously [29]. The longest TBC1D15 cDNA obtained contained 2002 bp of coding sequence followed by a stop codon and 174 bp of 3′ non-coding sequence. To obtain a full-length clone, a cDNA probe was generated by *Eco*RI and *Bam*HI double digestion of the TBC1D15 cDNA (in the pJG4-5 "prey" vector) obtained in the two-hybrid screen. This generated a 670 bp cDNA fragment corresponding to the 5′ end of the TBC1D15 clone. The cDNA probe was labeled with [^{32}P]dCTP using the Multiprime DNA labeling kit and purified using a Sephadex G-50 NICK column (Amersham Pharmacia Biotech). Library screening was performed using the cDNA Synthesis kit (Stratagene). The 3T3-L1 adipocyte library was inserted into the Uni-ZAP XR vector and 1×10^6 pfu were used to infect XL1-Blue MRF′ cells (Stratagene). Plaques were lifted onto Optitran BA-S 85 filters (Schleicher and Schuell) and hybridization of the radiolabeled TBC1D15 cDNA probe (400 ng) was performed according to the manufacturer's instructions. Two phages were isolated that contained an additional 16 bp of sequence at the 5′ end of the TBC1D15 cDNA, including an in-frame start codon. In addition, the coding sequence of TBC1D15 was confirmed by sequencing two murine EST clones (GenBank Accession Nos. AI036596 and AI315932). The full-length TBC1D15 cDNA clone was amplified by PCR using the primers 5′-CCGGAATTCATGGCGGCGGCGGGTGTTGTG and 5′-CCG CTCGAGTCATGCAGGTGTTAATCTGC, and ligated as an *Eco*RI–*Xho*I restriction fragment in-frame into the following expression vectors: pcDNA4 HisMax C (Invitrogen) for Xpress-tagged protein, pEGFP-C2 (Clontech Laboratories) for the GFP fusion protein, and pFASTBAC HT (Invitrogen) for baculovirus expression.

Cell culture, transfections, and fluorescence microscopy. COS1 and 293T cells were cultured at 37 °C in DMEM supplemented with 10% FBS (CSL Biosciences, Parkville, VIC, Australia) and 100 U/ml penicillin and streptomycin. Transient transfections were performed using Lipofectamine reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Fluorescence microscopy was performed as described previously [7].

Northern blotting. The murine TBC1D15 cDNA in pJG4-5 that was isolated in the two-hybrid screen was digested with *Bam*HI and *Xba*I to yield a ~900 bp cDNA fragment encoding amino acids 222–525 of TBC1D15. The cDNA probe was labeled with [^{32}P]dCTP using the Multiprime DNA labeling kit and purified using a Sephadex G-50 NICK column (Amersham Pharmacia Biotech). The radiolabeled probe was hybridized to a mouse MTN blot (Cat No. 7762-1; Clontech). Hybridization and washing were performed using the ULTRAhyb kit (Ambion) according to the manufacturer's instructions. The blot was exposed to X-ray film for 72 h at −80 °C prior to development.

Western blotting. A rabbit polyclonal antibody was prepared (Quality Controlled Biochemicals) that was raised against an 18-residue peptide (SEAKDDSPTQTLASPNAC) corresponding to amino acids 649–666 of murine TBC1D15. To prepare detergent-solubilized testis extract, the tissue was dissected from a freshly sacrificed mouse and homogenized using a Polytron in lysis buffer (2% Triton X-100, 0.15 M NaCl, 2 mM EDTA, 1 mM DTT, and 50 mM Hepes, pH 7.2) supplemented with a protease inhibitor cocktail (10 μM leupeptin, 7 μM pepstatin A, 20 μM AEBSF, and 0.7 μM aprotinin). Extracts from transiently transfected 293T cells were prepared 40 h post-transfection as described above. The testis and 293T cell extracts were centrifuged at 20,000*g* for 10 min and the supernatants were diluted into SDS-sample buffer, resolved by 8% SDS–PAGE, and subjected to immunoblotting using the TBC1D15 antiserum (1:1000). An HRP-conjugated anti-rabbit IgG (Pierce) was used as secondary antibody and the blot was developed using enhanced chemiluminescence.

GTP binding and hydrolysis assays. TBC1D15 was expressed in Sf21 cells using the BAC-TO-BAC baculovirus expression system (Invitrogen) and purified according to the manufacturer's instructions. Histidine-tagged Rab proteins were expressed in *E. coli* as described previously for Rab11 [7]. The activities of the purified Rabs were initially determined using [α-^{32}P]GTP-binding assays. The Rabs (1.6 or 3.3 μM) were incubated with 2 μM [α-^{32}P]GTP in buffer A (0.01%, BSA, 0.15 M NaCl, 2 mM EDTA, 1 mM DTT, and 25 mM Tris–HCl, pH 7.4) for 30 min at 30 °C. To terminate binding, samples (20 μl) were removed from the incubations and diluted into 5 ml ice-cold stop buffer (0.15 M NaCl, 25 mM $MgCl_2$, and 25 mM Tris–HCl, pH 7.4). Bound [α-^{32}P]GTP was measured by filter binding (0.45 μm membrane, Millipore) followed by liquid scintillation counting. For the GTPase assays, Rab7 (0.69 μM active protein) was incubated at 30 °C for 30 min with 0.05 μM [γ-^{32}P]GTP in buffer A supplemented with 15 mM ATP. To promote GTP hydrolysis, the samples were diluted 8-fold with buffer A in the presence or absence of purified TBC1D15

(0.7 μM), adjusted to 20 mM $MgCl_2$, and incubated for 0, 2, 10, 15, 30, 60, and 90 min at 30 °C. At each time point, the reactions were terminated by dilution of duplicate samples (25 μl) into 0.75 ml ice-cold 5% charcoal in 20% NaH_2PO_4. Following extraction of the solid phase by centrifugation, the amount of [^{32}P]Pi released was determined by liquid scintillation counting. To examine the substrate specificity of TBC1D15, the GTPase assays were performed as above using an equivalent concentration of active Rab4, Rab6, Rab7, and Rab11.

Results

Identification of a novel TBC domain-containing protein

In order to isolate factors that interact with the SNARE protein, VAMP2 in non-neuronal cells we utilized the yeast two-hybrid system [30] to screen a mouse 3T3-L1 adipocyte cDNA library using the cytoplasmic domain of VAMP2 as bait. Sequencing of the positive clones revealed that one of the interacting factors identified encoded a novel protein with a TBC domain. By sequencing available expressed sequence tags as well as an additional clone identified by screening of a 3T3-L1 adipocyte cDNA library, we were able to determine the full-length murine sequence. A start (ATG) codon was identified that conforms to the consensus translation initiation sequence ([A/G]XXAUGG; [31]) and precedes an open reading frame encoding 671 amino acids (deposited under GenBank Accession No. DQ054831). During the course of this work, a predicted murine protein (GenBank Accession No. Q9CXF4) was deposited in the database that is identical to the primary sequence of the novel protein that we had cloned with the exception of a single amino acid substitution (Ala364 to Asp). The predicted protein Q9CXF4 was denoted as TBC1 domain family, member 15 (TBC1D15) by the HUGO Gene Nomenclature Committee (http://www.gene.ucl.ac.uk/nomenclature/), and we therefore assigned this name to the novel protein identified in our study.

The TBC domain of TBC1D15 contains six sequence motifs, A–F (Fig. 1), that were identified previously based on sequence homology [12]. Within these motifs, TBC1D15 contains residues that are strictly conserved, such as Arg245 in motif A; Ile394, Asp397, and Arg400 in motif B; and Glu453, Phe457, and Trp458 in motif D. Importantly, the conserved Arg present in motif B of TBC1D15 has been shown, based on the mutational analysis of Gyp1p and Gyp7p, to be essential for Rab-GAP activity [11]. GenBank database searches using BLAST (Basic Local Alignment Search Tool) revealed that among the known members of this family the TBC domain of TBC1D15 exhibits the highest degree of homology (25% identity, 37% similarity) with that of *S. cerevisiae* Gyp7p, suggesting that it might be a mammalian ortholog of this Rab-GAP.

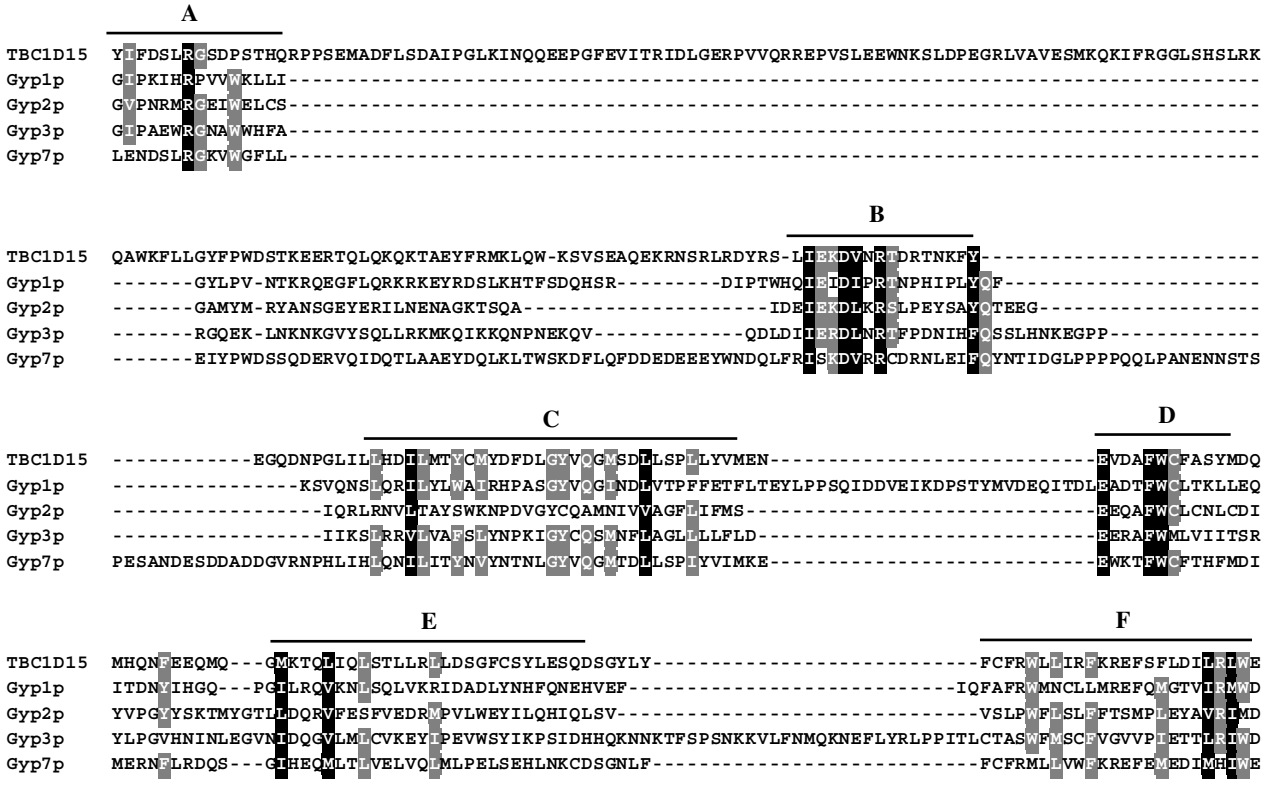

Fig. 1. Alignment between the TBC domain of TBC1D15 and yeast Rab-GAP proteins. The TBC domain of murine TBC1D15 (amino acids 238–533) was aligned with that of the indicated *S. cerevisiae* Rab-GAPs using the ClustalW program [34]. Shared motifs A–F as described previously [12] were aligned manually. Residues that are identical and conserved across a minimum four sequences are highlighted in black and gray, respectively.

Predicted proteins corresponding to human TBC1D15 were identified in BLAST searches (GenBank Accession Nos. AAH28352, Q8TC07, NP073608, and BAB13971), which are 88% identical and 92% similar to the murine ortholog (Fig. 2). In addition, BLAST searches revealed that TBC1D15 is highly homologous to another member of the TBC domain family, TBC1D17 (Fig. 2). The human forms of TBC1D15 and TBC1D17 exhibit 46% identity and 63% similarity. TBC1D17, like TBC1D15, has not been characterized previously and therefore it remains to be determined whether this TBC domain family member functions as a Rab-GAP in mammalian cells.

Tissue distribution of TBC1D15

Northern blotting using a radiolabeled cDNA probe encoding amino acids 222–525 of mouse TBC1D15 revealed the presence of two mRNA transcripts at ~2.2 and ~3.3 kb in most tissues examined (Fig. 3A). Both mRNAs are most abundant in heart, liver, and testis, with the ~2.2 kb mRNA species being particularly abundant in the latter tissue. Although the reason for the existence of two mRNAs encoding TBC1D15 is unclear, the data suggest that it is ubiquitously expressed. We raised a polyclonal antibody against a C-terminal peptide of murine TBC1D15. Western blots detected a robust band of 70–76 kDa in whole cell extracts of 293T cells transfected with an expression vector encoding full-length TBC1D15, but not in control cells transfected with the empty vector (Fig. 3B; lanes 2 and 3). In addition, a single prominent band was detected in mouse testis tissue extracts that migrated on SDS–PAGE with a molecular mass equivalent to that of the recombinant protein and consistent with its predicted molecular weight of 76.5 kDa (Fig. 3B; lane 1).

Subcellular localization of TBC1D15

Although the polyclonal anti-TBC1D15 antibody detected the endogenous protein on immunoblots, we were unable to observe a signal above background in a

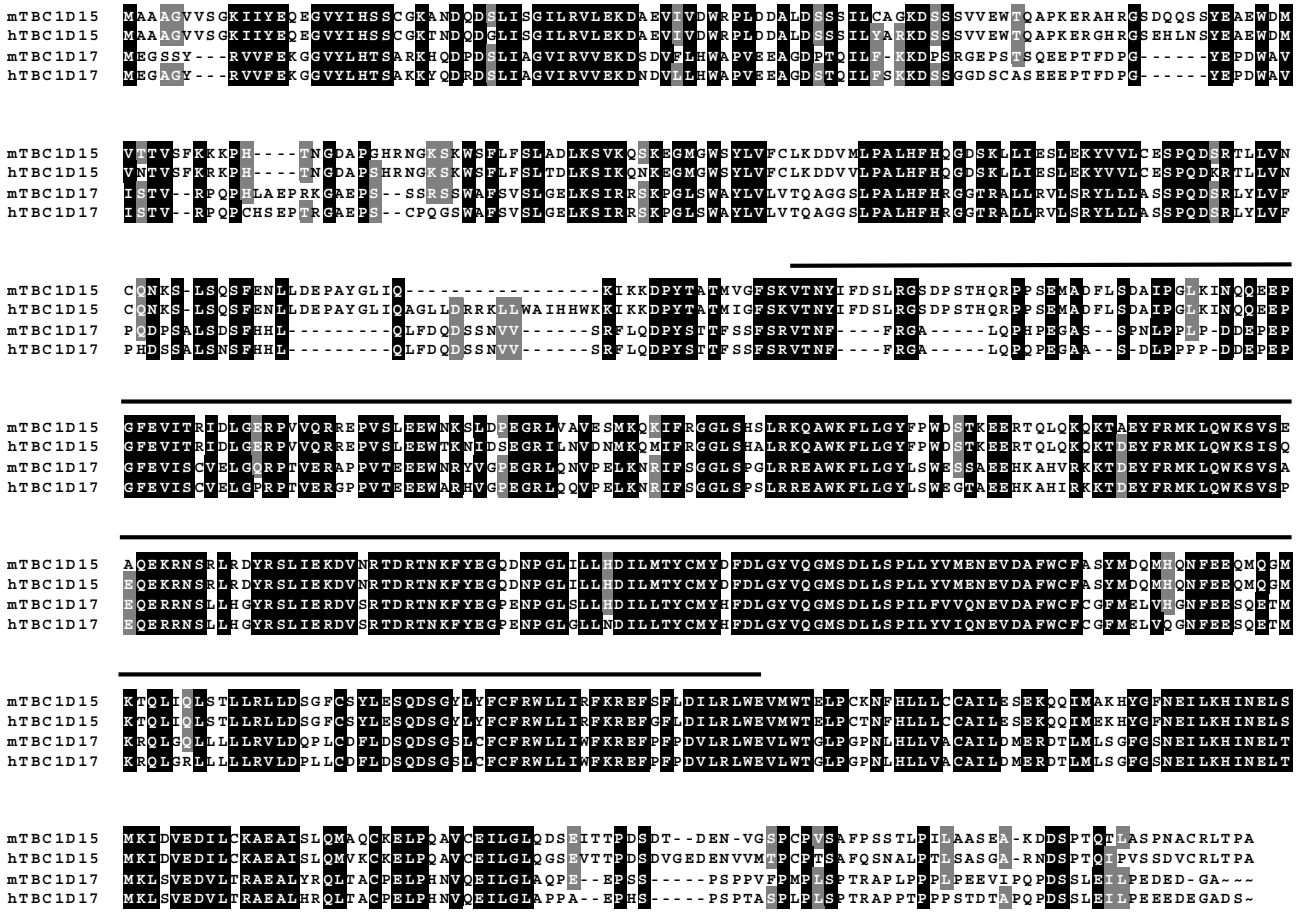

Fig. 2. Alignment between the novel TBC domain family members, TBC1D15 and TBC1D17. The primary sequences of murine TBC1D15 (mTBC1D15; GenBank Accession No. DQ054831) and human TBC1D15 (hTBC1D15; GenBank Accession No. Q8TC07), as well as murine TBC1D17 (mTBC1D17; GenBank Accession No. Q8BYH7) and human TBC1D17 (hTBC1D17; GenBank Accession No. Q9HA65) were aligned using the ClustalW program [34]. The positions of the TBC domains are indicated with a bar. Residues that are identical and conserved across a minimum four sequences are highlighted in black and gray, respectively.

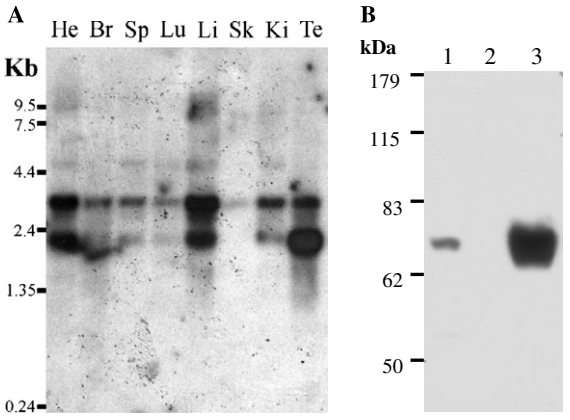

Fig. 3. Tissue distribution of TBC1D15. (A) Northern blotting analysis of TBC1D15 expression in multiple mouse tissues. A radiolabeled cDNA fragment encoding amino acids 222–525 of TBC1D15 was used to probe a commercial multiple tissue Northern blot. Subsequently, the blot was washed and subjected to autoradiography. The tissues are as follows: He (heart); Br (brain); Sp (spleen); lu (lung); Li (liver); Sk (skeletal muscle); Ki (kidney); and Te (testis). The positions of RNA molecular weight standards are indicated. Two mRNA species were detected across multiple tissues. (B) TBC1D15 expression examined by Western blotting. Protein extracts from mouse testis tissue (lane 1), and 293T cells transfected with either control vector (lane 2) or a TBC1D15 expression vector (lane 3) were analyzed by Western blotting using a primary antibody raised against the C-terminus of TBC1D15. The positions of protein molecular weight standards (kDa) are indicated.

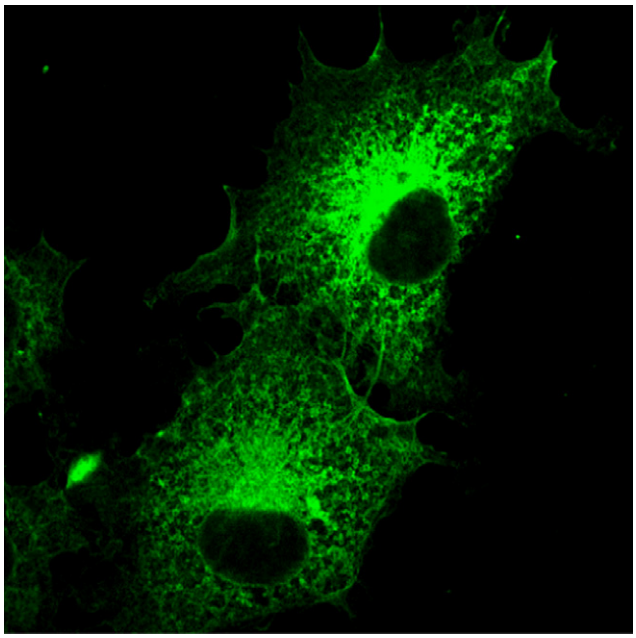

Fig. 4. Subcellular localization of TBC1D15. COS1 cells were transfected with an expression vector encoding a GFP–TBC1D15 fusion protein. The cells were subsequently fixed and examined by confocal fluorescence microscopy. The diffuse intracellular fluorescence observed is consistent with soluble cytoplasmic localization. A similar distribution of GFP–TBC1D15 was observed in multiple mammalian cell lines, and also when the GFP sequence was substituted for an N-terminal Xpress epitope tag.

panel of cell lines by fluorescence microscopy using the same antibody. Therefore, we examined the subcellular localization of overexpressed GFP-tagged TBC1D15. In transfected COS1 cells (Fig. 4), GFP–TBC1D15 exhibited diffuse intracellular fluorescence, suggesting that it is predominantly a cytosolic protein (consistent with the fact that it lacks a putative transmembrane domain). A similar cytosolic localization was observed using an alternative construct encoding TBC1D15 with an Xpress epitope tag, and also when a panel of cell lines were transfected with expression vectors encoding Xpress- or GFP-tagged TBC1D15. This result is in conflict with the fact that TBC1D15 was isolated in a yeast two-hybrid screen based on its interaction with the intracellular transmembrane protein, VAMP2. To investigate the potential interaction further, we performed protein:protein interaction assays. While binding between the recombinant proteins was observed using a GST pull-down assay following transient transfection of cultured cells, we were unable to demonstrate an interaction between the endogenous proteins in co-immunoprecipitation experiments using detergent-solubilized lysates of 3T3-L1 adipocytes (data not shown).

Rab-GAP activity and substrate specificity of TBC1D15

To investigate whether TBC1D15 functions as a Rab-GAP, we expressed the His6-tagged full-length recombinant protein using the baculovirus system and purified it from the soluble fraction on Nickel beads. The protein was isolated from insect cells at a relatively high level of purity as assessed by SDS–PAGE (Fig. 5A). In addition, Rabs4, 6, 7, and 11 were synthesized in *E. coli* and purified from the soluble fraction in order to examine whether they behave as substrates for TBC1D15 in vitro. Rab7 was included in the panel because of the relatively high level of homology between the catalytic domains of TBC1D15 and Gyp7p, a yeast Rab-GAP with a substrate preference for Ypt7p (the yeast ortholog of Rab7) [19]. The activities of the purified Rab proteins were initially determined using [α-^{32}P]GTP-binding assays, and for each Rab protein an equivalent concentration of active GTP-binding sites was added subsequently to the [γ-^{32}P]GTP hydrolysis assays. As shown in Figs. 5B and C, Rab7 showed a low intrinsic rate of GTP hydrolysis (0.583 fmol/min). However, when TBC1D15 was supplemented into the assay we found that it stimulated the GTPase activity of Rab7 by 90-fold roughly (44.61 fmol/min), confirming that it functions as a GAP for Rab7. In parallel incubations, TBC1D15 accelerated the intrinsic rate of GTP hydrolysis by Rab11 (8-fold), but was essentially inactive towards Rab4 (2-fold) and Rab6 (3-fold). The lower fold stimulation of GTP hydrolysis by Rab11 compared to Rab7 was due in large part to the significantly higher intrinsic GTPase activity

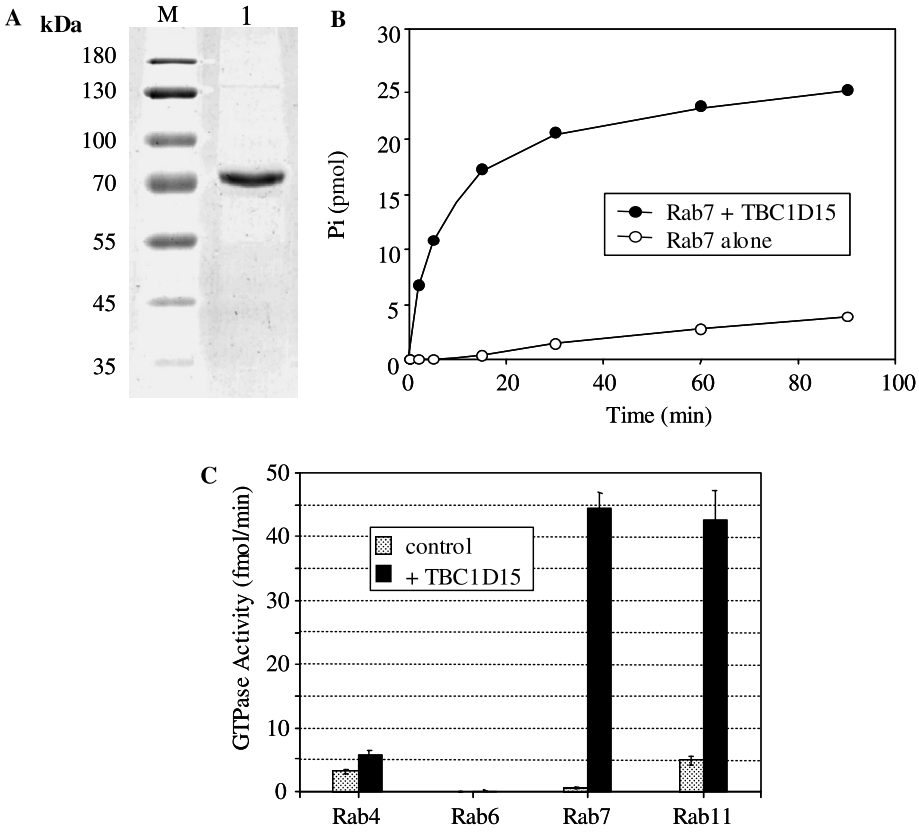

Fig. 5. TBC1D15 functions as a Rab-GAP protein. (A) Analysis of the purified His6-TBC1D15 protein (1 μg; lane 1) by SDS–PAGE (Coomassie-stained gel). The positions of the molecular weight markers (M) are shown. (B) Time-course of GTP hydrolysis by Rab7 in the absence and presence of purified TBC1D15 measured using a charcoal-binding assay. Purified Rab7 was pre-loaded with [γ-^{32}P]GTP and incubated at a final concentration of 0.087 μM in the absence or presence of TBC1D15 (0.7 μM) for the indicated time points at 30 °C. The amount of [^{32}P]Pi released at each time point was determined by liquid scintillation counting. (C) GTPase activities of purified Rab4, 6, 7, and 11 in the absence (control) and presence of purified TBC1D15. In this experiment, the GTPase assays were performed for 0, 30, and 60 min at 30 °C, and the rates of [γ-^{32}P]GTP hydrolysis were calculated following the determination of [^{32}P]Pi released at each time point. These experiments were repeated at least three times and representative data are shown.

(4.86 fmol/min) of the former. Taken together, these data indicate that TBC1D15 is a novel mammalian Rab-GAP with a substrate preference for the Rab7 GTPase.

Discussion

Here, we report the isolation and characterization of a novel mammalian TBC domain family member. TBC1D15 was identified in a yeast two-hybrid screen based on an interaction with the cytoplasmic domain of VAMP2. Although an association between recombinant TBC1D15 and VAMP2 was observed in biochemical GST pull-down assays, we were unable to confirm the interaction by co-immunoprecipitation of the endogenous proteins. Therefore, the physiological significance of the interaction is unclear. In fluorescence microscopy experiments, overexpressed TBC1D15 exhibited a predominantly cytosolic localization. This result is surprising, given that the substrate molecules for this protein family, GTP-bound Ypt/Rabs, are predominantly membrane-associated. For example, the yeast TBC domain-containing protein, Gyp1p, colocalizes with its substrate Ypt/Rab, Ypt1p on the Golgi apparatus [20], and two additional family members, Gyp5p and Gyl1p, colocalize with their substrate Ypt/Rab protein, Sec4p at bud emergence sites during cytokinesis [32]. The apparent lack of membrane association exhibited by TBC1D15 may be because: (a) it is not recapitulated by overexpression of the recombinant protein, (b) binding to membranes may be transient in nature, or (c) membrane association of TBC1D15 may be sensitive to the fixation conditions employed in our experiments, as reported previously in the case of Gyp1p [20].

TBC1D15 belongs to a large family of proteins containing TBC domains that are likely to function as Rab-GAPs. The presence in the TBC domain of TBC1D15 of an invariant arginine that has been shown previously to be required for the catalytic activity of

yeast Gyp1p and Gyp7p [11] is consistent with the observation that it behaves as a functional Rab-GAP. The human and *Drosophila* genomes predict 51 and 24 TBC domain family members, respectively, that may encode Rab-GAP proteins [21]. However, to date only three of the mammalian family members have been demonstrated to possess Rab-GAP activity: GAPCenA [22], RN-tre [24], and PRC17 [25]. Among the 10 TBC domain-containing proteins in yeast, the catalytic domain of TBC1D15 shows the highest degree of homology with Gyp7p. Furthermore, using in vitro GTPase assays we demonstrated that TBC1D15 functions as a Rab-GAP and displays a marked substrate preference for Rab7. This observation increases the number of mammalian TBC domain family members with demonstrated Rab-GAP activity to four. The substrate specificity of TBC1D15 is clearly distinct from those of the other mammalian Rab-GAPs characterized to date; GAPCenA, RN-tre, and PRC17 act preferentially as GAPs for Rab6, Rab5, and Rab5, respectively [22,24,25]. BLAST searches revealed that an additional mammalian TBC domain family member, TBC1D17, shows relatively high homology to TBC1D15. It will be interesting to determine whether the substrate specificities of these proteins are overlapping or distinct.

We found that TBC1D15 stimulates the intrinsic GTPase activity of Rab7, and to a lesser extent Rab11, but is essentially inactive towards Rab4 or Rab6 in vitro. In a previous study, it was reported that *S. cerevisiae* Gyp7p exhibits a substrate preference for Ypt7p, while it also accelerates the intrinsic rate of GTP hydrolysis by Ypt31p, Ypt32p, and Ypt1p, and has no effect on that of Ypt6p, Sec4p or H-ras [19]. The substrate preference for Ypt7p/Rab7 and lack of GAP activity towards Ypt6p/Rab6 are consistent with the possibility that Gyp7p and TBC1D15 represent orthologous proteins, although confirmation of this awaits analysis of their in vivo functions. Since Rab7 regulates late endosomal trafficking in mammalian cells [33], it is not unreasonable to speculate that TBC1D15 may be involved also in this pathway.

Eleven genes encode Ypt/Rab GTPases [1], and 10 encode TBC domain-containing proteins in *S. cerevisiae*, while the human genome predicts at least 60 Ypt/Rabs [2] and 51 TBC domain-containing proteins [21]. This suggests that members of the TBC domain family may exhibit a higher level of substrate specificity in vivo than observed in GTPase assays using purified proteins. One way this might be achieved is by selective colocalization with a substrate Ypt/Rab protein on a specific intracellular compartment. For example, Gyp1p stimulates the GTPase activity of multiple Ypt/Rab proteins in vitro, including Sec4p, Ypt1p, Ypt7p, and Ypt51p [15]. However, it colocalizes with Ypt1p on the Golgi apparatus and functions specifically as a negative regulator of Ypt1p in vivo [20]. Elucidation of the substrate specificities and in vivo functions of the mammalian TBC domain family members represents a major challenge for future studies.

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