Rab8b and Its Interacting Partner TRIP8b Are Involved in Regulated Secretion in AtT20 Cells*

Received for publication, November 30, 2000, and in revised form, January 16, 2001
Published, JBC Papers in Press, January 24, 2001, DOI 10.1074/jbc.M010798200

Shan Chen‡, Mui C. Liang‡, Jin N. Chia‡, Johnny K. Ngsee§, and Anthony E. Ting†‡

From the ‡Institute of Molecular and Cell Biology, 30 Medical Drive, Singapore 117609, Republic of Singapore and the §Department of Biochemistry, University of Ottawa, Loeb Health Research Institute, Ottawa, Ontario K1Y 4E9, Canada

Rab proteins are a family of small GTPases that regulate intracellular vesicle traffic. Rab8b, because of its homology with Rab8, has been suggested to function in vesicle transport to the plasma membrane. Using the yeast two-hybrid system, we identified a Rab8b interacting clone, termed TRIP8b, from a rat brain cDNA library. The gene encodes a 66-kDa protein with homology to the peroxisomal targeting signal 1 receptor. The interaction between Rab8b and TRIP8b was further verified by in vitro binding assays and co-immunoprecipitation studies. Additional experiments with Rab8b mutants demonstrated that Rab8b requires a guanine nucleotide but not prenylation for its interaction with TRIP8b. Western immunoblot analysis showed that TRIP8b was primarily expressed in brain. Subcellular fractionation of AtT20 cells revealed that TRIP8b was present in both cytosolic and membrane fractions. To investigate the function of Rab8b and TRIP8b in secretion, we examined the release of ACTH from AtT20 cells. Results from stable cell lines expressing Rab8b or TRIP8b indicated that both proteins had a stimulatory effect on cAMP-induced secretion of ACTH. In summary, these data suggest that Rab8b and TRIP8b interact with each other and are involved in the regulated secretory pathway in AtT20 cells.

Rab proteins are a family of small GTP-binding proteins that are important regulators of vesicle transport (1–3). They undergo nucleotide exchange to establish the active GTP-bound form and are incorporated onto transport vesicles either during or after vesicle formation. The GTP-bound form of Rab proteins recruit effectors, either directly or indirectly, to target vesicles to the appropriate sites on acceptor membranes. These effectors include motor proteins which link the vesicles to the cytoskeleton (4, 5), docking complexes which are recruited from the cytosol or tethering factors that mediate the initial contact of membranes that are destined to fuse (6, 7).

To date, about 40 distinct Rab proteins have been identified and each is believed to be specifically associated with a particular vesicle transport pathway (2, 8). However, only a fraction of known Rab proteins have their functions characterized in detail. Among these, Rab8 (which we term Rab8a) has been shown to be a key regulator of constitutive polarized vesicle transport to the dendrites in the neurons or to the basolateral membrane in epithelial cells (9–11). Using immunofluorescence and electron microscopy, Rab8a is localized to the Golgi region, cytoplasmic vesicular structures, and the plasma membrane of Madine-Darby canine kidney epithelial cells. When the wild type and dominant active mutant forms of Rab8a are overexpressed in baby hamster kidney fibroblast cells, a dramatic change in cell morphology occurs. The cells form elongated processes as a result of a reorganization of both their actin filaments and microtubules. In this case, newly synthesized vesicular stomatitis virus, a basolateral marker protein is preferentially delivered into these cell outgrowths.

While the role of Rab8a in vesicle traffic has been elucidated, the function of Rab8b, a closely related isoform, remains unclear. Rab8b was first identified during a library screen to look for novel Rabs involved in secretory granule fusion in mast cells and basophils (12). Rab8b has an overall amino acid identity of 80% with Rab8a. While the NH2 termini are highly conserved, the COOH-terminal domain of Rab8b is substantially different from that of Rab8a. Transient expression of the Myc-tagged Rab8b fusion protein in neuroblastoma and basophilic leukemia cells shows staining of both the plasma membrane and ill-defined vesicular structures. When overexpressed, Rab8b induces striking plasma membrane outgrowth, similar to that observed for Rab8a. Northern immunoblot analysis shows the highest expression level of Rab8b in spleen and brain. In contrast, Rab8a has very low expression levels in spleen and brain. From these data, we postulated that like Rab8a, Rab8b may also play a similar role in the vesicle traffic from the Golgi apparatus to the plasma membrane. Furthermore, because of the substantial COOH-terminal differences between Rab8b and Rab8a and their different tissue expression patterns, we also suspected that Rab8b may be involved in roles in secretion that are not shared or substituted by Rab8a.

Our study was aimed to further characterize Rab8b’s function in the secretory pathway. Here, we report the cloning and characterization of a Rab8b interacting protein named TRIP8b and the function of Rab8b and TRIP8b in the regulated secretion of adrenocorticotropic hormone (ACTH) secretion in AtT20 cells.

EXPERIMENTAL PROCEDURES

Cloning of Rab8b—Rat Rab8b cDNA was cloned from a rat brain cDNA library by polymerase chain reaction screening using primers specific to the 5′ and 3′ sequences of Rab8b (12). The forward primer used was 5′-CGGGATCCATGGCGAAGACGTAC-3′ and the reverse

* This work was supported by the National Science and Technology Board of Singapore. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
‡ To whom all correspondence should be addressed: Institute of Molecular and Cell Biology, 30 Medical Dr., Singapore 117609, Republic of Singapore. Tel.: 65-874-3780; Fax: 65-779-1117; E-mail: mcbaet@mcb.nus.edu.sg.
§ The abbreviations used are: ACTH, adrenocorticotropic hormone; HA, hemagglutinin; GST, glutathione S-transferase; TPR, tetra-tricopeptide repeat; Neo, neomycin; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; HEK, human embryonic kidney; GDI, GDP dissociation inhibitor; ABI, ACTH biosynthetic intermediate; PEX5, peroxisomal targeting signal 1 receptor; FBS, fetal bovine serum; GTPγS, guanosine 5′-3′-O-(thio)triphosphate; PAGE, polyacrylamide gel electrophoresis.
primer was 5′-CGGGATCTTACAGGAGAAACA-3′. For subsequent cloning, both primers were designed to contain a BamHI restriction site. The polymerase chain reaction products were cloned into pBlueScript vector (Stratagene) and clones were screened with a 32P-labeled primer (5′-GGTGCGCAGTGAATAACAGA-3′) corresponding to the C terminus of Rab8b. Positive colonies were confirmed by DNA sequencing.

**GAL4-based Yeast Two-hybrid Screening**—For two-hybrid screens, Rab8b was cloned into the pAS2-1 vector at the EcoRI and BamHI sites. The Rab8b construct used was previously described (13). Rab8a (kindly provided by Johan Perren) was cloned into pAS2-1 at the EcoRI and NcoI sites, while Rab9a (kindly provided by Wolfgang Schliebs) was cloned into the NcoI and XhoI sites. The expression of the GAL4BD fusion proteins were confirmed by immunoblotting before starting the library screen.

An oligo(dT) rat brain cDNA library was cloned into the pGAD424X vector as described (14). Rab8b and the rat brain cDNA library were co-transformed into the yeast strain Y190 according to manufacturer’s instructions (CLONTECH).

**Cloning of TRIP8b**—To obtain the amino-terminal TRIP8b sequence, a TRIP8b PstI fragment derived from the original R38 clone that was isolated in the yeast two-hybrid system was used to screen a rat brain cDNA phage library (Stratagene). Several clones containing the NH2-terminal sequence were identified. Enzyme digestion and cloning strategies were used to produce a full-length TRIP8b clone whose sequence has been deposited to the GenBankTM data bank (accession number AF234454).

**DNA Constructs and Mutagenesis**—Rab8b mutants were created using the Stratagene Quikchange Site-directed Mutagenesis kit. These mutants include: Rab8bQ67L, Rab8bT22N, Rab8bC204S, and Rab8bN12I. All the mutations were confirmed by DNA sequencing. These mutants were either cloned into pAS2-1 (CLONTECH) for two-hybrid studies, pXJ40-Myc or pXJ40-hemagglutinin (HA) (15) for immuno-fluorescence staining and pXJ40-glutathione S-transferase (GST) (16) for *in vitro* translation and binding studies.

The full-length TRIP8b was cloned into pBlueScript at the EcoRI and XhoI sites. Digestion with EcoRI, BglII, and XhoI produced a 570-base pair NH2-terminal fragment (TRIP8b-N) and a 1.3-kilobase fragment containing the tetratricopeptide repeat (TPR) domain (TRIP8b-TPR). TRIP8b-N, TRIP8b-TPR, and TRIP8b-TPR were cloned separately into pGEX4T-1 (Amersham Pharmacia Biotech) for GST fusion protein production and pXJ40-Myc or pXJ40-HA vectors for transient transfection and immuno-fluorescence staining. TRIP8b and TRIP8b-N were also cloned into pJX41-neomycin (neo) (16) for stable transfection of AtT20 cells and immunofluorescence staining. TRIP8b and pXJ40-Myc or pXJ40-HA vectors for transient transfection and pXJ40-glutathione-S-transferase (GST) (16) for *in vitro* translation and binding studies.

**Co-immunoprecipitation of Rab8b and TRIP8b**—The full-length TRIP8b was cloned into pBlueScript vector and then into pXJ40-Myc-Rab8b, 20 μl of Superfectin (Qiagen), and 300 μl of serum-free Dulbecco’s modified Eagle’s medium were incubated at room temperature for 15 min before another 2.5 ml of growth media was added. The total immunoprecipitation mixture was added to a 10-cm tissue culture plate containing HEK 293T cells and incubated at 37 °C for 3 h. The media was removed and the cells were grown overnight in 10 ml of fresh growth media. The next day, the cells were briefly and gently washed with PBS before cellular proteins were extracted at 4 °C with 10 mM Hepes-OH, pH 7.3, 150 mM NaCl, 0.1% Nonidet P-40, 2 mM MgCl2, 0.5 mM EGTA, 1 mM dithiothreitol, and 1 mM PMSF. The extract was centrifuged at 14,000 rpm for 15 min at 4 °C and the supernatant was incubated with 20 μl of protein A beads (Roche Molecular Biochemicals) at 4 °C for 1 h, spun down, and the beads discarded. The supernatant was then incubated with neither μg of anti-HA polyclonal antibody (Y-11, Santa Cruz Biotechnology) or 1 μg of rabbit IgG (Sigma) at 4 °C for 2 h. Then 20 μl of protein A beads were added and incubated with the supernatant for 30 min. The beads were recovered by brief centrifugation and washed 5 times with PBS containing 10 mM Hepes-OH, pH 7.3, 150 mM NaCl, and 0.05% Nonidet P-40. The amount of immunoprecipitated TRIP8b was determined using an anti-HA monoclonal antibody (F-7, Santa Cruz Biotechnology) and the amount of Rab8b that co-immunoprecipitated with TRIP8b was observed with an anti-Myc monoclonal antibody (9E10, Santa Cruz Biotechnology).

**Immunofluorescence Staining of AtT20 Cells**—AtT20 cells were plated onto 2-well chamber slides (Nunc) and transiently transfected the next day with Superfectin. The day after the transfection, the cells were washed 2 times with PBS and fixed with 3.7% formaldehyde in PBS at room temperature for 10 min or with −20 °C acetone at room temperature for 2 min. Then the cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min and blocked with 1% bovine serum albumin in PBS for 30 min. Subsequently, the cells were incubated with the primary antibody in blocking buffer for 1 h at room temperature, washed three times with blocking buffer, and incubated with a fluorescent secondary antibody for 30 min. The rabbit polyclonal TRIP8b antibody was used at a dilution of 1:100 and the monoclonal anti-Myc antibody was used at 1:500. The anti-rabbit Cy3-conjugated antibody (Sigma) was used at 1:1,200 and the fluorescein isothiocyanate-conjugated anti-mouse antibody (Jackson) was used at 1:1,000.

**Subcellular Analysis of AtT20 Cells**—A 10-cm plate of confluent AtT20 cells was scraped in PBS, centrifuged at 2,000 rpm for 5 min, and the cell pellet resuspended in ice-cold homogenization buffer containing 250 mM sucrose, 10 mM Hepes-OH, pH 7.3, 2 mM EDTA, 1 mM DTT, and 1 mM PMSF and lysed by sonication. An aliquot of the homogenized fraction was taken for Western immunoblot analysis and the remainder
Rab8b and TRIP8b Stimulate ACTH Release

centrifuged at 200,000 × g in a TLA 100.2 rotor (Beckman) at 4 °C for 1 h. The pellet was solubilized with 20 μl of 2 × SDS gel loading buffer. Proteins in the supernatant were precipitated with 10% trichloroacetic acid solution and washed twice with 20 μl of 2 × SDS gel loading buffer. The homogenate, pellet, and supernatant fractions were loaded onto a 10% SDS-PAGE gel, transferred to a nitrocellulose membrane, incubated with anti-TRIP8b sera or anti-Rab8 monoclonal antibody (Transduction Laboratories), and detected using goat anti-mouse or anti-rabbit horse-radish peroxidase-conjugated secondary antibody and Pierce Supersignal. Note, the Rab8 antibody reacts against both Rab8a and Rab8b, while reverse transcriptase-polymerase chain reaction of poly(A) RNA revealed that similar amounts of Rab8a and Rab8b messenger RNA were present in AtT20 cells (data not shown).

For the extraction studies, AtT20 membrane pellets were resuspended in 0.1 mM Na3CO3, pH 11, or 1% Triton X-100 as described (17). 40 μg of membrane fraction was incubated on ice for 1 h with 0.1 mM Na3CO3, pH 11, or 1% Triton X-100 in 20 mM Tris-HCl, pH 7.5, 1 mM EGTA, and 1 mM EDTA. Following the incubation, the fractions were centrifuged at 200,000 × g for 30 min. The supernatant fractions were precipitated with 10% trichloroacetic acid and the resulting precipitates were solubilized with 2 × SDS loading buffer. While the pellet fractions were resuspended with 2 × SDS loading buffer. Both fractions were subjected to Western immunoblot analysis as described above.

Solubilization of TRIP8b with GDP Dissociation Inhibitor (GDI)—GST-GDI1 protein was expressed and purified as described above except for the absence of detergent in the wash and elution buffers. Instead, the wash buffers were 25 mM Tris-HCl, pH 7.5, 10% glycerol, and 1 mM diithiothreitol with 500 or 150 mM NaCl. The protein was eluted with the 150 mM NaCl wash buffer containing 7.5 mM reduced glutathione.

An AtT20 membrane fraction was prepared as described (17) and the membrane pellet resuspended in 10 mM Tris-HCl, pH 7.5, and 300 mM sucrose by sonication. 25 μg of resuspended membranes were preincubated with 1 mM GDP for 20 min at 37 °C and then incubated with 400 nm GST-GDI1 in the presence of 1 mM GDP and analyzed as described above.

Stable Transfection of AtT20 Cells—AtT20 cells were plated out onto a 6-well plate with a density of 4 × 104 cells/ml, 2 ml/well. The next day, cells were transfected with pXJ41-neo-Myc-Rab8b, TRIP8b, or TRIP8b-N. Briefly, 2 μg of DNA, 5 μl of Superfectin, and 100 μl of serum-free media were mixed and incubated at room temperature for 20 to 30 min before another 600 μl of growth media was added. Then the 900-μl mixture was added drop by drop onto each well of cells. Cells were incubated for 4 to 5 h, changed to new growth media, and grown at 37 °C overnight in a 15% CO2 incubator. The next day, each well was trypsinized and the cells plated out into a 150-mm plate, and 105 cells/ml, 2 ml/well. The next day, each well was trypsinized and the cells plated out onto a 150-mm plate, and 105 cells/ml, 2 ml/well. The next day, each well was trypsinized and the cells plated out onto a 150-mm plate.

ACTH Release Assay—The experiment was performed as described with modifications (18). Cells were grown to 50–75% confluence in 12-well plates for 3 days before the assay. The cells were washed three times with serum-free media and then incubated in 150 mM NaCl, 20 mM Hepes-OH, pH 7.3, 0.7 mM CaCl2, 5 mM KCl, 1 mM MgCl2, and 0.1 mg/ml bovine serum albumin, with or without 100 mM 8-Br-cAMP at 37 °C for 1 h. The media fraction from each well was collected and PMSF was immediately added. The cells were washed with ice-cold PBS and the wash combined with the media fraction. The media fraction was precipitated with 4 × volume −20 °C acetone overnight. The cells were extracted with Hepes-OH, pH 7.3, 0.7 mM CaCl2, 5 mM KCl, 1 mM MgCl2, and 0.1 mg/ml bovine serum albumin, with or without 100 mM 8-Br-cAMP at 37 °C for 1 h. The media fraction from each well was collected and PMSF was immediately added. The cells were washed with ice-cold PBS and the wash combined with the media fraction.

Identification of a Rab8b-interacting Clone in the Yeast Two-hybrid System—Over 2 × 106 clones from a rat brain cDNA library were screened using the GAL4-based yeast two-hybrid system (14) with either Rab8b or the GTPase-defective mutant (Rab8b-Q67L) to identify Rab8b interacting proteins. A single clone was observed to interact with both proteins. A full-length clone (R38) was obtained by screening a rat brain cDNA library and predicted to encode a hydrophilic 66-kDa protein. BLASTP analysis of the R38 protein sequence against GenBankTM reveals that the R38 had 44% identity with the peripheral targeting signal 1 receptor (also referred to as PEX5), a protein involved in the transport of peroxisomal enzymes. Analysis of the R38 protein sequence revealed that the most significant homology (66% identity) between the two proteins occurs at the COOH-terminal half of R38 where six TPR motifs are located to form a TPR domain (Fig. 1). TPR motifs are 34-amino acid repeats that are observed in a variety of proteins and are implicated in protein-protein interaction (19–21). In the case of PEX5, its TPR domain consists of seven TPR motifs and is involved in the binding of peroxisomal enzymes containing the tripeptide motif SKL at their carboxyl terminus (22–25).

We further analyzed the interaction of R38 with several other mutant forms of Rab8b with properties described for a number of Rab proteins, including Rab8a (26) (Table I). In addition to Rab8b and Rab8b-Q67L, R38 was able to interact with Rab8b-T22N (a form of Rab8b which is predominantly in the GDP-bound state) and Rab8b-C204S (a mutant which abolishes the terminal cysteine required for isoprenylation) constructs. No binding to R38 was observed with the dominant negative nucleotide-free mutant, Rab8b-N121I. Finally, we examined the interaction of two other Rab proteins, Rab8a and Rab3a, with R38. While Rab8a has over 80% homology with Rab8b, no binding of R38 was observed with Rab8a as well as Rab3a (Table I). These results suggested that the binding of Rab8b to R38 was specific, independent of the nucleotide state and prenylation status of Rab8b. We have therefore termed R38 as TRIP8b, for TPR-containing Rab8b interacting protein.

In addition to the interaction of TRIP8b with Rab8b mutants, the two-hybrid system was also employed to characterize the interaction of Rab8b with different regions of TRIP8b, the amino-terminal half (TRIP8b-N) and the COOH-terminal half (TRIP8b-TPR) that contains the TPR domain. As displayed in Table I, Rab8b interacts only with TRIP8b constructs containing the TPR domain (TRIP8b and TRIP8b-TPR). This result is similar to that observed for PEX5, which only requires its TPR domain to bind to enzymes that have the tripeptide motif SKL as their COOH-terminal residues (22). Because of these results and the homology between TRIP8b and PEX5, the interaction between Rab8b, which has the tripeptide motif SLL at its COOH terminus, and PEX5 was also examined. However, no
interaction was observed (Table I), indicating that the SLL motif of Rab8b cannot be recognized by the TPR domain of PEX5.

**Interaction of Rab8b and TRIP8b Using In Vitro Translation and Co-immunoprecipitation**—To further characterize the interaction of Rab8b and TRIP8b, TRIP8b was *in vitro* translated and the binding of TRIP8b to bacterially expressed GST fusion proteins was examined (Fig. 2A). Similar to what was observed in the yeast two-hybrid studies, TRIP8b was able to bind to GST-Rab8b. Because PEX5 only requires the COOH-terminal SKL motif to bind proteins, we also examined the interaction of TRIP8b with GST containing SLL as the last three COOH-terminal amino acids (as is the case for Rab8b) and Rac2, another small GTP-binding protein with the last three COOH-terminal amino acids being SLL. No interaction was observed with these proteins suggesting that the mechanism of interaction between TRIP8b with Rab8b is not similar to that observed for PEX5 with SKL-containing peroxisomal enzymes. We also examined the interaction of GST fusion proteins expressed in mammalian cell cultures, which allows prenylation of Rab proteins. As shown in Fig. 2B, binding of TRIP8b to Rab8b bound with either GDP or GDP was observed, consistent with the results from the bacterial and yeast binding studies. In the presence of 10 mM EDTA, which chelates the free magnesium results from the bacterial and yeast binding studies. In the

**TABLE I**

**Interaction of Rab8b with TRIP8b in the yeast two-hybrid system**

Bait and prey constructs were co-transformed into yeast Y190. The strength of the interaction between bait and prey constructs was determined by the β-galactosidase activity of the transformants. Rab8b mutants were prepared as described under “Experimental Procedures.” TRIP8b-N represents a construct that encodes the NH2-terminal 190 amino acids of TRIP8b, while the TRIP8b-TPR construct encodes the COOH-terminal of TRIP8b containing the TPR domain.

| Bait       | Prey       | β-Galactosidase activity |
|------------|------------|-------------------------|
| Rab8b      | TRIP8b     | ++++                    |
| Rab8b-Q67L | TRIP8b     | ++++                    |
| Rab8b-T22N | TRIP8b     | +++                    |
| Rab8b-C204S| TRIP8b     | +                      |
| Rab8b-N121I| TRIP8b     | −                      |
| Rab8a      | TRIP8b     | −                      |
| Rab8a      | TRIP8b     | −                      |
| Rab8b      | TRIP8b     | ++                     |
| Rab8b     | TRIP8b     | +                      |
| Rab8b     | TRIP8b-N   | −                      |
| Rab8b     | TRIP8b-TPR | ++++                   |
| Rab8b     | PEX5       | −                      |

**TRIP8b Is Associated with a Membrane Fraction via Its Interaction with Rab8b**—Immunofluorescence staining of endogenous TRIP8b in the AtT20 cells revealed punctate structures distributed over the entire cell body as well as a concentration of TRIP8b at the perinuclear and cell tips (Fig. 5A). These results suggest that TRIP8b can interact with membrane structures. Additionally, this staining pattern is similar to that observed for Rab8b (12) as well as other proteins involved in vesicle traffic to the plasma membrane such as Rab3a (13).

The distribution of Rab8b and TRIP8b in AtT20 cells was also examined in subcellular fractions using Western immunoblot analysis. As shown in Fig. 5B, both Rab8b and TRIP8b were detected in the supernatant and pellet fractions, indicating that both are present in cytosolic and membrane compartments. While Rab8b contains a prenylation site that enables it to interact with membranes, TRIP8b contains no hydrophobic domains and may therefore interact with membranes through another protein such as Rab8b or be peripherally associated. To further investigate this interaction, the membrane fraction was incubated with Na2CO3, pH 11, or Triton X-100 to determine the nature of TRIP8b binding to membranes. As shown in Fig. 5C, treatment with high pH caused TRIP8b to appear in the soluble fraction suggesting that the membrane-bound TRIP8b is peripherally associated. In addition, incubation in the presence of detergent also released TRIP8b into the soluble fraction. One possibility is that the association of TRIP8b with membranes is via its interaction with Rab8b. To examine this, the membrane fraction was incubated with GD11 that can remove Rab proteins from membranes (27). As expected, some of the membrane-bound Rab8b now appeared in the soluble fraction (Fig. 5D). More importantly, a fraction of TRIP8b also appeared in the soluble fraction. These results indicate that the membrane association of TRIP8b is through its interactions with Rab8b and that removal of Rab8b from the membrane results in solubilization of TRIP8b.

**TRIP8b and Myc-Rab8b Co-localize and Co-immunoprecipitate in the AtT20 Cells**—Both the constitutive and regulated secretion of the hormone ACTH have been well studied in AtT20 cells (28–30). To further examine the relationship between Rab8b and TRIP8b and their role in membrane traffic to the plasma membrane, AtT20 cells stably expressing vector only, Rab8b, TRIP8b, or a truncated TRIP8b containing the amino-terminal 190 amino acids (TRIP8b-N) were established and examined for their ability to release ACTH. AtT20 cells were stably transfected with Rab8b, TRIP8b, or TRIP8b-N and their protein expression confirmed by Western immunoblot analysis (data not shown). The expression of Rab8b and TRIP8b in the AtT20 stable cells was also examined by immunofluorescence staining (Fig. 6). As previously observed, the overexpression of Rab8b resulted in an altered morphology in the AtT20 cells, with a dramatic increase in the length of the cell processes compared with the vector only cells (Fig. 6A). Co-staining with antibodies against ACTH also reveals that Rab8b has significant co-localization with ACTH, in particular at the perinuclear region and cell tips where release of ACTH vesicles occurs (Fig. 6A). While in the cells expressing TRIP8b, co-localization was primarily observed at the cell tips.

To examine the co-localization of Rab8b and TRIP8b, Rab8b expressing cells were stained for both Rab8b and endogenous TRIP8b. As shown in Fig. 6B, there was significant overlap observed in the staining patterns of Rab8b and TRIP8b suggesting that the two proteins are present on similar membranes. However, while co-localization was observed, this does not mean that the proteins are interacting. To establish whether Rab8b and TRIP8b interact when expressed in cells, we used antibodies against TRIP8b to co-immunoprecipitate Rab8b from the Rab8b stable cells. As shown in Fig. 7, TRIP8b was able to specifically co-immunoprecipitate with Rab8b. Thus, immunofluorescence and co-immunoprecipitation data further confirm that Rab8b and TRIP8b interact in cells.
Rab8b and TRIP8b Stimulate Secretion of ACTH from AtT20 Cells—

Post-translation processing of proopiomelanocortin leads to a 30-kDa ABI product that is eventually cleaved to produce a 13-kDa mature ACTH product (31). Studies on the secretion of ABI and ACTH have indicated that the ABI is constitutively secreted while the secretion of ACTH is regulated (30). The release of ACTH and ABI from AtT20 cells were measured in the presence or absence of the secretagogue 8-Br-cAMP (Table II). In the vector only cells, no increase in secretion of ABI was observed after incubation with 8-Br-cAMP when compared with unstimulated cells. In contrast, ACTH product had a 10-fold increase in secretion after stimulation with 8-Br-cAMP. These results are consistent with other studies (13, 18, 28) and indicate that the ABI is secreted via a constitutive-like process, while ACTH is secreted in a regulated manner (28).

As shown in Table II, all the stable cell lines displayed cAMP (Table II). In the vector only cells, no increase in secretion of ABI was observed after incubation with 8-Br-cAMP when compared with unstimulated cells. In contrast, ACTH product had a 10-fold increase in secretion after stimulation with 8-Br-cAMP. These results are consistent with other studies (13, 18, 28) and indicate that the ABI is secreted via a constitutive-like process, while ACTH is secreted in a regulated manner (28).
Rab8b and TRIP8b Stimulate ACTH Release

When stimulated. In summary, these data demonstrate that Rab8b and TRIP8b stimulate the regulated but not the constitutive secretion of ACTH products in AtT20 cells. Furthermore, the stimulation effect of TRIP8b was not due to its NH2-terminal domain because TRIP8bN clones did not affect the secretion of mature ACTH.

DISCUSSION

We have identified a protein that interacts with Rab8b, termed TRIP8b, that contains six TPR motifs within the COOH-terminal half of the protein. Based on several in vitro and cell based approaches, the binding of Rab8b to TRIP8b appears to be independent of the nucleotide bound to Rab8b, and does not require the prenylated form of Rab8b (Table I, Figs. 2–4). The interaction with TRIP8b was specific as no interaction was observed for several other small GTP-binding proteins such as Rac2, Rab3a, or even the homolog Rab8a (Table I). Recently, the sequence of several orthologs of TRIP8b have been deposited in GenBank (accession numbers CAC01120, NP_057643, NP_067458, and BAA92878) and based on their homology, termed PEX5 related protein or peroxisomal targeting signal 1 receptor-like. However, our data suggests that TRIP8b is not involved in peroxisomal function. In contrast to PEX5, which binds to peroxisomal enzymes with COOH-terminal residues of SKL (22–25), no interaction was observed between TRIP8b and GST-SLL (Fig. 2) or GST-SKL (data not shown). Furthermore, the interaction of TRIP8b with Rab8b can occur in the absence of the last three COOH-terminal residues of Rab8b. In fact, this is normally the case for mature prenylated Rab proteins, which undergo proteolytic cleavage of the last three residues after prenylation of the terminal cysteine residue (32, 33). Finally, immunostaining of AtT20 cells against the peroxisomal enzyme catalase revealed a different staining pattern when compared with TRIP8b (data not shown).

Proteins containing TPR motifs are observed in a variety of species from bacteria to human and are involved in several protein-protein interactions (19–21). Theses 34 amino acid repeats are frequently observed in tandem and observed to form a structural lattice consisting of two antiparallel α helices linked by a short loop (34–36). In fact, the interaction the small GTP-binding protein Rac2 to phox67 is dependent on the TPR motifs of phox67. Recently, the crystal structure of that interaction has been determined (36). The structural data indicates that the residues of phox67 responsible for the interaction occur between the loops that connect TPR1 with TPR2 and TPR2 with TPR3 as well as a β-hairpin insertion that occurs...
Semiconfluent AtT20 cells grown in 12-well plates were washed with media and incubated for 60 min in media in the presence or absence of 5 mM 8-Br-cAMP. The media and cells were collected separately, the proteins precipitated, and the amount of ABI and ACTH released determined as described under "Experimental Procedures." The stable clones N4, F6, WT3, and WT6 represent high-expression single clones, while Nm1, Nm2, Fm1, and Fm2 represent mixed clonal populations. The fold stimulation value represents the amount of ABI or ACTH released in the presence of 8-Br-cAMP over the amount released in the absence of secretagogue. Values represent the average ± S.E. of three independent experiments.

### Table II

| Vector | N4 | Nm1 | Nm2 | F8 | Fm1 | Fm2 | WT3 | WT6 |
|--------|----|-----|-----|----|-----|-----|-----|-----|
| ABI    | 0.8 ± 0.2 | 1.1 ± 0.2 | 1.1 ± 0.2 | 1.3 ± 0.3 | 1.2 ± 0.1 | 0.9 ± 0.1 | 1.1 ± 0.1 | 0.9 ± 0.2 | 1.2 ± 0.7 |
| ACTH   | 10.8 ± 0.9 | 10.3 | 12.9 ± 0.6 | 10.8 ± 0.9 | 17.4 ± 1.1a | 19.4 ± 1.7a | 19.7 ± 2.3a | 16.8 ± 1.0a | 32.5 ± 2.1a |

*p < 0.05 in unpaired Student’s t test.

between TPR3 and TPR4. Similarly, the interaction of Rab8b with TRIP8b requires the TPR motifs. When TRIP8b was separated into two domains, only the COOH-terminal domain containing the TPR domain was capable of interacting with Rab8b. Based on the Rac2/phox67 model, it would be of interest to further dissect the interaction of these TPR motifs with Rab8b.

In contrast to Rab8b, which is ubiquitously expressed (12), Western immunoblot analysis of TRIP8b reveals it to be exclusively expressed in brain. When we evaluated several cell lines for the expression of TRIP8b, only the neuroendocrine cell line, AtT20, was found to contain TRIP8b. Subcellular fractionation of these cells indicated that a significant percentage of TRIP8b was membrane bound even though the predicted protein sequence contains no hydrophobic domains. TRIP8b is most likely peripherally associated with membranes, as it was capable of being extracted by high pH from the membrane fraction. In fact, further experiments with isolated membranes revealed that the binding of TRIP8b to membranes occurs through its interaction with Rab8b. When Rab8b was removed from the membrane using GDI1, a fraction of TRIP8b was observed in the supernatant fraction. Thus, Rab8b acts as a recruitment factor in this case, bringing TRIP8b to membranes.

The presence of TRIP8b in AtT20 cells allowed us to examine the effects of overexpression of TRIP8b on both constitutive and regulated secretion. The processing of the proopiomelanocortin leads to ABI that is constitutively secreted and ACTH, which is leads to ABI that is constitutively secreted and ACTH, which is likely of being extracted by high pH from the membrane fraction.

The majority of studies on Rab proteins and regulated secretion have focused on the Rab3 family members. The Rab3 family consists of four highly homologous isofoms associated with secretory granules and synaptic vesicles. Overexpression of each of the four members of the Rab3 family inhibited secretion (37). Moreover, knockout of Rab3A increased the quantal release of synaptic vesicles (39). In contrast to the inhibitory effect of Rab3 family on regulated secretion, overexpression of Rab8b exerted a stimulatory effect and suggests that several Rab may converge to regulate secretion.

While this is the first study to implicate Rab8b in regulated secretion, a previous study indicated that the overexpression of Rab8b in rat basophilic leukemia cells, a model system for mast cells, resulted in significant plasma membrane extension (12). These results are similar to the effect observed in the AtT20 stable cells lines Rab8b-WT3 and Rab8b-WT6. One possibility is that the effects on cell morphology observed by the overexpression of Rab8b are related to the stimulatory effects on the regulated secretion of ACTH. In the AtT20 stable cells expressing TRIP8b, only a slight increase in plasma membrane extensions was observed, however, the expression of TRIP8b was several fold less than observed for the Rab8b stable cell lines (data not shown). Since it is known that the dense core secretory granules accumulate at the tips of these processes, the morphological changes enabled by Rab8b expression may be related to the mechanism by which regulated secretion is enhanced. In fact, similar results have been observed with other proteins involved in ACTH secretion. Kalirin is an interacting partner of peptidylglycine α-amidating monoxygenase (40), an enzyme essential for the proteolytic processing of ACTH in AtT20 cells (41). In addition to peptidylglycine α-amidating monoxygenase, kalirin also can interact with the small GTPase Rac1 and serve as a guanine nucleotide exchange factor (42). The expression of kalirin in AtT20 cells also affects the cytoskeleton and causes the elongation of the cell processes, the same phenomena observed in the Rab8b stable cells. It was also demonstrated that exogenous expression of kalirin could restore the regulated ACTH secretion that is inhibited by the overexpression of peptidylglycine α-amidating monoxygenase (40). It is interesting to speculate that these two systems (kalirin/peptidylglycine α-amidating monoxygenase/Rac1 and TRIP8b/Rab8b) may utilize similar pathways to regulate the secretion of ACTH. Further exploration of this system may unveil the communication between Rab proteins on secretory vesicles and the Rho family proteins that regulate the cytoskeletal network and lead to a greater understanding of the regulation of the secretory pathway.

Acknowledgments—We thank Wanjin Hong and Ed Manser for ad-

---

**Table II**

Fold stimulation of ACTH release in AtT20 clones

| Vector | N4 | Nm1 | Nm2 | F8 | Fm1 | Fm2 | WT3 | WT6 |
|--------|----|-----|-----|----|-----|-----|-----|-----|
| ABI    | 0.8 ± 0.2 | 1.1 ± 0.2 | 1.1 ± 0.2 | 1.3 ± 0.3 | 1.2 ± 0.1 | 0.9 ± 0.1 | 1.1 ± 0.1 | 0.9 ± 0.2 | 1.2 ± 0.7 |
| ACTH   | 10.8 ± 0.9 | 10.3 | 12.9 ± 0.6 | 10.8 ± 0.9 | 17.4 ± 1.1a | 19.4 ± 1.7a | 19.7 ± 2.3a | 16.8 ± 1.0a | 32.5 ± 2.1a |

*p < 0.05 in unpaired Student’s t test.
Rab8b and TRIP8b Stimulate ACTH Release

REFERENCE

vic and critical comments on the manuscript. We thank Louis Lim for kindly providing the Rac2 cDNA.

1. Gonzalez, L. J., and Scheller, R. H. (1999) Cell 96, 755–758
2. Martinez, O., and Goud, B. (1998) Biochim. Biophys. Acta 1404, 101–112
3. Schimmoller, F., Simon, I., and Pfeffer, S. R. (1998) J. Biol. Chem. 273, 22161–22164
4. Echard, A., Jollivet, F., Martinez, O., Lacapere, J. J., Rousselet, A., Janoueix-Lerosey, I., and Goud, B. (1998) Science 279, 580–585
5. Kato, M., Sasaki, T., Ohyba, T., Nakazawa, H., Nishioka, H., Imamura, M., and Takai, Y. (1996) J. Biol. Chem. 271, 31775–31778
6. Guo, W., Roth, D., Walch-Solimena, C., and Novick, P. (1999) EMBO J. 18, 1071–1080
7. Waters, M. G., and Pfeffer, S. R. (1999) Curr. Opin. Cell Biol. 11, 453–459
8. Ollkonen, V. M., and Stemman, K. (1997) Int. Rev. Cytol. 176, 1–85
9. Huber, L. A., Pimplikar, S., Parton, R. G., Virta, H., Zerial, M., and Simons, K. (1999) J. Cell Biol. 123, 35–45
10. Huber, L. A., Dupree, P., and Dotti, C. G. (1995) Mol. Cell. Biol. 15, 918–924
11. Peranen, J., Auvinen, P., Virta, H., Wepf, R., and Simons, K. (1996) J. Cell Biol. 135, 153–167
12. Armstrong, J., Thompson, N., Squire, J. H., Smith, J., Hayes, B., and Solari, R. (1996) J. Cell Sci. 109, 1265–1274
13. Ngsee, J. K., Fleming, A. M., and Scheller, R. H. (1993) Mol. Biol. Cell 4, 747–756
14. Martinic, I., Peralta, M. E., and Ngsee, J. K. (1997) J. Biol. Chem. 272, 26991–26998
15. Manser, E., Huang, H. Y., Loo, T. H., Chen, X. Q., Dong, J. M., Leung, T., and Lim, L. (1997) Mol. Cell. Biol. 17, 1129–1143
16. Zhao, Z. S., Manser, E., Chen, X. Q., Chong, C., Leung, T., and Lim, L. (1998) Mol. Cell. Biol. 18, 2153–2163
17. Hutt, D. M., Da Silva, L. F., Chang, L. H., Prosser, D. C., and Ngsee, J. K. (2000) J. Biol. Chem. 275, 18511–18519
18. Baldini, G., Wang, G., Weber, M., Zeever, M., Bareggi, R., Witkin, J. W., and Martelli, A. M. (1998) J. Cell Biol. 140, 305–313
19. Ponting, C. C., and Phillips, C. (1996) Biochem. J. 314, 1053–1054
20. Lamb, J. R., Tugendreich, S., and Hieter, P. (1995) Trends Biochem. Sci. 20, 257–259
21. Blatch, G. L., and Laslele, M. (1999) Bioessays 21, 932–939
22. Otera, H., Okumoto, K., Tateishi, K., Ikoma, Y., Matsuda, E., Nishimura, M., Toukamotu, T., Osumi, T., Ohashi, K., Higuchi, O., and Fujiki, Y. (1998) Mol. Cell. Biol. 18, 388–399
23. Dotti, G., and Gould, S. J. (1996) J. Cell Biol. 135, 1763–1774
24. Gould, S. J., Keller, G. A., and Subramani, S. (1988) J. Cell Biol. 107, 897–905
25. Gould, S. J., Keller, G. A., Hosken, N., Wilkinson, J., and Subramani, S. (1998) J. Cell Biol. 108, 1657–1664
26. Ren, M., Zeng, J., De Lemos-Chiarandini, C., Rosenfeld, M., Adesnik, M., and Sabatini, D. D. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 5151–5155
27. Takai, Y., Kaibuchi, K., Kikuchi, A., Sasaki, T., and Shirataki, H. (1993) Ciba Found. Symp. 176, 128–38
28. Matsuuchi, L., and Kelly, R. B. (1991) J. Cell Biol. 112, 843–852
29. Moore, H. P., Gumbiner, B., and Kelly, R. B. (1983) Nature 302, 434–436
30. Dummermuth, E., and Moore, H. P. (1998) Methods 16, 188–197
31. Schnabel, E., Mains, R. E., and Farquhar, M. G. (1989) Mol. Endocrinol. 3, 1225–1235
32. Kinsella, B. T., and Malteese, W. A. (1991) J. Biol. Chem. 266, 8540–8544
33. Casey, P. J., and Seabra, M. C. (1996) J. Biol. Chem. 271, 5289–5292
34. Das, A. K., Cohen, P. W., and Barford, D. (1998) EMBO J. 17, 1192–1199
35. Scheufler, C., Brinker, A., Burenkov, G., Pogoraro, S., Moroder, L., Bartunik, H., Hartl, F. U., and Moarefi, I. (2000) Cell 101, 199–210
36. Lapeurge, K., Smith, S. J., Walker, P. A., Gamblin, S. J., Smerdon, S. J., and Rittinger, K. (2000) Mol. Cell 6, 899–907
37. Chung, S. H., Joberty, G., Gelino, E. A., Macara, I. G., and Holz, R. W. (1999) J. Biol. Chem. 274, 18113–18120
38. Matsuuchi, L., Buckley, K. M., Lowe, A. W., and Kelly, R. B. (1988) J. Cell Biol. 106, 239–251
39. Geppert, M., Goda, Y., Stevens, C. F., and Sudhof, T. C. (1997) Nature 387, 810–814
40. Mains, R. E., Alam, M. R., Johnson, R. C., Darlington, D. N., Back, N., Hand, T. A., and Eipper, B. A. (1999) J. Biol. Chem. 274, 2929–2937
41. Ciccozsto, G. D., Schiller, M. R., Eipper, B. A., and Mains, R. E. (1999) J. Cell Biol. 144, 459–471
42. Penzes, P., Johnson, R. C., Alam, M. R., Kamamtpati, V., Mains, R. E., and Eipper, B. A. (2000) J. Biol. Chem. 275, 6395–6403
Rab8b and Its Interacting Partner TRIP8b Are Involved in Regulated Secretion in AtT20 Cells
Shan Chen, Mui C. Liang, Jin N. Chia, Johnny K. Ngsee and Anthony E. Ting

J. Biol. Chem. 2001, 276:13209-13216.
doi: 10.1074/jbc.M010798200 originally published online January 24, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M010798200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 42 references, 28 of which can be accessed free at http://www.jbc.org/content/276/16/13209.full.html#ref-list-1