THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 272, No. 10, Issue of March 7, pp. 6097–6100, 1997

Printed in U.S.A.

Communication

The Tuberous Sclerosis 2 Gene Product, Tuberin, Functions as a Rab5 GTPase Activating Protein (GAP) in Modulating Endocytosis*

(Received for publication, November 20, 1996, and in revised form, January 16, 1997)

Guang-Hui Xiao‡, Fariba Shoarinejad‡, Fang Jin, Erica A. Golemis§, and Raymond S. Yeung¶

From the Divisions of Medical and §Basic Sciences, Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111

The tuberous sclerosis complex 2 (*TSC2*) is a tumor suppressor gene that plays a causative role in the autosomal dominant syndrome of tuberous sclerosis. The latter is characterized by the development of hamartomas and occasional malignancies. Expression of the wild-type gene in *TSC2* mutant tumor cells inhibits proliferation and tumorigenicity. This "suppressor" activity is encoded by functional domain(s) in the C terminus that contains homology to Rap1GAP. Using a yeast two-hybrid assay to identify proteins that interact with the C-terminal domain of tuberin, the product of *TSC2*, a cytosolic factor, rabaptin-5, was found to associate with a distinct domain lying adjacent to the *TSC2* GAP homology region. Rabaptin-5 also binds the active form of GTPase Rab5. Immune complexes of native tuberin, as well as recombinant protein, possessed activity to stimulate GTP hydrolysis of Rab5. Tuberin GAP activity was specific for Rab5 and showed no cross-reactivity with Rab3a or Rab6. Cells lacking tuberin possessed minimal Rab5GAP activity and were associated with an increased uptake of horseradish peroxidase. Re-expression of tuberin in *TSC2* mutant cells reduced the rate of fluid-phase endocytosis. These findings suggest that tuberin functions as a Rab5GAP *in vivo* to negatively regulate Rab5-GTP activity in endocytosis.

Tumor suppressor genes consist of a diverse group of genetic elements that encode proteins whose normal functions are to suppress cell proliferation and tumor formation. Their inactivation often plays a critical role in neoplastic transformation and is responsible for the initiation of the majority of hereditary cancers in humans. The *TSC2* gene is a new member of the tumor suppressor gene family that is involved in the autosomal dominant syndrome of tuberous sclerosis (TSC)[1] (1). The latter is a multi-organ disease of benign tumors (*i.e.* hamartomas) and malformations affecting tissues of mesodermal and ectodermal derivation (2). Occasionally, additional tumorigenic events can lead to malignant transformation affecting mainly the kidneys (3).

The study of the Eker rat model of hereditary cancer has provided additional evidence for the tumor suppressor role of *TSC2* (4, 5). These animals carry a germline mutation of *TSC2*, and tumors arising from the kidneys and uterus showed frequent loss of heterozygosity at this locus resulting in loss of protein expression (6, 7). Introduction of a wild-type *TSC2* gene or its 3′ region into the Eker tumor cell lines inhibited cell proliferation and tumorigenicity, thus providing direct experimental evidence for tuberin tumor suppressor function (8).

The *TSC2* gene, identified through positional cloning, encodes an open reading frame of 1870 amino acids with a region of sequence homology with the catalytic domain of Rap1GAP near the C terminus (1). Multiple splice variants that are conserved between rodents and human have been identified and are differentially expressed in adult tissues (9, 10). The ~190-kDa protein product, tuberin, is widely expressed and separates with the membrane/particulate (100,000 × *g* pellet) fraction (1, 4, 11). Immunofluoresence analysis has sublocalized the protein to the perinuclear region where it co-localized with Rap1 (12). Biochemical analysis has demonstrated *in vitro* GAP activity toward Rap1a, but the degree of stimulation of the intrinsic GTPase by tuberin is weak (11). While the physiologic significance of this activity *in vivo* remains undefined, it has been postulated that tuberin defective in its GAP activity could lead to the constitutive activation of Rap1a or other monomeric GTPase proteins which may result in deregulated mitogenic signaling in the target cells. Such a model would be analogous to the role of mutant neurofibromin in modulating Ras GTPase activity in schwannomas (13).

The Ras superfamily of small GTP-binding proteins are central to a wide variety of cellular processes and are regulated by different classes of proteins that determine the "on-off" state of the GTPases. GAPs stimulate the intrinsic rate of GTPases and serve as negative regulators of these binary switches. Unique GAPs exist for specific families of GTPases and perhaps for each member of the family. Substrate specificity exhibited by GAP proteins appears quite stringent. The $p120^{GAP}$ specifically stimulates GTPase activities of Ha-Ras, N-Ras, Ki-Ras and R-Ras, but not those of Rho, Rac, or Rab (14). Rap1a, a closely related member of Ras, can bind to $p120^{GAP}$, but its GTPase activity is unaffected (15). Proteins that activate GTPase of Rap1 include Rap1GAP, Spa1, and tuberin, but they share no similarity with $p120^{GAP}$. To understand the mechanism of tuberin function, studies were undertaken to identify proteins that interact with the C-terminal fragment containing the GAP homology domain. Surprisingly, we identified a tuberin-binding molecule that associates with the small GTPase Rab5 and demonstrated specific GAP activity of tuberin toward Rab5. These findings have implications for tuberin function in the endocytic pathway.

* This work was supported by National Institutes of Health Grant CA61889, American Cancer Society Grant CN-161, National Tuberous Sclerosis Association Grant 95-3, and an appropriation from the Commonwealth of Pennsylvania. 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.

‡ These authors contributed equally to this work.

¶ To whom correspondence should be addressed: Divisions of Medical and Basic Sciences, Fox Chase Cancer Center, 7701 Burholme Ave., Philadelphia, PA 19111. Tel.: 215-728-3117; Fax: 215-728-3574; E-mail: r_yeung@fccc.edu.

[1] The abbreviations used are: TSC, tuberous sclerosis complex; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; GST, glutathione *S*-transferase; GAP, GTPase activating protein; HRP, horseradish peroxidase; DTT, dithiothreitol.

EXPERIMENTAL PROCEDURES

Materials and Cell Lines—The rat *TSC2* cDNA clones and cell lines with *TSC2* mutation (LEF2, 18M) were as described (4, 8). Embryonic fibroblast cells (EEF4, EEF8) were derived from passages 12 to 14 of primary explants of embryos from a single (Ek/+ × Ek/+) mating. Horseradish peroxidase (HRP) type II was purchased from Sigma. HeLa and 136 cell lines were from ATCC (Rockville, MD).

Yeast Two-hybrid Assay—The 3′ fragment of the rat *TSC2* gene (nucleotides 4351–5352) was subcloned into the *Bam*HI site of the *LexA* fusion expression plasmid pJK202 and used as "bait" to screen a human fetal brain acid fusion library (gift of R. Brent, MGH, Boston, MA) in a yeast two-hybrid system as described (16). For domain mapping, deletion subclones of the original *TSC2* bait were generated by polymerase chain reaction and cloned into *Bam*HI/*Eco*RI sites of pJK202. Positive interactions were identified by growth on Leu^--Ura^--His^--Trp^- plates in the presence of galactose and by strong β-galactosidase activity. Individual positive clones were sequenced using the ABI373A automated DNA sequencer and analyzed for homology with sequences in the GenBank™ data base using the BLAST algorithm.

Antibodies—GST-fusion proteins were expressed in bacteria using pGEX constructs containing the C-terminal fragment of tuberin (L3, residues 1429–1761) and an N-terminal fragment of rabaptin-5 (B9, residues 455–717). For L3, insoluble recombinant protein was separated by SDS-PAGE and the gel slices containing the 65-kDa product were used to immunize New Zealand White rabbits along with Freund's adjuvant. For B9, soluble GST-fusion protein, purified using glutathione affinity chromatography, was used as immunogen. Polyclonal IgG was isolated from rabbit sera by binding to protein A and tested for specificity. For anti-L3, Western blot identified an ~190-kDa fragment which cross-reacts with a known antibody, anti-TubC (raised against the human *TSC2* product) in immunoprecipitation/immunoblot analyses (not shown) (11). Further, this band is absent in *TSC2* mutant cell lysate and not detected by preimmune sera. Anti-B9 antisera were tested for specificity using preimmune serum and blocking experiments with the purified antigen.

Co-immunoprecipitation Analysis—For *in vivo* binding assay, expression constructs for full-length rat *TSC2* and partial rabaptin-5 cDNAs were prepared in pcDNA3 (Invitrogen). Transfections into COS-7 cells were performed using the calcium phosphate precipitation method. Cells were collected 48 h after transfection and lysed in TNE buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 5 μg/ml leupeptin). One mg of protein was used for immunoprecipitation with 5 μl of antisera raised against rabaptin-5, anti-B9. The immune complexes were analyzed on 7% SDS-PAGE and electroblotted onto Hybond ECL nitrocellulose membrane (Amersham Life Science, Inc.). The blots were blocked overnight in 5% non-fat dry milk in TBS-T buffer, incubated with 1:2000 antisera for tuberin, anti-L3, and detected by the ECL Western blotting analysis system (Amersham Life Science). For co-immunoprecipitation of endogenous proteins, 500-μl lysates from HeLa and human sarcoma cell lines 136 were immunoprecipitated with 5 μl of anti-L3 antiserum. Samples were separated and immunoblotted with anti-B9 antiserum.

In Vitro GAP Assay—The GAP activities of immunoprecipitated tuberin and purified recombinant protein were measured using a nitrocellulose filter binding assay. Anti-L3 antibody was used to immunoprecipitate endogenous tuberin from lysates of HeLa (3×10^6 cells) prepared in 1 ml of lysis buffer (20 mM Tris-HCl, pH 7.5, 20 mM EDTA, 2 mM DTT, 0.2% (v/v) Nonidet P-40, 10 μg/ml aprotinin, 10 μg/ml leupeptin). The immune complexes were washed extensively with lysis buffer and resuspended in 10 μl of lysis buffer. Recombinant GST-tuberin fusion protein (amino acids 1429–1761) was extracted from *Escherichia coli* lysates as inclusion bodies, solubilized in 6 M guanidine HCl, 50 mM HEPES (pH 7.5), 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, dialyzed extensively in 25 mM HEPES (pH 7.5), 200 mM NaCl, 1 mM DTT, and affinity-purified by GST-glutathione affinity system. Rab5 and Rab6 were purified as described (17). Rab3a and Ha-Ras were purchased from PanVera Corp., Madison, WI. 10 μM Rab5, Rab3a, or Rab6 was loaded with 0.17 μM [γ-^{32}P]GTP (6000 Ci/mmol, DuPont NEN) in 50 mM Tris-HCl, pH 8.0, 2 mM EDTA, 1 mM DTT, 1 mg/ml bovine serum albumin, 0.05% (w/v) polyoxyethylene ether W-1 (Sigma), and 0.3% (w/v) CHAPS in a volume of 10 μl and incubated at 30 °C for 10 min. This mixture was diluted 6-fold in cold GAP buffer (loading buffer with 5 mM MgCl and 10 μM unlabeled GTP). Fifty-μl reactions containing 10 μl of diluted [γ-^{32}P]GTP-loaded protein, 10 μl of tuberin preparation, and 30 μl of GAP buffer were incubated at 20 °C for 30 min. At each time point, 10-μl aliquots of duplicate reactions were passed through nitrocellulose filters and washed with ice-cold buffer containing 20 mM Tris-HCl, pH 7.5, 10 mM MgCl, 100 mM NaCl. The amount of Rab5-bound [γ-^{32}P]GTP in each sample was quantitated by scintillation counting. For Ha-Ras, *in vitro* GAP assay was performed as described (11).

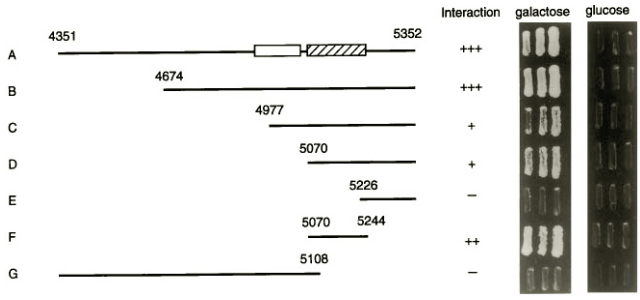

FIG. 1. **Rabaptin-5-binding domain of *TSC2* lies adjacent to but distinct from the GAP homology domain.** Deletion derivatives (*A–G*) of *TSC2* were fused to the DNA-binding domain of *LexA* and tested for interaction with the N-terminal portion of rabaptin-5 (residues 455–717) fused to the transcriptional activation domain in a yeast two-hybrid system. Colony growth on Leu^--Ura^--His^--Trp^- medium containing galactose or glucose after 72 h is shown on the *right-hand columns*. Positive interaction is indicated by selective growth on galactose and not glucose-containing medium. Parallel results were obtained from assaying β-galactosidase activity. *Hatched box*, rabaptin-5-binding domain corresponds to clone F; *open box*, *TSC2* GAP homology domain (1).

To analyze products of GTP hydrolysis, thin layer chromatography was performed as described (27). Briefly, Rab5 was preloaded with [α-^{32}P]GTP (3,000 Ci/mmol, DuPont NEN), as above and incubated with immunoprecipitated tuberin or controls for 5 min at 20 °C. After filtration and washing, the filters were suspended in 500 μl of 0.1 N HCl to elute guanine nucleotides from Rab5. The extract was neutralized to a pH of 7.2 with 130 μl of 0.5 M Tris and mixed immediately with 1 mM each of GTP and GDP. The samples were spotted on polyethyleneimine cellulose plates and chromatographed in sealed chamber filled with 1 M LiCl. GDP and GTP were detected by autoradiography and quantitated using a phosphorimage analyzer (BAS1000, Fuji, Japan).

HRP Uptake—Measurements of fluid-phase endocytosis was essentially as described (18). Cells were exposed to horseradish peroxidase (5 mg/ml, type II, Sigma) for 1 h at 37 °C and rinsed with phosphate-buffered saline, 0.5% bovine serum albumin. Cell pellets were lysed in 170 μl of 0.2% Triton X-100 and 10 mM HEPES, pH 7.2. The post-nuclear fractions (20 μl) were exposed to 1 ml of 0.01% *o*-dianisidine (Sigma), 50 mM sodium phosphate, 0.003% H_2O_2, 0.1% Triton X-100 in the dark for 1 h. HRP internalization was determined by the absorbance at 455 nm and adjusted for the amount of protein (mg) in the sample.

RESULTS AND DISCUSSION

We focused on the tuberin C-terminal region encompassing the GAP domain as a probe to identify potential binding molecules. An ~1-kilobase *Bam*HI fragment of the 3′ end of the rat *TSC2* gene (nucleotides 4351–5352) was cloned into pJK202 as bait vector in a yeast two-hybrid system (16). Homology search of 4 positive, overlapping clones revealed identity with a partial human cDNA sequence of unknown function (GenBank™ accession number X77723) and a recently cloned gene, rabaptin-5 (GenBank™ accession number X91141). The latter was isolated independently from an "interaction" search in a yeast two-hybrid system using the GTP-bound Rab5 as bait (19). Of the four partial rabaptin-5 clones identified in our screen, the smallest overlapping region corresponded to nucleotides 455–717 (amino acids 90–176) near the 5′ end of the gene. In contrast, Stenmark *et al.* (19) reported interaction of GTP-Rab5 with the C terminus of rabaptin-5 amino acids 551–862 (19). Thus there exist at least two protein-protein-binding domains in rabaptin-5. To define the structural requirement of tuberin for rabaptin-5 binding, deletion subclones of the *LexA-TSC2* constructs were tested for β-galactosidase activity and leucine auxotrophy. The smallest region of overlap was mapped to a 59-amino acid fragment near the C terminus of tuberin amino

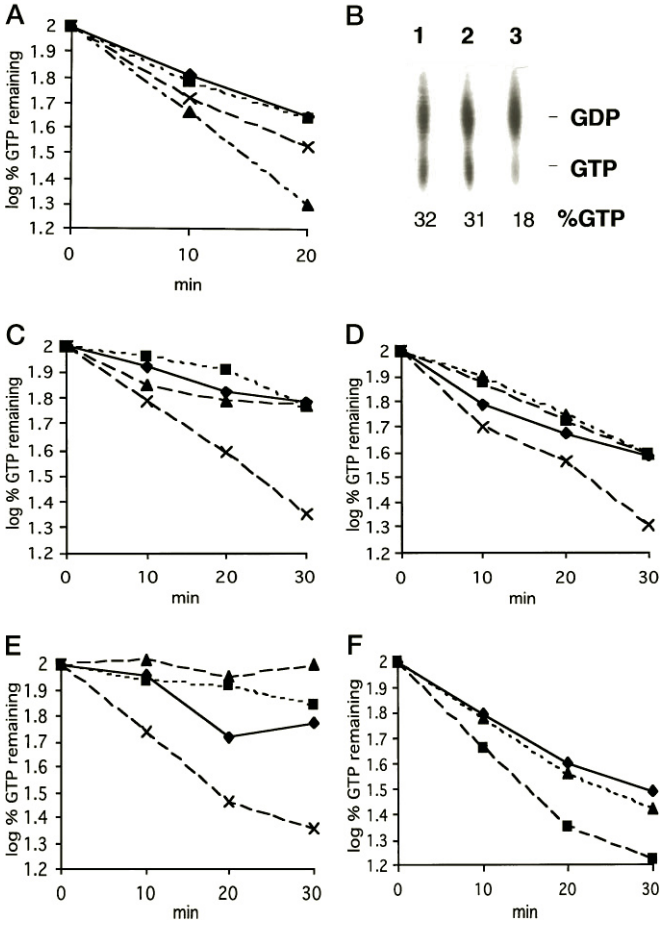

FIG. 2. **Tuberin possesses specific GAP activity for Rab5.** *A*, activation of Rab5 intrinsic GTPase activity by native tuberin was determined by nitrocellulose filter assay and expressed as the $\log_{10}$ fraction of GTP remaining bound to Rab5 as a function of time. ♦, buffer alone; ■, immunoprecipitate of preimmune serum; ▲, native tuberin immunoprecipitated by anti-L3; X, HeLa cell lysate. *B*, analysis of the protein-bound nucleotide by thin layer chromatography showed that the [α-^{32}P]GTP-bound Rab5 was hydrolyzed specifically to GDP. The percentages of bound GTP remaining after a 5-min incubation with buffer alone (*lane 1*), preimmune precipitate (*lane 2*), and native tuberin (*lane 3*) were determined by phosphorimage analysis and adjusted for the phosphorus content of GDP. *In vitro* GAP assay using native tuberin failed to show activity toward Ha-Ras (*C*), Rab3a (*D*), and Rab6 (*E*). *Symbols* as in *A*. Different intrinsic GTPase activities were reflected in the slopes of each assay. Consistent results were obtained in two to three independent experiments. *F*, whole cell lysates of EEF4 (■) and EEF8 (▲) were assayed for Rab5 GAP activity. EEF8 cells were deficient of endogenous tuberin (see Fig. 3*B*) and possessed minimal activity compared with buffer alone (♦).

acids 1668–1726 (Fig. 1). The *TSC2*-GAP homology domain lies adjacent but distinct from this region. Of significance, there exist at least two examples of missense mutations within this rabaptin-5-binding domain in two affected TSC individuals (20).

To further investigate *in vivo* tuberin-rabaptin-5 interaction in eukaryotic cells, binding assays were conducted following transient transfection in COS-7 cells and with endogenous proteins in human-derived cell lines. A polyclonal anti-rabaptin-5 antiserum, anti-B9, directed against the *TSC2*-binding domain of rabaptin-5 (residues 455–717) was used to immunoprecipitate lysate from COS-7 cells transfected with full-length rat *TSC2* and/or partial rabaptin-5 cDNAs (data not shown). The resultant protein complexes were resolved on SDS-PAGE and analyzed on immunoblot developed with anti-tuberin antibody, anti-L3. The ~190-kDa tuberin band co-purified with rabaptin-5 in cells transfected with both *TSC2* and rabaptin-5 expression vectors and not when either one is omitted. The ability of endogenous tuberin to stably bind to rabaptin-5 was assessed in HeLa and CCL-136 (human sarcoma cell line, ATCC, Rockville, MD) cells. Lysates were immunoprecipitated with anti-L3, and the products were analyzed for the presence of rabaptin-5 by immunoblot detection. The anti-L3 purified tuberin complexed to the 115-kDa rabaptin-5 that was not evident in the preimmune sera immunoprecipitate (data not shown). These results established that tuberin physically associates with rabaptin-5 *in vivo*.

As a membrane-bound GTPase activating protein, tuberin may promote the hydrolysis of Rab5-GTP to its inactive GDP form via its interaction with rabaptin-5. To test this hypothesis, *in vitro* GAP activity of tuberin was examined using purified Rab5 as the substrate. Native tuberin immunoprecipitated from HeLa cells with anti-L3 showed substantial levels of GAP activity when incubated with [γ-^{32}P]GTP-Rab5 (Fig. 2*A*). While the intrinsic GTPase activity of Rab5 *in vitro* was high as was previously shown (21, 22), the rate of GTP hydrolysis was further accelerated by the anti-L3 immunoprecipitate and not preimmune control (Fig. 2*B*). Tuberin GAP activity toward Rab5 was specific since no activation of GTPase activity was noted for Ras and other members of the Rab family including Rab3a and Rab6 (Fig. 2, *C–E*). GAP activity was also detected, albeit weaker, using purified recombinant GST-fusion protein consisting of the C-terminal region of tuberin, in the absence of rabaptin-5 (data not shown). This suggests that the latter is not required for GTPase activation and may function as an adapter protein to recruit Rab5-GTP to tuberin. Recent evidence reported that in the relative abundance of rabaptin-5, the rate of nucleotide triphosphate hydrolysis by Rab5 *in vitro* was reduced (22). This would suggest that the binding of excess amounts of rabaptin-5 to both Rab5 and tuberin might hinder the interaction between the GTPase and its GAP.

Rap1a has been also implicated as a substrate for tuberin GAP activity *in vitro*, but the magnitude of activation is weak (11). Under conditions of our assay, the observed GAP activity of tuberin on Rap1a was noted to a lesser extent than that of Rab5 (data not shown), suggesting that Rab5 may be the primary target for tuberin GAP function in HeLa cells. To determine the *in vivo* relevance of the observed Rab5GAP activity of tuberin, we examined the relative levels of GAP activity in cells with and without endogenous tuberin. The latter consisted of embryonic cultured cells derived from an Eker heterozygous mating. Total cell lysate from *TSC2*−/− embryo fibroblasts possessed minimal GAP activity toward Rab5 compared with *TSC2*+/+ cell lysate (Fig. 2*F*); thus, total cellular Rab5GAP activity correlated with tuberin expression. The evidence for a biochemical interaction between tuberin, Rab5, and Rap1 highlights the unique substrate specificities of tuberin toward two dissimilar GTPases. This is surprising in light of the stringent specificity of GAP proteins. Rab5 and Rap1a belong to different families of the Ras-like GTPases and share only 33% identity. While Rab5 has a unique function in the endocytic pathway, the physiologic role of Rap1a is less well defined. However, both Rab5 and Rap1 have been detected in endosomal compartments (Rap1 also resides in the Golgi) and is consistent with the perinuclear localization of tuberin (12, 23, 24). Our results suggest a potential role of *TSC2* product in the regulation of endocytosis.

The Rab5 GTPase is a critical and rate-limiting component of the docking and fusion process of the endocytic pathway (23). This suggests that proteins governing the nucleotide-bound state of Rab5 (*e.g.* guanine dissociation inhibitors, guanine exchange factors, and GAPs) may have a regulatory role in endocytosis. To assess the potential function of tuberin in ve-

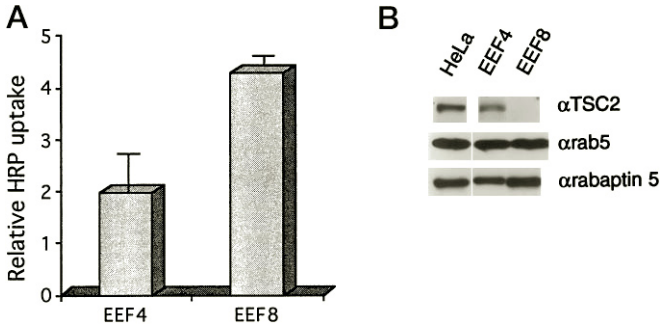

FIG. 3. **Tuberin reduces the rate of fluid-phase endocytosis.** *A*, internalization of HRP in Eker rat-derived embryo fibroblasts, EEF4 (*TSC2*+/+) was compared with that of EEF8 (*TSC2*−/−), following a 1-h exposure. Results (absorbance at A_{455}/mg of protein) were normalized to HeLa controls and expressed as the mean ± S.D. from two independent experiments with duplicate samples. *B*, both cell lines expressed equal amounts of Rab5 and rabaptin-5, but only EEF4 expressed tuberin. Total cell lysates were immunoblotted with indicated antibodies (anti-L3 for *TSC2*; anti-B9 for rabaptin-5; 4F11 monoclonal antibody for Rab5).

sicular transport, we examined the effects of endogenous *TSC2* gene expression in fluid-phase HRP uptake in *TSC2* mutant cells derived from the Eker rat. Upon transient exposure to HRP (5 mg/ml), embryo fibroblasts from *TSC2*−/− fetuses endocytosed significantly greater amounts of HRP *in vitro* compared with those of *TSC2*+/+ genotype (Fig. 3*A*). These cells, while differing in tuberin protein levels, expressed equal amounts of Rab5 and rabaptin-5 (Fig. 3*B*). This finding, in conjunction with the contrasting endogenous GAP activities for Rab5 of these two cell lines (see Fig. 2*F*), is consistent with a negative regulatory role of tuberin on Rab5 activity *in vivo*. Furthermore, in transient transfection studies, tuberin re-expression in *TSC2*−/− tumor cells reduced the rate of HRP uptake (data not shown). It remains to be defined which process tuberin mediates during endocytosis.

Intracellular trafficking is highly specific and directional. It has been postulated that unique sets of effectors may exist for individual Rab proteins to account for the required specificity. Our findings have identified tuberin as a protein with substantial Rab5-GAP activity and demonstrated that tuberin association with Rab5 is mediated by an intermediate adapter-like molecule, rabaptin-5. These data add to the current model of the early endocytic pathway in which a Rab5-GTP bound vesicle recruits rabaptin-5 to the cytosolic surface of the membrane. This, in turn, targets the vesicle to a tuberin-bound organelle where proper docking and fusion can take place. A second function of tuberin is to stimulate hydrolysis of Rab5-GTP, thereby releasing the GDP-Rab5 and rabaptin-5 into the cytosol for recycling. The specificity of this interaction is governed by at least two mechanisms. Rabaptin-5 displays specific binding for Rab5 and not other related GTPases (19), and secondly, tuberin GAP activity does not cross-react with other Rab-GTPases besides Rab5. Thus, the functional interplay between Rab5, rabaptin-5, and tuberin provides one level of specificity that may operate in concert with the SNAP receptors to ensure compartment-specific docking. Recent evidence suggested that while Rab5 is necessary for early endosome fusion, GTP hydrolysis by Rab5 is not required in this process, but rather the fusion reaction is dependent on certain cytosolic factors (22). Whether the Rab5-rabaptin-5-tuberin association is sufficient for vesicle docking is not known, but tuberin is capable of interacting with other proteins that may serve as additional components of the fusion machinery.[2] This is consistent with fractionation data suggesting that rabaptin-5 may exist in a high molecular weight multi-protein complex (19).

The mechanism by which a tumor suppressor gene, such as *TSC2*, causes the development of hamartoma and neoplasia based on its effects on protein trafficking remains speculative. One hypothesis is to suggest that the loss of tuberin Rab5GAP activity would interfere with the docking, fusion, and processing of the Rab5-GTP-associated early endosomes. Perturbation of the endocytic pathway could lead to missorting of internalized growth factor receptors or other signal-mediated membrane-bound molecules that would otherwise undergo lysosomal degradation. For example, cells expressing non-internalizing epidermal growth factor receptors behaved in ways similar to transformed cells (25). Recent evidence also points to the critical role of endocytic trafficking not only in down-regulating epidermal growth factor receptor signaling but also in controlling specific signaling pathways (26). It remains to be determined if tuberin dysfunction could lead to aberrant turnover of ligand-activated receptor tyrosine kinases during tumorigenesis. The relative importance of Rab5GAP and Rap1GAP activities in governing *TSC2*-related tumor suppression is yet to be defined.

Acknowledgments—We thank A. G. Knudson and J. Chernoff for discussion and comments on the manuscript. We also thank J. DeClue and R. Wienecke for help with GAP assays. We are grateful to C. Der for providing expression constructs of Rab5 and Rab6, R. Brent for the human central nervous system fusion library, and M. Zerial for the Rab5 (4F11) antibody.

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