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)

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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 TSC2, a 190-kDa protein product, tuberin, is widely expressed and 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)†. 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). Immunofluorescence analysis has sublocalized the protein to the perinuclear region where it co-localized with Rab1 (12). Biochemical analysis has demonstrated in vitro GAP activity toward Rap1α, but the degree of stimulation of the intrinsic GTPase by tuberin is weak (11). While the physiological 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 Rap1α 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 p120GAP specifically stimulates GTPase activities of Ha-Ras, N-Ras, Ki-Ras and R-Ras, but not those of Rho, Rac, or Rab (14). Rap1α, a closely related member of Ras, can bind to p120GAP, but its GTPase activity is unaffected (15). Proteins that activate GTPase of Rap1 include Rap1GAP, Spa1, and tuberin, but they share no similarity with p120GAP. 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.

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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.
Tuberin Possesses Rab5GAP Activity

EXPERIMENTAL PROCEDURES

Materials and Cell Lines—The rat TSC2 cDNA clones and cell lines with TSC2 mutation (LEF2, 15M) were as described (4, 8). Embryonic fibroblast cells (E14F4, E14F8) were derived from passages 12 to 14 of primary explants of embryos from a single (E1+/×E1+/) mating. Horseradish peroxidase (HRP) type II was purchased from Sigma. HeLa and 136 cell lines were from ATCC (Rockville, MD).

Two-hybrid Assay—The 3’ fragment of the TSC2 gene (nucleotides 4351–5352) was subcloned into the BamHI site of the LexA fusion expression plasmid pJK202 and used as “bait” to screen a human fetal brain cDNA 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 SacHI/EcoRI 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 GenBankTM 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 using Western blotting. Anti-Tuberin antibodies were raised against a known antibody, anti-TubC (raised against the human TSC2 product) in immunoprecipitation/immunoblot analysis (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.

Immunoprecipitation Analysis—For in vitro 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 lysis 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 anti antisera raised against rabaptin-5, anti-B9. The immunocomplexes 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, Inc.). Co-immunoprecipitation of partial rabaptin-5 (residues 455–717) was fused to the DNA-binding domain of LexA (GenBankTM accession number X91141). The latter was isolated independently from an “interaction” search in a yeast two-hybrid system.

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 100 µl of 0.5 M Tris and mixed immediately with 1 mM H2O2. 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).

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 BamHI 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 (GenBankTM accession number X77723) and a recently cloned gene, rabaptin-5 (GenBankTM accession number X91144). 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-terminal 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...
Tuberin possesses specific GAP activity for Rab5. A, activation of Rab5 intrinsic GAP 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. **A**, buffer alone; **B**, immunoprecipitate of preimmune serum; **C**, native tuberin immunoprecipitated by anti-L3; X, HeLa cell lysate. **B**, analysis of the protein-bound nucleotide by thin layer chromatography showed that the [{\textsuperscript{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 GAP 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. 3B) 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 GAPase 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 [{\textsuperscript{32}}P]GTP-Rab5 (Fig. 2A). While the intrinsic GAP 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. 2B). Tuberin GAP activity toward Rab5 was specific since no activation of GAPase activity was noted for Ras and other members of the Rab family including Rab3a and Rab6 (Fig. 2C–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 GAPase 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 GAPase 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. 2F); 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 GAPases. 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 GAPase 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-
sicul 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. 3A). These cells, while differing in tuberin protein levels, expressed equal amounts of Rab5 and rabaptin-5 (Fig. 3B). This finding, in conjunction with the contrasting endogenous GAP activities for Rab5 of these two cell lines (see Fig. 2F), is consistent with a negative regulatory role of tuberin on Rab5 activity in vivo. Furthermore, in transient transfection studies, tuberin re-expression in TSC2−/− 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. 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 mis-sorting 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. Wiennecke 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 Rab8 (4F11) antibody.

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