Characterization of the Cytosolic Tuberin-Hamartin Complex

TUBERIN IS A CYTOSOLIC CHAPERONE FOR HAMARTIN®

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Tuberous sclerosis (TSC) is an autosomal dominant disorder characterized by a broad phenotypic spectrum that includes seizures, mental retardation, renal dys- function and dermatological abnormalities. Mutations to either the TSC1 or TSC2 gene are responsible for the disease. The TSC1 gene encodes hamartin, a 130-kDa protein without significant homology to other known mammalian proteins. Analysis of the amino acid sequence of tuberin, the 200-kDa product of the TSC2 gene, identified a region with limited homology to GTPase-activating proteins. Previously, we demonstrated direct binding between tuberin and hamartin. Here we investigate this interaction in more detail. We show that the complex is predominantly cytosolic and may contain additional, as yet uncharacterized components alongside tuberin and hamartin. Furthermore, because oligomerization of the hamartin carboxyl-terminal coiled coil domain was inhibited by the presence of tuberin, we propose that tuberin acts as a chaperone, preventing hamartin self-aggregation.

Tuberous sclerosis (TSC) is characterized by the development of hamartomatous growths in many tissues and organs (1). In particular, the brain and skin are affected, leading to the classic phenotype of seizures, mental retardation, and facial and ungual angiofibromas. Renal, cardiac, and retinal tissues are also often affected. The majority of cases of TSC are sporadic, caused by a de novo mutational event. However, in familial cases the disease segregates as an autosomal dominant trait, linked either to chromosome 9q34 (TSC1) or to chromosome 16p13.3 (TSC2). The genes mapping to these loci and identified (2, 3). Screening large numbers of TSC patients indicates that the majority of patients carry TSC2 mutations. The ratio of TSC1 to TSC2 mutations in familial cases is approximately equal (4).

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The TSC1 gene codes for hamartin, a novel 130-kDa protein. Hamartin has a relatively high proportion of hydrophilic amino acids and a large region close to the carboxyl terminus that is predicted to form coiled coil structures (amino acids 719–998). Coiled coils mediate many protein-protein interactions (5). In addition, hamartin contains a putative transmembrane domain, and it has been suggested that hamartin is associated with membranes (6). The TSC2 gene encodes tuberin, a 200-kDa protein that contains a stretch of 163 amino acids with homology to the ras GTPase-activating proteins rap1GAP (3), p130(14–1) (7), and Drosophila rapGAP1 (8). Tuberin has been shown to have GAP activity toward rap1 (9) and rab5 (10), another small GTPase of the Ras superfamily. Despite the reported GAP activity, the exact function of tuberin is not yet clear. Possible roles in endocytosis (10), cell cycle regulation (11), differentiation (12), and steroid receptor modulated transcription (13) have all been proposed.

Tuberin and hamartin form a complex in vivo (6, 14). Studies using the yeast two-hybrid system indicate that the first of the tuberin coiled coil regions (amino acids 346–371) and the first heptad repeats of the hamartin coiled coil domain are involved in the association between the two proteins (14). The demonstration that tuberin and hamartin bind directly and the fact that mutations to either gene lead to the same phenotypic spectrum suggest that both proteins are required for the correct function of the tuberin-hamartin complex, and that it is the inactivation of the complex that leads to TSC. Here we investigate the formation, composition, and subcellular localization of the tuberin-hamartin complex and demonstrate that tuberin may act as a chaperone, preventing self aggreation of hamartin via its carboxyl-terminal coiled coil domain.

EXPERIMENTAL PROCEDURES

Generation of Constructs and Antisera—The full-length TSC1 and TSC2 expression constructs have been described previously (14), although in this study an untagged TSC2 expression construct (in pcDNA3.1) was also used. Truncated TSC1 and TSC2 expression constructs were derived by appropriate restriction digestion of the full-length cDNAs. Truncated TSC2 cDNAs encoding amino acids 1–1240, 1–1099, 1–1099, 1–607, 607–1099 were cloned into pcDNA3.1-derived expression vectors containing either an amino-terminal Xpress epitope tag or a carboxyl-terminal myc epitope tag (Invitrogen). The rabaptin cDNA was isolated in a yeast two-hybrid screen with rab4 as bait and cloned into pcDNA3.1His. Antiserum has been described previously (21). Transferrin-Texas Red (Invitrogen) was used. Truncated TSC1 and TSC2 expression constructs were transfected into COS-7 cells with LipofectAMINE and PLUS reagent.

Transfections and Immunocytochemistry—Expression constructs were transfected into COS-7 cells with LipofectAMINE and PLUS reagent.
gent, as recommended by the manufacturer (Life Technologies). For immunocytochemistry, cells on coverslips were fixed with 3% paraformaldehyde for 10 min at room temperature and permeabilized in either 0.1% Triton X-100 for 5 min or methanol for 20 min. The coverslips were incubated with primary antibodies diluted 1:100 in phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin and 0.1% Tween 20, followed by fluorescein isothiocyanate- or rhodamine isothiocyanate-coupled secondary antibodies. Images were captured using the PowerGene fluorescence in situ hybridization system on a Leica DM RXA microscope. Images were processed using a filter wheel (Chroma Technology) and the Adobe Photoshop software package.

**RESULTS**

**Tuberin and Hamartin Cofractionate**—We first analyzed the localization of tuberin and hamartin by subcellular fractionation. HeLa cells were homogenized and fractionated by high speed centrifugation into pellet and supernatant fractions. As shown in Fig. 1, immunoblot analysis demonstrated that both tuberin and hamartin were present exclusively in the supernatant fraction. Extraction of the pellet with either 3-[3-cholamidopropyl(dimethylammonio)-1-propanesulfonic acid or Triton X-100 did not alter the fractionation profile of either protein. After high-speed centrifugation, both tuberin and hamartin were recovered in the pellet fraction. In contrast, resuspension of the pellet in either high ionic strength (0.5 m NaCl) or high pH (10 mM Na2CO3, pH 10.7) buffers resulted in partial solubilization of both proteins. Cofractionation of tuberin and hamartin in more detail, flotation gradient density centrifugation was performed. The postnuclear supernatant from HeLa cell homogenates were fractionated by high speed centrifugation as described under "Experimental Procedures." The pellet was extracted with salt or detergent and recentrifuged. The first supernatant fraction (S1) and final pellet (P2) and supernatant (S2) fractions were analyzed for the presence of tuberin and hamartin by immunoblotting.

**Tuberin and Hamartin Are Localized Predominantly to the Cytosol**—To investigate the putative cytosolic localization of tuberin and hamartin in more detail, flotation gradient density centrifugation was performed. The postnuclear supernatant from a HeLa cell homogenate was brought to a concentration of 50% sucrose and overlaid with a 15–40% (w/v) sucrose gradient. After centrifugation at 42,000 rpm for 14 h in a Beckman SW50.1 ultracentrifuge rotor at 4 °C, the supernatant was then spun at 38,000 rpm for 60 min in a Beckman ultracentrifuge SW50.1 rotor at 4 °C. The supernatant was recovered, and the pellet was suspended in homogenization buffer alone or homogenization buffer containing one of the following: 10 mM Na2CO3, pH 10.7, 1% Triton X-100, 1% 3-[3-cholamidopropyl(dimethylammonio)-1-propanesulfonic acid, 1% Triton X-100 plus 0.15 M NaCl, or 0.5 M NaCl. The suspension was then resuspended in 38,000 rpm for 60 min in a Beckman SW50.1 ultracentrifuge rotor at 4 °C, and the pellet and supernatant fractions were recovered and analyzed by immunoblotting.

**Cytosol**—

Postnuclear supernatants were prepared from three confluent 10-cm plates of HeLa cells as described under "Subcellular Fractionation." The postnuclear supernatant was brought to 50% (w/v) sucrose and overlaid with a 15–40% (w/v) sucrose gradient. After centrifugation at 42,000 rpm for 14 h in a Beckman SW50.1 ultracentrifuge rotor at 4 °C, 0.2-mI gradient fractions were analyzed for the presence of hamartin and tuberin by immunoblotting.

**RESULTS**

**Tuberin and Hamartin Cofractionate**—We first analyzed the localization of tuberin and hamartin by subcellular fractionation. HeLa cells were homogenized and fractionated by high speed centrifugation into pellet and supernatant fractions. As shown in Fig. 1, immunoblot analysis demonstrated that both tuberin and hamartin were present exclusively in the supernatant fraction. Extraction of the pellet with either 3-[3-cholamidopropyl(dimethylammonio)-1-propanesulfonic acid or Triton X-100 did not alter the fractionation profile of either protein. After high-speed centrifugation, both tuberin and hamartin were recovered in the pellet fraction. In contrast, resuspension of the pellet in either high ionic strength (0.5 M NaCl) or high pH (10 mM Na2CO3, pH 10.7) buffers resulted in partial solubilization of both proteins. Cofractionation of tuberin and hamartin is consistent with the two proteins binding together in a complex, whereas solubilization by 10 mM Na2CO3 but not by 1% Triton X-100 suggests that the complex is unlikely to be associated with membranes and therefore that tuberin and hamartin are more likely to be cytosolic proteins. Coimmunoprecipitation of tuberin and hamartin from the high salt- and high pH-solubilized fractions with antisera specific for either protein indicated that, under these conditions, tuberin and hamartin remained bound together in a complex (data not shown).

**Tuberin and Hamartin Are Localized Predominantly to the Cytosol**—To investigate the putative cytosolic localization of tuberin and hamartin in more detail, flotation gradient density centrifugation was performed. The postnuclear supernatant from a HeLa cell homogenate was brought to a concentration of 50% sucrose and overlaid with a 15–40% sucrose gradient of decreasing density. After high speed centrifugation at 42,000 rpm for 14 h in a Beckman SW50.1 rotor, fractions were collected from the top of the gradient and analyzed by immunoblotting. Organelles and membranes disperse along the gradient according to their buoyant density, whereas cytosolic proteins remain in the bottom layer. As shown in Fig. 2, the vast majority of tuberin and hamartin remained in the lower cytosolic fractions, whereas the lysosomal enzyme α-glucosidase was present in the upper half of the gradient (data not shown). This result strongly suggested that tuberin and hamartin colocalise to the cytosol.
A very small proportion (<1%) of both tuberin and hamartin consistently cofractionated with a membrane fraction. This suggests that the tuberin-hamartin complex might associate transiently with a specific membrane. Whether the tuberin-hamartin complex shuttles between membrane-bound and cytosolic states and the possible significance of this function of the complex are currently under investigation.

Localization of Tuberin and Hamartin by Immunofluorescence Microscopy—Because our antibodies against tuberin and hamartin did not enable us to define reliably the localization of the endogenous proteins by immunocytochemistry, we expressed tuberin and hamartin exogenously, in transfected COS cells. First we transfected a TSC2 expression construct. Tuberin was distributed diffusely in the cytoplasm and was not obviously associated with intracellular membranes. Because tuberin had been reported to be associated with the Golgi complex (15), we used wheat germ agglutinin as a marker for the Golgi complex. As shown in Fig. 3, we did not detect any association between tuberin and the Golgi complex. Our results are therefore not in agreement with those of Wienecke and co-workers (15) but are consistent with the finding that tuberin binds rabaptin, a cytosolic effector of rab5 (10, 16). We performed double label immunofluorescence and coimmunoprecipitation studies on cells cotransfected with both TSC2 and rabaptin cDNAs. As shown in Fig. 4, both proteins gave a very similar diffuse, cytoplasmic labeling pattern. However, despite this colocalization and in contrast to the findings of Xiao and co-workers (10), we could find no evidence for a direct association between tuberin and rabaptin in coimmunoprecipitation experiments. Yeast two-hybrid assays for interaction between tuberin and rabaptin were also negative (data not shown).

Next we investigated the expression of hamartin in transfected COS cells. In contrast to the diffuse labeling of tuberin, hamartin was predominantly localized to distinct, punctate cytoplasmic structures. Furthermore, unlike tuberin, which is partially recruited to the hamartin structures (14), we could find no association between rabaptin and hamartin either by double label immunofluorescence, as shown in Fig. 4, or by coimmunoprecipitation (data not shown). Additional transfection experiments to express a series of truncated hamartin variants indicated that amino acids 788–1153, containing a large proportion of the predicted coiled coil domain, caused the punctate labeling. Hamartin constructs lacking this domain did not result in the same distinctive labeling pattern (data not shown). These results suggested that the predicted coiled coil domain was required either for association of hamartin with a membrane-bound organelle or alternatively for the formation of large, hamartin-containing protein aggregates.

To investigate the subcellular localization of hamartin in more detail, we performed a series of double label immunofluorescence experiments with marker proteins for the central vacuolar system. One possibility was that the punctate labeling reflected association with a membrane bound organelle. However, hamartin did not co-localize with the endoplasmic reticulum marker protein disulphide isomerase, with the Golgi complex-specific lectin wheat germ agglutinin, with the early endosomal and late endosomal markers rab5 and rab7, or with lysosomal acid α-glucosidase (data not shown). Moreover, the structures did not label with fluorescently labeled transferrin, a marker for early endocytic compartments. To investigate the alternative explanation that the punctate structures were attributable to specific self-aggregation of hamartin caused by homomeric interaction of the carboxyl-terminal coiled coil domain, yeast two-hybrid assays were performed. A CDNA encoding the hamartin coiled coil region (amino acids 334–1153) was cloned into Gal4 activation (pGADGH) and binding (pGBT9) domain constructs and tested for interaction using the β-galactosidase assay. As shown in Fig. 5, a strong induction of β-galactosidase activity was detected, suggesting that the hamartin coiled coil domains had the ability to form homodimers. No β-galactosidase activity was detected with either activation or repression.
Hamartin and Tuberin Cofractionate by Velocity Gradient Centrifugation—The fact that hamartin interacted with itself in yeast two-hybrid assays suggested that the labeling pattern observed in cells transfected with TSC1 might indeed represent hamartin self-aggregation. We took a biochemical approach to investigate this question. COS cells transfected with TSC1 cDNA were lysed in 0.2% Nonidet P-40, and the lysates were cleared by centrifugation for 10 min at 10,000 rpm. The resulting supernatant was then overlaid onto a 5–25% sucrose gradient. Identical results were obtained with detergent and salt-resistant protein complexes.

As shown in Fig. 6b, using the same sucrose gradient centrifugation procedure on detergent lysates of COS cells transfected with TSC2, we detected >95% of tuberin approximately halfway along the gradient. Only a minor proportion (<5%) was detectable in the pellet fraction, suggesting that most if not all of the overexpressed tuberin was not associated with the dense, detergent-resistant fraction and was therefore, in contrast to hamartin, not part of an insoluble protein complex. The different sedimentation profiles of tuberin and hamartin reflected the distinct immunofluorescent labeling patterns; overexpressed tuberin produced a diffuse cytosolic labeling, in contrast to the punctate labeling of hamartin. To gain more insight into the intracellular localization of the tuberin-hamartin complex, high speed velocity gradient centrifugation was performed on detergent lysates of nontransfected HeLa cells. As shown in Fig. 6c, tuberin and hamartin co-migrated through the gradient. Similar results were obtained with COS cells cotransfected with TSC1 and TSC2 (data not shown). Neither protein was present in the pellet fraction, suggesting that tuberin and hamartin form a soluble complex, and that tuberin may therefore prevent the formation of large, insoluble hamartin aggregates, possibly by interfering with hamartin-hamartin binding.

The Tuberin-Hamartin Complex Forms Rapidly after Translation—Because tuberin binding prevented the formation of large, detergent-insoluble hamartin aggregates, we reasoned that tuberin may act as a chaperone, maintaining hamartin in a soluble form in the cytosol by preventing hamartin from binding to itself. According to this idea, the association between tuberin and hamartin would be expected to occur rapidly after hamartin is synthesized. To investigate this issue we transfected COS cells with TSC1 and TSC2 cDNAs and metabolically labeled the proteins for 10 min with [35S]methionine and [35S]cysteine (Trans). The cells were subsequently chased in media lacking labeled amino acids for between 0 and 180 min. The cells were then lysed, tuberin and hamartin were immunoprecipitated from the cleared lysates, and coimmunoprecipitating proteins were resolved by SDS-polyacrylamide gel electrophoresis. As shown in Fig. 7, even at the earliest chase times tuberin and hamartin coimmunoprecipitated. Because protein synthesis occurs at a rate of ~5–10 amino acids per second (17), it takes ~5 min to synthesize full-length tuberin (1784 amino acids) and hamartin (1164 amino acids); therefore, the two proteins must associate during or very soon after synthesis.

Gel Filtration Analysis Indicates That the Molecular Size of the Tuberin-Hamartin Complex is >450 kDa—To determine the size of the tuberin-hamartin complex, we performed gel filtration on HeLa cell detergent lysates. Samples were loaded on a Superose 6 column, and fractions eluting from the column were analyzed by immunoblot. As shown in Fig. 8, tuberin and hamartin co-eluted in fractions 14–18, between the thyroglobulin (660 kDa; fraction 12) and catalase (240 kDa; fraction 22) molecular size markers. The elution positions of the marker proteins are indicated with arrowheads. Co-elution of tuberin and hamartin helps confirm that both proteins are in a complex and provides a first indication of the size of this complex. We
tin form a complex, and it is difficult to reconcile the differing docking (6). Rule out a role for the tuberin-hamartin complex in vesicle significance of this finding still has to be confirmed, it does not majority of the tuberin-hamartin complex is cytosolic. How ever, approaches, indicate that this is indeed the case, and that the proteins should co-localize to the same intracellular compart ment(s). Our data, from a variety of different biochemical ap proaches, suggest that the two proteins bind each other, and because inactivation of either gene causes the same disease, it follows that the tuberin-hamartin complex is required for whatever func tion is disrupted in TSC. Furthermore, it implies that the two proteins should co-localize to the same intracellular compart ment(s). Our data, from a variety of different biochemical ap proaches, indicate that the two proteins bind each other, and because inactivation of either gene causes the same disease, it follows that the tuberin-hamartin complex is required for whatever function is disrupted in TSC. Furthermore, it implies that the two proteins should co-localize to the same intracellular compart ment(s). Our data, from a variety of different biochemical ap proaches, indicate that the two proteins bind each other, and because inactivation of either gene causes the same disease, it follows that the tuberin-hamartin complex is required for whatever function is disrupted in TSC. Furthermore, it implies that the two proteins should co-localize to the same intracellular compart ment(s). Our data, from a variety of different biochemical ap proaches, indicate that the two proteins bind each other, and because inactivation of either gene causes the same disease, it follows that the tuberin-hamartin complex is required for whatever function is disrupted in TSC. Furthermore, it implies that the two proteins should co-localize to the same intracellular compart ment(s). Our data, from a variety of different biochemical ap proaches, indicate that the two proteins bind each other, and because inactivation of either gene causes the same disease, it follows that the tuberin-hamartin complex is required for whatever function is disrupted in TSC. Furthermore, it implies that the two proteins should co-localize to the same intracellular compart ment(s). Our data, from a variety of different biochemical ap proaches, indicate that the two proteins bind each other, and because inactivation of either gene causes the same disease, it follows that the tuberin-hamartin complex is required for whatever function is disrupted in TSC. Furthermore, it implies that the two proteins should co-localize to the same intracellular compart men...
In summary, we have shown that tuberin and hamartin colocalize, and that the tuberin-hamartin complex is cytosolic and may contain additional components. In addition, our results indicate that both tuberin and hamartin are required for the correct localization and function of the complex. Binding between tuberin and hamartin occurs rapidly after synthesis of the two proteins and prevents hamartin self-aggregation caused by intermolecular homomeric binding of the coiled coil domain. We are continuing with a more detailed analysis of the tuberin-hamartin complex, its localization, function, and additional component parts.

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REFERENCES
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