

Characterization of the Cytosolic Tuberin-Hamartin Complex

TUBERIN IS A CYTOSOLIC CHAPERONE FOR HAMARTIN*

(Received for publication, August 9, 1999)

Mark Nellist, Marjon A. van Slegtenhorst‡, Miriam Goedbloed, Ans M. W. van den Ouweland, Dicky J. J. Halley§, and Peter van der Sluijs||

Department of Clinical Genetics, Erasmus University, 3015 GE Rotterdam, The Netherlands and ||Department of Cell Biology, Utrecht University School of Medicine, 3584 CX Utrecht, The Netherlands

Tuberous sclerosis (TSC) is an autosomal dominant disorder characterized by a broad phenotypic spectrum that includes seizures, mental retardation, renal dysfunction 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)¹ 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 mutated in familial as well as sporadic cases of TSC have been 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).

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 rap1 GTPase-activating proteins rap1GAP (3), p130^{spa-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 aggregation 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, 1–607, and 1–252 plus 1536–1784, 1125–1784, and 607–1099 were cloned behind an amino-terminal polyhistidine epitope tag in a pSG5-derived expression vector. Partial *TSC1* cDNAs encoding amino acids 228–572, 466–788, and 788–1153 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 rapabtin cDNA was isolated in a yeast two-hybrid screen with rab4 as bait and cloned into pcDNA3.1His.² Antisera specific for human tuberin and hamartin have been described (14); antibodies against Xpress, myc (Invitrogen), and polyhistidine (Qiagen) epitope tags were purchased from the manufacturers. Acid α -glucosidase expression constructs and antibodies have been described previously (21). Transferrin-Texas Red conjugate was purchased from Molecular Probes (Leiden, The Netherlands).

Transfections and Immunocytochemistry—Expression constructs were transfected into COS-7 cells with LipofectAMINE and PLUS rea-

* This research was supported by grants from Noortman b.v., (Maas-tricht, The Netherlands), the Dutch Epilepsiefonds, the Nederlands Organisatie voor Wetenschappelijk Onderzoek, and the Dutch Cancer Society. 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.

‡ Present address: Birdshall Research Bldg., Mayo Clinic, Jacksonville, FL 32224.

§ To whom correspondence should be addressed: Dept. of Clinical Genetics, Erasmus University, P. O. Box 1738, 3000 DR Rotterdam, The Netherlands. Tel.: 31-10-4087197; Fax: 31-10-4089489; E-mail: halley@kgen.fgg.eur.nl.

¹ The abbreviations used are: TSC, tuberous sclerosis; PBS, phosphate-buffered saline.

² B. Nagelkerken and P. van der Sluijs, submitted for publication.

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 0.15% glycine, followed by fluorescein isothiocyanate- or rhodamine isothiocyanate-coupled secondary antibodies. Images were captured using the Power Gene 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.

Yeast Two-hybrid Constructs and Assays—GAL4 activation and binding domain constructs encoding amino acids 334–1153 of hamartin (EE1a and EE1b, respectively) were as described previously (14). Yeast host strain Y190 was cotransformed with 2.5 μ g of each plasmid according to the method of Klebe and co-workers (23). Transformants were plated on media lacking tryptophan and leucine and on media lacking histidine, tryptophan, and leucine. Colonies were tested for β -galactosidase activity by a filter assay.

Subcellular Fractionation—HeLa cells (three confluent 10-cm plates) were washed with PBS and collected by scraping into PBS. The cells were pelleted by centrifugation at 2500 rpm for 5 min at 4 °C and resuspended in homogenization buffer (3 mM imidazole, pH 7.0, 250 mM sucrose, 0.5 mM EDTA containing protease inhibitors (Complete; Roche Molecular Biochemicals)). The cell suspension was homogenized by repeated (10 times) passage through a 25-gauge hypodermic needle and respun as above to remove the nuclei and unbroken cells. The postnuclear 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 resuspended in homogenization buffer alone or homogenization buffer containing one of the following: 10 mM Na_2CO_3 , 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 respun at 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.

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

Sucrose Velocity Gradient Centrifugation—HeLa (confluent 10-cm plate) or transfected COS (6-cm plate) cells were washed with PBS, collected by scraping, and pelleted by centrifugation at 2500 rpm for 5 min at 4 °C. The cells were lysed in 250 μ l of TEN buffer (50 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 150 mM NaCl, 0.2% Nonidet P-40) containing protease inhibitors (Complete; Roche Molecular Biochemicals). After centrifugation at 10,000 rpm for 10 min at 4 °C, the cleared detergent extract was layered on top of a 5–25% (w/w) sucrose gradient in TEN buffer (3.5 ml) over a 0.5-ml 40% (w/w) sucrose cushion and spun at 42,000 rpm for 14 h in an SW50.1 rotor at 4 °C. Fractions (0.2 ml) were collected from the top and analyzed by immunoblotting with antibodies against hamartin and tuberin.

Gel Filtration—Postnuclear supernatants were prepared from HeLa cells lysed in TEN buffer as described above. Gel filtration was then performed on a Superose 6 column using the SMART fast protein liquid chromatography system, according to the manufacturer's guidelines (Amersham Pharmacia Biotech), and 50- μ l fractions were collected and analyzed by immunoblotting. Thyroglobulin (660 kDa), catalase (240 kDa), bovine serum albumin (67 kDa), and chymotrypsinogen A (25 kDa) were run as protein molecular size markers.

Pulse-Chase Labeling Experiments—Twenty-four hours after transfection, COS cells (T25 culture flask) were washed twice with 5 ml of PBS and incubated at 37 °C for 30 min in 1 ml of serum-free medium lacking methionine and cysteine (labeling medium). This medium was then replaced with 0.5 ml of labeling medium containing 0.4 mCi/ml ^{35}S label (ICN). After pulse labeling by incubation for 10 min at 37 °C, the cells were washed extensively and chased in Dulbecco's modified Eagle's medium containing 5% fetal calf serum for 0, 10, 30, 60, 120, and 180 min. The cells were then transferred to ice, washed twice with 5 ml of PBS, and lysed in TEN buffer containing protease inhibitors (Complete; Roche Molecular Biochemicals). The cell lysates were cleared by centrifugation at 10,000 rpm for 10 min at 4 °C. The cleared detergent lysates were transferred to 3 μ l of antiserum, incubated on ice for 60 min, and transferred to 35 μ l of a 50% protein A-Sepharose bead suspension. The beads were rotated gently at 4 °C for

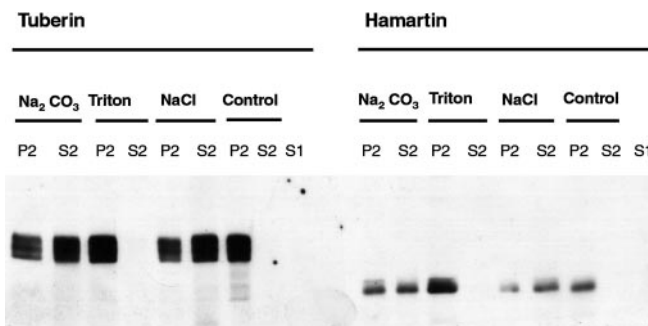


FIG. 1. Subcellular localization of the tuberin-hamartin complex. The postnuclear supernatants from HeLa cell homogenates were fractionated by high speed centrifugation as described under "Experimental Procedures." The pellet was extracted with salt or detergent and recentrifuged. The first supernatant fraction (S1) and final pellet (P2) and supernatant (S2) fractions were analyzed for the presence of tuberin and hamartin by immunoblotting.

60 min, washed extensively with TEN buffer and high salt buffer (50 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 500 mM NaCl, 1% Nonidet P-40), and resuspended in Laemmli gel-loading buffer (24). The immunoprecipitates were separated according to standard procedures on 8% denaturing polyacrylamide gels. The gels were fixed, dried, and exposed to x-ray film.

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 pellet 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 Na_2CO_3 , 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 Na_2CO_3 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 colocalize to the cytosol.

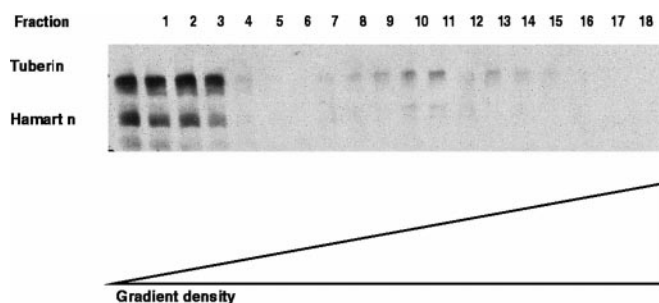


FIG. 2. Flotation gradient centrifugation of tuberin and hamartin. A HeLa cell homogenate was brought to 50% (w/w) sucrose and overlaid with a 15–40% (w/w) sucrose gradient. After centrifugation at 42,000 rpm for 14 h at 4 °C in a Beckman SW50.1 rotor, 0.2-ml fractions were analyzed by immunoblotting. Tuberin and hamartin are indicated. The bar indicates increasing sucrose density.

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

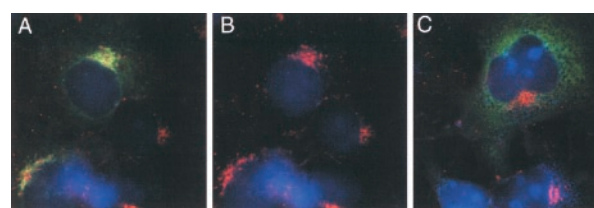


FIG. 3. Localization of tuberin and wheat germ agglutinin in COS cells. A, COS cells transfected with an α -glucosidase expression construct, showing clear overlap between the expression of acid α -glucosidase (green fluorescence) and the distribution of the Golgi complex, as revealed by a wheat germ agglutinin-rhodamine conjugate (red fluorescence). Acid α -glucosidase is transported through the Golgi complex en route to the lysosome. B, same image as in A, without the α -glucosidase signal to indicate the Golgi-specific wheat germ agglutinin signal (red fluorescence). C, COS cells transfected with TSC2. The distribution of tuberin (green fluorescence) is clearly distinct from that of the Golgi marker wheat-germ agglutinin (red fluorescence).

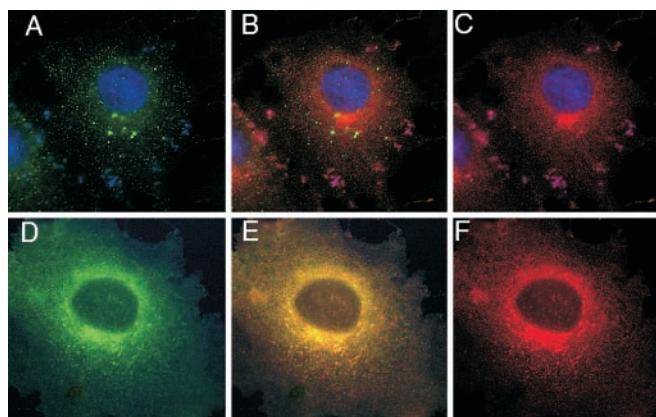


FIG. 4. Localization of tuberin, hamartin, and rabaptin in COS cells. Double label immunofluorescence microscopy was performed on co-transfected COS cells overexpressing hamartin and Xpress-tagged rabaptin (A–C) and tuberin and Xpress-tagged rabaptin (D–F). A, hamartin expression, showing the characteristic punctate labeling pattern in the cytoplasm (green fluorescence). B, combined double label image showing hamartin, as in A (green fluorescence) and rabaptin (red fluorescence). Rabaptin shows the expected diffuse, cytoplasmic labeling pattern, and there is minimal overlap of the hamartin and rabaptin signals. C, rabaptin expression (red fluorescence). D, tuberin expression, showing typical diffuse cytoplasmic staining (green fluorescence). E, combined double label image showing tuberin, as in D (green fluorescence), and rabaptin (red fluorescence). Yellow indicates significant overlap of the tuberin and rabaptin signals. F, rabaptin expression (red fluorescence).

orescence 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

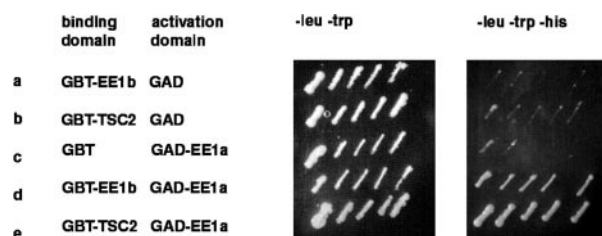


FIG. 5. Yeast two-hybrid analysis of homomeric binding of the hamartin coiled coil domain. Amino acids 334–1153 of hamartin fused to the GAL4 DNA-binding and trans-activation domains (*GBT-EE1b* and *GAD-EE1a*, respectively) were tested for interaction by colony growth on selective medium. *a*, *GBT-EE1b* and *GAD* control cotransformation; no growth on *-leu -trp -his* plates. *b*, *GBT-TSC2* and *GAD* control cotransformation; no growth on *-leu -trp -his* plates. *c*, *GBT* and *GAD-EE1a* control cotransformation; no growth on *-leu -trp -his* plates. *d*, *GBT-EE1b* and *GAD-EE1a* test cotransformation; growth on *-leu -trp -his* plates indicates homomeric binding between the hamartin activation and binding domain constructs. *e*, *GBT-TSC2* and *GAD-EE1a* positive control co-transformation; growth on *-leu -trp -his* plates confirms the interaction between tuberin and hamartin described previously (14).

binding domain-hamartin fusion construct alone.

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 and subjected to high speed centrifugation at 42,000 rpm in a Beckman SW50.1 rotor for 14 h. As shown by the immunoblot analysis in Fig. 6*a*, hamartin was confined to the pellet fraction and was not detectable in the gradient. This suggested that hamartin was present in detergent-resistant protein complexes that were unlikely to be associated with membranes, because the latter were solubilized by the 0.2% Nonidet P-40 in the lysis buffer. Identical results were obtained with detergent lysates prepared from COS cells expressing a truncated hamartin protein containing amino acids 788–1153 only (data not shown), whereas addition of 0.5 M NaCl to the lysate did not affect the distribution of hamartin. Therefore, using three independent assays, we showed that the coiled coil domain of hamartin promotes self-aggregation into insoluble, detergent- and salt-resistant protein complexes.

As shown in Fig. 6*b*, 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. 6*c*, 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,

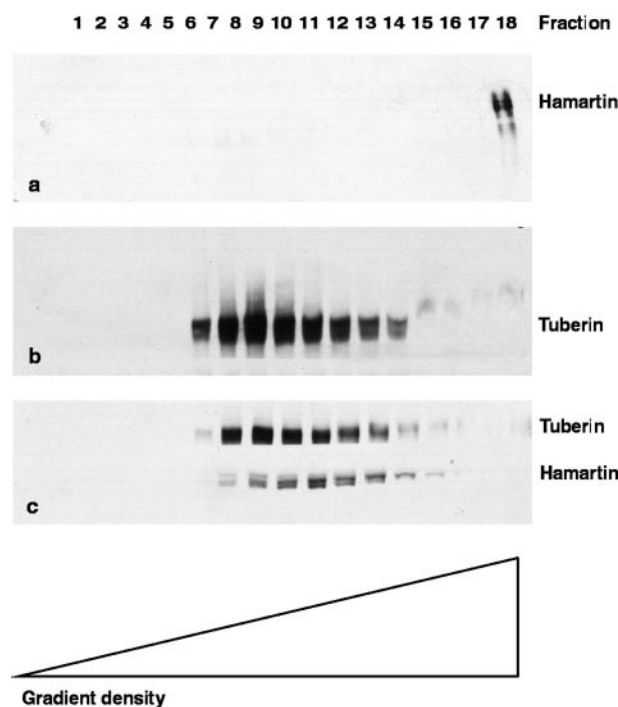


FIG. 6. Velocity gradient centrifugation of tuberin and hamartin. High speed centrifugation through sucrose gradients was performed on lysates prepared from COS cells overexpressing hamartin (*a*) and tuberin (*b*). Tuberin and hamartin were detected by immunoblotting. (*c*), Lysates prepared from nontransfected HeLa cells were analyzed according to the same protocol. The bar indicates the direction of the gradient.

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

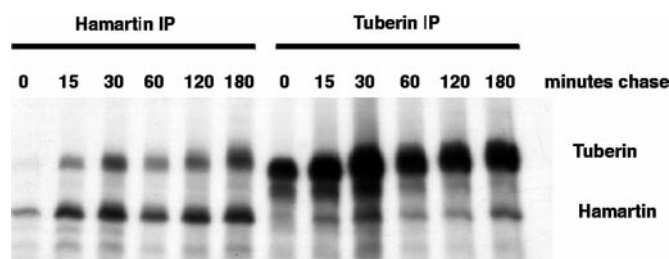


FIG. 7. **Pulse-chase coimmunoprecipitation of metabolically labeled tuberin and hamartin.** COS cells cotransfected with TSC1 and TSC2 were pulse-labeled for 10 min and then chased for different periods as described under "Experimental Procedures." After each chase, hamartin was immunoprecipitated (IP) from the cleared cell lysate. Co-immunoprecipitated tuberin was detected at each time point. Similarly, hamartin was co-immunoprecipitated when tuberin was immunoprecipitated.

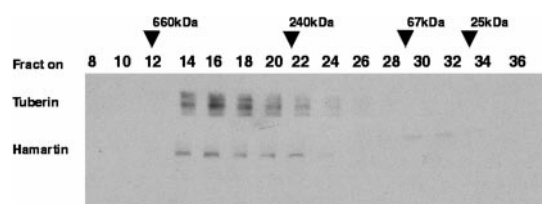


FIG. 8. **Gel filtration analysis of the tuberin-hamartin complex.** A HeLa cell lysate was separated on a Superose 6 gel filtration column according to the manufacturer's recommendations. Fractions were collected, and tuberin and hamartin expression in each fraction was monitored by immunoblotting. Molecular size marker proteins were run on the same column under identical buffer conditions. The positions of thyroglobulin (660 kDa; fraction 12), catalase (240 kDa; fraction 21), bovine serum albumin (67 kDa; fraction 29) and chymotrypsinogen A (25 kDa; fraction 33) are indicated (arrowheads). Endogenous tuberin and hamartin both eluted in the early, high molecular size fractions (peak fraction 16; estimated molecular size, >450 kDa).

estimate that the molecular size of the tuberin-hamartin complex is >450 kDa. The molecular sizes of tuberin and hamartin are 200 and 130 kDa, respectively. Therefore, co-elution of the two proteins is consistent with the presence of a ~330-kDa tuberin-hamartin dimer but not a 2:2 tuberin-hamartin tetramer, which would be expected to elute in fraction 12, together with the 660-kDa thyroglobulin marker. Because we estimate that the molecular size of the complex is >450 kDa, it seems likely that the tuberin-hamartin complex contains additional, as yet unidentified, protein components. The resolution of the Superose 6 column was not sufficient to exclude 2:1 or 1:2 tuberin-hamartin heterotrimers (~530 and ~460 kDa, respectively).

DISCUSSION

Current evidence indicates that many of the lesions characteristic of TSC arise when a second somatic mutation abolishes completely the expression of either hamartin or tuberin (18). Because 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 compartment(s). Our data, from a variety of different biochemical approaches, indicate that this is indeed the case, and that the majority of the tuberin-hamartin complex is cytosolic. However, we also obtained evidence that at steady state a very small proportion is associated with membranes. Although the significance of this finding still has to be confirmed, it does not rule out a role for the tuberin-hamartin complex in vesicle docking (6).

We (14) and others (6) have shown that tuberin and hamartin form a complex, and it is difficult to reconcile the differing

localizations of tuberin and hamartin reported in the literature with these findings, as well as with the co-fractionation results detailed here. We could not confirm the reported punctate distribution of hamartin in cells expressing endogenous levels of the protein (6). Furthermore, in line with others (10), we could not support the proposed localization of tuberin to the Golgi (15). One possible explanation is that the formation and localization of the tuberin-hamartin complex is different in different cell types, because Planck and co-workers (6) studied human kidney 293 cells, and Wienecke and co-workers (15) investigated the localization of tuberin in different renal tumor, adenocarcinoma, and astrocytoma cell lines, whereas most of our studies were performed in HeLa cells.

The tuberin-hamartin complex is large (we estimate that the molecular size is >450 kDa), and binding between the two proteins is resistant to detergent extraction, high salt, and high pH. Resistance to detergent indicates that membrane association is not required for formation of the complex, consistent with earlier yeast two-hybrid assays (14), whereas solubility of the complex in a high pH sodium carbonate buffer indicates that the tuberin-hamartin complex is not an integral membrane complex. Partial solubilization in high salt is consistent with association between the tuberin-hamartin complex and additional cellular components. However, we could find no evidence that rabaptin was associated with tuberin, hamartin, or the tuberin-hamartin complex, suggesting that further searches for proteins that interact with either tuberin or hamartin are necessary to unravel the function of the tuberin-hamartin complex.

Hamartin can form homomeric protein complexes. Whether this interaction is important with respect to the formation of the tuberin-hamartin complex or for the pathogenesis of TSC is not yet clear. Our results suggest that the punctate labeling pattern observed in cells overexpressing hamartin is attributable to oligomerization of the coiled coil domain and that tuberin may interfere with this process, because the gel filtration and ultracentrifugation fractionation profiles of hamartin in the presence of tuberin are different from those in the absence of tuberin. Therefore, tuberin may act as a chaperone for hamartin. Demonstration that the tuberin-hamartin complex forms during or very soon after protein synthesis provides support for this idea.

We could find no evidence for hamartin-hamartin aggregation in cells expressing normal levels of hamartin (and tuberin), and the punctate labeling and fractionation profile may therefore be caused by overexpression. However, it will be interesting to study the fractionation of hamartin from cells in which tuberin expression has been lost, in Eker rat-derived tumor cell lines for example. Analogous to the disruption caused by the aggregation of insoluble proteins containing long glutamine stretches (19), aggregation of hamartin may also interfere with normal neuronal cell function.

Considering the consequences of TSC2 or TSC1 inactivation, the data presented here suggest that the formation and localization of the complex is critically dependent on the direct interaction between tuberin and hamartin. Because loss of either protein leads to TSC, and because the activity of tuberin may be altered when associated with hamartin, the important function with respect to the pathogenesis of the disease may be that of the hamartin-containing complex. Reports on the putative functions of tuberin have generally focused on tuberin alone and have not taken hamartin into account (9–13, 20). This may partly explain the diverse range of proposed functions and suggests that the true function of the tuberin-hamartin complex, with particular regard to the pathogenesis of TSC, has yet to be established.

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.

Acknowledgments—We thank A. Hoogeveen, R. Koppenol, and T. de Vries Lentsch for assistance with the figures and L. van Unen for help with the SMART system. I. Braakman is acknowledged for helpful suggestions.

REFERENCES

- Gomez, M. R. (1980) *Tuberous Sclerosis*, 2nd Ed., Raven Press, New York
- The European Chromosome 16 Tuberous Sclerosis Consortium (1993) *Cell* **75**, 1305–1315
- van Slegtenhorst, M., De Hoogt, R., Hermans, C., Nellist, M., Janssen, L. B. J., Verhoef, S., Lindhout, D., van den Ouweland, A. M. W., Halley, D. J. J., Young, J., Burley, M., Jeremiah, S., Woodward, K., Nahmias, J., Fox, M., Ekong, R., Osborne, J., Wolfe, J., Povey, S., Snell, R. G., Cheadle, J. P., Jones, A. C., Tachataki, M., Ravine, D., Sampson, J. R., Reeve, M. P., Richardson, P., Wilmer, F., Munro, C., Hawkins, T. L., Sepp, T., Ali, J. B. M., Ward, S., Green, A. J., Yates, J. R. W., Kwiatkowska, J., Henske, E. P., Short, M. P., Haines, J. H., Jozwiak, S., and Kwiatkowski, D. J. (1997) *Science* **277**, 805–807
- Jones, A. C., Daniells, C. E., Snell, R. G., Tachataki, M., Idziaszczyk, S. A., Krawczak, M., Sampson, J. R., and Cheadle, J. P. (1997) *Hum. Mol. Genet.* **6**, 2155–2161
- Lupas, A., van Dyke, M., and Stock, M. (1991) *Science* **252**, 1162–1164
- Plank, T. L., Yeung, R. S., and Henske, E. P. (1998) *Cancer Res.* **58**, 4766–4770
- Kurachi, H., Wada, Y., Tsukamoto, N., Maeda, M., Kubota, H., Hattori, M., Iwai, K., and Minato, N. (1997) *J. Biol. Chem.* **272**, 28081–28088
- Chen, F., Barkett, M., Ram, K. T., Quintanilla, A., and Hariharan, I. K. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 12485–12490
- Wienecke, R., Konig, A., and DeClue, J. E. (1995) *J. Biol. Chem.* **270**, 16409–16414
- Xiao, G.-H., Shoarinejad, F., Jin, F., Golemis, E. S., and Yeung, R. S. (1997) *J. Biol. Chem.* **272**, 6097–6100
- Soucek, T., Pusch, O., Wienecke, R., DeClue, J. E., and Hengstschlager, M. (1997) *J. Biol. Chem.* **272**, 29301–29308
- Soucek, T., Holzl, G., Bernaschek, G., and Hengstschlager, M. (1998) *Oncogene* **16**, 2197–2204
- Henry, K. W., Yuan, X., Koszewski, N. J., Onda, H., Kwiatkowski, D. J., and Noonan, D. J. (1998) *J. Biol. Chem.* **273**, 20535–20539
- van Slegtenhorst, M., Nellist, M., Nagelkerken, B., Cheadle, J. P., Snell, R. G., van den Ouweland, A. M. W., Reuser, A. J. J., Sampson, J. R., Halley, D. J. J., and van der Sluijs, P. (1998) *Hum. Mol. Genet.* **7**, 1053–1057
- Wienecke, R., Maize, J. C., Jr., Shoarinejad, F., Vass, W. C., Reed, J., Bonifacino, J. S., Resau, J. H., de Gunzburg, J., Yeung, R. S., and DeClue, J. E. (1996) *Oncogene* **13**, 913–923
- Stenmark, H., Vitale, G., Ullrich, O., and Zerial, M. (1993) *Cell* **83**, 423–432
- Braakman, I., Hoover-Litty, H., Wagner, K. R., and Helenius, A. (1991) *J. Cell Biol.* **114**, 401–411
- Sampson, J. R., and Harris, P. C. (1994) *Hum. Mol. Genet.* **3**, 1477–1480
- Perutz, M. F. (1999) *Trends Biochem. Sci.* **24**, 58–63
- Tsuchiya, H., Orimoto, K., Kobayashi, T., and Hino, O. (1996) *Cancer Res.* **56**, 429–433
- Hermans, M. M. P., Kroos, M. A., Van Beeuman, J., Oostra, B. A., and Reuser, A. J. J. (1993) *J. Biol. Chem.* **266**, 13507–13512
- Deleted in proof
- Klebe, R. J., Harris, J. V., Sharp, Z. D., and Douglas, M. G. (1983) *Gene* **25**, 333–341
- Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York