Similarities and differences in the subcellular localization of hamartin and tuberin in the kidney

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Murthy, Vanishree, Luciana A. Haddad, Nicole Smith, Denise Pinney, Robert Tyszkowski, Dennis Brown, and Vijaya Ramesh. Similarities and differences in the subcellular localization of hamartin and tuberin in the kidney. Am J Physiol Renal Physiol 278: F737–F746, 2000.—Tuberous sclerosis complex (TSC) is an autosomal dominant disorder characterized by hamartomas in multiple organs, notably the brain and kidneys. The disease is caused by mutations in TSC1 or TSC2 genes, coding hamartin and tuberin, respectively. Immunofluorescence analysis of tuberin and hamartin performed here demonstrates that both proteins are specifically expressed in the distal urinary tubule, comprising the distal tubules, connecting segment, and collecting ducts. Hamartin, distinct from tuberin, is expressed in the thick ascending limbs of Henle and in juxtaplagomular cells, where it colocalizes with renin. In positive epithelial cells, tuberin localizes to the cytoplasm as well as the apical membrane. Hamartin, however, preferentially localizes to the apical membrane. The two proteins colocalize at the apical membrane of type A intercalated cells and connecting tubule cells, whereas in type B intercalated cells they reveal a variable pattern of expression. The cell-specific expression of tuberin and hamartin described here will provide critical insight into the cell types that give rise to kidney lesions, and the tumor suppressor role of these proteins in TSC.

TUBEROUS SCLEROSIS COMPLEX (TSC) is an autosomal dominant disorder resulting in abnormal cellular differentiation and proliferation. It is characterized by the development of hamartomas in multiple organs, mainly in the brain, retina, skin, kidneys, and heart (15). The disease has an estimated prevalence of 1:10,000 and involves two genes, TSC1 and TSC2 (11, 33), believed to function as classic tumor suppressors (10, 16, 17).

The TSC2 gene, located on chromosome 16p13.3, encodes the 1804-amino acid protein tuberin (13), for which Rap1 and Rab5 GTPase activating protein activities have been demonstrated (41, 43). Tuberin has been shown to interact with rabaptin 5, a binding partner for Rab5, which plays a role in regulation of endocytosis (43). Tuberin also appears to function as a regulator of the cell cycle as reported by studies in mammalian cells, and Drosophila (20, 35, 36). In addition, loss of tuberin leads to inactivation of p27kip1, a cyclin-dependent kinase inhibitor, due to its mislocalization from the nucleus to the cytoplasm (37). The COOH terminus of tuberin has been shown to interact specifically with steroid hormone receptors (19).

The TSC1 gene maps to chromosome 9q34, and encodes the 1164-amino acid protein hamartin with no known function. Hamartin shows limited homology to a hypothetical yeast protein and has a large putative coiled-coil domain (34). The coiled-coil domain of hamartin interacts with a coiled-coil domain near the amino-terminus of tuberin suggesting that they function as partners in a common cellular pathway (28, 34). Immunofluorescence analysis in cultured cells reveals tuberin and overexpressed hamartin in the Golgi complex and in cytoplasmic vesicles respectively (28, 34, 40). Subcellular fractionation studies have shown both hamartin and tuberin to be associated with the P100 particulate/membrane fractions (28, 41).

The most severe clinical features of TSC are mental retardation and seizures due to the presence of brain cortical tubers, and renal failure secondary to kidney cystic disease or renal cell carcinoma (RCC) (15). The renal tumors seen in TSC patients include angiomyolipomas, renal cysts and less frequently renal cell carcinomas. Angiomyolipomas, the most common renal lesions associated with TSC in adulthood, are mixed tumors composed of adipocytes, blood vessels and smooth muscle cells and can arise in the renal cortex or medulla (39). Some patients present a severe cystic disease due to contiguous deletions comprising both the TSC2 gene and polycystic kidney disease gene 1 (PKD1) (5). About 3% of TSC patients also develop RCC, which occurs at an earlier age than sporadic RCC, and is observed mainly in women (3).

Unlike the TSC phenotype in humans where angiomyolipomas are the most common renal tumors, renal carcinoma is the most common tumor in the Eker rat, a naturally occurring animal model for TSC2 (12). The Eker rat harbors a germline insertion in the TSC2 gene leading to inactivation of the gene (21, 44). Although the mutation is embryonically lethal in homozygous rats, rats heterozygous for the Eker mutation develop spontaneous kidney tumors and are hypersensitive to carcinogen and radiation-induced renal carcinomas.
MATERIALS AND METHODS

Recently a TSC2 knockout mouse model has been described with similar renal tumors as those observed in the Eker rat (22). While TSC1 mutations are known to occur in chemically-induced RCC from non-Eker rats (32), there is no animal model available yet for TSC1.

Immunohistochemical studies have shown that tuberin expression in kidney is restricted to the distal tubules (42). It is critical to understand the distribution of the TSC proteins at the subcellular level in kidney, which will provide information regarding their normal function(s), and how their absence leads to kidney tumor development. Employing antibodies specific for tuberin and hamartin, we have performed immunostaining on normal rat kidney sections. We show here that tuberin and hamartin are strongly expressed in intercalated cells of the collecting duct, distal tubules, connecting segment, and in the connecting tubule cells. While tuberin is predominantly expressed in type A intercalated cells, hamartin is strongly expressed in both A- and B-intercalated cells. Hamartin and tuberin colocalize to the apical part of the cells that are known to be active in endocytosis. Hamartin is also expressed in the thick ascending limbs of Henle, and cells of the juxtaglomerular apparatus as demonstrated by its colocalization in renin-containing cells. Our results on the distribution of both proteins in the kidney will provide important insight into the cell types that may give rise to TSC-related kidney tumors as well as the cellular functions of tuberin and hamartin in tuberous sclerosis.

Cell lines and constructs. Cell lines 293T and NIH3T3 were maintained in DMEM supplemented with 10% fetal calf serum. HeLa cells were grown in EMEM supplemented with 10% fetal calf serum. Full-length human hamartin was cloned into a 5′FLAG-tagged expression vector pCDNA3, and transfected into Cos-7 cells employing the LipofectAMINE transfection protocol from GIBCO-BRL (Life Technologies, Bethesda, MD).

Antibodies. An antihamartin monoclonal antibody, 1B2, was generated against exon 15 of human hamartin (amino acids 480–666). For preparation of 1B2, a GST-fusion construct of exon 15 in pGEX4T was bacterially expressed, purified with glutathione sepharose, and injected intraperitoneally into BALB-C female mice. After the final injection, responding mice were killed and spleen cells were harvested and fused with SP2 myeloma cells. Fusions and selections were carried out according to standard protocols (18). Class and subclass of the MAb 1B2 were determined to be IgG1 and subclass of the MAb 1B2 were determined to be IgG1 by immunoblotting.

Immunoblotting. Protein lysates were prepared from cell lines in buffer containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% NP40, and a cocktail of protease inhibitors (Boehringer Mannheim, Indianapolis, IN). After 30 min on ice the extracts were centrifuged at 12,000 g for 10 min and supernatants were stored at −70°C. Cell lysates containing about 100 µg were electrophoresed on a 6% SDS-polyacrylamide gel and transferred to nitrocellulose filters (Bio-Rad, Melville, NY). Blots were then probed with anti-tuberin antibody C20 or anti-hamartin antibody 1B2. Proteins were visualized with anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibodies and the ECL chemiluminescence system (Amersham, Arlington Heights, IL).

Immunoprecipitation. For each immunoprecipitation, HeLa cells from one 15-cm plate were harvested by scraping, washed in PBS, and protein lysates were made in buffer as described earlier but using 1% Brij96 instead of NP-40 as a detergent. Lysates were preceeded with normal rabbit serum and then incubated with primary antibody and a 1:1 mixture of protein A/G-agarose overnight at 4°C. The immunoprecipitated complexes were washed with lysis buffer and analyzed by immunoblotting.

Western blots and undiluted for immunostaining. Tissue sections were cut at 4°C in PLP. The following day tissues were washed three times in PBS and quenched in 50 mM NH4Cl in PBS for 1 h. Tissues were washed three times with PBS and stored in the same buffer containing 0.02% sodium azide until further use.

For immunocytochemical staining, tissues were cryoprotected by immersion in 0.9M (30%) sucrose in PBS overnight, mounted in OTC medium (Tissue-Tek, Miles), frozen, and 5-µm cryosections were cut by using a Reichert Frigocut Cryostat 2800. Tissue sections were blocked with 5% normal donkey serum in PBS (10 mM sodium phosphate buffer containing 0.9% NaCl, pH 7.4) and 0.02% sodium azide.

Histological processing. Tissue sections were dehydrated through a graded series of ethanol and infiltrated with paraffin. Sections were mounted in Superfrost Plus charged microscope slide (Fisher Scientific, Pittsburgh, PA), and the slides were then used for immunostaining.

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Immunostaining. Tissue sections were rinsed in PBS twice for 5 min each, pretreated with 0.1% SDS for 5 min to retrieve antigenic sites (8), washed twice in PBS, and incubated with PBS containing 0.1% BSA-5% normal goat serum for 30 min. Depending on the antibodies, sections were incubated with the primary antibodies for 1–2 h at room temperature or overnight at 4°C. After each antibody incubation, sections were washed once for 5 min in high-salt PBS (PBS containing 2.7% NaCl) to diminish nonspecific staining and twice for 5 min in PBS. The respective secondary antibodies were then applied for 1 h at room temperature. For double staining, sections were incubated separately with two primary antibodies in succession followed by incubation with the respective secondary antibodies. The slides were then mounted in Vectashield H-1000 mounting medium (Vector Laboratories, Burlingame, CA) diluted 1:1 in 1.5 M Tris·HCl, pH 8.8, and reviewed by using a Nikon FXA fluorescence microscope. Blocking of staining by C20 antibody was observed by staining sections with C20 that had been preincubated at room temperature for 1 h with 1 µg of blocking peptide/0.4 µg of the antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Blocking of staining by 1B2 antibody was performed after preincubation of 1B2 with GST-hamartin fusion protein (8 µg of fusion protein/µl of 1B2 ascites) at 4°C overnight.

Black and white images were taken on Kodak TMax 400 film push processed to 1600 ASA. Color images were collected digitally by using an Optronix 3-bit color charge-coupled device camera a coupled to IP Lab Spectrum software (Scanalytics, Fairfax, VA). Images from FITC and Cy3 double-stained sections were collected separately and digitally merged in IP Lab Spectrum before exporting into Adobe Photoshop for final printing.

RESULTS

Characterization of the anti-hamartin antibody 1B2. The antihamartin antibodies 1B2 and HF6 recognize a protein at ∼160 kDa on Western blots. As shown in Fig. 1A, 1B2 detects endogenous hamartin in 293T, NIH3T3, and HeLa cells as well as exogenously expressed full-length hamartin at a size of ∼160 kDa. We have observed similar expression of hamartin in a variety of cell lines and tissues with 1B2, HF6 and other anti-hamartin antibodies (data not shown).

To further characterize 1B2 and confirm its specificity for hamartin, we performed immunoprecipitation experiments with this antibody followed by Western blotting with HF6 antibody and observed that immunoprecipitated hamartin migrates at ∼160 kDa (Fig. 1B). Previous studies have demonstrated that the TSC2 protein tuberin interacts in vivo with the TSC1 protein hamartin (28, 34). We have seen that hamartin can be coimmunoprecipitated with tuberin (Fig. 1C), further confirming the specificity of the anti-hamartin antibodies 1B2 and HF6.

Distribution of hamartin in adult rat kidney. To study the distribution of hamartin, we used the anti-hamartin monoclonal antibody 1B2 to stain different regions of perfused normal adult rat kidney. Hamartin showed a distinct apical membrane localization in cells of the distal tubules, connecting segment, and collecting ducts (Fig. 2, A, D and G). It was also seen in the thick...
ascending limbs of Henle where tuberin staining was not observed (Fig. 2D). Occasionally, a basolateral staining was observed in some cells where the apical staining was present (Fig. 2A). However, basolateral staining of hamartin was never seen without apical staining in any cell. Although hamartin apical staining was often observed as a sharp signal, in some instances the staining extended to the cytoplasm underneath the apical membrane (Fig. 2, A and G). Preadsorption of 1B2 with polypeptide encoded by exon 15 showed significant reduction in staining (results not shown).

In addition to the staining pattern described above, a few cells adjacent to the glomeruli stained positive with the 1B2 antibody (Fig. 2A). Interestingly, no membrane staining was observed in these cells. Due to the localization of these cells close to the glomerulus and from their punctated pattern of staining, we postulated that hamartin was probably expressed in juxtaglomerular cells known to secrete renin (2). To test this hypothesis, we costained kidney sections with an antirenin polyclonal antibody, and the 1B2 antibody. As seen in Fig. 3C, hamartin and renin partially colocalized in the renin-secreting cells of the adult rat kidney. Because it is known that renin is stocked in secretion granules in the cytoplasm (25), it is likely that hamartin can be present in organelles related to exocytosis.

Distribution of tuberin in adult rat kidney. Tuberin is expressed at ~200 kDa in many cell lines and tissues including adult rat kidney (Ref. 41 and our unpublished data). Employing the antituberin antibody C20, we analyzed the distribution of tuberin in sections of perfused kidney taken from the same adult rat kidney used for hamartin staining. Tuberin was predominantly seen in specific regions of the urinary tubule, namely, the collecting duct and distal tubules, both in the cortex and the medulla, and in regions of the connecting segment in the cortex (Fig. 2B, E, and H). Other regions of the nephron such as the glomerulus, the proximal tubules, and the limbs of Henle revealed no tuberin staining. Expression of tuberin in the medullary or cortical collecting ducts and distal convoluted tubules appeared to be restricted to specific cell types. However, it should be noted that not all cells were equally stained (Fig. 2, E and H). At the subcellular level tuberin revealed apical membrane localization along with cytoplasmic staining (Fig. 2E). Thus the expression of tuberin was not limited to the cytoplasm as has been reported previously by immunocytochemistry in cultured cells (40). Occasionally, tuberin expression was also detected in the basolateral membrane. In some instances, both basolateral and apical staining along with cytoplasmic staining could be observed in the same cells. Tuberin staining with C20 was completely blocked when preincubated with specific blocking peptide (data not shown).
Colocalization of tuberin and hamartin. As described, hamartin and tuberin expression overlapped in regions such as the collecting duct, distal tubules, and connecting segments. To identify cells in which they colocalized, we performed double immunofluorescence staining of the same section of kidney with antibodies, 1B2 and C20. Interestingly, although they colocalized in many cells of the medullary or cortical collecting duct, or distal tubules, other cells showed immunoreactivity only for hamartin with no detectable tuberin expression (Fig. 2C, F, and I). Hamartin almost invariably localized to the apical membrane domain with a few cells showing submembranous cytoplasmic localization, or basolateral distribution. Compared with hamartin, tuberin displayed a more diffuse cytoplasmic distribution with a few cells showing apical membrane localization.

Cell type-specific expression in distal tubules and collecting ducts. The distal convoluted tubules and collecting ducts are essentially composed of two cell types, intercalated and principal cells in the collecting ducts, and mainly distal convoluted tubule cells in the distal tubule, whereas the connecting segment is composed of connecting tubule cells and intercalated cells. To identify which cell types express tuberin and/or hamartin in the distal tubules and collecting ducts, specific transport proteins were selected as markers to costain rat kidney sections. These markers, the anion exchanger 1 (AE1 or Band 3), and the vacuolar H\textsuperscript{+}-adenosine triphosphatase (H\textsuperscript{+}-ATPase) are expressed mainly in intercalated cells (6). Although anti-AE1 antibody also recognizes the anion exchanger AE2, it will be referred to here as AE1 antibody. The acid-secreting type A intercalated cells show basolateral AE1 and apical H\textsuperscript{+}-ATPase, whereas bicarbonate-secreting type B intercalated cells show basolateral H\textsuperscript{+}-ATPase but no detectable AE1 in any membrane domain (1, 7). Therefore, types A and B of intercalated cells can be differentiated according to the position of such markers in the respective membrane domains. We have used polyclonal antibodies against AE1 and the 31-kDa subunit of the H\textsuperscript{+}-ATPase to double label kidney sections along with either C20 or 1B2 antibodies.

The staining with C20 and H\textsuperscript{+}-ATPase, showed that tuberin is expressed predominantly in intercalated cells of the collecting duct and distal tubules. When kidney sections were stained for both tuberin (C20) and H\textsuperscript{+}-ATPase, apical colocalization was observed in type A intercalated cells for both proteins (Fig. 4, A and B, Fig. 5A) in the medullary and cortical collecting duct and distal tubule, with the same A cells also showing a diffuse cytoplasmic staining for tuberin. In contrast, B intercalated cells showed much less staining for tuberin than did A cells or principal cells (Fig. 4, A and B, Fig. 5A), although occasional B cells did show some staining. In the renal cortex a wider distribution of tuberin was observed in a range of cell types, where its expression was not only restricted to the intercalated cells but also seen in cortical principal cells (Fig. 4, A and B). Note that in some parts of the distal nephron...
and connecting segment, some nonintercalated cells also show a band of H\textsuperscript{+}-ATPase staining which is weaker than that seen in intercalated cells.

Double staining performed with hamartin (1B2) and H\textsuperscript{+}-ATPase revealed that as seen for tuberin, hamartin is expressed in type A intercalated cells. However, in cells that had diffuse or basolateral H\textsuperscript{+}-ATPase staining (Fig. 4D), hamartin showed either apical or basolateral expression, indicating that B cells can also express hamartin [Fig. 4C (arrowheads), Fig. 5B]. Double-staining with AE1 and 1B2 showed a basolateral signal with the AE1 antibody and apical localization for hamartin in certain cells from distal tubules and collecting ducts confirming that hamartin is expressed in typeA intercalated cells (Fig. 6). Moreover, hamartin was also detected in cells that were negative for markers, AE1 and H\textsuperscript{+}-ATPase, suggesting that it is expressed in some principal cells as well. Significant hamartin expression was also observed in the cells of the thick ascending limbs of Henle. Since the smooth and rough cell types of the thick ascending limbs of Henle can be differentiated only by electron microscopy (24) in our present study we have not attempted to identify the specific cell types involved. In summary, hamartin appears to be more widely distributed than tuberin in the kidney.

The connecting segment is a short region of the cortical nephron that links the distal tubule to the collecting duct. Because it is difficult to distinguish the connecting segment from distal tubules and collecting ducts by standard light microscopy (27), we used a cell marker calbindin-D that can identify connecting segments in the renal cortex (29, 31). In the cortex, calbindin-D is a specific marker for the connecting
tubule cells of the connecting segment, and does not stain other cortical intercalated cells. Our studies with an anti-calbindin-D monoclonal antibody (Calb-D) showed that calbindin-D colocalized with either tuberin (Fig. 7) or hamartin in the connecting segments of the rat kidney (data not shown). Thus in addition to the intercalated and principal cells, tuberin and hamartin are also expressed in connecting tubule cells.

DISCUSSION

Tuberous sclerosis is a disease with significant renal involvement. In the present study we have examined the normal cell-specific expression of the two TSC proteins hamartin and tuberin in the adult rat kidney. For the first time, we demonstrate that tuberin and hamartin are expressed predominantly in regions of the kidney that include distal tubules, the connecting segment, and collecting ducts. Hamartin in addition displays distinct expression in the thick ascending limbs of Henle and juxtaglomerular cells. By the use of specific markers, the cell types of the adult nephron that express both tuberin and hamartin were identified. Predominant expression of both proteins was observed in type A intercalated cells. Tuberin and hamartin are also expressed in cells negative for AE1 or H^+^-ATPase, which are probably principal cells. In both medulla and cortex, tuberin expression was the strongest in type A intercalated cells in contrast to hamartin, which is strongly expressed in both A and B cells. The heterogenous distribution of hamartin and tuberin in intercalated cell subtypes may be related to the well-described differential abilities of these cells to insert proton pumps into different membrane domains (1, 7).

Hamartin and tuberin association with the plasma membrane is consistent with their presence in the particulate (P100) fractions (28, 41). It is likely that tuberin and hamartin localization to the cell membrane may occur only in polarized cells. Published reports suggest that these proteins interact in a common pathway involved in regulation of vesicular trafficking (34, 43). Tuberin has been shown to regulate Rab5.

Fig. 5. Detail of Fig. 4 displaying pseudocolor and merged images. A: tuberin (red) and H^+^-ATPase (green). B: hamartin (red) and H^+^-ATPase (green). In A, tuberin is colocalized with H^+^-ATPase at apical pole of type A intercalated cell (a, yellow) but not in type B intercalated cell (b). In B, hamartin and H^+^-ATPase are colocalized at apical pole of an A cell (a, yellow). Hamartin is also expressed at apical and lateral regions of a B cell (b). Scale bar, 1 cm = 5 µm.

Fig. 6. Localization of hamartin in type A intercalated cells of collecting duct. A: hamartin staining of apical membrane observed with FITC. B: anion exchanger 1 (AE1) staining of basolateral membrane with Cy3. C: both proteins are strongly expressed in type A intercalated cells in opposite membrane domains. Scale bar, 1 cm = 20 µm.
activity in endocytosis through its interaction with rabaptin-5. Tsc2 mutant cells derived from Eker rats have minimal Rab5-GAP activity with an increased rate of fluid-phase endocytosis (43). Studies using FITC-dextran as a marker of fluid-phase endocytosis in the kidney have identified a high rate of apical endocytosis in both A and some B intercalated cells. Type A intercalated cells are particularly active in the apical recycling of H<sup>+</sup>-ATPase. The endocytosis process involved in H<sup>+</sup>-ATPase recycling does not involve clathrin or caveolin, and the molecular mechanism underlying this internalization process is unknown (4). The apical colocalization of both tuberin and hamartin with H<sup>+</sup>-ATPase in A type intercalated cells raises the question of whether either or both the TSC proteins could be active players in the recycling of H<sup>+</sup>-ATPase in this cell type. In this context it is interesting to note that proximal tubules that are active in clathrin-dependent apical membrane recycling (9) lack expression of hamartin and tuberin. Thick ascending limbs of Henle, in which hamartin staining was detected, are also known to be active in apical membrane recycling (26). In addition, colocalization of hamartin with renin in juxtaglomerular cells is further evidence that hamartin may be part of the exocytosis machinery, because renin is in a punctate pattern that may reflect its presence in membrane-bound secretion granules. Thus our studies here indicate that tuberin and hamartin, besides having shared functions in a common biochemical pathway, may also possess unique functions in different cell compartments.

The most frequent nephrological manifestations in TSC are angiomyolipomas and epithelial cysts (11). Although angiomyolipomas occur in ~80% of TSC patients, the incidence of TSC associated renal cysts is close to 20%, with most cysts occurring in childhood and arising from any region of the tubule (14). These cysts may be indistinguishable from the cysts seen in polycystic kidney disease patients (30). In the autosomal dominant polycystic kidney disease (ADPKD), the loss of the proteins polycystin 1 and 2 mislocalizes the basolateral Na<sup>+</sup>-K<sup>+</sup>-ATPase to the apical membrane domain in cysts from the medullary collecting ducts, thus interrupting the cell polarity (9). The establishment and maintenance of cell polarity is essential for the integrity and function of epithelial cells and is particularly critical in the kidney. The localization of tuberin and hamartin in specific membrane domains of normal kidney epithelial cells suggests a functional role of either or both of these proteins in these regions. It would be of great interest to determine whether the loss of tuberin or hamartin in TSC associated renal cysts could result in altered cell polarity and defective cellular function.

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**Fig. 7.** Identification of cell types in connecting segment expressing tuberin, using calbindin-D as a marker. Tuberin staining was detected by CY3 (A), whereas Calb-D was detected with FITC (B), and double staining was observed in C. Colocalization of tuberin and Calb-D in connecting tubule cells is indicated by thin arrow (C), whereas cell types expressing only tuberin are indicated with thick arrow (C). Scale bar: 1 cm = 30 µm.
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