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Short Communication

Expression of the *TSC2* Product Tuberin and Its Target Rap1 in Normal Human Tissues

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The tuberous sclerosis-2 (TSC2) gene is linked to tuberous sclerosis (TSC), a dominantly inherited genetic syndrome in which inactivation of the normal TSC2 allele is associated with the development of mostly benign tumors and focal dysplasias. TSC2 encodes the protein tuberin, which is a widely expressed 180-kd polypeptide that exhibits specific GTPase activating activity toward Rap1 in vitro and co-localizes with Rap1 in cultured cells. In this study, we have performed immunohistochemical analyses, using affinity-purified anti-tuberin antibodies, to study the distribution of tuberin in a panel of normal human organs that are commonly affected by TSC. Cryosections indicated that tuberin is widely expressed at low levels. More intense staining of tuberin, in the cryosections and in paraffin sections, was observed in the small blood vessels of many organs, including the kidney, skin, and adrenal gland. High levels of tuberin were also detected in cortical neurons and cerebellar Purkinje cells. These findings imply that loss-of-function mutations in TSC2 might lead to the development of highly vascularized tumors, subcortical tubers, and focal atrophy of the cerebellar cortex, which are features commonly associated with TSC. Moreover, Rap1 was also found to be highly expressed in many of the same cells that contained high levels of tuberin, suggesting a functional interaction between tuberin and Rap1 in these tissues. (Am J Pathol 1997, 150:43–50)

Tuberous sclerosis (TSC) is a genetic syndrome with a prevalence of 1 in 10,000 that is inherited as an autosomal dominant disorder and displays a highly variable phenotype.[1–3] Accordingly, the clinical presentation of TSC can range from mild dermatological involvement to severe neurological impairment, such as intractable epilepsy or mental retardation.[4] Common pathological manifestations include angiofibromas and ash-leaf-shaped, hypopigmented patches of the skin, focal cortical dysplasias (cortical tubers), focal cerebellar atrophy, multiple renal angiomyolipomas, and cardiac rhabdomyomas.[5–7] Neoplasms of the brain and the kidneys also occur at a low frequency.[8]

TSC displays genetic heterogeneity and has been linked to two separate human chromosomal loci. One gene (*TSC1*) localizes to chromosome 9 but has not yet been identified, whereas a second gene (*TSC2*) is found on chromosome 16 and encodes the 180-kd protein tuberin.[9–12] Loss of heterozygosity at the *TSC2* locus has been shown in tumors of patients with TSC and in sporadic tumors of non-TSC patients (eg, astrocytomas of the brain and hemangiolipomas of the kidney), which suggests that *TSC2* functions as a tumor suppressor.[13,14] In support of this model, we recently reported that the re-expression of *TSC2* in *TSC2*-negative rat tumor cell lines inhibited the

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growth of these cells and suppressed their tumorigenicity.[15]

Analysis of the amino acid sequence of tuberin revealed a short region of homology to the Rap1 GTPase accelerating protein (GAP), Rap1GAP. Rap1 is a small Ras-related GTP-binding protein[16] that can promote DNA synthesis and morphological changes when microinjected into Swiss 3T3 cells.[17] Like Ras, Rap1 is active in the GTP-bound state and is inactive in the GDP-bound state. GAP proteins such as Rap1GAP act as negative regulators by catalyzing the hydrolysis of GTP to GDP. We have raised antisera against tuberin and used them to identify the protein in cell extracts. These studies also demonstrated that tuberin exerts a weak but specific GAP activity toward Rap1 *in vitro*, as originally suggested by its homology to Rap1GAP.[12] Moreover, we have observed that tuberin and Rap1 co-localize *in vivo* in the Golgi apparatus of several different cultured human cell lines.[18] It is therefore likely that one important physiological function of tuberin may be the negative regulation of Rap1-GTP levels.

Using anti-tuberin sera, we have analyzed the expression of tuberin in extracts of human cell lines and organs. Combined immunoprecipitation/Western blot analyses revealed that tuberin is present in most human cell lines, whereas Western blot analyses of normal human tissue demonstrated the expression of tuberin in every tissue tested.[18] Thus, tuberin is a widely expressed protein. However, consistent with the disease phenotype, the highest levels of tuberin expression were found in extracts of adult human brain, heart, and kidney.[18]

In addition to documenting the expression of tuberin in human tissues by Western blot, we wished to investigate the localization of tuberin within different human organs. In particular, we undertook to study those organs that are primarily affected by TSC. To do this, we performed immunohistochemical analyses of brain (cerebral cortex and cerebellum), heart, skin, and kidney using several different affinity-purified anti-tuberin antibodies. We found that, although low levels of tuberin expression could be detected in most cell types, the highest levels could be detected in the neurons of the cerebral cortex, the Purkinje cells of the cerebellum, and the smooth muscle cells lining the small blood vessels in many organs. We also examined the expression pattern of Rap1, the small GTPase target for tuberin, in a variety of organs. High levels of Rap1 were found in a distribution similar to that of tuberin (ie, vascular smooth muscle cells) in several organs. The co-expression of tuberin and Rap1 provides additional evidence for a physiologically important interaction between these proteins *in vivo*.

Materials and Methods

Tissue Samples

Slides containing paraffin-embedded sections of normal human organs including skin, heart, pancreas, adrenal gland, brain, and kidney were purchased from Dako (Carpinteria, CA). Cryosections of normal human skin, kidney, human cerebral cortex, and cerebellum tissue, taken from the margins of surgical specimens, were provided by Dr. Abhijit Guha (University of Toronto) and Dr. Ray Yeung. Cryosections of tissue from human hearts explanted from patients undergoing heart transplantation were provided by Dr. Loren J. Field (Krannert Institute of Cardiology, Indianapolis, IN).

Antibodies

Immunohistochemical analyses were performed using several different affinity-purified primary polyclonal rabbit antibodies. For tuberin labeling, we used anti-TubD1 (0.2 μg/ml), anti-TubD2 (3 μg/ml)[18] or the affinity-purified anti-tuberin C20 (0.2 μg/ml) (Santa Cruz Biotechnology, Santa Cruz, CA). Rap1 staining was detected using an affinity-purified anti-Rap1 polyclonal antibody (1 μg/ml).[19]

Immunohistochemistry

Paraffin sections were deparaffinized and rehydrated. Cryosections were fixed in 4% phosphate-buffered paraformaldehyde for 10 minutes. All sections were permeabilized for 2 minutes in 0.15% Triton X-100. The tissue sections were blocked for 20 minutes in Tris-buffered saline (50 mmol/L Tris, 150 mmol/L NaCl) with 20% fetal calf serum. The sections were incubated overnight at 4°C in a humidified chamber with the primary antibody diluted in Tris-buffered saline containing 2% fetal calf serum. For detection, a modified alkaline phosphatase/anti-alkaline phosphatase method was performed,[20] except for Figure 1, A–C, in which an avidin-biotin complex method was used. New fuchsin (Fluka Chemical Corp., Ronkonkoma, NY)/naphthol-AS-bi-phosphate (Sigma Chemical Co., St. Louis, MO) was used as the chromogenic substrate in all sections, except for Figure 1, A–C, in which a naphthol AS-MX-PO_4 (Sigma)/Fast Red TR (Amresco, Solon, OH) substrate was used as chromogen. Endogenous alkaline phosphatase was blocked using levamisol (Sigma).

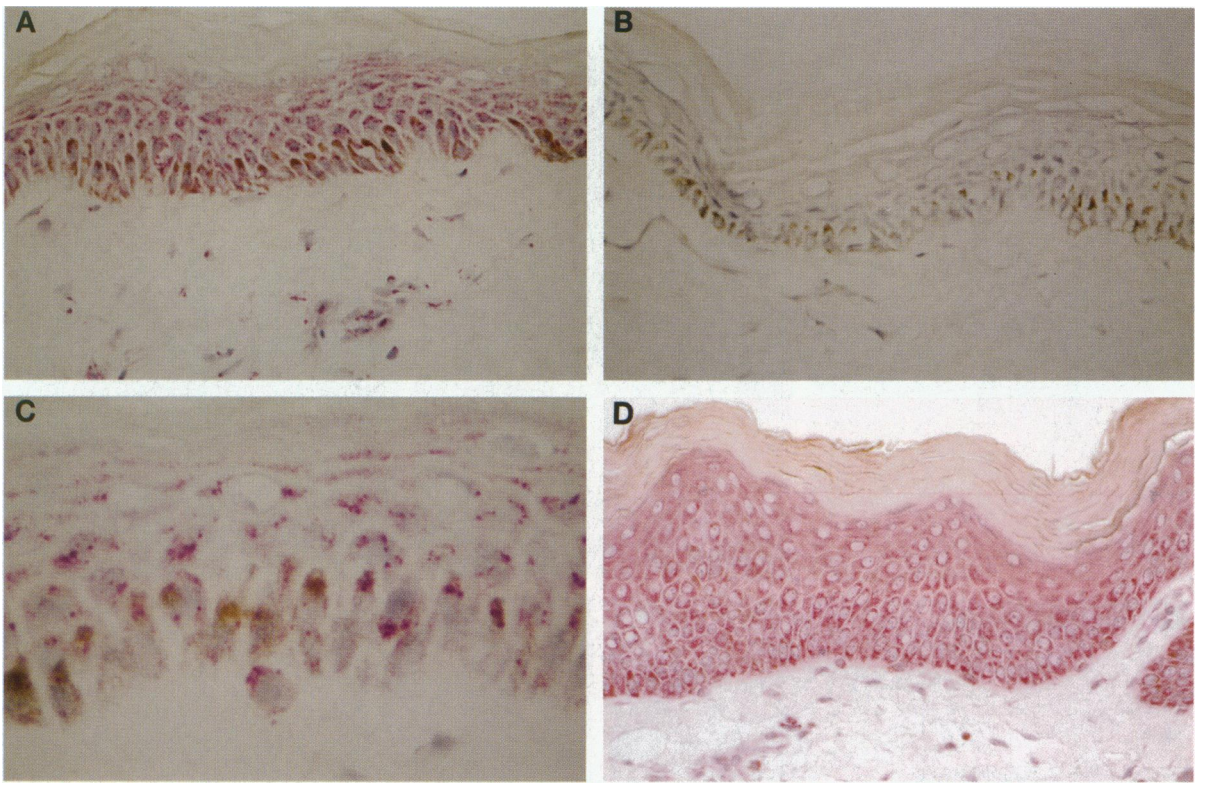

Figure 1. *Immunohistochemical analyses of cryosections of human skin, using anti-TubD1* (A *and* C), *preimmune IgG* (B), *or a paraffin section using anti-TubD2* (D). *The preimmune IgG yields no staining, whereas specific immunoreactivity of anti-TubD1 was detected in keratinocytes, melanocytes, fibroblasts, and endothelial cells of the blood vessels. Staining with anti-TubD2 resulted in a similar pattern, both in dermal and epidermal cells, although the background staining appears stronger in this section. All panels were hematoxylin counterstained. Magnification,* ×*200* (A, B, *and* D) *and* ×*500* (C).

Sections were briefly counterstained with Mayer's hematoxylin.

Results

Two different preparations of affinity-purified polyclonal antibodies were generated against amino acids 1165 to 1393 of human tuberin. The production and characterization of these antibodies (designated anti-TubD1 and anti-TubD2) is described elsewhere.[18] Both antibodies recognize a single band of 180 kd in Western blots both of human tissue homogenates and of human cell lysates. Anti-TubD1 also yields a specific tuberin signal in immunofluorescence applications and was used previously to detect the Golgi localization of tuberin in cultured human cells.[18] For the current studies, tuberin expression in various human tissues was detected principally with anti-TubD1 in an immunolabeling procedure. Staining with preimmune IgG was consistently negative. Anti-tuberin C20 (commercially obtained) is an affinity-purified antibody directed against amino acids 1765 to 1784 of human tuberin. This antibody was also tested by Western blot and detected tuberin as the predominant band (data not shown). Anti-TubD2 and anti-tuberin C20 occasionally displayed some nonspecific background, and therefore were used mainly to confirm the staining with anti-TubD1. When anti-TubD1 was used in cryosections, most cells showed some tuberin immunoreactivity, usually in a perinuclear location consistent with the Golgi apparatus. In paraffin sections, staining was mostly evident in the particular cells that express tuberin at the highest levels.

Our investigation focused on the organs that are most commonly involved in the phenotype of TSC patients. As patients are often afflicted with skin lesions, such as angiofibromas, subungual fibromas, and hypomelanotic macules,[21] we examined tuberin expression in normal human skin. Cryosections of skin were immunolabeled using anti-TubD1 (Figure 1, A and C). Tuberin staining was observed in many different cell types including keratinocytes, melanocytes, fibroblasts, and smooth muscle cells of dermal small blood vessels. Tuberin expression in keratinocytes was confirmed by immunofluorescence analysis and Western blot analysis of cultured primary keratinocytes (data not shown). High levels of

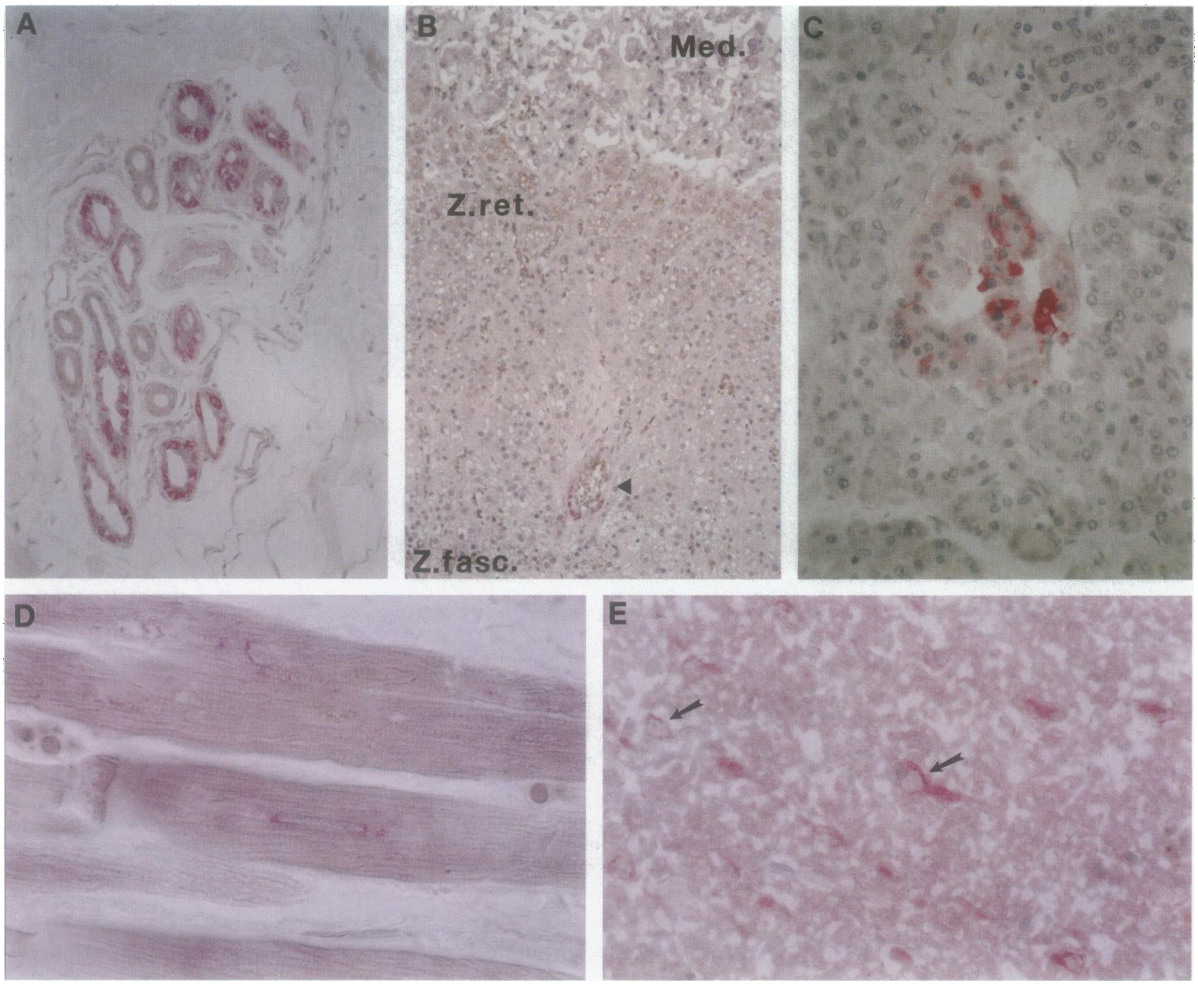

Figure 2. *Analysis of different organs using anti-TubD1* (**A** *to* **C** *and* **E**) *and anti-Tuberin C20* (**D**) *to detect tuberin expression. Tuberin was found to be highly expressed in the eccrine sweat glands of the skin* (**A**)*, suggesting a possible involvement in secretory processes. In the adrenal gland* (**B**)*, a weak reactivity was detected in the zona reticularis* (*Z. ret.*)*, whereas greater reactivity was found in the arterioles* (**arrowhead**)*. In the pancreas* (**C**)*, tuberin was expressed at high levels in certain cells of the islets of Langerhans. In normal heart tissue* (**D**)*, tuberin was detected in the cardiac myocytes. The final image* (**E**) *represents the gray matter of human cerebral cortex; note strong staining of the cortical neurons* (**arrows**) *and weak staining of the surrounding matter. All panels were hematoxylin counterstained. Magnification,* ×*100* (**A** *and* **B**)*,* ×*200* (**C** *and* **E**)*, and* ×*500* (**D**).

tuberin were found particularly in vascular cells and cells of the eccrine sweat glands (Figures 1A and 2A). The specificity of the staining was evident as incubation of the tissue with the same concentration of preimmune IgG yielded no staining (Figure 1B). In addition, preadsorption of anti-TubD1 against GST-TubD eliminated the anti-TubD1 immunoreactivity, whereas preadsorption against GST alone did not alter the specific staining pattern of the antibody (data not shown). The pattern of tuberin expression observed using anti-TubD1 was confirmed using anti-TubD2. The staining patterns produced by these two antibodies were almost identical, the main difference being that there was a background of cytoplasmic staining seen with anti-TubD2 (Figure 1D), likely due to the higher concentration of anti-TubD2 required for detection. Staining of skin with affinity-purified anti-Rap1 antibody revealed a location of Rap1 mainly in vascular smooth muscle cells (data not shown).

Endocrine glands have been reported to be affected in some patients with TSC.[22] Staining of the adrenal gland revealed that the highest levels of tuberin expression were in the arterioles (Figure 2B). In addition, a weak cytoplasmic tuberin reactivity was detected in the zona reticularis (Figure 2B) and the zona glomerulosa (not shown). In the pancreas, some cells in the islets of Langerhans displayed high levels of tuberin (Figure 2C).

Cardiac rhabdomyomas are a very common feature of TSC.[23] Figure 2D depicts the expression of tuberin in human heart tissue as detected by anti-tuberin C20. Tuberin was present at the nuclear poles of the cardiac myocytes, suggesting a Golgi

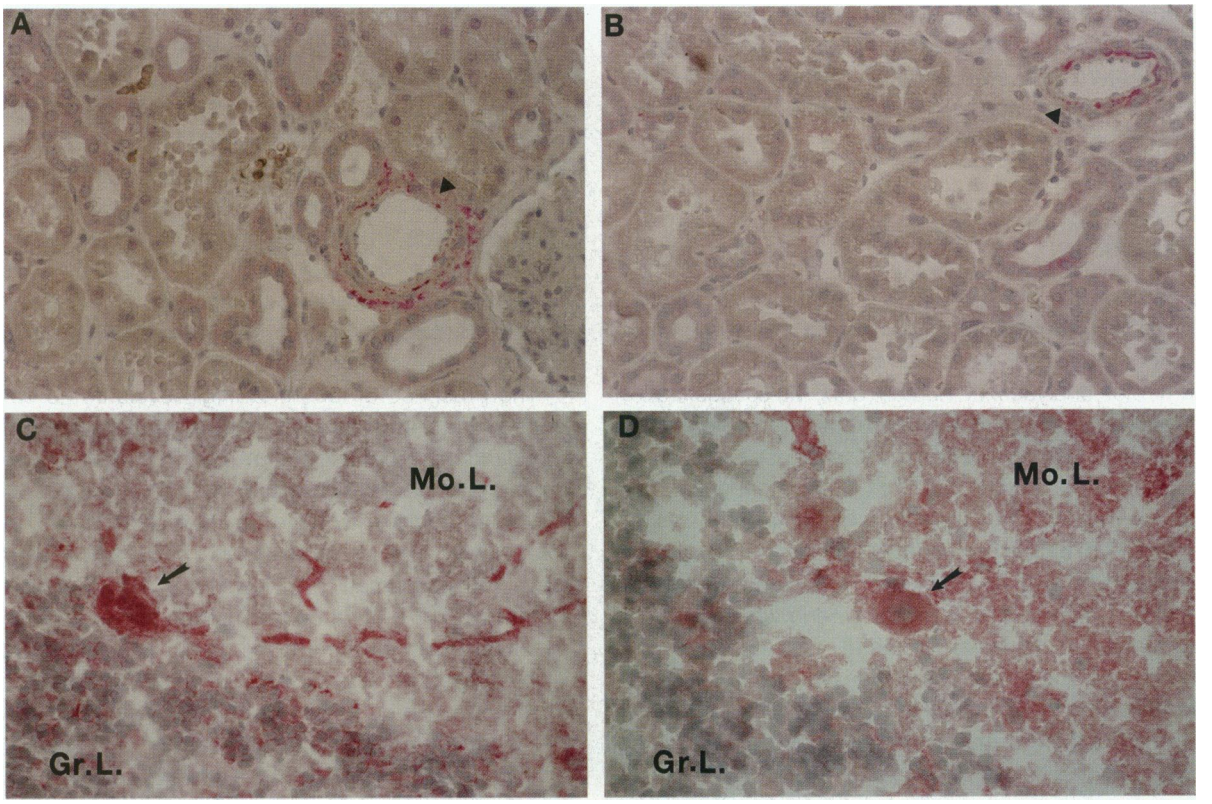

Figure 3. *Immunohistochemical analyses of human kidney* (A *and* B) *and human cerebellum* (C *and* D) *for the expression of tuberin* (A *and* C) *and Rap1* (B *and* D). *High levels of expression of tuberin and Rap1 are visible in the small blood vessels* (arrowhead) *of the kidney and in the cerebellar Purkinje cells* (arrow). *Weak expression of tuberin was found in the renal tubules and in the granular layer* (*Gr.L.*) *and molecular layer* (*Mo.L.*) *of the cerebellum. All panels were hematoxylin counterstained. Magnification,* ×*200.*

localization of tuberin in these cells. Tuberin was also found in the smooth myocytes of arterioles within the heart (not shown). This staining pattern was confirmed with cryosections of heart tissue that were incubated with anti-TubD1.

Patients with TSC frequently develop seizures, most often due to focal developmental abnormalities of the cortex and occasionally to the development of giant cell astrocytoma.[24] In normal human brain, tuberin was detected mainly in a diffuse pattern, in the gray matter of the cortex (Figure 2E). At high magnification, the immunoreactivity of the cortical tissue was most prominent in the perikaryons and processes of neurons (Figure 2E), whereas other cell types such as astrocytes contained much lower levels. These data are in good agreement with the pattern of expression of *TSC2* mRNA in neurons and astrocytes, as determined by *in situ* hybridization.[25] Rap1 was also detected in the neurons and in the gray matter (data not shown).

The kidneys are frequently affected in patients with TSC; manifestations include benign angiomyolipomas and, much more rarely, renal cell carcinoma.[6] Immunohistochemical analysis of the renal cortex using anti-TubD1 revealed weak staining of cells, mainly in the distal tubules (Figure 3A). A significantly higher level of tuberin expression was found in the small blood vessels of the kidney. Analyses with the antibodies anti-TubD2 and anti-tuberin C20 confirmed the expression of tuberin in these cells. Once again, paralleling the expression of tuberin, Rap1 was also found to be expressed at high levels in the small blood vessels (Figure 3B).

Although patients with TSC do not show clinical signs of cerebellar dysfunction, focal atrophy of the cerebellar cortex and a paucity of Purkinje cells have been documented in these patients.[24,26,27] The distribution of tuberin in the cerebellum was examined using anti-TubD1 (Figure 3C). Diffuse staining of the molecular layer, which contains neuronal processes, and perinuclear staining in cells of the granular cell layer was observed. The Purkinje cells exhibited striking immunoreactivity in their perikaryons and dendritic processes. This pattern of protein expression agrees with a previous report that identified high levels of *TSC2* mRNA within the Purkinje cells.[25] The cerebellar expression of tuberin was confirmed using anti-TubD2 (data not shown). Staining of the

cerebellum for Rap1 revealed a similar pattern to that of tuberin within the molecular and granular cell layers (Figure 3D). In the Purkinje cells, high levels of Rap1 staining was seen in a perinuclear location within the soma (Figure 3D).

Discussion

The common manifestations of TSC involve several organs and a variety of cell types. Previous reverse transcriptase polymerase chain reaction and Western blot analyses of human and rat tissues have identified *TSC2* mRNA and tuberin in numerous cell lines and organs, although the levels of expression varied considerably.[18,25,28,29] Consistent with the above results, the immunohistochemical analyses presented here showed that tuberin is widely expressed, at least at a low level, in nucleated cells. In humans, the highest levels of tuberin were found in tissue extracts from brain, heart, and kidney, all of which are commonly affected in TSC patients.[18] An important finding of the current study is that high levels of tuberin, when they were present, were usually confined to a specific subset of cells within a tissue, which may provide clues toward understanding the pathogenesis of TSC lesions. In these cells that would normally express high levels of tuberin, inactivation or loss of tuberin might lead to increased cell proliferation and thereby to the development of hamartomas.

One particularly striking result of this study was the consistently high level of tuberin expression found in vascular smooth muscle cells of the small arteries in many organs, particularly the kidney. This pattern of tuberin expression suggests that loss of tuberin in these vascular cells in patients with TSC might contribute to the formation of the highly vascularized renal angiomyolipomas that are an especially frequent manifestation of TSC.[6,7] Additional support for this hypothesis can be found in studies that have shown loss of heterozygosity at *TSC2* in sporadic and TSC-linked angiomyolipomas of the kidney.[13] Furthermore, nonrandom X chromosome inactivation was demonstrated in these tumors, suggesting a clonal origin, despite the presence of several cell types.[30] Thus far, it has not been determined whether these tumors arise from a common precursor cell or whether the vascular smooth muscle cells are specifically involved.

In the skin, tuberin expression was found in several cell types, including endothelial cells of the small blood vessels, fibroblasts, melanocytes, and keratinocytes, and at especially high levels in the cells of the eccrine sweat glands. Cutaneous manifestations of TSC include a variety of hamartomas such as benign angiofibromas (eg, adenoma sebaceum), fibromas (eg, Koenen tumors of the nail, shagreen patches, and forehead plaques), and soft fibromas.[5,7,21] Loss or inactivation of tuberin in fibroblasts or endothelial cells might contribute to the formation of these tumors. Remarkably, all of these tumors develop at very distinct locations (eg, angiofibromas of the face and subungual fibromas), suggesting that the inactivation of *TSC2* in cells present at specific sites is critical for tumor development.

The presence of tuberin at high levels in the eccrine sweat glands and in the islets of Langerhans of the pancreas may suggest a role for tuberin in secretory processes. In addition to its possible secretory role, tuberin may have an antiproliferative function in the islets, as patients with TSC occasionally develop insulinomas.[22,31] However, it remains to be determined whether the tuberin-expressing cells are identical to the insulin-secreting β-cells. Our results also suggest a role for tuberin in other secretory tissues, for example, the adrenal gland. In the adrenal gland, we found some staining in the steroid-hormone-secreting cells of the cortex, although the highest staining was seen in the small arteries of the gland. Interestingly, TSC patients sometimes develop angiomyolipomas of the adrenal gland and, very rarely, steroid-hormone-producing adrenal tumors.[22]

In the adult heart, as in other tissues, expression of tuberin was detected in the smooth muscle cells of the arteries but also at the nuclear poles within the cardiac muscle cells. Expression of *TSC2* mRNA was recently demonstrated in cardiac muscle cells by *in situ* hybridization.[25] The main cardiac manifestation of TSC in the heart is the development of rhabdomyomas, which are usually present at birth and are less prevalent in adult patients with TSC, suggesting that the growth-suppressive function of tuberin may be most critical during periods of organ development and growth.[32]

In neuronal tissue, tuberin is highly expressed in the cortical neurons and Purkinje cells of the cerebellum and, to a lesser extent, in other neurons and glial cells. Loss of tuberin in the precursors of the cortical neurons might lead to defects in differentiation and/or migration, thereby contributing to the formation of the characteristic cortical tubers. Focal atrophy and disorganization of the cerebellar cortex has been noted in patients with TSC, although patients usually fail to develop cerebellar symptoms.[24] The specific role of tuberin in the cerebellum remains to be determined.

Two different intracellular patterns of tuberin were observed in these studies. In the majority of cells, tuberin was seen in a perinuclear and punctate pattern consistent with a Golgi localization. However, tuberin staining was predominantly cytoplasmic in neuronal cells and cells involved in secretory functions, such as those of the eccrine sweat glands and the islets of Langerhans. In these cells, tuberin may have a significant association with other cellular constituents (eg, secretory vesicles) in addition to the Golgi.

Tuberin has been shown to possess GAP activity toward the small GTP-binding protein Rap1 *in vitro*.[12] Moreover, tuberin and Rap1 have been shown to co-localize in the Golgi compartment *in vivo*.[18] In this study, we found that tuberin and Rap1 have similar patterns of expression in certain tissues, for example, in vascular smooth muscle cells of the kidney and in the Purkinje cells of the cerebellum. In these cells, both tuberin and Rap1 are expressed at high levels, suggesting that the interaction of these proteins is physiologically relevant. There is evidence that Rap1 serves a positive mitogenic function as Rap1 has been shown to induce DNA synthesis when microinjected into Swiss 3T3 cells[17] and to directly activate the B-Raf serine/threonine kinase *in vitro*.[33] These findings raise the possibility of a positive role for Rap1 in cellular growth regulation. In this context, the inactivation of tuberin would leave Rap1 in the active GTP-bound state and allow for the transmission of mitogenic signals, possibly through B-Raf. However, contradictory to a positive function of Rap1, there are also data that suggest a negative function of Rap1 in cell growth.[34,35] Such a model would depict a function for tuberin as an effector for Rap1, which would then mediate a negative growth signal. Although there is ample evidence for an important interaction between tuberin and Rap1 *in vivo*, further investigation is necessary to provide a better understanding of the exact nature of this interaction and its role in the pathogenesis of TSC.

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