Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 9154–9159, August 1996
Medical Sciences

Suppression of tumorigenicity by the wild-type tuberous sclerosis 2 (*Tsc2*) gene and its C-terminal region

(Eker rat/renal carcinoma/hereditary cancer/inducible expression)

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Communicated by Alfred G. Knudson, Fox Chase Cancer Center, Philadelphia, PA, May 28, 1996 (received for review March 1996)

ABSTRACT **The *Tsc2* gene, which is mutationally inactivated in the germ line of some families with tuberous sclerosis, encodes a large, membrane-associated GTPase activating protein (GAP) designated tuberin. Studies of the the Eker rat model of hereditary cancer strongly support the role of *Tsc2* as a tumor suppressor gene. In this study, the biological activity of tuberin was assessed by expressing the wild-type *Tsc2* gene in tumor cell lines lacking functional tuberin and also in rat fibroblasts with normal levels of endogenous tuberin. The colony forming efficiency of Eker rat-derived renal carcinoma cells was significantly reduced following reintroduction of wild-type *Tsc2*. Tumor cells expressing the transfected *Tsc2* gene became more anchorage-dependent and lost their ability to form tumors in severe combined immunodeficient mice. At the cellular level, restoration of tuberin expression caused morphological changes characterized by enlargement of the cells and increased contact inhibition. As with the full-length *Tsc2* gene, a clone encoding only the C terminus of tuberin (amino acids 1049–1809, including the GAP domain) was capable of reducing both colony formation and *in vivo* tumorigenicity when transfected into the Eker rat tumor cells. In normal Rat1 fibroblasts, conditional overexpression of tuberin also suppressed colony formation and cell growth *in vitro*. These results provide direct experimental evidence for the tumor suppressor function of *Tsc2* and suggest that the tuberin C terminus plays an important role in this activity.**

Tuberous sclerosis (TSC) is an autosomal dominant disease that affects ≈1 in 10,000 persons (1). The clinical manifestations, which are highly variable, include neurological dysfunction (e.g., epilepsy, mental retardation), cutaneous lesions (e.g., facial angiofibroma, "ashleaf" macules), and visceral tumors (e.g., cardiac rhabdomyoma, renal angiomyolipoma, retinal hamartoma) (2). TSC lesions probably arise from several defects such as abnormal cellular migration, proliferation, and differentiation, resulting in the development of benign tumors (hamartomas) and malformations (hamartias) in tissues of mesodermal and ectodermal origin. A small proportion (3–5%) of TSC patients develop TSC-related malignancies, mainly in the kidneys and nervous system. In light of these facts, TSC can be regarded as an hereditary cancer-predisposing syndrome, much like other phakomatoses such as neurofibromatosis type 1 and 2 (NF1 and NF2) and von Hippel–Lindau disease. For all of these disorders, the susceptibility genes belong to a group of genes designated tumor suppressor genes, which are mutationally inactivated in a wide variety of human cancers (3).

Studies focusing on the Eker rat model of hereditary cancer have provided an animal model for analyzing the tumor suppressor function of the tuberous sclerosis 2 (*Tsc2*) susceptibility gene. This rat strain is predisposed to the development of multiple neoplasias in the kidney, uterus, and spleen due to a germ-line mutation in the rat homolog of the human *Tsc2* gene (4, 5). Specifically, the Eker strain bears an intragenic insertion of a rat endogenous retroviral element into *Tsc2*, leading to the inactivation of its gene product, tuberin, by virtue of a functional deletion of the C terminus (6). Sequence analysis of *Tsc2* revealed that this C-terminal region of tuberin displays limited homology to a GTPase activating protein (GAP) for the small GTPase Rap1 (7, 8). We recently demonstrated that this portion of tuberin possesses Rap1–GAP activity, suggesting that this C-terminal region of tuberin might be important for its putative tumor suppressor function (9). Loss of heterozygosity (LOH) analysis has shown specific deletion of the wild-type *Tsc2* allele, with retention of the germ-line mutant allele, in a high percentage of Eker rat-derived tumors, as well as in hamartomas of human TSC patients (10, 11). These findings support the contention that *Tsc2* functions as a tumor suppressor in humans and in the Eker rat, in accordance with Knudson's "two-hit" hypothesis for carcinogenesis (12).

One common measure of the function of tumor suppressor genes is their ability, when reintroduced or overexpressed in cells, to inhibit cell proliferation or tumorigenicity (13, 14). To address this aspect of the tumor suppressor activity of *Tsc2*, we examined the biological effects following introduction of the wild-type *Tsc2* gene in cells lacking tuberin expression, as well as in cells with normal endogenous levels of tuberin. We show that reintroduction of *Tsc2* into Eker rat-derived cell lines lacking the wild-type gene was effective in suppressing *in vitro* and *in vivo* growth and tumorigenicity. Furthermore, it was found that a clone encoding the C-terminal GAP domain of tuberin was sufficient for this suppressive function. Overexpression of tuberin in a cultured rat fibroblast line also reduced its proliferation, suggesting that the growth inhibitory function of tuberin is active in a variety of cell types, consistent with the diverse clinical manifestations characteristic of this syndrome.

MATERIALS AND METHODS

Plasmids. The full-length cDNA of rat *Tsc2* was derived from a rat kidney cDNA library as described (8) and cloned into two sets of expression vectors. For the purpose of transfection into the Eker-derived cells, the *Tsc2* clone including the translation initiation sequence was blunt-end ligated into a retroviral-based vector, pLXSN, which also contains the neomycin (G418) selectable marker (15). Both the sense (pTsc2-S) and antisense (pTsc2-AS) constructs were confirmed by direct

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Abbreviations: GAP, GTPase activating protein; TSC, tuberous sclerosis; LOH, loss of heterozygosity; NF1, neurofibromatosis 1 gene; SCID, severe combined immunodeficient.

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partial sequencing. To create a clone encoding the tuberin C terminus and GAP domain (amino acids 1049–1809), a 2.4-kb *Eco*RI/*Xho*I fragment was ligated into pLXSN, creating pTsc2–GAP with an in-frame initiator methionine. For tetracycline-regulated expression of tuberin, full-length rat *Tsc2* cDNA was cloned into pUHD10-3 downstream of the *tet*-operator and cytomegalovirus core promoter sequences. The Tk–hygromycin plasmid contains the hygromycin resistance gene under the control of the thymidine kinase (TK) promoter. pUHD15-1 contains the tTA transactivator gene and a neomycin (G418) resistance gene (16). The tTA transactivator gene encodes a chimeric protein (tTA) composed of the tetracycline repressor fused to the activation domain of the herpes virus VP16 transactivator, and functions as transactivator in the absence of tetracycline.

Cell Lines and Transfection. The Eker tumor cell lines, LExF2 and ERC18M, were derived from independent renal cell carcinomas and passaged in hormone-supplemented media as described (4). ERC18M was rederived from parental ERC18 following passage of the tumor line through the severe combined immunodeficient (SCID) mouse. Both of the cell lines were genotyped for the *Tsc2* locus, which confirmed loss of the wild-type allele. Cells were transfected with 1.5–3 mg of purified plasmid DNA using the lipofectin method (GIBCO/BRL) according to the manufacturer's instructions. Selection with G418 (400–600 mg/ml) began 48 h after transfection. For the colony formation assay, cells were selected in G418 for 3 weeks, then stained with methylene blue in 50% ethanol. R12 cells, a derivative of Rat1 fibroblasts containing a stable copy of pUHD15-1 (16), were obtained from S. I. Reed (Scripps Research Institute, La Jolla), and were cultured in DMEM containing 10% fetal calf serum (FCS), 350 μg of G418 per ml, and 2 μg of tetracycline per ml (to block the transactivating activity of tTA). These cells were then cotransfected with 4 μg pUHD10-3–Tsc2 and 0.2 μg Tk–hygromycin plasmid by Tfx-50 (Promega), and selected with 350 μg of G418 per ml and 150 μg of hygromycin per ml. Individual cell clones were selected based on tuberin expression in the absence of tetracycline. The Tub6 clone expressed similar levels of tuberin as R12 in the presence of tetracycline and showed a 2- to 5-fold increase in the absence of tetracycline.

***In Vitro* Growth Assays.** The Tub6 line, in which expression of the *Tsc2* cDNA is induced by the removal of tetracycline from the culture medium, and the parental R12 cells were seeded (10^3 cells) in duplicate into 60-mm dishes in the presence of 10% FCS and 2 μg of tetracycline per ml. The next day, the medium was changed and the cells were grown for 6 days in the presence or absence of 2 μg of tetracycline per ml in DMEM containing 2% FCS. The number and size of colonies were assessed following methylene blue-carbon fuchsia staining. In a separate experiment, 10^4 Tub6 cells were seeded in duplicate and grown in the presence or absence of tetracycline, and the number of cells on days 1, 2, and 6 were counted with a hematocytometer. To assay anchorage-independent growth, stable clones derived from the ERC18M line following transfection with empty vector, pTsc2-AS, or pTsc2-S were suspended (10^4 cells) in medium with 0.35% agarose and seeded on 0.6% agarose-medium plates. Fresh medium was added every 5 days. Growth in soft agarose was assessed after 4 weeks by scoring macroscopically visible colonies.

***In Vivo* Tumorigenicity Assay.** Four-week old male CB17SCID mice were injected with 2×10^6 cells from stable transfectants of LExF2-V, LExF2-S(F), and LExF2-S(GAP) into the dorsal subcutaneous tissue. Tumor growth was measured after 3 weeks. In the absence of gross tumor, the injection site was examined under the dissecting microscope. Gross tumors were analyzed histologically with hematoxylin/eosin staining.

Immunoblotting. Expression of the *Tsc2* product tuberin was analyzed by Western blotting of extracts from cell lines. Briefly, cells were lysed in lysis buffer (10 mM Tris·HCl, pH 7.8/150 mM NaCl/1 mM EDTA/1% Nonidet P-40) containing 10 μg of aprotinin per ml and 5 μg of leupetin per ml. Portions of each lysate containing equal amounts of protein were subjected to SDS/PAGE on 6.5% gels or 4–20% gradient gels, and the proteins were transferred to Immobilion-P membranes (Millipore) according to the manufacturer's instructions. Tuberin was detected using a rat cross-reactive anti-human (TubC) antiserum (1:3000), followed by an anti-rabbit peroxidase-based ECL system (9). The antibody recognizes the 180-kDa tuberin protein, and also a nonspecific 75-kDa band.

RESULTS

***In Vitro* Growth Inhibition of *Tsc2* Mutant Cells.** The derivation of renal carcinoma cell lines from rats of the Eker strain has been described elsewhere (17, 18). Two independently-derived tumor lines (designated LExF2 and ERC18M) were employed for these studies. Both of these lines have lost or undergone mutation of both alleles of the *Tsc2* locus and fail to express detectable tuberin protein (data not shown). The premise that the absence of functional tuberin governs tumor development in the Eker rat suggests that the reintroduction of wild-type *Tsc2* into these cells might inhibit their proliferation. In two independent experiments, the full-length rat *Tsc2* cDNA in a plasmid carrying the neomycin-resistance gene, as well as an isogenic plasmid encoding only the 3′ GAP domain (ΔGAP), were individually transfected into LExF2 and ERC18M, and the ability of the transfected cells to form G418-resistant colonies was analyzed. Transfection of cells with pTsc2-S (the expression vector containing *Tsc2* in the sense orientation) reproducibly yielded a lower number of cell colonies compared with insertless vector and antisense (pTsc2-AS) controls. Following 3 weeks of selection, the relative colony-forming efficiencies of LExF2 cells transfected with the pTsc2-S and pTsc2–GAP constructs were only 25% and 18% respectively, compared with cells transfected with the insertless expression vector (pLXSN) (Fig. 1*a*). Similarly, colony formation by ERC18M cells transfected with pTsc2-S and pTsc2–GAP was reduced by 44% and 67%, respectively, relative to vector control transfectants.

To further characterize the properties of the transfected Eker tumor cell lines, stable transfectants were isolated and expression of the exogenous *Tsc2* gene was analyzed by immunoblotting with rabbit antituberin serum (Fig. 1*b*). As expected, no tuberin was detected in LExF2 cells transfected with the antisense or vector controls; in contrast, the ≈180-kDa product was readily detected in the G418-resistant subclone of LExF2 derived from transfection with pTsc2-S. A similar pattern of expression of the transgene was noted for an ERC18M subclone and cell lines transfected with pTsc2–GAP (data not shown). The rate of cell growth was reduced in the tuberin-expressing LExF2 subclone compared with the controls, but proliferation was not completely inhibited (Fig. 1*c*). The transfectants were also tested for their anchorage-independent growth in soft agar suspensions. Of the two parental cell lines, only ERC18M was capable of growth in soft agar (at a frequency of 0.5–1.0% compared with <0.01% for LExF2), so we compared transfectants derived from this line (Fig. 1*d*). The colony-forming efficiency of ERC18M was reduced by 63% (compared with the vector control) following transfection with pTsc2-S; no inhibition of colony formation was observed in cells transfected with the antisense (pTsc2-AS) control.

Altered Morphology of *Tsc2*-Transfected Eker Tumor Cells. The cytological appearance of the parental Eker tumor cell lines in culture consists of pleiomorphic polygonal cells, with

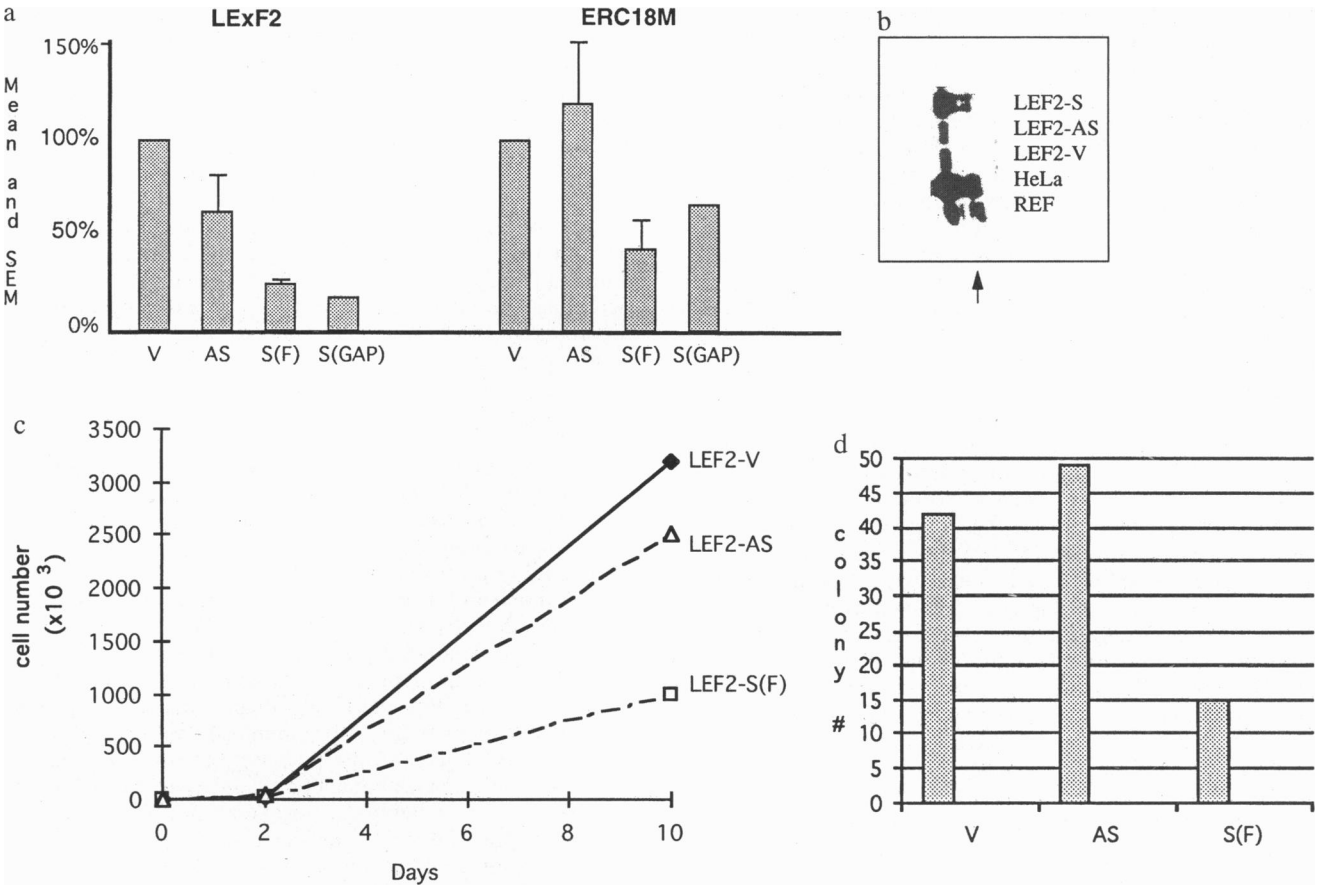

FIG. 1. *In vitro* growth inhibition following replacement of the *Tsc2* gene into *Tsc2* deficient Eker-derived renal carcinoma cell lines, LExF2 and ERC18M. (*a*) Colony formation after 3 weeks of G418 selection demonstrates growth inhibition by full-length *Tsc2* and by its C terminus GAP domain. Results are normalized to vector control and represent the average of two independent experiments. V, vector alone; AS, antisense; S, full-length sense; GAP, GAP domain (amino acids 1049–1809). (*b*) Immunoblot of tuberin expression in a stable transfectant, LExF2-S, demonstrates a tuberin band of 180 kDa (arrow) not seen in vector, LExF2-V, nor antisense, LExF2-AS, controls. Tuberin is expressed in both of the control cells: HeLa, human cervical carcinoma cell line; REF, rat embryo fibroblasts. (*c*) Cell proliferation of LEF2-S(F) stable transfectant was significantly reduced compared with antisense (LEF2-AS) and vector (LEF2-V) controls. Cells (4×10^3) of each were seeded on day 0 and cultured under identical conditions. (*d*) Macroscopic colony counts of ERC18M cells transfected with vector (V), antisense *Tsc2* (AS), and full-length sense *Tsc2* [S(F)] after 3 weeks of growth in soft agarose.

uniform nuclei that lack features of severe dysplasia. Their morphology is consistent with the well-differentiated histology of the primary chromophobic tumors. A feature of the cells in these established tumor lines is their tendency to form multilayer foci resulting in papillary projections (Fig. 2). This absence of contact-inhibition was dramatically reversed in LExF2 cells transfected with the pTsc2-S plasmid. These cells were flatter, displayed an accumulation of cytoplasm, and were much more monolayer-restricted in their growth. The accumulation of cytoplasm in these slowly proliferating cells may reflect a defect or block in cell cycle progression, a possibility that is consistent with the finding of binucleation in many of these enlarged cells, reminiscent of postmitotic arrest during anaphase. In contrast, tumor cells transfected with the antisense construct retained the parental morphology, while those expressing the *Tsc2*–GAP domain showed an intermediate appearance consistent with partial morphological reversion. The ability of normal tuberin or its GAP domain to suppress the transformed morphology *in vitro* was also apparent with transfectants derived from the ERC18M line (data not shown). A recent study using yet a different Eker tumor cell line transfected with a tetracycline-responsive *Tsc2* gene construct also suggested a change in morphology following withdrawal of tetracycline (19).

Inhibition of *in Vivo* Tumorigenicity by Reintroduction of *Tsc2*. The suppression of *in vitro* growth properties in the Eker cell lines following transfection with *Tsc2* suggested that reintroduction of this gene might also suppress *in vivo* tumor growth. As subcutaneous injection of parental LExF2 cells into SCID mice resulted in tumor formation within 2 weeks, we tested stable transfectants of LExF2 [LExF2-V, LExF2-S(F), LExF2-S(GAP)] for their *in vivo* tumorigenicity. The respective cells were propagated until 80% confluent and 2×10^6 cells of each transfectant were implanted into the subcutaneous flank tissue of individual animals. Trypan blue staining showed that >90% of the cells of each sample were viable. At the end of 3 weeks, large tumors (2–3 cm diameter) developed in each of the two mice that had received control LExF2-V cells (Fig. 3). Grossly, these tumors invaded the underlying muscle and ulcerated the overlying skin. The histology was consistent with adenocarcinoma, and resembled the primary tumor of origin. Of the remaining two test groups [LExF2-S(F), $n = 3$; LExF2-S(GAP), $n = 3$], none of the animals developed evidence of tumor (Fig. 3). Examination of the injection sites under the dissection microscope failed to identify visible tumor in all six mice. Autopsy of these animals did not uncover evidence of tumor elsewhere. Thus the suppression of tumor in cells transfected with sense *Tsc2* constructs was complete. These results indicate that expression of normal tuberin or the C-terminal GAP domain region in *Tsc2* mutant tumor cells can inhibit *in vivo* tumorigenicity.

Growth Inhibition by Conditional Overexpression of *Tsc2* in Rat1 Fibroblasts. Many tumor suppressor genes possess the

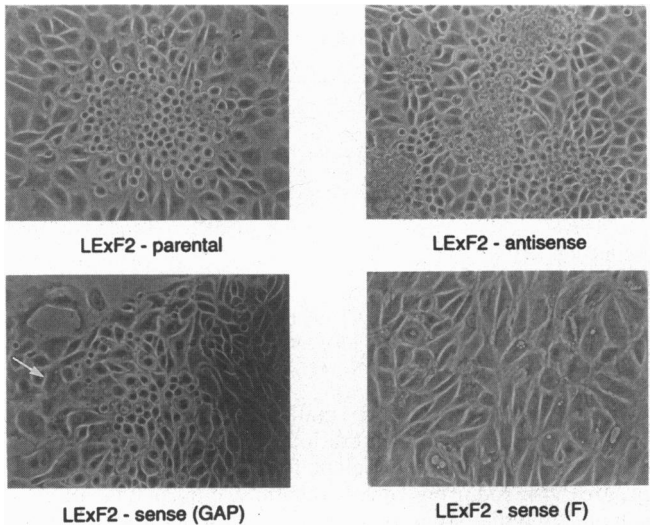

FIG. 2. Morphology of LExF2 cells before and after transfection of *Tsc2* or its 3′ region. Papillary foci are seen in parental and antisense cultures; enlargement of the cells and increased contact inhibition are observed in full-length sense transfectants. Cells expressing the GAP domain showed an intermediate phenotype. Arrow indicates a typical binucleated cell found in *Tsc2*-transfected cells. (×100.)

ability to inhibit the growth of a variety of cell types, indicating that the mechanisms underlying their function are basic to all or most cells. Given that the clinical spectrum of TSC is diverse, tuberin might be expected to display growth inhibitory activity in cells other than the Eker rat kidney lines. To test this hypothesis, the *in vitro* growth properties of Rat1 fibroblast cells were examined following expression of exogenous *Tsc2*. To minimize effects stemming from clonal variation, the full-length rat *Tsc2* gene was placed under the control of a regulatable tetracycline promoter and introduced into a strain of Rat1 fibroblasts (R12) stably expressing the tetracycline repressor/VP16 transactivator expression vector (16). This line was designated Tub6. After removal of tetracycline from Tub6 cells for 4 to 6 days, we observed a substantial increase in tuberin over the basal expression level (Fig. 4*a*). In contrast, the control R12 cell line lacking the *Tsc2* plasmid showed no difference in tuberin expression in the presence or absence of tetracycline. Removal of tetracycline also caused specific inhibition of the growth of Tub6 cells, over the same time course as tuberin expression was elevated. The formation of single cell-derived colonies was suppressed, and both the number and size of colonies were significantly reduced in the absence of tetracycline (Fig. 4*b*). The overall number of colonies decreased by almost 40%, and the percentage of colonies that were smaller than 1 mm in diameter was 72% when *Tsc2* was expressed versus 59% when repressed, suggesting a significant association between colony size and tuberin expression ($\chi^2 = 16.5$, $P < 0.0001$) (Table 1). Furthermore, proliferation of Tub6 cells was retarded upon expression of *Tsc2* such that by day 6 the number of cells growing in the absence of tetracycline was only 59% of that of cells maintained in the presence of tetracycline (Fig. 4*c*). A similar reduction in growth was reported in an Eker tumor cell line containing the *tet*-inducible *Tsc2* gene (19).

DISCUSSION

Evidence for the tumor suppressor function of *Tsc2* gene first arose from studies of the Eker rat model of hereditary cancer, and the from the genetic analysis of lesions from human TSC patients. Our early observation that the multiplicity of kidney tumors in the Eker rat carriers increased linearly with radiation dose suggested that only one rate limiting step (i.e., a somatic

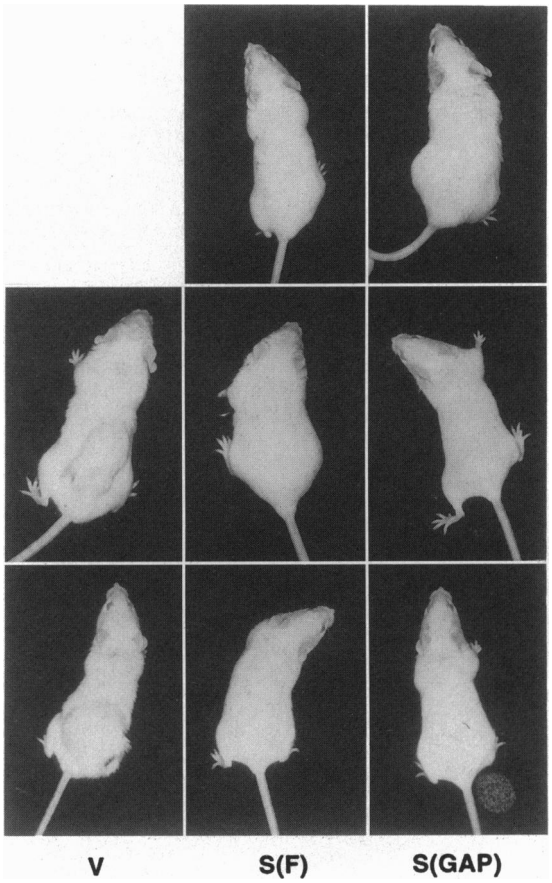

FIG. 3. *In vivo* tumor growth of LExF2 transfectants (2×10^6 cells) in SCID mice at 3 weeks postinoculation. No visible tumor was detected in any of the animals in the S(F) and S(GAP) groups. V, vector; S(F), full-length sense; S(GAP), C-terminal GAP domain region.

mutation) was necessary for tumor formation (17). Subsequently, we demonstrated that the Eker-derived tumors (with the exception of splenic hemangiosarcoma) showed frequent LOH at the *Tsc2* locus (10). In cases of LOH, invariably it was the wild-type allele that was lost, meaning that the tumor cells would completely lack tuberin expression. Indeed, immunoblots of cell lysates from such tumors using antituberin antibodies directed against the C terminus confirmed the absence of the ≈180-kDa protein (R.W. and R.S.Y., unpublished observations). Furthermore, studies describing LOH in hamartomas from TSC patients have also supported the tumor suppressor paradigm of the initiating (*Tsc2*) gene (11). All of these findings are consistent with the two-hit hypothesis proposed for the inactivation of tumor suppressor genes (12).

Here we have provided direct experimental evidence for the tumor suppressor function of the *Tsc2* gene through an *in vivo* tumorigenicity assay, and demonstrated the growth-inhibitory activities of this gene *in vitro*. Recent preliminary data reported by Orimoto *et al.* (19) also lend support to our observations. In their study, derepression of the *tet*-inducible rat *Tsc2* gene in an Eker renal carcinoma cell line resulted in morphologic change and a reduction in *in vitro* proliferation. By assessing additional properties of *in vitro* growth, we establish that the wild-type *Tsc2* gene is capable of suppressing colony formation and anchorage-independent growth in soft agar. More importantly, the evidence that reintroduction of the normal gene into *Tsc2*-mutant tumor cell lines can profoundly inhibit *in vivo* tumorigenicity provides the strongest argument to date that *Tsc2* functions as a tumor suppressor gene. This biological activity has important implications with respect to *TSC2*-

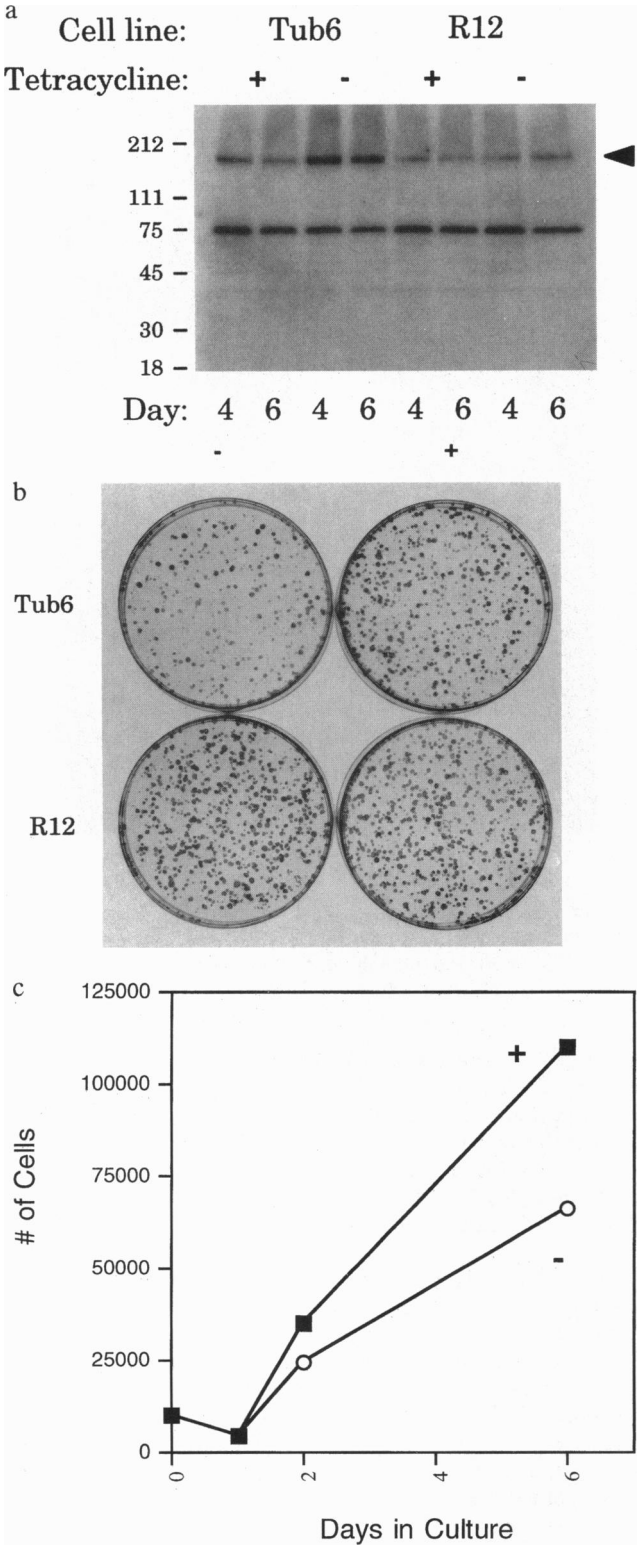

FIG. 4. Inhibition of growth and colony formation upon conditional overexpression of tuberin by the removal of tetracycline. Tub6 and R12 cells contain the tetracycline repressor/VP16 transactivator construct. Tub6 cells were transfected with full-length *Tsc2* under the control of a tetracycline-repressible promoter. (*a*) Conditional overexpression of tuberin in R12 and derivatives. Tuberin protein (arrowhead) was assayed by immunoblot after cells were grown in the presence (+) or absence (−) of tetracycline for 4 or 6 days. The reactivity of the nonspecific band at ≈75 kDa indicates equal loading of protein. (*b*) Colony formation of Tub6 cells (*Upper*) and R12 cells (*Lower*). An equal number of cells was seeded and grown in the absence (*Left*) or presence (*Right*) of tetracycline. After 6 days the plates were stained with methylene blue-carbon fuchsia. (*c*) Growth of Tub6 cells in the presence (+) or absence (−) of tetracycline. Slower growth of Tub6 cells in the absence of tetracycline mirrors increased tuberin expression noted above.

Table 1. Number and size of colonies formed by Rat1-derived fibroblast lines grown in the presence (+) or absence (−) of tetracycline

	Tub6		R12	
Colony Size	−	+	−	+
<1 mm	261	347	325	354
≥1 mm	101	243	297	291
Total	362	590	622	645
Reduction in the absence of tetracycline, %	38.6		3.6	

Growing cultures of R12 and Tub6 maintained in tetracycline were trypsinized and seeded (10^3) in 60-mm plates, in the presence or absence of tetracycline. Colonies were scored after 6 days.

dependent disorders. Since malignant tumor development involves multiple genetic alterations, the loss or inactivation of *Tsc2* is likely to be insufficient for neoplastic or malignant progression, requiring other somatic events in the initiated cells (20). While some of these additional changes might in theory become autonomous to those of the initiating mutation, our data from the Eker rat-derived tumor cell lines demonstrate that the restoration of *Tsc2* function was sufficient to inhibit their growth *in vitro* and *in vivo*. Thus, the malignant phenotype in the Eker lines appears to be dependent on the continuous disruption of the tuberin signaling pathway. If this paradigm holds for malignancies of TSC patients as well, the restoration of tuberin activity by gene therapy, or through biochemical inhibitors might prove clinically useful.

Another aspect of these studies is the finding that a C-terminal tuberin construct, encoding amino acids 1049–1809 including the GAP domain, was capable of inhibiting growth and suppressing tumorigenicity in the Eker rat lines. This demonstrates a tumor suppressor role for this region of tuberin. Although there is a paucity of information regarding the regulation and biochemical function of tuberin, we have recently shown that the tuberin C terminus possesses *in vitro* GAP activity for the Ras-related protein Rap1A (9). These findings point to an analogy between tuberin and the role of the *NF1* product neurofibromin in NF1 patients, where the loss of Ras–GAP activity by neurofibromin correlates with increased Ras activity and malignant tumor development (21, 22). Although the exact biological role of Rap1 is unclear, microinjection of this protein has been shown to induce DNA synthesis (23), and a recent report demonstrated direct activation of the Raf family member B-raf by interaction with Rap1 (24). Thus the regulation of Rap1–GTP levels may be a primary biochemical function of tuberin, linked to its tumor suppressor function, although it is possible that a different small GTPase might also be regulated by tuberin. In support of the former possibility, we have recently found significant colocalization of tuberin and Rap1 in cultured cells, using confocal microscopy (25). An alternative mechanism of tuberin function was recently proposed based on the presence of transcriptional activation domains in the C terminus suggesting that the Eker *Tsc2* gene product may directly deregulate transcription of downstream effectors (26).

These studies touch on several outstanding questions regarding the clinical manifestations of tuberous sclerosis, including the systemic nature of the disease, the wide spectra of organs affected, and the clinical heterogeneity of TSC. We have observed tuberin-induced growth inhibition in the Eker rat kidney tumor cells, which lack endogenous tuberin, and in rat fibroblasts containing normal tuberin levels. The impor-

tance of *Tsc2* in controlling cell growth and proliferation in diverse cell types is in keeping with the systemic nature of TSC and the multiple neoplasias found in the Eker rat. Analysis of the effects of *Tsc2* overexpression in tumors derived from these various (nonkidney) sites will help to define the generality of its tumor suppressor function. A generalized role for *Tsc2* as a tumor suppressor is also suggested by the wide tissue distribution of *Tsc2* expression including the brain, heart, kidney, lung, and skin (4, 8, 27). The fact that *Tsc2* overexpression inhibited growth (though perhaps not as efficiently) in cells already containing normal tuberin levels suggests that the negative effect exerted by normal tuberin levels is not present in excess. Thus it is possible that the half-dosage of the gene found in cells of TSC patients may affect the growth or differentiation of a variety of cells during development, setting the stage for occasional inactivation of the normal *Tsc2* allele and hamartoma development. The infrequent and random nature of the second somatic event at the predisposing locus may explain, in part, the clinical heterogeneity in TSC. The systems described here to demonstrate growth inhibition and tumor suppression by *Tsc2* should provide a valuable bioassay for evaluating other mutants of the gene and to gain further insight into the molecular pathogenesis of TSC.

We gratefully acknowledge A. G. Knudson and D. R. Lowy for critical reading of the manuscript and continued support of this project. R12 cells were kindly provided by S. I. Reed, and T. Herzinger provided helpful advice for working with them. We thank A. Lerro, S. Howard, and W. C. Vass for technical assistance. This work was support in part by grants from National Institute of Health, the W. W. Smith Foundation, the Markey Charitable Trust, and an appropriation from the Commonwealth of Pennsylvania. R.S.Y. is a recipient of the Council for Tobacco Research Scholar Award. R.W. is a fellow of the Deutsche Forschungsgemeinschaft (Wi 1302/1-1). J.C.M. is a National Institutes of Health/Howard Hughes Medical Institute Research Scholar.

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