Expression of wild type and mutant TSC2, but not TSC1, causes an increase in the G1 fraction of the cell cycle in HEK293 cells

L Khare, A Astrinidis, W Senapedis, P D Adams, E Petri Henske

Tuberous sclerosis complex (TSC) is a tumour suppressor gene syndrome whose manifestations include seizures, mental retardation, autism, and tumours of the brain, retina, kidney, heart, and skin. Mutations in two tumour suppressor genes, TSC1 on chromosome 9q34 and TSC2 on chromosome 16p13, cause TSC. TSC2 encodes tuberin, a 190 kDa protein with homology to the catalytic domain of a GTPase activating protein (GAP) for Rap1. TSC1 encodes hamartin, a 130 kDa protein. Tuberin and hamartin have been shown to directly interact, both in mammalian cells and in Drosophila. This is consistent with the nearly identical spectrum of disease seen in humans with TSC1 mutations, and with the identical phenotypes of tuberin and hamartin shown to increase the G1 fraction of cells transiently expressed in mammalian cells. Site directed PCR based mutagenesis was performed using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) to generate TSC2 mutants which were then confirmed by DNA sequencing. To generate the exon 16 (G1832A) TSC2 mutant construct, the primers were 5′-GAG CAG CAT CCA GCT GCA GGC C-3′ and 5′-GAC TAC GAG TGC-3′. The G1832A missense change in exon 16 changes the amino acid at position 611 from arginine to glutamine (R611Q). This is a frequent naturally occurring germline mutation in TSC patients and has recently been found as a somatic mutation in tumour cells from women with the sporadic form of pulmonary lymphangioleiomyomatosis. To generate the exon 38 TSC2 mutant construct (C5024T), the primers were 5′-CAT CCA GCT GCA GGC C-3′ and 5′-GCA CTC GTA GTC-3′. Constructs were cloned into pcDNA 3.1+ (Invitrogen, Carlsbad, CA). The C5024T missense change in exon 38 changes the amino acid at position 1675, within the GAP related domain, from proline to leucine (P1675L) (fig 1B). This mutation occurs at a highly conserved residue and has been reported in at least 10 unrelated TSC patients (see the TSC Variation Website (http://expmed.bwh.harvard.edu/ts/) for specific references and further details for both mutations).

Overexpression of the appropriate protein(s) was confirmed by western immunoblot analysis (fig 2A). A polyclonal tuberin C20 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), a polyclonal affinity purified hamartin antibody developed in our laboratory, a p27(KIP1) antibody (Oncogene Research Products, San Diego, CA), and an actin antibody (Sigma, St Louis, MO) were used to detect proteins by enhanced chemiluminescence (Amersham, Piscataway, NJ).

Key points

- To understand the relevance of p27 and cell cycle regulation to the clinical manifestations of TSC, we analysed HEK293 cells overexpressing wild type TSC1, wild type TSC2, or disease causing mutant forms of TSC2.
- Expression of wild type TSC2, but not TSC1, caused an increase in the G1 fraction of the cell cycle. Expression of the TSC2 mutants also resulted in a G1 fraction increase.

MATERIAL AND METHODS

We cotransfected cells with TSC1 and/or TSC2 in excess of a construct encoding the cell surface protein CD19, and sorted transfected cells by FACS using an FITC conjugated anti-CD19 antibody. Transfections were performed using calcium phosphate precipitation. The TSC1 and/or TSC2 constructs were transfected in five-fold molar excess of the expression vector encoding the cell surface protein CD19, as previously described. After transfection, the cells were washed with phosphate buffered saline and cultured in fresh media until trypsinisation at 48 hours post-transfection. A construct encoding the cyclin dependent kinase inhibitor p21(WAF1/CIP1) was used as a positive control for an increase in the G1 phase of the cell cycle. A summary of the constructs used in these experiments is shown in fig 1A. The C-terminus construct has been previously shown to reduce the in vitro proliferation and in vivo tumorigenicity of Eker rat renal carcinoma cells. Site directed PCR based mutagenesis was performed using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) to generate TSC2 mutants which were then confirmed by DNA sequencing. To generate the exon 16 (G1832A) TSC2 mutant construct, the primers were 5′-GAG CAG CAT CCA GCT GCA GGC C-3′ and 5′-GAC TAC GAG TGC-3′. The G1832A missense change in exon 16 changes the amino acid at position 611 from arginine to glutamine (R611Q). This is a frequent naturally occurring germline mutation in TSC patients and has recently been found as a somatic mutation in tumour cells from women with the sporadic form of pulmonary lymphangioleiomyomatosis. To generate the exon 38 TSC2 mutant construct (C5024T), the primers were 5′-CAT CCA GCT GCA GGC C-3′ and 5′-GCA CTC GTA GTC-3′. Constructs were cloned into pcDNA 3.1+ (Invitrogen, Carlsbad, CA). The C5024T missense change in exon 38 changes the amino acid at position 1675, within the GAP related domain, from proline to leucine (P1675L) (fig 1B). This mutation occurs at a highly conserved residue and has been reported in at least 10 unrelated TSC patients (see the TSC Variation Website (http://expmed.bwh.harvard.edu/ts/) for specific references and further details for both mutations).

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Two colour fluorescence activated cell sorting (FACS) was used to determine the DNA content of cells transiently transfected with plasmids encoding CD19 and TSC1 or TSC2. After harvest, the cell pellet was resuspended in 100 µl of phosphate buffered saline containing 5 µl of FITC conjugated anti-CD19 antibody (Caltag Laboratories, Burlingame, CA), incubated on ice for one hour, and washed twice with PBS containing 0.1% BSA. The cells were then fixed with 70% ethanol at 4°C. Before flow cytometry analysis, the cells were pelleted, washed in phosphate buffered saline, and stained with 20 µg/ml of propidium iodide (Sigma) containing 9.5 mg/ml RNase (Sigma). Flow cytometry analysis was performed on a Becton-Dickinson FACScan machine. The intensity of propidium iodide staining was analysed on FITC positive cell populations. Dickinson FACScan machine. The intensity of propidium iodide (Sigma) containing 9.5 mg/ml RNase (Sigma). Flow cytometry analysis was performed on a Becton-Dickinson FACScan software version 3.1 (Verity Inc, Topsham, ME). Statistical analysis was performed using a one sided Student's t test. The intensity of PI staining was analysed in the CD19 positive cells and used to determine the percentages of transfected cells in the G1, S, and G2/M phases of the cell cycle.

RESULTS AND DISCUSSION
We initially analysed U2OS cells, which were derived from a human osteosarcoma. In U2OS cells, the expected increase in G1 was seen with p21(WAF1/CIP1) transfection, which was used as a positive control, but no change in G1 was found with transfection of TSC1, TSC2, or both (data not shown).

We then studied HEK293 cells, which were derived from human embryonic kidney cells. A small but significant increase in the G1 phase of the cell cycle was observed when wild type TSC2 was overexpressed in HEK293 cells (fig 3). The percentage of cells in G1 was 33.2% for cells transfected with CD19 and the empty pcDNA vector, compared with 40.6% for p21(WAF1/CIP1) transfection, 40.9% for TSC2, and 39.4% for TSC1 plus TSC2. In each case, the increase in the G1 fraction was accompanied by a decrease in the G2 fraction. No significant change in the G1 fraction was found when TSC1 (30.1% in G1) or the carboxy-terminus of TSC2 (35.2% in G1) were overexpressed. This is, to our knowledge, the first time that the cell cycle effects of hamartin have been studied using transient, rather than stable, expression, and the first time that the effects of TSC1 and TSC2 have been directly compared. Our data suggest that hamartin, not hamartin, is responsible for G1 regulation by the hamartin-tuberin complex.

We chose to study HEK293 cells in part because the kidney is a major site of disease in TSC patients. Renal disease in TSC can include angiomylipomas, cysts, and renal cell carcinomas, with angiomylipomas by far the most common renal manifestation. Renal disease is an important cause of mortality in TSC patients. The fact that we observed an effect of TSC2 in HEK293 cells but not U2OS cells could reflect intrinsic differences between these cell types, and these differences could be related to the high incidence of renal manifestations among patients with TSC. It is more likely, though, that U2OS cells contain mutations in other genes that block the effect of TSC2. Similar differences between cell lines have been observed for many tumour suppressor genes, including RB. In some cases, the downstream genes that distinguish “sensitive” cells from insensitive cells have been elucidated.

The effect of TSC2 on the G1 phase of the cell cycle that we observed in HEK293 cells was substantially enhanced by treating the cells with the anti-microtubule agent nocodazole, which induces a G2/M block. The addition of nocodazole to the culture medium for 18–24 hours allowed, after nocodazole treatment, the vector transfected control cells accumulated in G2/M (as...
expected) accompanied by a decrease in the G1 population, while the TSC2 transfected cells had a significant accumulation in G1 (figs 3 and 4). The percentage of cells in G1 was 4.9% for cells transfected with CD19 and the empty pcDNA vector, compared with 28.5% for p21 (WAF1/CIP1), TSC2, TSC1 plus TSC2, the TSC2 exon 16 mutant (TSC2 Mut Ex16), and the TSC2 exon 38 mutant (TSC2 Mut Ex38). There was no change in the G1 fraction for cells transfected with TSC1 or the TSC2 carboxy-terminus (TSC2 C-term). *p<0.05 relative to CD19/pcDNA control. (B) With nocodazole treatment, the majority of the pcDNA/CD19 control transfected cells were in G2/M, as expected. An increase in the G1 fraction was seen in cells transfected with p21 (WAF1/CIP1), TSC2, TSC1 plus TSC2, the TSC2 exon 16 mutant (TSC2 Mut Ex16), and the TSC2 exon 38 mutant (TSC2 Mut Ex38). There was no change in the G1 fraction for cells transfected with TSC1 or the TSC2 carboxy-terminus (TSC2 C-term). *p <0.05 relative to the CD19/pcDNA control.

Figure 3  TSC2 expression causes an increase in cells in the G1 phase of the cell cycle. (A) The fraction of cells in the G1, S, and G2 phases of the cell cycle for each transfection is indicated. The percentage of cells in G1 is indicated by the bar height. The standard error of the mean (SEM) is shown in white. An increase in the G1 fraction was seen in cells transfected with p21 (WAF1/CIP1), TSC2, TSC1 plus TSC2, the TSC2 exon 16 mutant (TSC2 Mut Ex16), and the TSC2 exon 38 mutant (TSC2 Mut Ex38). There was no change in the G1 fraction for cells transfected with TSC1 or the TSC2 carboxy-terminus (TSC2 C-term). *p<0.05 relative to CD19/pcDNA control. (B) With nocodazole treatment, the majority of the pcDNA/CD19 control transfected cells were in G2/M, as expected. An increase in the G1 fraction was seen in cells transfected with p21 (WAF1/CIP1), TSC2, TSC1 plus TSC2, the TSC2 exon 16 mutant (TSC2 Mut Ex16), and the TSC2 exon 38 mutant (TSC2 Mut Ex38). There was no change in the G1 fraction for cells transfected with TSC1 or the TSC2 carboxy-terminus (TSC2 C-term). *p <0.05 relative to the CD19/pcDNA control.

We hypothesised that naturally occurring TSC2 missense mutations would not result in the G1 increase seen with wild type TSC2 if perturbation of this pathway is a primary event in disease pathogenesis. Approximately 20% of germinal TSC2 mutations are missense changes. For reasons that are not yet understood, all identified TSC1 mutations result in premature protein truncation, with no known missense mutations. The functional characterisation of mutant forms of TSC2 is at a very early stage. In our study, expression of two of the most common naturally occurring TSC2 missense mutants, in exons 16 (R611Q) and 38 (P1675L), resulted in an increase in the G1 fraction of similar magnitude to that mediated by wild type TSC2. As with wild type TSC2, this effect was enhanced by exposure of the cells to nocodazole (figs 3 and 4). The percentage of cells in G1 with nocodazole treatment was 4.9% for the pcDNA/CD19 control, 17.4% for wild type TSC2, 16.6% for the
TSC2 exon 16 mutation, and 13.6% for the TSC2 exon 38 mutation (p<0.05 for each construct compared with the vector control). These results support recent work in which three mutations within the GAP domain (N1643K, N1651S, and N1681K) were found to have effects on the cell cycle that were similar to those of wild type TSC2, consistent with our results for the P1675L GAP domain mutant. Because these mutations occurred in patients with TSC, these data suggest that cell cycle regulation is not the primary mechanism through which tuberin functions as a tumour suppressor gene.

In addition to cell cycle regulation, mammalian tuberin and/or hamartin appear to be involved in other cellular pathways that may contribute to tumorigenesis: Rap1 activation, vesicular trafficking, transcriptional activation by steroid hormones, and focal adhesion formation. The in vivo significance of these pathways and their contribution to the abnormal cell proliferation and abnormal cell cycle kinetics in TSC are not yet known. Clearly, it will be important to test the function of naturally occurring TSC2 missense mutations in these pathways.

While the exon 16 and exon 38 TSC2 mutants resulted in an increase in cells in the G1 phase of the cell cycle, the carboxy-terminus construct containing the wild-type GAP domain did not. An identical carboxy-terminus construct has been shown previously to reduce the in vitro proliferation of two Eker rat derived cell lines at a level similar to that of full length TSC2, and suppressed the in vivo tumorigenicity of one of the cell lines (as did full length TSC2). Our data may indicate that the carboxy-terminus of TSC2 is not required for the cell cycle effects of TSC2 overexpression, but is involved in growth suppression through other cellular pathways.

Other groups have observed increases in p27(KIP1) protein levels when TSC1 or TSC2 is overexpressed. We also found increases in p27(KIP1) levels when TSC1 or TSC2 was expressed (fig 2B), although only when the cells were exposed to nocodazole. The relative increase in p27(KIP1) compared with the control transfected cells, as determined by densitometry, was 3.3 for TSC1 transfection, 2.5 for TSC2 transfection, and 3.9 for the TSC2 exon 16 mutant transfection. An increase in p27(KIP1) was not seen when the TSC2 carboxy-terminus or the TSC2 exon 38 mutant construct was transfected. Similar results were seen in multiple independent transfections. Interestingly, in our study the p27(KIP1) increase was seen in TSC1 transfected cells, despite the fact that TSC1 did not affect the cell cycle profile. The exon 38 mutant (P1675L), which resulted in an increase in the G1 fraction, did not affect the p27(KIP1) level. In contrast, the exon 16 mutant (R611Q) increased both G1 and p27(KIP1). These results functionally separate cell cycle regulation (predominantly mediated by tuberin and intact in the GAP domain mutant construct) from p27(KIP1) up regulation (mediated by tuberin and hamartin and lost in the GAP domain mutant construct).

In summary, we examined the cell cycle impact of transient expression of TSC1 and/or TSC2 using a two colour FACS assay. We found that expression of wild type TSC2, but not TSC1, increased the percentage of cells in the G1 phase of the cell cycle. No additive or synergistic effect occurred when TSC1 and TSC2 were coexpressed. This is the first time that the cell cycle effects of TSC1 and TSC2 have been studied in parallel, and suggests that the cell cycle effects of the tuberin-hamartin complex derive primarily from tuberin, not hamartin. We also tested two naturally occurring mutant forms of TSC2, one within the region of GAP homology (P1675L) and one in exon 16 (R611Q). We found that expression of either mutant also induced an increase in the G1 fraction, similar in magnitude to that of wild type TSC2. A similar result was recently reported for three other mutations in the GAP homology domain. There are several competing hypotheses that could explain the unexpected finding that expression of mutant forms of TSC2 increases the G1 fraction. First, it is possible that the domains affected by these mutations (including the GAP domain) are not involved in tuberin’s cell cycle regulation. Second, overexpression of the mutant proteins could compensate for decreased activity in vivo at endogenous expression levels. Finally, it is possible that the G1 increase seen with expression of mutant TSC2 (as well as wild type) indicates that disruption of the cell cycle is not an important pathway of tumorigenesis in TSC, despite a number of studies indicating that wild type TSC2 and TSC1 regulate the G1 phase of the cell cycle.
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