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TSC2 missense mutations inhibit tuberin phosphorylation and prevent formation of the tuberin–hamartin complex

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Tuberous sclerosis (TSC) is an autosomal dominant disorder characterized by a broad phenotypic spectrum that includes seizures, mental retardation, renal dysfunction and dermatological abnormalities. Inactivating mutations to either of the *TSC1* and *TSC2* tumour suppressor genes are responsible for the disease. *TSC1* and *TSC2* encode two large novel proteins called hamartin and tuberin, respectively. Hamartin and tuberin interact directly with each other and it has been reported that tuberin may act as a chaperone, preventing hamartin self-aggregation and maintaining the tuberin–hamartin complex in a soluble form. In this study, the ability of tuberin to act as a chaperone for hamartin was used to investigate the tuberin–hamartin interaction in more detail. A domain within tuberin necessary for the chaperone function was identified, and the effects of *TSC2* missense mutations on the tuberin–hamartin interaction were investigated to allow specific residues within the central domain of tuberin that are important for the interaction with hamartin to be pin-pointed. In addition, the results confirm that phosphorylation may play an important role in the formation of the tuberin–hamartin complex. Although mutations that prevent tuberin tyrosine phosphorylation also inhibit tuberin–hamartin binding and the chaperone function, our results indicate that only hamartin is phosphorylated in the tuberin–hamartin complex.

INTRODUCTION

Tuberous sclerosis (TSC) is characterized by the development of hamartomatous growths in many tissues and organs. Brain and skin involvement leads to the classic phenotype of seizures, mental retardation and facial and ungual fibromas. Renal, cardiac and retinal tissues are also often affected (1).

TSC is an autosomal dominantly inherited disorder caused by inactivation of either the *TSC1* gene on chromosome 9q34 or the *TSC2* gene on chromosome 16p13.3 (2,3). Evidence from loss of heterozygosity studies of TSC-associated lesions, as well as from TSC animal models, supports the hypothesis that *TSC1* and *TSC2* are tumour suppressor genes, and that it is the absence of the *TSC1* or *TSC2* gene products that underlies the pathogenesis of TSC (4–8).

The *TSC1* gene codes for hamartin, a novel 130 kDa protein containing a putative coiled coil domain (amino acids 719–998) (2). This region has been shown to mediate binding between different hamartin molecules and it has been proposed that this homomeric interaction explains the distinctive punctate expression pattern of hamartin when the protein is over-expressed in COS cells (9). The *TSC2* gene codes for tuberin, a 200 kDa protein containing a small region of homology to the rap1 GTPase activating protein, rap1GAP (amino acids 1593–1631) (3). There is good evidence that tuberin and hamartin form a complex *in vivo* (10,11) and function within the same pathway(s) regulating cell growth (6–8). It has been suggested that formation of the tuberin–hamartin complex prevents tuberin ubiquitination (12) and that tuberin phosphorylation regulates its interaction with hamartin (13).

The demonstration that tuberin and hamartin bind each other directly and the fact that mutations to either *TSC1* or *TSC2* lead to the same disease, suggest that both proteins are required for the correct function of the tuberin–hamartin complex and that inactivation of the complex leads to many of the lesions associated with TSC. We examined the effect of *TSC2* missense changes on the interaction between tuberin and hamartin and identified a domain within tuberin that regulates the formation and localization of the tuberin–hamartin complex. We pin-pointed amino acid residues that are not only critical for tuberin–hamartin binding but also regulate the phosphorylation status of tuberin. Our results suggest that phosphorylation may regulate both the formation and activity of the tuberin–hamartin complex.

RESULTS

Tuberin is a chaperone for hamartin

The expression of hamartin and tuberin in transfected COS cells was investigated by immunofluorescence microscopy and western blotting. Immunofluorescence microscopy showed that the expression pattern of hamartin was tuberin-dependent. In cells transfected with a *TSC1* expression construct, hamartin was expressed in the cytoplasm in the distinctive punctate pattern shown in Figure 1A. However, in cells co-transfected with both *TSC1* and *TSC2* expression constructs, co-expression

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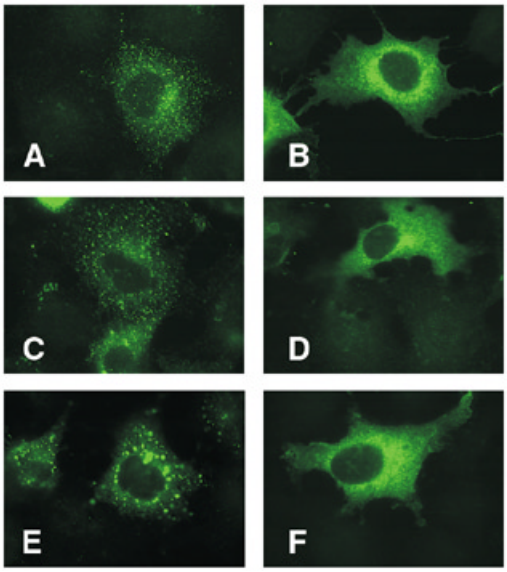

Figure 1. Effect of tuberin expression on the expression pattern of hamartin. Immunofluorescence microscopy was performed on COS cells overexpressing hamartin alone (**A**), hamartin with wild-type tuberin (**B**) and hamartin with different tuberin-derived variant proteins (**C**–**F**). (A) Punctate expression of hamartin in the absence of tuberin. (B) Homogeneous, diffuse expression of hamartin in the presence of tuberin. (C) Punctate expression of hamartin in the presence of a truncated tuberin protein (amino acids 1–607). A similar punctate hamartin expression pattern was observed in the presence of proteins containing tuberin amino acids 607–1099, 1125–1784, 1–252 and 1535–1784 (not shown). (D) Diffuse expression of hamartin in the presence of a truncated tuberin protein containing amino acids 1–1099. A similar pattern was observed in the presence of truncated tuberin proteins containing amino acids 1–1712, 1–1240 and 1–1133 (not shown). (E) Punctate expression of hamartin in the presence of the tuberin R611Q variant. A similar hamartin expression pattern was also observed in the presence of the R611W, A614D, F615S, C696Y and V769E tuberin variants (not shown). (F) Diffuse expression of hamartin in the presence of the tuberin R905Q variant. A similar effect on the expression pattern of hamartin was observed with the N525S and K599M tuberin variants (not shown).

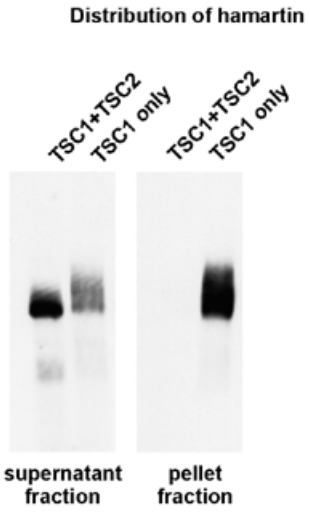

Figure 2. Effect of tuberin expression on the subcellular distribution of hamartin. Detection of hamartin in the supernatant and pellet fractions of lysates prepared from cells expressing either hamartin alone (TSC1 only) or both tuberin and hamartin (TSC1 + TSC2). Co-expression of tuberin causes a significant re-localization of hamartin from the insoluble pellet fraction to the soluble supernatant fraction.

of tuberin resulted in the expression pattern of hamartin becoming more homogeneous and diffuse (Fig. 1B). The expression pattern of hamartin was therefore altered in the presence of tuberin.

The homogeneous expression pattern of hamartin in the co-transfected cells was indistinguishable from the expression pattern of tuberin, consistent with the proposal that hamartin and tuberin interact to form a complex and that tuberin is a chaperone for hamartin (9). To investigate whether the observed tuberin-dependent change in the expression pattern of hamartin really reflected a cytosolic chaperone function of tuberin, detergent lysates of the cells transfected with the *TSC1* and *TSC2* expression constructs were analysed by western blotting (Fig. 2). From cells co-expressing hamartin and tuberin, hamartin was detected in the soluble, supernatant fraction of the detergent lysate. However, from cells expressing hamartin only, the amount of hamartin in the supernatant fraction was reduced and the bulk of the protein was present in the insoluble pellet fraction. Tuberin was detected in both the supernatant and pellet fractions and co-expression of hamartin did not affect the proportion in either fraction (Fig. 4).

The results of the western blot analysis were consistent with the immunofluorescence microscopy analysis, which had shown a significant change in the expression pattern of hamartin in the presence of tuberin. Therefore, the western blot analysis provided further support for the proposal that tuberin is a cytosolic chaperone for hamartin, maintaining the tuberin–hamartin complex in a soluble form (9).

The C-terminal domain of tuberin is not required for the chaperone function

To investigate whether the tuberin-dependent change in the expression pattern and localization of hamartin was mediated by a specific domain within tuberin, COS cells were co-transfected with expression constructs encoding full-length hamartin and a series of truncated tuberin-derived proteins. The expression pattern of hamartin in the presence of the truncated tuberin-derived proteins was determined by immunofluorescence microscopy.

In cells expressing hamartin and a protein consisting of tuberin amino acids 1–1099, the hamartin labelling was homogeneous, identical to the labelling pattern of hamartin in the presence of full-length tuberin (Fig. 1B and D). Therefore, deletion of amino acids 1100–1784 from the C-terminal of tuberin did not affect the ability of tuberin to cause a change in the expression pattern of hamartin. Similar results were obtained when hamartin was co-expressed with truncated proteins lacking amino acids 1713–1784, 1241–1784 and 1134–1784 of tuberin. In each case, hamartin was homogeneously distributed throughout the cytoplasm in a diffuse pattern (data not shown). In contrast, when co-expressed with a protein consisting of only the first 607 amino acids of tuberin, hamartin was detected in discrete punctate structures. The expression pattern was the same as in the absence of tuberin (Fig. 1C and A). Similarly, in the presence of a tuberin in-frame deletion variant lacking amino acids 253–1535, or when

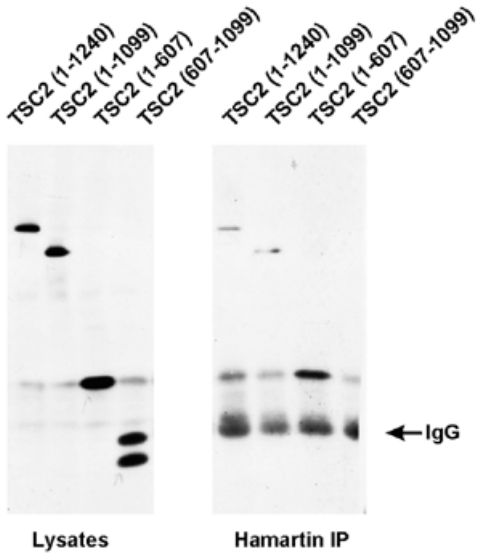

Figure 3. Detection of truncated tuberin proteins in hamartin immunoprecipitates: Tuberin amino acids 1–607 are sufficient for hamartin binding. Hamartin was immunoprecipitated from COS cells co-expressing hamartin and truncated tuberin proteins encoding amino acids 1–1240, 1–1099, 1–607 and 607–1099. The truncated proteins in the immunoprecipitates were detected with an epitope tag antibody (Qiagen). The proteins encoded by tuberin amino acids 1–1240, 1–1099 and 1–607 are detected in both the cell lysates and the hamartin immunoprecipitates, indicating that all three proteins can bind hamartin. Similar results were obtained with a protein encoding tuberin amino acids 1–1712. The protein encoded by amino acids 607–1099 is detected as two bands in the cell lysate but is not present in the hamartin immunoprecipitate and therefore does not bind hamartin. Truncated tuberin proteins encoding amino acids 1125–1784 and 1–252 plus 1535–1784 were also absent from the hamartin immunoprecipitates (not shown). Cross-reacting immunoglobulins present in the immunoprecipitates are indicated.

proteins containing only amino acids 607–1099, or amino acids 1125–1784 of tuberin were co-expressed, hamartin was detected in the same punctate pattern (data not shown). In each case, the truncated tuberin-derived proteins gave a homogeneous, diffuse labelling pattern, similar to full-length tuberin. The expression patterns of the truncated proteins were not affected by co-expression of hamartin. This analysis indicated that both the central region of tuberin, encompassing amino acids 607–1099, and the N-terminal domain consisting of amino acids 1–607, were necessary for the chaperone function.

Tuberin–hamartin binding is not sufficient for the tuberin chaperone function

To determine whether the tuberin chaperone function was dependent on tuberin–hamartin binding, hamartin was immunoprecipitated from cells co-expressing hamartin and the truncated tuberin proteins. The hamartin immunoprecipitates were then analysed for the presence of the truncated tuberin proteins. As shown in Figure 3, the 607 N-terminal residues were co-immunoprecipitated by the hamartin-specific antibodies, indicating that although this domain was not sufficient for the tuberin chaperone function, it was sufficient for the binding between tuberin and hamartin. Truncated tuberin proteins lacking the N-terminal domain (amino acids 1–607) were not detected in the hamartin immunoprecipitates.

Tuberin amino acid substitutions affect the localization of exogenously expressed hamartin

To investigate the tuberin–hamartin interaction in more detail, COS cells were co-transfected with expression constructs encoding full-length hamartin and nine different full-length tuberin variants (N525S, K599M, R611Q, R611W, A614D, F615S, C696Y, V769E and R905Q). Each tuberin variant contained a different single amino acid substitution that had been identified previously in one or more TSC patients (14–19). Further, all of the amino acid substitutions were located within the N-terminal half of tuberin that had been shown to be essential for the chaperone function. No differences in expression of the different tuberin variants were observed by immunofluorescence microscopy. None of the variants could be distinguished from wild-type tuberin using this technique.

The effect of each of the tuberin variants on the expression pattern of hamartin was also investigated by immunofluorescence microscopy. Amino acid substitutions N525S, K599M and R905Q did not interfere with the tuberin-dependent change in the expression pattern of hamartin. In each case, the hamartin labelling was homogeneously distributed throughout the cytoplasm, identical to the pattern in the presence of wild-type tuberin (Fig. 1B and F). In contrast, no tuberin-dependent change in the expression pattern of hamartin was observed when hamartin was co-expressed with the R611Q, R611W, A614D, F615S, C696Y and V769E variants. The hamartin labelling was localized to discrete punctate structures similar to the pattern observed in the absence of tuberin (Fig. 1A and E). The immunofluorescence microscopy experiments indicated that tuberin amino acids R611, A614, F615, C696 and V769 were all necessary for the chaperone function.

Tuberin amino acid substitutions disrupt the chaperone function of tuberin

To confirm the results of the immunofluorescence microscopy analysis, the cells expressing hamartin and the different tuberin variants were analysed by western blotting. As shown in Figure 4A, co-expression of hamartin with the N525S, K599M and R905Q variants gave patterns similar to that of wild-type tuberin: hamartin was detected as an ~130 kDa band in the supernatant fraction from detergent lysates of the co-transfected cells. In contrast, when expressed with the R611Q, R611W, A614D, F615S, C696Y and V769E variants, hamartin was detectable in the supernatant fractions as a series of weak bands, similar to when hamartin was expressed in the absence of tuberin. Therefore, the tuberin variants unable to cause the change from the punctate hamartin expression pattern to the diffuse expression pattern (as revealed by immunofluorescence microscopy) were also less capable of retaining hamartin in the soluble supernatant fraction of a detergent lysate.

The results of the western blot analysis were consistent with the immunofluorescence microscopy experiments. Hamartin was detectable when co-expressed with the tuberin variants N525S, K599M and R905Q that were able to change the punctate hamartin expression pattern to a homogeneous pattern. Upon co-expression of the tuberin variants R611Q, R611W, A614D, F615S, C696Y and V769E that were unable to alter the hamartin expression pattern in the immunofluorescence microscopy experiments, reduced levels of hamartin were detected in the lysate supernatants by western blotting. This analysis pin-pointed

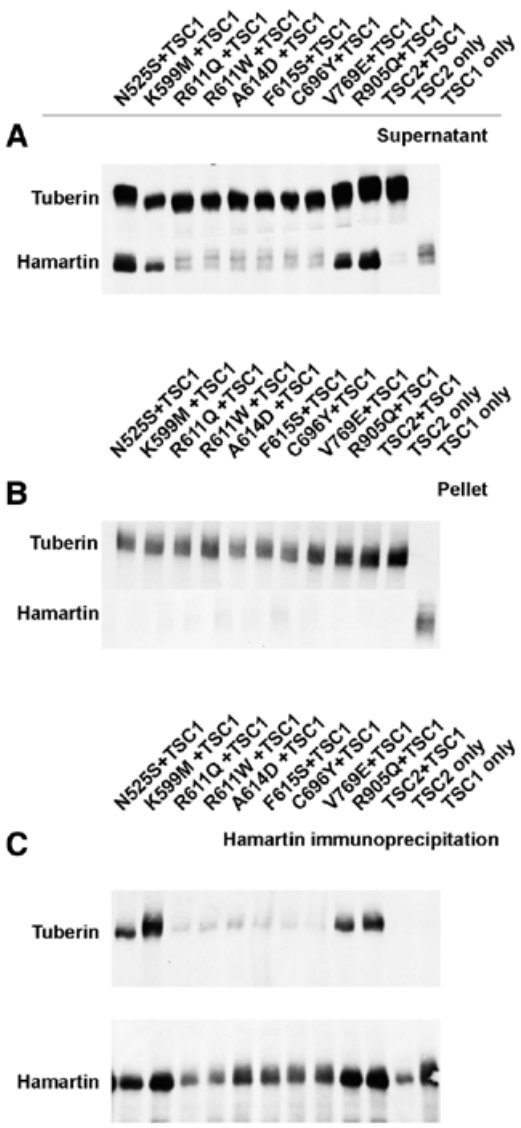

Figure 4. Amino acid substitutions destroy the tuberin chaperone function and inhibit tuberin–hamartin binding. COS cell lysates expressing hamartin and different tuberin variants were analysed by western blotting and immunoprecipitation. (**A**) Detection of tuberin and hamartin in the soluble (supernatant) fraction. Wild-type tuberin (TSC2) and the different tuberin variants (N525S, K599M, R611Q, R611W, A614D, F615S, C696Y, V769E and R905Q) were co-expressed with hamartin (+TSC1). COS cells expressing tuberin alone (TSC2 only) and hamartin alone (TSC1 only) were included as controls. The amount of hamartin in the supernatant fraction is increased in the presence of wild-type tuberin and the N525S, K599M and R905Q variants relative to when tuberin is either completely absent or when the R611Q, R611W, A614D, F615S, C696Y and V769E variants are expressed. The hamartin signal visible in the COS cells transfected with TSC2 only is due to cross-reaction of the antiserum with the endogenous COS hamartin. (**B**) Detection of tuberin and hamartin in the insoluble (pellet) fraction. Hamartin was detected in the pellet fraction of the cells expressing hamartin alone (TSC1 only) but not in any of the co-transfected cells. (**C**) Detection of the tuberin–hamartin complex by immunoprecipitation. The supernatant fractions from (A) were immunoprecipitated with the anti-hamartin antiserum. Only wild-type tuberin and the N525S, K599M and R905Q variants were readily detected in the immunoprecipitates, consistent with formation of the tuberin–hamartin complex being necessary for the chaperone function of tuberin. The hamartin immunoprecipitated from the COS cells transfected with TSC2 only is due to cross-reaction of the antiserum with the endogenous COS hamartin. This cross-reaction may also account for a significant proportion of the immunoprecipitated hamartin in the co-transfected cells.

amino acids R611, A614, F615, C696 and V769 as being critical for the chaperone function of tuberin.

Hamartin was detected in the pellet fraction of the cells transfected with *TSC1* only (Fig. 4B) whereas co-expression of wild-type tuberin or the N525S, K599M and R905Q variants resulted in an increased amount of hamartin in the supernatant fraction, consistent with the results shown in Figure 2. Suprisingly, although no differences in hamartin expression between cells transfected with TSC1 only and cells co-transfected with *TSC1* and the R611Q, R611W, A614D, F615S, C696Y and V769E *TSC2* variants were observed by immunofluorescence microscopy, hamartin was not detected in the pellet fraction when co-expressed with any of the tuberin variants.

Tuberin expression affects the migration of hamartin through SDS–PAGE gels

The western blot analysis shown in Figure 4A indicated that co-expression of tuberin affected the migration of hamartin through SDS–PAGE gels. In the presence of wild-type tuberin and the N525S, K599M and R905Q variants, hamartin migrated as an ~130 kDa band whereas in the absence of tuberin, or the presence of the R611Q, R611W, A614D, F615S, C696Y and V769E variants, additional slowly migrating bands were detected. These additional bands could not be efficiently immunoprecipitated with the hamartin-specific antiserum (Fig. 4C).

Tuberin amino acid substitutions disrupt tuberin–hamartin binding

It was suggested that interaction between tuberin and hamartin was critical for the chaperone function of tuberin (9). To investigate whether disruption of the tuberin chaperone function by the R611Q, R611W, A614D, F615S, C696Y and V769E variants was because tuberin–hamartin complex formation was prevented, co-immunoprecipitation experiments were performed using hamartin-specific antibodies. As shown in Figure 4C, wild-type tuberin and the N525S, K599M and R905Q variants were readily detected in the immunoprecipitates. However, significantly less of the R611Q, R611W, A614D, F615S, C696Y and V769E variants were detectable indicating that these amino acid substitutions interfere with formation of the tuberin–hamartin complex and that formation of this complex is necessary for the chaperone function of tuberin. The amount of hamartin in the immunoprecipitates of the R611Q, R611W, A614D, F615S, C696Y and V769E variants was also reduced, most likely because these variants are unable to stabilize hamartin in the supernatant fraction (Fig. 4A).

Amino acid substitutions affect the migration of tuberin through SDS–PAGE gels

On western blots, tuberin was detected as a series of bands migrating between ~180 and ~200 kDa. However, western blot analysis of the tuberin variants used in this study revealed two distinct patterns (Fig. 5). The N525S, K599M and R905Q variants were detected as multiple bands indistinguishable from wild-type tuberin, whereas the R611Q, R611W, A614D, F615S, C696Y and V769E variants lacked the more slowly migrating forms. Therefore, the N525S, K599M and R905Q variants that were able to act as chaperones for hamartin,

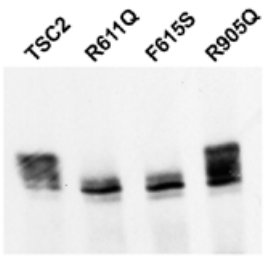

Figure 5. Amino acid substitutions regulate the post-translational modification of tuberin. Western blot analysis of the supernatant fractions from COS cell lysates expressing wild-type tuberin (TSC2) and the R611Q, F615S and R905Q variants. Multiple bands are visible in the wild-type and R905Q lysates, and a similar pattern was observed with the N525S and K599M variants (not shown). In contrast, the R611Q and F615S variants migrate as one major band and two closely-spaced minor bands. A similar migration pattern was observed with the R611W, A614D, C696Y and V769E variants (not shown).

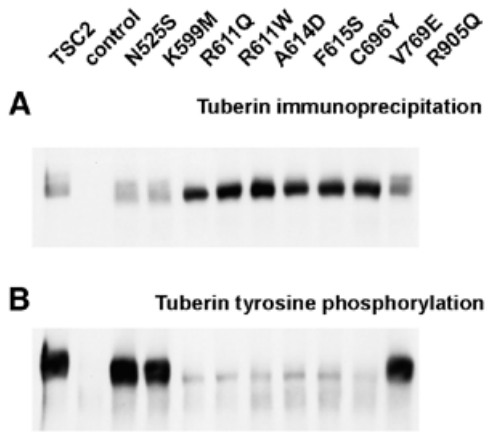

Figure 6. Amino acid substitutions inhibit tuberin tyrosine phosphorylation. Western blot analysis of tuberin immunoprecipitated from COS cells expressing wild-type tuberin (TSC2) and the N525S, K599M, R611Q, R611W, A614D, F615S, C696Y, V769E and R905Q variants. Untransfected COS cells (control) are included to control for cross-reaction of the tuberin-specific antiserum with endogenous COS tuberin. Immunoprecipitation was performed as described in Materials and Methods using a polyclonal antiserum specific for human tuberin. (**A**) Immunoprecipitation of the different tuberin variants. Wild-type tuberin and all the different variants are readily detected. Wild-type tuberin and the N525S, K599 and R905Q variants are visible as multiple, slowly migrating forms whereas the R611Q, R611W, A614D, F615S, C696Y and V769E variants migrate as a single band. The presence of multiple tuberin species may account for the reduction in the intensity of the protein bands in the wild-type, N525S, K599M and R905Q samples. The endogenous COS tuberin is not recognized efficiently by the antiserum used in these experiments. (**B**) Detection of tuberin tyrosine phosphorylation with the PY99 phosphotyrosine-specific antibody in immunoprecipitates of the different tuberin variants. Wild-type tuberin (TSC2) and the N525S, K599M and R905Q variants show significantly more tyrosine phosphorylation than the R611Q, R611W, A614D, F615S, C696Y and V769E variants.

migrated through an SDS–PAGE gel in the same way as wild-type tuberin, whereas the tuberin variants unable to perform the chaperone function had an abnormal migration pattern. The migration patterns of the different tuberin variants were unaffected by co-expression of hamartin (not shown), indicating that the different migration patterns were more likely to be due to the effects of the amino acid substitutions themselves on tuberin, than to be a consequence of the interaction with hamartin.

Amino acid substitutions affect tuberin tyrosine phosphorylation

Aicher *et al.* (13) had shown that tuberin phosphorylation regulates its interaction with hamartin and that the phosphorylation could also explain the multiple forms of tuberin detected on western blots. To investigate whether the abnormal SDS–PAGE migration patterns of the R611Q, R611W, A614D, F615S, C696Y and V769E variants used in this study were due to phosphorylation changes, the different variants were immunoprecipitated with an antiserum specific for tuberin and the immunoprecipitates probed with the phosphotyrosine-specific antibody PY99. As shown in Figure 6, the R611Q, R611W, A614D, F615S, C696Y and V769E variants were less strongly phosphorylated than wild-type tuberin and the N525S, K599M and R905Q variants. Tyrosine phosphorylation of wild-type tuberin and the N525S, K599M and R905Q variants, but not the R611Q, R611W, A614D, F615S, C696Y and V769E variants correlated with the two distinct SDS–PAGE migration patterns and indicated that missense mutations to tuberin amino acids R611, A614, F615, C696 and V769 inhibit tuberin tyrosine phosphorylation.

Phosphorylation of the tuberin–hamartin complex: hamartin-bound tuberin is not phosphorylated on tyrosine residues; tuberin-bound hamartin is phosphorylated on tyrosine residues

The tuberin R611Q, R611W, A614D, F615S, C696Y and V769E substitutions inhibited tuberin–hamartin binding, the tuberin chaperone function and tuberin phosphorylation. This suggested that phosphorylation of specific tyrosine residues within tuberin may be necessary for formation of the tuberin–hamartin complex, as proposed by Aicher *et al.* (13). To address this question, tuberin and the tuberin–hamartin complex were immunoprecipitated from the supernatant fractions of detergent lysates of COS cells transfected with full-length *TSC1* and *TSC2* expression constructs. The immunoprecipitates were tested for the presence of tuberin and hamartin and analysed with the phosphotyrosine-specific antibody. The results of this analysis are summarized in Figure 7. With an antiserum specific for tuberin, tyrosine-phosphorylated tuberin was immunoprecipitated from cells expressing tuberin alone and from cells expressing both tuberin and hamartin. As expected, hamartin in a complex with tuberin was co-immunoprecipitated from the cells expressing both proteins (Fig. 7A, Tuberin IP). Interestingly, the co-immunoprecipitated hamartin reacted with the phosphotyrosine-specific antibody, indicating that when hamartin is bound to tuberin it is phosphorylated (Fig. 7B, Tuberin IP). With the hamartin-specific antiserum, hamartin could only be efficiently immunoprecipitated from the cells expressing both tuberin and hamartin since tuberin is required to retain hamartin in the soluble supernatant fraction (9; and see above). Indeed, co-immunoprecipitated, hamartin-bound tuberin was readily detected (Fig. 7A, Hamartin IP). Again, the immunoprecipitated hamartin was phosphorylated (Fig. 7B, Hamartin IP), confirming the result of the tuberin immunoprecipitation experiment. However, the co-immunoprecipitated tuberin did not react with the phosphotyrosine-specific antibody, suggesting that although free tuberin undergoes tyrosine phosphorylation, dephosphorylation occurs

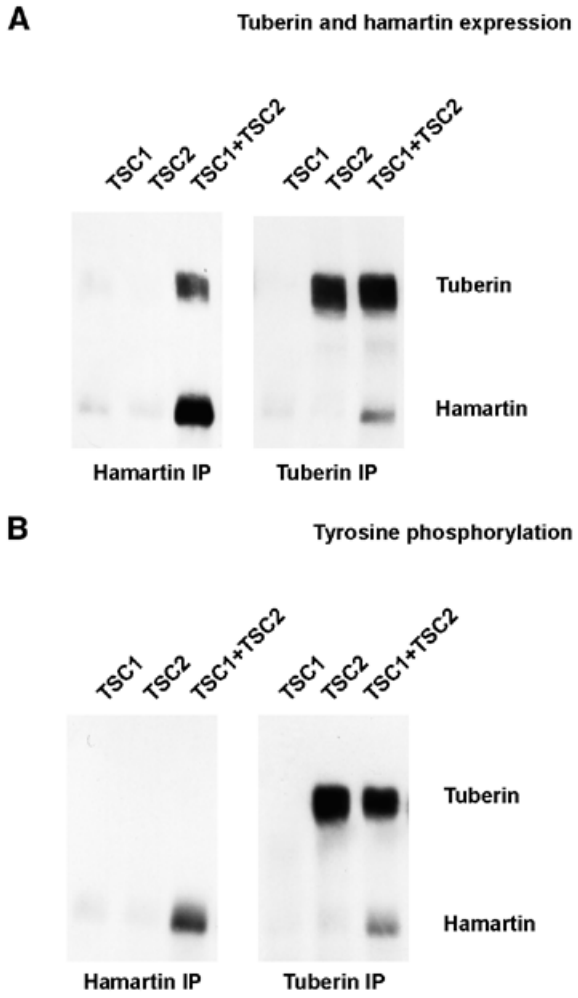

Figure 7. Tyrosine phosphorylation of the tuberin–hamartin complex. Western blot analysis of the tuberin–hamartin complex immunoprecipitated from COS cells expressing hamartin (TSC1), tuberin (TSC2) or both proteins (TSC1 + TSC2). (**A**) Expression of tuberin and hamartin. Immunoprecipitation was performed using antisera specific for hamartin (left, Hamartin IP) and for tuberin (right, Tuberin IP). The immunoprecipitates were subsequently probed for the presence of tuberin and hamartin using the same antisera. As expected, tuberin and hamartin could be co-immunoprecipitated from the cells expressing both proteins with either antiserum. The amount of hamartin immunoprecipitated from the cells expressing hamartin only is reduced relative to the co-expressing cells because tuberin is absent and therefore hamartin is not retained in the soluble fraction. (**B**) Tyrosine phosphorylation of the tuberin–hamartin complex. The immunoprecipitates from (A) were probed with the PY99 phosphotyrosine-specific antibody. Phosphorylated tuberin, not bound to hamartin, could be detected after direct immunoprecipitation with the tuberin-specific antiserum (Tuberin IP, middle lane) but not after co-immunoprecipitation with the hamartin-specific antiserum (Hamartin IP, right lane). In contrast, hamartin bound to tuberin was phosphorylated (Hamartin IP, right lane; Tuberin IP right lane).

when tuberin interacts with hamartin in the complex. Because wild-type tuberin could be tyrosine phosphorylated and form a complex with hamartin, while the tuberin variants that could not be phosphorylated were unable to form a complex with hamartin, the absence of phosphorylated tuberin in the tuberin–hamartin complex was unexpected. However, it was noted that tuberin co-immunoprecipitated by the hamartin-specific antiserum resembled the fast migrating, non-phosphorylated R611Q, R611W, A614D, F615S, C696Y and V769E variants. This would be consistent with the absence of tuberin tyrosine phosphorylation in the tuberin–hamartin complex.

Preliminary experiments suggest that the N525S, K599M and R905Q tuberin variants that are able to act as chaperones for hamartin and undergo tyrosine phosphorylation are also dephosphorylated when bound to hamartin and do not affect the phosphorylation status of hamartin (not shown).

DISCUSSION

Expression of tuberin caused a change in the expression pattern of hamartin. Hamartin was distributed homogeneously throughout the cytoplasm instead of in discrete, punctate structures and solubility, as measured by the amount of hamartin detectable by western blotting, was increased. These observations are consistent with the proposal that the tuberin–hamartin interaction is necessary to retain hamartin in a soluble form in the cell cytoplasm, and that tuberin has a chaperone function (9).

The interaction between tuberin and hamartin was investigated using nine different tuberin missense variants, each carrying a single amino acid substitution, and eight truncated tuberin proteins. The positions of the missense changes and extent of the truncated proteins are shown in Figure 8. The C-terminal half of tuberin (amino acids 1100–1784), including the rap1GAP-related domain, was not necessary for either formation of the tuberin–hamartin complex or the chaperone function, consistent with previous work (10,12). However, a truncated protein consisting of the N-terminal 1099 amino acids of tuberin was sufficient for the chaperone function. A protein containing only the first 607 amino acids of tuberin bound hamartin. However, this protein did not affect the expression pattern of hamartin, indicating that amino acids between residues 607 and 1099 of tuberin are essential for the chaperone function. A protein encoding only amino acids 607–1099 of tuberin was unable to either bind hamartin or change its expression pattern. Taken together these results suggest that tuberin–hamartin binding is necessary for the chaperone function but not sufficient. The N-terminal 607 amino acids of tuberin are sufficient to bind hamartin whereas amino acids 607–1099 are necessary for the chaperone function. The reported binding of hamartin with a domain within the N-terminal third of tuberin (10,12) is consistent with the conclusion that this region is required for formation of the tuberin–hamartin complex and the chaperone function of tuberin.

The tuberin missense changes analysed in this study were all identified in TSC patients (14–19). In each case, the mutated amino acid is completely conserved between humans, rats and mice and 5/8 of the residues are also conserved in fruitflies and the pufferfish, as shown in Table 1. All the substitutions were described as being responsible for TSC, except the F615S change which was identified as a polymorphic variant (14). However, since this variant prevents tuberin phosphorylation and inhibits formation of the tuberin–hamartin complex, the possibility that the F615S substitution is responsible for TSC in the original patient should be re-considered. Indeed, Gilbert *et al*. could not exclude completely the possibility that the F615S change was in fact a pathogenic mutation (J.Gilbert, personal communication).

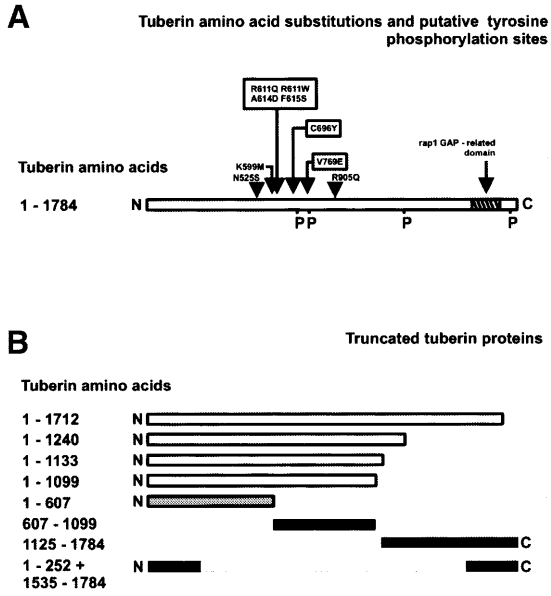

Figure 8. Tuberin missense variants and truncated tuberin proteins. Schematic diagram indicating the positions of the tuberin amino acid substitutions (**A**) and the extent of the truncated tuberin proteins (**B**) used in this study. (A) Tuberin amino acid substitutions and putative tyrosine phosphorylation sites. The relative positions of the N525S, K599M, R611Q, R611W, A614D, F615S, C696Y, V769E and R905Q substitutions are indicated. The variants that prevented both tyrosine phosphorylation of tuberin and the interaction with hamartin are boxed. Putative tyrosine phosphorylation sites are indicated with the letter P. N and C represent the tuberin N- and C-termini, respectively; the extent of the rap1GAP-related domain is indicated by cross-hatching. (B) Truncated tuberin proteins. Truncated proteins able to bind hamartin and perform the chaperone function are represented as open squares; the proteins able to bind hamartin but unable to perform the chaperone function are indicated by the shading; proteins unable to either bind or perform the chaperone function are shown as closed squares. The extent of the internal in-frame deletion is indicated by a dashed line. The tuberin N- and C-termini are represented by N and C, respectively.

Table 1. Cross-species comparison of the tuberin variants[a]

Species	Amino acid							
Human	**N**525	**K**599	**R**611	**A**614	**F**615	**C**696	**V**769	**R**905
Human (variants)	S	M	Q/W	D	S	Y	E	Q
Mouse	**N**	**K**	**R**	**A**	**F**	**C**	**V**	**R**
Rat	**N**	**K**	**R**	**A**	**F**	**C**	**V**	**R**
Fugu	T	**K**	**R**	V	**F**	**C**	**V**	**R**
Drosophila	M	E	**R**	I	**F**	**C**	**V**	**R**

[a]The amino acid substitutions analysed in this study are indicated. Amino acids conserved in the mouse, rat, pufferfish (*Fugu*) and fruitfly (*Drosophila*) are highlighted in bold.

The functional analysis carried out in this study confirms that the R611Q, R611W, A614D, C696Y and V769E substitutions are disease-causing. Each of these changes destroyed the interaction between tuberin and hamartin and prevented tyrosine phosphorylation of tuberin. The N525S, K599M and R905Q substitutions did not affect either tuberin–hamartin binding, the chaperone function of tuberin or the degree of tyrosine phosphorylation. Therefore, *TSC2* mutations that do not affect the tuberin–hamartin interaction still cause TSC. The N525S, K599M and R905Q substitutions, as well as the C-terminal domain of tuberin encompassing amino acids 1100–1784, must be vital for other aspects of tuberin function not addressed in this study, and highlight interesting areas for further functional analysis. One possibility is that mutations that do not prevent the tuberin–hamartin interaction may be associated with a less severe TSC phenotype. Indeed, the N525S and R905Q substitutions were originally described in familial TSC cases and it was suggested that these mutations may be associated with a milder phenotype than other *TSC2* mutations and therefore be more likely to be inherited through several generations (15). Another possibility was that both changes represented rare polymorphisms segregating with TSC. However, the K599M substitution was described in a sporadic case of TSC, where both parents tested negative for the mutation (15) and the R905Q substitution, as well as a R905W substitution, have since been identified as *de novo* mutations in several sporadic cases of TSC (16; M.Goedbloed, unpublished data). The R611W, R611Q and V769E mutations have all been described in both sporadic as well as familial cases of TSC, whereas the A614D substitution has been found in a single *de novo* case. The parents of the patient with the C696Y substitution have not been tested (19). Therefore, in the present study, there was no correlation between familial cases of TSC and *TSC2* missense mutations that did not affect the tuberin–hamartin interaction. However, only a very small number of mutations were analysed and very limited clinical information on the patients carrying these mutations was available. A more detailed comparison of the TSC phenotypes of these patients, and functional analysis of additional missense mutations to the *TSC2* gene in other TSC patients may identify new genotype–phenotype correlations.

Co-immunoprecipitation experiments indicated that tuberin amino acids 1–607 were sufficient to bind hamartin. However, amino acid substitutions outside this domain were sufficient to disrupt the interaction between tuberin and hamartin. It is possible that the R611Q, R611W, A614D, F615S, C696Y and V769E substitutions alter the conformation of tuberin and thereby inhibit tuberin–hamartin binding. However, inhibition of the tuberin chaperone function by these substitutions reduces the amount of hamartin in the supernatant fraction used for the immunoprecipitation experiments. Therefore, an alternative explanation is that it is the inhibition of the chaperone function that reduces the amount of immunoprecipitable tuberin–hamartin complex.

Tuberin contains four potential tyrosine kinase phosphorylation sites, encompassing amino acids 712–719, 784–790, 1240–1250 and 1753 –1760, respectively (Fig. 8A). None of the missense changes analysed in this study lie within these sequences and it is therefore likely that conformational changes or other indirect effects of the substitutions are responsible for preventing tyrosine kinase-mediated phosphorylation of these predicted sites. Aicher *et al.* (13) examined the effects of two different amino acid substitutions,Y1571H and P1675L, on tuberin phosphorylation and formation of the tuberin–hamartin complex. Both of these variants showed a reduction in phosphorylation, although the interaction with

hamartin was not prevented (13). This is consistent with our finding that tuberin amino acids 1–1099 are sufficient for the chaperone function. In contrast to Aicher *et al.* (13) the substitutions analysed in the present study are all located in the central one-third of the tuberin molecule (amino acids 525–905). In six cases (R611Q, R611W, A614D, F615S, C696Y and V769E) dramatic reductions in both tuberin tyrosine phosphorylation and formation of the tuberin–hamartin complex were observed. The fact that amino acid substitutions to different regions of tuberin have a similar effect on tuberin function is consistent with the suggestion that conformational changes may play an important role in regulating tuberin phosphorylation.

The tuberin variants that were unable to interact with hamartin were not phosphorylated. This suggested that phosphorylation of tuberin may be a prerequisite for the interaction with hamartin. Therefore, it was surprising that tyrosine phosphorylation of tuberin in the tuberin–hamartin complex could not be detected. However, this apparent paradox may be explained by the finding that hamartin tyrosine residues are phosphorylated when hamartin is bound to tuberin. It is possible that the phosphate group on tuberin is transferred to hamartin upon formation of the complex. This would be consistent with the observation that the tuberin variants unable to be phosphorylated are also unable to interact with hamartin since no phosphate transfer could occur. Tuberin may also act as a hamartin-specific tyrosine kinase and it may be the phosphorylation of hamartin that is critical for the observed localization changes. Alternatively, hamartin may have phosphatase activity, catalysing the release of the tuberin-bound phosphate.

Previous work had suggested that formation of the tuberin–hamartin complex occurred almost immediately after translation (9) and in the experiments detailed in this report, kinase activity was stimulated 42 h after transfection. Therefore, a more likely and more simple explanation for the finding that tuberin is not phosphorylated when bound to hamartin is that the tuberin–hamartin interaction blocks the access of tyrosine kinases to the tuberin phosphorylation sites. This would provide a mechanism for hamartin-mediated regulation of tuberin function because binding of hamartin to tuberin could prevent kinase-dependent activation/inactivation of tuberin.

The tuberin missense variants R611Q, R611W, A614D, F615S, C696Y and V769E are not phosphorylated and do not interact with hamartin. If conformational changes caused by these substitutions prevent recognition of tuberin by a specific tyrosine kinase, the same changes may also prevent hamartin binding. In this way hamartin may regulate tuberin activity by competing with a tyrosine kinase for tuberin binding. Initial experiments suggest that although the endogenous tuberin–hamartin complex is not highly tyrosine phosphorylated, the hamartin component can be phosphorylated on tyrosine residues (not shown).

Prosite analysis indicates that hamartin contains a single putative tyrosine kinase phosphorylation site, encompassing amino acids 500 and 508, which may therefore be a good candidate region for further study of hamartin function. If phosphate transfer between tuberin and hamartin is necessary for the chaperone function, the phosphorylation sites encompassing tuberin amino acids 712–719 and 784–790 may be critical since only amino acids 1–1099 of tuberin are required for the chaperone function. Deletion of tuberin amino acids 1100–1784, including the phosphorylation sites encompassing amino acids 1240–1250 and 1753–1760, does not affect the chaperone function.

When hamartin and tuberin were co-expressed, the hamartin isoforms that migrated more slowly through a SDS–PAGE gel were not detected. It has been suggested that hamartin augments the expression of tuberin by inhibiting tuberin ubiquitination and that tuberin stabilises hamartin, that is itself weakly ubiquitinated in overexpressing cells (12). Therefore, the slowly migrating isoforms may represent ubiquitinated isoforms of hamartin that are rapidly degraded. This is consistent with the absence of hamartin from the pellet fractions of the co-transfected cells (Fig. 4B). Our results also suggest that the presence of the R611Q, R611W, A614D, F615S, C696Y and V769E tuberin variants may lead to more rapid hamartin turn-over, possibly by interfering with the reported hamartin–hamartin interaction (9). Our data support the suggestion that the tuberin–hamartin interaction stabilizes both proteins (12) and also suggest that phosphorylation may account for the different migration patterns of hamartin.

We have investigated the tuberin–hamartin interaction in detail and studied the effects of tuberin amino acid substitutions on this interaction. Tuberin amino acids 1–607 are sufficient for binding hamartin whereas amino acids 1–1099 are necessary for the chaperone function, to maintain hamartin in a soluble cytosolic complex. Substitutions to amino acids R611, A614, F615, C696 and V769 prevent the phosphorylation of tuberin tyrosine residues, inhibit formation of the tuberin–hamartin complex and destroy the tuberin chaperone function. Unexpectedly, we found that although substitutions that prevented tyrosine phosphorylation of tuberin also prevented the interaction with hamartin, of the two proteins in the tuberin–hamartin complex, only hamartin contained phosphorylated tyrosine. Our data provide an explanation for why several *TSC2* missense mutations cause TSC and suggest that phosphorylation of tyrosine residues on both tuberin and hamartin may play a critical role in the formation of the tuberin–hamartin complex and possibly in the function of the complex in the cell.

MATERIALS AND METHODS

Generation of constructs and antisera

The full-length *TSC1* and *TSC2* expression constructs have been described by van Slegtenhorst *et al.* (10). Truncated *TSC1* and *TSC2* expression constructs were derived by restriction digestion of the full-length cDNAs or by PCR. Truncated *TSC2* cDNAs encoding amino acids 1–1240 (*Eco*47III truncation), 1–1099 (*Xma*I truncation), 1–607 (*Nru*I truncation), and 1–252 plus 1536–1784 (*Sac*I internal in-frame deletion) were cloned behind an N-terminal polyhistidine epitope tag derived from the pQE series of vectors (Qiagen) in the pSG5 expression vector. The expression constructs encoding amino acids 607–1099 and 1125–1784 were created by *Nru*I–*Xma*I double digestion of the full-length *TSC2* cDNA followed by cloning of the released cDNA fragments into the polyhistidine-tagged expression construct. An additional truncated *TSC2* cDNA encoding amino acids 1–1712 (*Eco*RV truncation) was cloned into a pcDNA3.1 expression construct containing a C-terminal myc epitope tag (Invitrogen). Missense mutations (N525S, K599M,

R611Q, R611W, A614D, F615S, C696Y, V769E and R905Q) were introduced into the *TSC2* full-length cDNA by site-directed mutagenesis using the Stratagene QuickChange kit. A frameshift (3413insG) leading to premature truncation of tuberin at amino acid 1134 was introduced using the same procedure. All constructs were sequenced completely. Primer sequences used for site-directed mutagenesis are available on request.

Polyclonal rabbit antisera specific for human tuberin and hamartin have been described by van Slegtenhorst *et al.* (10). Briefly, two histidine-tagged fusions proteins encoding tuberin amino acids 1558–1807 and hamartin amino acids 543–1087 were purified under denaturing conditions according to the manufacturer's protocol (Qiagen), suspended in Freud's complete adjuvant and injected into New Zealand white rabbits at 4 week intervals. Mouse monoclonal antibodies against Xpress, myc (Invitrogen) and polyhistidine (Qiagen) epitope tags were purchased from the manufacturers. The phosphotyrosine-specific antibody, PY99, was purchased from Santa Cruz Biotechnology.

Immunocytochemistry

Expression constructs were transfected into COS-1 cells seeded at 50–70% confluency on glass coverslips using lipofectAMINE and PLUS reagent, as recommended by the supplier (Life Technologies). Forty-two hours after transfection the cells were washed with phosphate-buffered saline (PBS), fixed with 3% paraformaldehyde for 10 min at room temperature and permeabilized with 0.1% Triton X-100 for a further 5 min at room temperature. Primary antibodies, diluted 1:100 in PBS containing 0.5% bovine serum albumin and 0.15% glycine, were incubated with the coverslips for 60–120 min at room temperature, followed by fluoroescein isothiocyanate or Texas Red isothiocyanate-coupled secondary antibodies against mouse or rabbit immunoglobulins (DAKO), for 60 min at room temperature. The coverslips were washed extensively in PBS, briefly in deionized water and mounted on glass microscope slides using Vectashield mounting medium (Vector Laboratories). Images were captured using the Power Gene fluorescence *in situ* hybridization system on a Leica DM RXA microscope. Images were processed using a filter wheel (Chroma technology) and Adobe Photoshop software.

Immunoblotting

COS cells (30 cm^2 culture dishes), were washed with PBS and harvested by scraping into 0.5 ml of PBS. The cells were collected by centrifugation at 2500 *g* for 5 min at 4°C and resuspended in 0.4 ml of TNE lysis buffer [50 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.5 mM EDTA, 1% (v/v) Triton X-100 containing a protease inhibitor cocktail (Complete; Roche Molecular Biochemicals)]. Cells were lysed for 10 min on ice and cleared by centrifugation at 10 000 *g* for 10 min at 4°C. The soluble supernatant fractions were separated by 6% SDS–PAGE and analysed by immunoblotting. For immunoprecipitation of tuberin–hamartin complexes, 1 µl of tuberin- or hamartin-specific antiserum was added to the supernatant fractions and incubated on ice for 90 min before the addition of 20 µl of a 50% suspension of Protein A–Sepharose beads. After gentle rotation for 90 min at 4°C, the beads were washed extensively with TNE lysis buffer, resuspended in Laemmli loading buffer and separated by SDS–PAGE. Proteins were transferred to nitrocellulose membranes according to standard procedures (20) and detected using the appropriate antibodies and enhanced chemiluminescence protocol, as described by the manufacturer (Amersham).

Phosphorylation analysis

Forty-two hours after transfection COS cells (30 cm^2 culture dishes) were incubated with 100 µM pervanadate for 30 min, washed with PBS and lysed in TNE buffer containing 1 mM sodium vanadate. Tuberin–hamartin complexes were immunoprecipitated from the cleared lysates, separated by SDS–PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 10 mM Tris–HCl pH 7.4, 150 mM NaCl, 0.2% Tween 20 containing 7.5% BSA prior to incubation with the PY99 antibody and detection by enhanced chemiluminescence.

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