Regulation of TSC2 by 14-3-3 Binding*

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Mutation in either the TSC1 or TSC2 tumor suppressor gene is responsible for the inherited genetic disease of tuberous sclerosis complex. TSC1 and TSC2 form a physical and functional complex to regulate cell growth. Recently, it has been demonstrated that TSC1-TSC2 functions to inhibit ribosomal S6 kinase and negatively regulate cell size. TSC2 is negatively regulated by Akt phosphorylation. Here, we report that TSC2, but not TSC1, associates with 14-3-3 in vivo. Phosphorylation of Ser1210 in TSC2 is required for its association with 14-3-3. Our data indicate that 14-3-3 association may inhibit the function of TSC2 and represents a possible mechanism of TSC2 regulation.

Tuberous sclerosis complex (TSC)1 is a relatively common genetic disorder. TSC is caused by mutation in either the TSC1 (hamartin) or TSC2 (tuberin) gene, of which each contributes to 50% of the genetic defects (1, 2). Studies of TSC patients and animal models support the hypothesis that TSC1 and TSC2 are tumor suppressor genes. Homozygous deletion of either TSC1 or TSC2 in mice produces an embryonic lethal phenotype, suggesting an essential function in development. As predicted, heterozygous deletion of either TSC1 or TSC2 in mice results in a significant increase of carcinomas in many tissues with a 100% incidence of renal carcinomas (3, 4).

Mutation of TSC1 or TSC2 results in similar phenotypes, suggesting that the two proteins function in the same pathway. Biochemical studies have shown that TSC1 and TSC2 form a stable complex (5). Genetic studies in Drosophila have demonstrated that TSC1-TSC2 plays a major negative role in the regulation of cell growth. Mutation of either TSC1 or TSC2 results in a significant increase of cell mass in Drosophila (6–8). Overexpression of either TSC1 or TSC2 in Drosophila produces little phenotype, while co-expression of both TSC1 and TSC2 causes a significant reduction of cell size. Furthermore, genetic epistatic studies indicate that TSC1/TSC2 acts downstream of the insulin receptor (6–8).

Recently, we and other groups have demonstrated that TSC1-TSC2 functions to inhibit S6K activation (9–14). In TSC1−/− or TSC2−/− cells, S6K is highly activated. S6K activation requires phosphorylation of multiple sites. Interestingly, TSC1-TSC2 specifically inhibits the phosphorylation of Thr389, but not phosphorylation of Thr421 and Ser424 in S6K. Thr389 is the primary site phosphorylated by mTOR (mammalian target of rapamycin) (15). Furthermore, TSC1/TSC2 also inhibits phosphorylation of 4E-BP1 (eukaryotic initiation factor 4E-binding protein 1) (16), which is also a mTOR target. Both genetic data and biochemical data indicate that TSC1-TSC2 inhibits the function of mTOR (9, 11). Several groups, including ours, have shown that TSC2 is directly phosphorylated and inhibited by Akt (11–13). These studies provide an important link between TSC2 and growth factor signaling.

In this report, we show that TSC2 interacts with 14-3-3, but not the binding-defective 14-3-3 mutant. This interaction is dependent on the phosphorylation of TSC2. We have identified that Ser1210 of TSC2 is phosphorylated in vivo and is the primary binding site of 14-3-3. In contrast, mutation of all AKT phosphorylation sites in TSC2 had no effect on its interaction with 14-3-3. Overexpression of 14-3-3 enhanced phosphorylation of both S6K and 4E-BP1. Furthermore we demonstrated that interaction between TSC2 and 14-3-3 is also modulated by serum starvation.

Experimental Procedures

Antibodies and Plasmids—Anti-phospho-S6K and anti-phospho-4E-BP2 were from Cell Signaling Inc. and anti-TSC2, anti-TSC2 blocking peptide, anti-14-3-3 (K-19), anti-14-3-3 (C-20), anti-14-3-3, anti-14-3-3, anti-14-3-3y were from Santa Cruz Biotechnology. Anti-HA and anti-Myc were from Covance; anti-FLAG and mouse IgG were purchased from Sigma. Rat TSC1 and TSC2 constructs were generously provided by Dr. Y. Xiong. HA-tagged S6K1 and all other DNA constructs including Myc-1433β, Myc-1433β-DN (dominant negative), and FLAG-4E-BP2 were laboratory stocks. Expressions of these plasmids are controlled by the pCMV promoter. Mutant constructs of TSC2 were created by PCR mutagenesis and verified by DNA sequencing.

Cell Culture, Transfection, and Immunoprecipitation—HEK293 cells and Phoenix (retrovirus packaging cells) were seeded and maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS). LExF2 (TSC2−/− cell line) were cultured in DMEM/F-12 containing 10% FBS (16). Transfections were performed using LipofectAMINE2000 Reagent (Invitrogen) following the manufacturer’s instructions. Transiently transfected cells were lysed in lysis buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1% Nonidet P-40, 1% Triton X-100, 50 mM NaF, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin) and immunoprecipitated with the indicated antibodies. Immunocomplexes were subjected to SDS-PAGE.

Metabolic Labeling and Two-dimensional Phosphopeptide Mapping—HEK293 cells were co-transfected with the indicated plasmids. The serum-starved cells were washed twice with phosphate-free DMEM and incubated with 0.25 μCi/ml [32P]orthophosphate (ICN) for 4 h. HA-tagged TSC2 was immunoprecipitated, resolved by SDS-PAGE, and transferred to a polyvinylidene difluoride membrane. Phosphorylated TSC2 was visualized by autoradiography. Phosphopeptide mapping was performed as described (11).

Stable Expression of TSC2 in LExF2 Cells by Retrovirus Infection—The TSC2 cDNA was subcloned to the retroviral vector pPGS-CMV.
with 14-3-3 under physiological conditions, we immunoprecipitated endogenous TSC2 from 293 cells. Western blot with the anti-14-3-3β (K-19) antibody, which recognizes all 14-3-3 isoforms, indicated that TSC2 associates with 14-3-3 under physiological conditions (Fig. 1c). Preincubation of the TSC2 antibody with a competing peptide completely eliminated TSC2 and the co-precipitated 14-3-3 (Fig. 1c). These results demonstrated that TSC2 is associated with 14-3-3 under physiological conditions and suggest that 14-3-3 may play a role in the regulation of TSC2.

TSC2, but Not TSC1, Interacts with 14-3-3—TSC1 and TSC2 form a physical and functional complex in vivo. To determine whether the TSC1-TSC2 complex, TSC1 or TSC2 alone interacts with 14-3-3, HEK293 cells were transfected with these constructs, and co-immunoprecipitation studies were performed. Immunoprecipitation of 14-3-3 β did not bring down TSC1 alone. Co-precipitation of 14-3-3 and TSC1 was observed only when TSC2 was present in the transfection (Fig. 2a), indicating that TSC1 alone cannot interact with 14-3-3. In contrast, 14-3-3 co-immunoprecipitated with TSC2 regardless of the presence of TSC1. These results demonstrate that 14-3-3 can interact with TSC2 alone or the TSC1-TSC2 complex. The interaction between transfected TSC2, but not TSC1, and endogenous 14-3-3 further confirmed that 14-3-3 interacts with TSC2 or with the TSC1-TSC2 complex, but not with TSC1 (Fig. 2b). Interestingly, 14-3-3 interacts with TSC2 stronger than with the TSC1-TSC2 complex (Fig. 2). We tested the effect of 14-3-3 on the TSC1-TSC2 complex and found that 14-3-3 had no significant effect on the complex formation between TSC1 and TSC2 (data not shown).

Mapping of the 14-3-3 Interaction Domain in TSC2—Serial
deletions of TSC2 were constructed to locate the domain responsible for 14-3-3 interaction. Our results showed that fragments 1–608, 1–1080, 1–1200, and 1321–1765 did not bind 14-3-3, while fragments 1–1320, 1101–1320, 1080–1765 interacted with 14-3-3 at a level similar to the wild type TSC2 (Fig. 3a). These data demonstrate that the 14-3-3 binding site in TSC2 is localized between residues 1101 and 1320. It has been well established that 14-3-3 binds to phosphorylated residues with a consensus recognition sequence (18). We first tested whether the Akt phosphorylation sites are required for 14-3-3 binding. The TSC2–6A mutant, which has all six predicted Akt phosphorylation sites (residues 939, 1086, 1088, 1378, 1422, 1756) substituted by alanine (11), still binds to 14-3-3 (Fig. 4a). These results strongly indicate that Ser 1210 is an Akt phosphorylation site. Phosphorylation of GST-TSC2 is evident by an increased electrophoretic mobility of the protein (Fig. 4a). The purified GST-TSC2 was treated with λ-phosphatase as indicated. The immunoprecipitated GST-TSC2 present in the anti-Myc-14-3-3 immunoprecipitates was detected by Western blot. b, Ser1210 of TSC2 is phosphorylated. TSC2 and the TSC2/S1210A mutant was transfected into HEK293 cells and labeled with [32P]phosphate. Two-dimensional phosphopeptide mapping was performed. The circle indicates the phosphopeptide absent in the mutant, but present in wild type TSC2. c, inhibition of S6K in the LExF2 cells by TSC2. The LExF2 TSC2+/− cells were infected with wild type TSC2 or TSC2 (S1210A) mutant, and stably infected cells were selected. The phosphorylation status of endogenous S6K was determined by phospho-specific antibodies (pS6K(T389)). d, serum starvation decreased interaction between TSC2 and 14-3-3. HA-TSC2-transfected HEK293 cells were treated with PD98059 (50 µM, 90 min), wortmannin (100 nM, 30 min), rottlerin (5 µM, 60 min), or serum starvation (16 h). Co-immunoprecipitation of 14-3-3 was determined. e, expression of 14-3-3 enhanced phosphorylation of S6K and 4E-BP1. Increasing amounts of Myc-14-3-3β were transfected into HEK293 cells with HA-S6K or FLAG-4E-BP1. Basal phosphorylation of transfected S6K and 4E-BP1 was determined by anti-phospho-S6K and anti-phospho-4E-BP1. The expression of Myc-14-3-3β was also determined. IP, immunoprecipitation.

Fig. 4. Phosphorylated Ser1210 of TSC2 is the primary 14-3-3 binding site. a, phosphorylation of TSC2 is required for interaction with 14-3-3. GST-TSC2 was purified from transfected HEK293 cells and dephosphorylated with λ-phosphatase as indicated. The immunoprecipitated Myc-14-3-3β (from 300 µg of transfected cell lysates) was incubated with the purified GST-TSC2 (~10 ng). GST-TSC2 present in the anti-Myc-14-3-3 immunoprecipitates was detected by Western blot. b, Ser1210 of TSC2 is phosphorylated. TSC2 and the TSC2/S1210A mutant was transfected into HEK293 cells and labeled with [32P]phosphate. Two-dimensional phosphopeptide mapping was performed. The circle indicates the phosphopeptide absent in the mutant, but present in wild type TSC2. e, inhibition of S6K in the LExF2 cells by TSC2. The LExF2 TSC2+/− cells were infected with wild type TSC2 or TSC2 (S1210A) mutant, and stably infected cells were selected. The phosphorylation status of endogenous S6K was determined by phospho-specific antibodies (pS6K(T389)). d, serum starvation decreased interaction between TSC2 and 14-3-3. HA-TSC2-transfected HEK293 cells were treated with PD98059 (50 µM, 90 min), wortmannin (100 nM, 30 min), rottlerin (5 µM, 60 min), or serum starvation (16 h). Co-immunoprecipitation of 14-3-3 was determined. e, expression of 14-3-3 enhanced phosphorylation of S6K and 4E-BP1. Increasing amounts of Myc-14-3-3β were transfected into HEK293 cells with HA-S6K or FLAG-4E-BP1. Basal phosphorylation of transfected S6K and 4E-BP1 was determined by anti-phospho-S6K and anti-phospho-4E-BP1. The expression of Myc-14-3-3β was also determined. IP, immunoprecipitation.

Sequence analysis by scansite (www.scansite.mit.edu) (21) predicts that TSC2 contains several putative 14-3-3 binding sites. We created single and double mutations by substituting the top four predicted 14-3-3 binding sites (Fig. 3b). Therefore, TSC2 utilizes Ser1210 as the primary 14-3-3 binding site.

Phosphorylated Ser1210 of TSC2 Is the 14-3-3 Binding Site—We want to test whether the interaction between 14-3-3 and TSC2 requires the phosphorylation of TSC2. GST-TSC2 was expressed and purified from transfected HEK293 cells. Purified GST-TSC2 was treated with λ-phosphatase. Dephosphorylation of GST-TSC2 is evident by an increased electrophoretic mobility of the protein (Fig. 4a). The purified GST-TSC2 was incubated with immunoprecipitated Myc-14-3-3β and the co-precipitation of GST-TSC2 by Myc-14-3-3β was determined. Treatment with phosphatase completely eliminated the interaction between GST-TSC2 and Myc-14-3-3β (Fig. 4a).

To directly demonstrate the phosphorylation status of Ser1210 in TSC2, we performed in vivo 32P labeling and two-dimensional phosphopeptide mapping of TSC2. The TSC2/S1210A mutant eliminated a single phosphopeptide spot depicted by the arrow in Fig. 4b, while the rest of phosphopeptides were unchanged (Fig. 4b). These results strongly indicate that Ser1210 is an in vivo phosphorylation site in TSC2.

Binding of 14-3-3 may modulate the cellular function of TSC2. We have shown that one of the physiological functions of TSC1-TSC2 is to inhibit S6K activation. In TSC2−/− LExF2 cells, S6K is highly activated. The abilities of wild type and 14-3-3 binding-defective mutant TSC2 to inhibit S6K were tested in the TSC2−/− cells. We observed that both wild type
and the 14-3-3 binding-defective TSC2 could inhibit S6K (Fig. 4c). These data indicate that 14-3-3 binding may not modulate the ability of TSC2 to inhibit S6K. However, the lack of a difference between the wild type and the mutant TSC2 could be due to the fact that the majority of the expressed TSC2 is not phosphorylated on Ser\textsuperscript{1210}, therefore, free of 14-3-3 binding. Our two-dimensional phosphopeptide mapping data also indicates that the majority of TSC2 is not phosphorylated on Ser\textsuperscript{1210} because the intensity of this phosphopeptide is significantly weaker compared with the Akt phosphorylation site Ser\textsuperscript{939} (Fig. 4b) (11).

Sequences surrounding Ser\textsuperscript{1210} have limited resemblance to PKA and PKC-\beta phosphorylation sites. Inhibition of PKA (data not shown) or PKC-\beta by rottlerin had no effect on the interaction between TSC2 and 14-3-3 (Fig. 4d). We also observed that inhibition of the phosphatidylinositol 3-kinase-Akt pathway by wortmannin and the ERK pathway by PD90589 had no effect on the interaction (Fig. 4d), which suggests that the Akt phosphorylation sites are not involved. Interestingly, serum starvation resulted in a visible reduction of association between TSC2 and 14-3-3 (Fig. 4d). The above data indicate that the complex formation between TSC2 and 14-3-3 may be modulated by cell growth status.

**Increased Phosphorylation of S6K and 4E-BP1 by 14-3-3**—To test the effect of 14-3-3 on downstream effectors of TSC2, we examined the phosphorylation of S6K and 4E-BP1. Phosphorylation of these two proteins was inhibited by TSC1/TSC2. We discovered that co-expression of 14-3-3\beta elevated the Thr\textsuperscript{389} phosphorylation of S6K (Fig. 4e). Similarly, expression of 14-3-3\beta also enhanced the basal phosphorylation of 4E-BP1 (Fig. 4e). These observations indicate that 14-3-3 may negatively regulate the functions of TSC2.

The cellular functions of the TSC1-TSC2 tumor suppressor gene products have just begun to be elucidated. TSC1-TSC2 plays an important role in cell growth regulation and cell size control. Recent studies have demonstrated the TSC2 protein was phosphorylated and inhibited by Akt-dependent phosphorylation (11–13). In this report, we showed that TSC2 binds to 14-3-3 under physiological conditions. We have mapped a single site, Ser\textsuperscript{1210}, in TSC2 responsible for binding with 14-3-3 (22). During the preparation of this manuscript, Nellist et al. (22) also reported that TSC2 interacts with 14-3-3. However, they showed that 14-3-3 binds to multiple sites in TSC2 and concluded that the Akt phosphorylation sites in TSC2 are responsible for 14-3-3 binding (22). We have no obvious explanation why the data by Nellist et al. (22) are dramatically different from ours. Nevertheless, our data clearly indicate that one major 14-3-3 binding site exists in TSC2, and Akt phosphorylation sites are not responsible for 14-3-3 binding.

Overexpression of 14-3-3 results in elevated phosphorylation of S6K and 4E-BP1. In contrast, overexpression of TSC1/TSC2 suppresses the phosphorylation of these two proteins, indicating 14-3-3 and TSC1/TSC2 have opposite effects on S6K activation. Our results indicate that 14-3-3 binds to phosphorylated TSC2 and may suppress its activity. This interpretation is consistent with the fact that the interaction between TSC2 and 14-3-3 is decreased under serum-starved conditions. Serum starvation is predicted to activate TSC2 and suppress cell growth. The dissociation of 14-3-3 may partly contribute to TSC2 activation and S6K inhibition under conditions of serum starvation. However, we cannot exclude the possibility that the effect of 14-3-3 on S6K and 4E-BP1 may not be mediated by TSC2. It has been reported that 14-3-3 may positively modulate the function of TOR in yeast (23). 14-3-3 has also been shown to interact with mTOR (24). Therefore, 14-3-3 may regulate S6K and 4E-BP1 through multiple targets. Future studies to identify the kinase responsible for phosphorylation of Ser\textsuperscript{1210} will provide new insights into the mechanism of TSC2 regulation.

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