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Analysis of TSC1 truncations defines regions involved in TSC1 stability, aggregation and interaction

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ABSTRACT

Tuberous sclerosis complex (TSC) is an autosomal dominant disorder characterised by the development of hamartomas in a variety of organs and tissues. The disease is caused by mutations in either the *TSC1* gene on chromosome 9q34, or the *TSC2* gene on chromosome 16p13.3. The *TSC1* and *TSC2* gene products, TSC1 and TSC2, interact to form a protein complex that inhibits signal transduction to the downstream effectors of the target of rapamycin complex 1 (TORC1). Here we investigate TSC1 structure and function by analysing a series of truncated TSC1 proteins. We identify specific regions of the protein that are important for TSC1 stability, localisation, interactions and function.

1. Introduction

Tuberous sclerosis complex (TSC) is an autosomal dominant disorder characterised by the development of hamartomas in a variety of organs and tissues, including the brain, skin and kidneys [1]. Mutations in either the *TSC1* gene on chromosome 9q34 [2], or the *TSC2* gene on chromosome 16p13.3 [3] cause TSC.

The *TSC1* and *TSC2* gene products, TSC1 and TSC2, interact to form a protein complex [4]. TSC2 contains a GTPase activating protein (GAP) domain, and the TSC1–TSC2 complex has been shown to have GAP activity for a GTPase called ras homolog expressed in brain (RHEB). The TSC1–TSC2 complex stimulates the hydrolysis of RHEB-bound GTP to GDP, thereby inhibiting the RHEB-GTP-dependent stimulation of the target of rapamycin complex 1 (TORC1) [5]. TORC1 regulates a wide array of cellular processes, including transcription, translation and autophagy [6]. Inactivation of the TSC1–TSC2 complex results in the phosphorylation of TORC1 targets, including p70 S6 kinase (S6K) and elongation factor 4E binding protein 1 and, consequently, increased protein synthesis and cell growth [6]. TSC1 has limited homology with other proteins [2] and the exact role of TSC1 in the TSC1–TSC2 complex is not completely clear. Some studies have shown that TSC1 is required for TSC2 GAP activity [7–9], while others suggest that TSC1 is not essential for GAP activity but is necessary to maintain the stability, activity and correct intracellular localisation of the TSC1–TSC2 complex [10,11]. TSC1 has been shown to be involved in recruitment of the TSC1–TSC2 complex to cell membranes [12] and may integrate multiple inputs to help regulate TORC1 activity, or perform other, independent functions [13].

In the absence of TSC2, over-expressed TSC1 forms Triton X100-insoluble aggregates or inclusions due to interactions between the coiled coil regions (amino acids 719–998) of different TSC1 molecules [14]. Coexpression of TSC2 prevents the formation of these inclusions, resulting in a shift of TSC1 to Triton X100-soluble cell lysate fractions [15]. Stable TSC1–TSC2 complexes can be immunoprecipitated from these cell fractions, demonstrating that TSC2 acts as a molecular chaperone, maintaining TSC1 in a stable cytosolic form [14]. Recently, we identified TSC1 amino acid substitutions in TSC patients that prevented TSC1 inclusion formation *in vitro* [16,17]. Surprisingly, none of these changes mapped to the coiled coil region, indicating that other regions of TSC1 are also important for the expression and localisation of the protein.

To gain additional insight into the structure and function of TSC1, we compared full-length TSC1 to a series of TSC1 N- and C-terminal truncation proteins. Our *in vitro* analysis demonstrates that the N-terminal region is important for TSC1 stability and that both the N-terminal and coiled coil regions are required for TSC1 aggregation. Furthermore, we show that multiple regions of TSC1 are required for binding TSC2 and that some TSC1 truncations can co-operate with TSC2 to inhibit TORC1 activity. This information will be useful for the functional characterisation of unclassified *TSC1* variants identified in individuals with TSC.

Abbreviations: TSC, tuberous sclerosis complex; RHEB, ras homolog expressed in brain; TORC1, target of rapamycin complex 1; S6K, p70 S6 kinase; GAP, GTPase activating protein

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2. Materials and methods

2.1. Generation of constructs and antisera

To derive expression constructs encoding myc-tagged TSC1 C-terminal truncations a KpnI site was introduced into a full-length myc-tagged TSC1 expression construct by site-directed mutagenesis (QuikChange, Stratagene, La Jolla, CA, U.S.A.). After digestion with KpnI to excise the 3′ portion of the *TSC1* open reading frame (ORF), the vector and remaining ORF was re-circularised so that the epitope tag remained in-frame with the last codon of the truncated *TSC1* ORF. N-terminal truncations were derived in a similar way. A NheI site was introduced to allow excision of the 5′ portion of the *TSC1* ORF. In each case the complete open reading frame of the new construct was verified by sequence analysis. Expression constructs were derived for 4 C-terminal truncation proteins: Q900 (TSC1 amino acids 1–900, molecular mass 100 kDa), R692 (amino acids 1–692, 80 kDa), R509 (amino acids 1–509, 60 kDa) and T339 (amino acids 1–339, 35 kDa); one N-terminal truncation protein, M351 (amino acids 351–1164, 100 kDa), and one truncation protein, M351-Q900, lacking both termini (amino acids 351–900, 65 kDa). The pathogenic L117P and M224R missense changes were introduced by site-directed mutagenesis, as described previously [15]. An overview of the expressed TSC1 truncation proteins is shown in Fig. 1. Other constructs used in this study have been described elsewhere [4,15,18].

Antibodies were purchased from Cell Signaling Technology (Danvers, MA, U.S.A.), except for a mouse monoclonal antibody against TSC2 which was purchased from Zymed Laboratories (San Francisco, CA, U.S.A.), or were described previously [4]. Secondary antibodies for infra-red detection proteins on immunoblots were obtained from Li-Cor Biosciences (Lincoln, NE, U.S.A.).

2.2. Immunoblot analysis of cells over-expressing TSC1 truncations

HEK 293T cells in 3.5 cm dishes were transfected with expression constructs using polyethylenimine (Polysciences Inc., Warrington, PA, U.S.A.)[19]. Twenty-four hours after transfection the cells were washed with cold phosphate buffered saline (PBS) and lysed in 0.15 ml lysis buffer (50 mM Tris–HCl (pH 7.5), 100 mM NaCl, 50 mM NaF, 1% Triton X100 and a protease inhibitor cocktail (Complete, Roche Molecular Biochemicals)) for 10 min on ice. The cell lysates were cleared by centrifugation (10,000 *g* for 10 min at 4 °C) and the supernatant (Triton X100-soluble) and pellet (Triton X100-insoluble) fractions were recovered. The pellet fractions were resuspended in 0.15 ml lysis buffer and sonicated at 12 μm for 15 s prior to immunoblot analysis using the Criterion SDS-PAGE system (BioRad, Hercules, CA, U.S.A.). Protein expression levels were estimated by near infra-red detection and quantification of the blotted proteins on an Odyssey™ scanner (Li-Cor Biosciences).

To estimate the effect of the TSC1 truncation proteins on S6K T389 phosphorylation HEK 293T cells were transfected with a 4:2:1 mixture of the TSC1, TSC2 and S6K expression constructs and harvested as above. The Triton X100-soluble fractions were analysed by immunoblotting, as described previously [16,17].

2.3. Immunofluorescent detection of TSC1 truncations

HEK 293T cells were seeded onto glass cover slides coated with poly-L-lysine (Sigma-Aldrich, Carlsbad, CA, U.S.A.), transfected and processed for immunofluorescent microscopy as described previously [16]. Fixed, permeabilised cells were incubated with a primary mouse monoclonal antibody specific for the myc epitope tag, followed by a cyanine (Cy2)-coupled secondary antibody (DAKO,

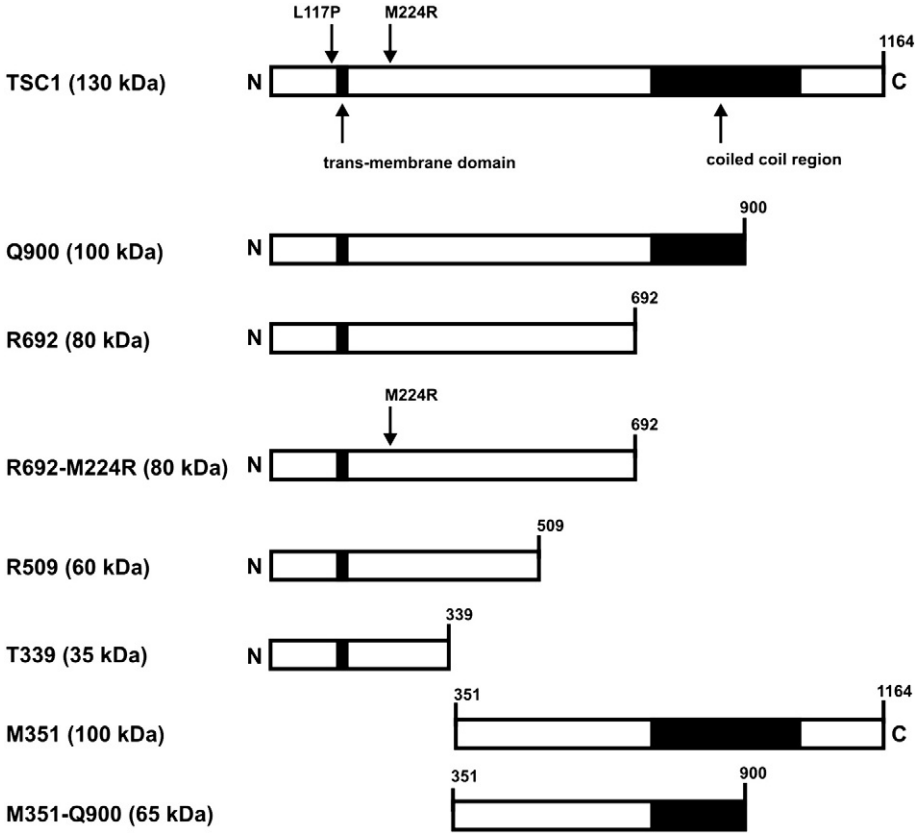

Fig. 1. TSC1 truncation proteins. Schematic diagram illustrating the TSC1 truncation proteins used as part of this study. The positions of the predicted transmembrane domain (amino acids 127–144), coiled coil region (amino acids 719–998), and the L117P and M224R missense mutations identified in individuals with TSC are indicated. Numbers indicate the positions of the first and last amino acids, relative to the full-length protein (top).