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Biochemical and Biophysical Research Communications 333 (2005) 818–826
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www.elsevier.com/locate/ybbrc

Phosphorylation and binding partner analysis of the TSC1–TSC2 complex

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Received 24 May 2005
Available online 13 June 2005

Abstract

Tuberous sclerosis complex (TSC) is an autosomal dominant benign tumour syndrome caused by mutations to either the *TSC1* or *TSC2* tumour suppressor gene. The *TSC1* and *TSC2* gene products, TSC1 and TSC2, form a protein complex that integrates inputs from multiple signalling cascades to inactivate the small GTPase rheb, and thereby inhibit mTOR-dependent cell growth. We have used matrix-assisted laser desorption/ionisation time-of-flight and Fourier transform mass spectrometry to identify TSC1 and TSC2 phosphorylation sites and candidate TSC1 and TSC2 interacting proteins. We identified three sites of TSC2 phosphorylation and a novel site of TSC1 phosphorylation, and investigated the roles of these sites in regulating the activity of the TSC1–TSC2 complex. In addition, we identified three TSC1–TSC2 interacting proteins, including DOCK7 a putative rhebGEF.

Keywords: Tuberous sclerosis complex; TSC1; TSC2; DOCK7; rheb; MALDI-TOF MS; MALDI-FTMS

In humans, germ-line mutations to the *TSC1* and *TSC2* tumour suppressor genes cause tuberous sclerosis complex (TSC), an autosomal dominant disease characterised by the occurrence of hamartomatous growths in various organs and tissues [1]. Affected cells show defects in growth, proliferation, migration, and differentiation [2].

The *TSC1* and *TSC2* gene products, TSC1 and TSC2 interact to form a heterodimeric protein complex [3] that restricts cell growth upstream of the mammalian 'target of rapamycin' (mTOR) [4]. mTOR plays a central role in controlling cell growth, proliferation, and metabolism [5]. Pathogenic *TSC1* and *TSC2* mutations identified in TSC patients inactivate the TSC1–TSC2 complex and prevent the TSC1–TSC2-dependent inhibition of mTOR [6]. mTOR-dependent signal transduction is activated by the active, GTP-bound form of the small GTPase rheb. Together, TSC1 and TSC2 form a GTPase activating protein (GAP) complex that catalyses the conversion of GTP-bound rheb to the inactive GDP-bound form, and thereby inhibits mTOR function [7–10]. A rheb-specific guanine nucleotide exchange factor (GEF) that catalyses rheb GDP–GTP exchange and antagonises TSC1–TSC2 activity has not yet been identified.

Multiple upstream signals, including growth factors and intracellular levels of ATP, regulate the activity of the TSC1–TSC2 complex through phosphorylation of either TSC1 [11] or TSC2. Phosphorylation of TSC2 by protein kinase B (PKB) relieves the

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0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.05.175

TSC1–TSC2-dependent inhibition of mTOR [12] while, in contrast, phosphorylation of TSC2 by AMP-dependent kinase (AMPK) enhances the activity of the TSC1–TSC2 complex, inhibiting signal transduction through mTOR [13].

Here, a combination of matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF MS), MALDI-Fourier transform mass spectrometry (MALDI-FTMS), and MALDI tandem mass spectrometry (MS/MS) was used to identify phosphorylation sites in both TSC1 and TSC2, and to identify candidate TSC1- and TSC2-interacting proteins, including a potential rhebGEF.

Materials and methods

DNA constructs. The wild-type TSC1, wild-type TSC2 and TSC2 R611Q, R611W, and R905Q variant expression constructs have been described elsewhere [14]. The other TSC1 and TSC2 variants were derived by site-directed mutagenesis using the Stratagene QuickChange kit. All constructs were sequenced completely. The wild-type TSC2 cDNA expression construct matches GenBank entry AAC34210 [15]; the wild-type TSC1 cDNA expression construct encodes GenBank entry T03814 [16]. The expression construct 2B4, encoding myc-tagged p70 S6 kinase (S6K) was provided by T. Nobokuni (Friedrich Miescher Institute, Basel, Switzerland).

Cell culture and transfections. Human embryonal kidney (HEK) 293 cells were cultured in Dulbecco's modified Eagle's medium containing 10% foetal calf serum, penicillin, and streptomycin. Transfections were performed in 10 cm dishes using polyethyleneimine [17]. Two days after transfection the cells were lysed in 50 mM Tris–HCl, pH 8.0, 100 mM NaCl, 50 mM NaF, 1 mM EDTA, and 1% Triton X-100 plus protease inhibitors (Roche), as described previously [6]. Immunoprecipitations were performed on the post-10,000*g* supernatant using antibodies specific for TSC2 or TSC1 [3]. After washing at least three times with 700 µl of lysis buffer, the immunoprecipitates were separated by 5% SDS–PAGE. Protein bands were visualised by Coomassie staining, excised, and digested with trypsin according to a standard protocol [18]. For immunoprecipitation of the endogenous TSC1–TSC2 complex, approximately 1×10^8 HEK 293 cells were used.

Mass spectrometry. A 1:1 mixture of the tryptic peptides and 10 mg/ml of 2,5-dihydroxybenzoic acid (DHB) in water was applied to the MALDI-TOF MS target plate. MALDI-TOF mass spectra and MS/MS spectra were obtained on a Bruker Ultraflex TOF/TOF mass spectrometer operated in the reflectron mode. Ions were generated by a nitrogen laser ($\lambda = 337$ nm) and accelerated to 25 keV. Multiple laser shots (>1000) were averaged to generate the mass spectra, and instrument (external) calibration was performed using a commercially available tryptic peptide mixture. Post-acquisition (internal) recalibration using theoretical masses of the tryptic peptides from the analyzed proteins was applied to increase mass accuracy [19]. Key experiments were repeated on a Bruker Apex Q 9.4 Tesla Fourier transform mass spectrometer (FTMS), using a broadband MS scan (m/z 800–4000 at 125,000 resolution at m/z 2000).

S6K-T389 phosphorylation assay. HEK 293 cells (3.5 cm dishes) were cotransfected with expression constructs encoding wild-type TSC2, or one of the TSC2 variants, myc-tagged TSC1, or one of the myc-tagged TSC1 variants, and myc-tagged S6K. After 24 h the cells were harvested directly in loading buffer and subjected to immunoblotting. S6K phosphorylation was determined using a phosphorylation-specific S6K-T389 antibody (Cell Signaling Technology).

Results

Identification and characterisation of TSC2 phosphorylation

TSC2 was over-expressed in HEK 293 cells and isolated by immunoprecipitation followed by SDS–PAGE (Fig. 1). The protein was excised from the gel, digested with trypsin, and subjected to MALDI-TOF MS. The resulting mass spectrum was searched against the GenBank protein database using the MASCOT software package (www.matrixscience.com). In multiple, separate experiments, matches between 39% and 50% of the theoretical TSC2 tryptic peptides, and mass peaks in the MALDI-TOF spectra were detected. Probability-based Mowse scores were all significant (>65 at a mass tolerance of 150 ppm). To confirm that the mass peaks corresponded to actual TSC2 tryptic peptides, the TSC2 mass spectra were re-analysed using MALDI-TOF tandem MS (MS/MS). The predicted amino acid sequences of selected mass peaks were confirmed from the MS/MS spectra, allowing accurate (<25 ppm) internal calibration of the mass spectra and the subsequent identification of candidate phosphopeptides purely by mass assignment. The presence of peptide fragments in the MS/MS spectra showing loss of 98 (and 80) Da was strong evidence for a phosphopeptide [20].

Monophosphorylated forms of three TSC2 tryptic peptides, SQSGTLDGESAAWSASGEDSR (amino acid residues 1395–1415), SSSSPELQTLQDILGDP GDK (TSC2 amino acid residues 1362–1381), and RLIS SVEDFTEFV (amino acid residues 1772–1784), were detected by MALDI-TOF MS. Experiments were performed using an isoform of TSC2 lacking the 23 amino

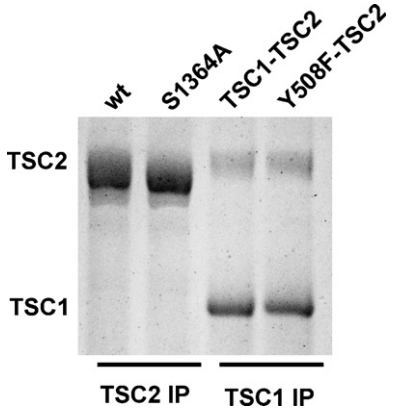

Fig. 1. Immunoprecipitation of TSC1 and TSC2 from HEK 293 cells. Representative Coomassie-stained gel showing wild-type TSC2 (wt) and the TSC2 S1364A variant (S1364A) immunoprecipitated with TSC2-specific antibodies (TSC2 IP); and wild-type TSC1–TSC2 and TSC1 Y508F variant-TSC2 complexes (Y508F-TSC2) immunoprecipitated with TSC1-specific antibodies (TSC1 IP).