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IDENTIFICATION OF NOVEL BINDING PARTNERS FOR TUBEROUS SCLEROSIS COMPLEX 2 (TSC2) BY YEAST TWO-HYBRID APPROACH

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Aim: **To identify novel tuberous sclerosis complex (TSC2) binding partners by yeast two-hybrid screening.** ***Methods:*** **The yeast two-hybrid system DupLEX-A™ developed by OriGene Technologies and Mouse embryo and HeLa cells cDNA libraries were used in this study. The "bait" constructs, containing full-length and truncated form of TSC2 were prepared. The expression of all constructs in yeast was confirmed by immunoblotting with specific anti-LexA antibodies. The suitability of generated constructs for screening was tested in autoactivation and nuclear translocation assays. Screening of mouse embryo and HeLa cDNA libraries with selected baits was carried out according to manufacturer's recommendations. Positive clones were selected using double selection procedure and further confirmed in mating assay. Isolated cDNA clones were identified by automated DNA sequencing and database searching.** ***Results:*** **Extensive screening of two cDNA libraries from mouse embryo and HeLa cells with TSC2 baits led to the isolation of 102 positives clones. The specificity of interaction between TSC2 and binding proteins of selected clones was confirmed by mating assay for 83 clones. Sequencing of these clones indicated that they encode already known and novel TSC2-binding partners.** ***Conclusion:*** **The isolation of several known TSC2-binding partners, such as several isoforms of 14-3-3, demonstrates the validity of generated bait constructs and screening conditions. In addition, we have found a number of novel interactors, which encode cytoskeletal proteins and signaling molecules, such as Ser/Thr phosphatases.**

Key Words: **tuberous sclerosis, TSC1/2, yeast two-hybrid system, protein-protein interactions.**

The study of hereditary cancer-predisposition syndromes is important in developing our present understanding of the rate-limiting steps in cancer progression, by facilitating the identification of a range of tumor-suppressor proteins that have important functions in normal cell growth, tissue homeostasis and response to genetic damage. Tuberous sclerosis complex (TSC) is a relatively common inheritable disorder that occurs in approximately 1 in 6000 of the population and is characterized by the development of hamartomas in different organs [1–3]. The growth of benign tumors is frequently associated with skin rashes, seizures and/or mental handicap. Patients may experience a few or all of the symptoms with varying degrees of severity. Mutations in either the TSC1 or TSC2 tumor suppressor genes are responsible for both the familial and sporadic forms of this disease [4].

Two loci for tuberous sclerosis have been found: the locus on chromosome 9 is linked to TSC1, and the locus on chromosome 16 is specific for TSC2 [5, 6]. It took four years to pin down a specific gene from the TSC1 region of chromosome 9, and in 1997 a promising candidate was found [5].

Called hamartin by the discoverers, it is similar to a yeast protein of unknown function, and appears to act as a tumor suppressor. Without TSC1, growth of cells proceeds in an unregulated fashion, resulting in tumor formation [7, 8]. TSC2 encodes for a signaling protein called tuberin, which possesses homology to GTPase-activation proteins [9, 10]. Hamartin and tuberin form a protein complex, which plays a major role in negative regulation of cell growth [7, 11] by inhibiting mTOR (mammalian target of rapamycin) and S6 kinase, while PI3-kinase-Akt signaling relieves this inhibition [12–17]. Although the TSC1 and TSC2 tumor suppressor proteins have been shown to be involved in the regulation of cell growth and cell size, the precise function of this signaling complex in the signal transduction and the molecular mechanism through which it functions as a tumor suppressor has not been elucidated [18–20]. So far, only few TSC2-binding partners have been identified, partially through the use of yeast two-hybrid system. These include signaling proteins rabaptin-5, 14-3-3 isoforms and other proteins (Pam, HPV16 E6) [29].

To identify novel TSC2 binding partner(s) and to study their role in the regulation of PI3-kinase-Akt and mTOR signaling pathways we have created full-length and truncated forms of TSC2, which were used as "baits" in a yeast two-hybrid search. Extensive screening of two cDNA libraries from mouse embryo and HeLa cells led us to the isolation of 102 positives clones. The specificity of interaction between TSC2 and selected clones was confirmed for 82 clones by mating assay in yeasts. The identification of isolated clones was carried out by automated DNA sequencing and database searching. This analysis revealed

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that isolated clones correspond to 24 genes, which encode cytoskeletal and signaling proteins, transcription factors and cell cycle regulators. Among isolated clones there are known TSC2-binding partners, such as 4 isoforms of 14-3-3, and novel potential regulators of TSC1/2 signaling pathway. The specificity of identified interactions and their physiological relevance is currently investigated, focusing mainly on proteins, which have the potential to regulate the stability and the phosphorylation status of TSC complex.

MATERIALS AND METHODS

The DupLEX-A™ yeast two-hybrid system was used in this study (OriGene Technologies, USA) [21]. The following components of the system have been used: yeast strains EGY48 and RFY206; bacterial strain *E.coli* KC8; reporter plasmids pSH18-34, PJK101; control plasmids pRHFM1, pSH17-4, pEG202-Max, pBait, pTarget; vectors for bait and prey constructs pEG202 and pJG4-5 respectively.

Creation of bait-constructs. To construct the LexA-fused "bait" proteins, the full-length (residues 1–1809) and the C-terminal region of rat TSC2 (residues 931–1809) were amplified using following primers (full length: forward 5′-TCCGCG CCATGG GCCAAA CCAACT AGCAAA GATTCAG-3′, reverse 5′-TCCGCC CTCGAG TCACAC AAACTC TGTGAA GTCATCC-3′; the C-terminal region: forward 5′-TCCGCG CCATGG GACAGT TTCAGA GCACGG AGCAC-3′, reverse 5′-TCCGCC CTCGAG TCACAC AAACTC TGTGAA GTCATCC-3′). All DNA fragments were amplified by Vent polymerase (New England BioLabs, UK) and cloned into the pEG202 vectors using ligation kit from Takara (Japan).

Transformation and selection of recombinant clones. Yeast cells were transformed with generated constructs and DupLEX-A™ system plasmids by PEG-lithium method as recommended by OriGene Technologies. Transformation of *E.coli* KC8 (Trp-) with DNA plasmids was performed by electroporation using Electroporator 2510 (Eppendorf, Germany) device under 14 kV/cm^2 voltage and 4-5 msec conditions. Selection of Trp$^+$-clones was performed as recommended by OriGene protocol.

SDS-PAGE electrophoresis and immunoblotting. Yeast protein extracts were prepared in accordance with CLONTECH Yeast Protocols Handbook. Cells were lysed on ice in equal volume of cracking buffer (8 M urea, 5% w/v SDS, 40 mM Tris-HCl (pH 6.8), 0.1 mM EDTA, 0,4 mg/ml bromophenol blue), which contained β-mercaptoethanol, a mix of protease inhibitors and glass beads. The mixture was vortexed for 2 min before centrifugation. Proteins from prepared lysates were separated on a 10% SDS-PAGE gel and transferred to PVDF-membrane using Semidry Trans-Blot SD device (BioRad, USA). Immunoblot analysis of separated protein has been performed with polyclonal anti-LexA antibodies, kindly provided by Dr. Erica Golemis (USA) [22]. The blot was developed using the ECL kit (Amersham, UK).

Transactivation and nuclear localization tests of TSC2-"baits". Autoactivation assay, testing for nuclear localization of the bait fusion proteins, selection of positive clones, and mating assay were performed as recommended by the manufacturer and described in [23].

Transactivation activity of the TSC2-LexA-fused proteins has been determined by the level of colony colouring in X-Gal containing medium and by growth in leucine-depleted medium. The ability of the TSC2-LexA-fused proteins to translocate to the nucleus and to bind to the LexA-specific DNA operator sequence was detected by b-galactosidase activity of LacZ reporter gene.

Yeast Two-hybrid Screening. The full-length and the C-terminal domain of TSC2 were used as "baits" to screen mouse embryo (OriGeneTM Technologies) and HeLa cells (Invitrogen) cDNA libraries. Bait constructs, reporter plasmids and cDNA libraries were sequentially transformed into EGY48 yeast strain using the method of yeast transformation in semi-fluid agarose [24].

The primary selection of positive yeast clones by Leu$^+$ phenotype was carried out on agarized galactose-containing Leu$^-$ minimal medium, which was selective for reporter, bait and prey plasmids (–Ura, –His, –Trp, respectively) as recommended by the protocol.

To fulfil colour selection of LacZ$^+$ phenotype, the colonies were overlaid with X-Gal containing solution of agarose. The blue colour selection was used to identify transformants in which the interaction between the TSC2 - LexA-fused proteins and library prey-proteins took place.

The secondary screening of transformants was carried out by comparative analysis on 4 types of media with or without galactose inductor: –HUTdex (–Ura, –His, –Trp, +glucose), –HULTdex (–Ura, –His, –Trp, –Leu, +glucose), –HUTgal (–Ura, –His, –Trp, +galactose), –HULTgal (–Ura, –His, –Trp, –Leu, +galactose). Positives were selected by their ability to grow on –HULTgal/not to grow on –HULTdex, and to become blue on –HUTgal/not to become blue on –HUTdex.

The quantitative analysis of LacZ$^+$ and Leu$^+$ phenotypes after galactose induction was performed in 96-well plates and calculated in following way: $N_{lacZ} = (Gal_{405}/Glu_{405}) \times (Glu_{620}/Gal_{620})$, where Gal and Glu-values of E_{405} and E_{620} wavelengths for galactose-and glucose-containing media, respectively.

$N_{leu} = (LEUGal_{620}/LEUGlu_{620}) \times (Glu_{620}/Gal_{620})$, where $LEUGal_{620}$, $LEUGlu_{620}$ are values of E_{620} of cell cultures in galactose-, glucose-containing media from Leu$^+$ phenotype analysis, Glu_{620}, Gal_{620} are values of E_{620} of cell cultures in galactose-, glucose-containing media from LacZ$^+$ phenotype analysis [25].

Analysis of plasmid DNAs. The analysis of isolated plasmid DNAs from (LacZ$^+$ Leu$^+$) yeast clones was performed according to yeast two-hybrid system protocol. *E.coli* strain (KC8) were transformed with isolated prey-DNA plasmids from libraries and grown on LB selective medium with 50 μg/ml of ampicillin.

HindIII/XhoI and EcoRI/XhoI restriction enzymes were used to digest plasmids isolated from HeLa and mouse embryo DNA plasmids, respectively. Comparative analysis of DNA restricted products was carried out by electrophoresis in 1.2% agarose gel.

Mating assay. RFY206 (MATa) yeast strain was transformed with isolated prey-plasmids and systemic pTarget plasmid. Mating between RFY206 (MATa) and EGY48 (MATα strains containing two variants of bait vectors and systemic pBait plasmid together with pSH18-34 reporter plasmid) was performed by the method adapted for 96-well plates. MATa and MATα transformed strains were grown on selective medium (–Trp for MATa and –Ura, –His for MATα) in plate wells to OD_{600} = 1. Later on, 25 ml of yeast suspension was mixed with enriched YPD medium in various combinations "MATa x MATα" and incubated for 36 h at 30 °C.

Cell suspensions were washed extensively in water and used for quantitative analysis of $LacZ^+$ and Leu^+ phenotypes of the mated clones, as described earlier, and on plates with agar selective medium according to OriGene protocol.

RESULTS AND DISCUSSION

The yeast two-hybrid system has proved to be an extremely effective method for studying protein-protein interactions [26]. It has been used successfully to study an array of defined protein-protein interactions as well as to identify proteins encoded by selected members of cDNA library that interact with a given protein of interest [27, 28]. One of the most important preconditions for successful yeast two-hybrid screening is the creation of "bait" constructs that should fulfil the demands of selected system. Therefore, we have created following TSC2 "bait" constructs in pEG202 vector: full-length TSC2 construct; truncated TSC2 construct, encoding the C-terminal region of tuberin (amino acids 931–1809), containing GTPase-activating protein (GAP) domain (Fig. 1).

The expression of recombinant LexA/"baits" was examined by SDS-PAGE electrophoresis under denaturating conditions and immunoblotting with anti-LexA antibodies. As shown in Fig. 2, total lysates of yeast cells transformed with pEG202/full-length TSC2 and pEG202/C-term TSC2 contain LexA fusion proteins of predicted sizes: 227 kDa for the full length LexA/TSC2 and 124 kDa for the LexA/TSC2 C-term. Next, we analysed the ability of created "bait" constructs to penetrate into the nucleus. The analysis indicated that LexA/C-term TSC2 is capable to translocate to the nucleus much better than LexA/full-length TSC2 (data not shown). Then we tested "bait"-constructs in an

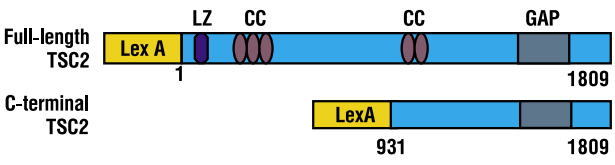

Fig. 1. Schematic representation of LexA/TSC2 fusion constructs in pEG202. TSC2 has a Leucine zipper region (LZ); two coiled-coiled domains (CC) and a GTPase-activating protein homology region (GAP)

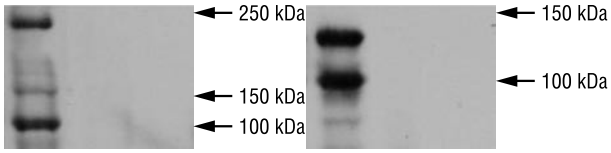

Fig. 2. The expression analysis of recombinant LexA/"baits" TSC2 in total lysates of yeast: *a*. The expression analysis of LexA/full-length TSC2; *b*. The expression analysis of LexA/C-term TSC2. Lane 1 —yeasts transformed with pEG202/full-length TSC2 or pEG202/C-term TSC2; Lane 2 — yeasts transformed with empty pEG202 plasmid; Lane 3 — untransformed yeasts

autoactivating assay and found that LexA/full-length TSC2 autoactivates the transcription of Leu2 reporter gene but not LacZ reporter gene, while LexA/C-term TSC2 does not autoactivate either LacZ or Leu2 reporter (data not shown).

Based on this analysis, we screened mouse embryo and HeLa cell libraries with the LexA/C-term TSC2 "bait" construct. In addition, we have also screened mouse embryo library with the LexA/full length TSC2 construct, whose suitability for yeast two-hybrid screening was uncertain. After transformation of yeast cells with cDNA libraries, colonies were grown on selective galactose-containing medium without leucine. The selection of positive clones by blue colour (in the presence of X-gal) and by their ability to grow on leucine-depleted medium was carried out. The primary transformants were identified and stroked simultaneously on four types of media. The selection was performed by the ability of yeast colonies to grow on leucine-depleted medium with galactose induction/not to grow on leucine-depleted medium with glucose and become blue on galactose medium/not to become blue on glucose medium. The quantitative assay of $Leu^+/LacZ^+$ phenotypes in the screening of mouse embryonic cDNA library allowed us to select 83 positive clones which interacted with LexA/C-TSC2 "bait" and 5 positive clones which interacted with LexA/full length TSC2 construct. We have also selected 14 clones from the screen by LexA/C-TSC2 construct of HeLa cDNA library. Further analysis of isolated clones in mating assay confirmed the specificity of interaction for 69 clones obtained by the screening of mouse embryo cDNA library with the C-terminal TSC2. Furthermore, from 14 clones isolated by the screening of HeLa cDNA library by the TSC2 C-term "bait" 13 were confirmed in mating assay.

The identity of isolated clones was found by automated DNA sequencing followed by database searching for homologous molecules, protein domains and regulatory motifs. This analysis indicated that 83 isolated clones encode 24 proteins. As shown in Table, among isolated clones there are 13 that encode different isoforms of 14-3-3 proteins, which are well-known binding partners for TSC2. It is important to note, that 14-3-3 clones were isolated from mouse embryo and HeLa cells libraries by screening with both the full-length and the C-terminal "bait" of TSC2. Altogether, we have isolated 4 isoforms of 14-3-3 in these screens: zeta, epsilon, eta and theta.

Table. TSC2-binding partners obtained in yeast two-hybrid system

Bait	Prey	cDNA library	Number of isolated clones	Function
C-term TSC2	14-3-3 protein eta	Mouse	1	Cell signaling
C-term TSC2	14-3-3 protein epsilon	Mouse	4	Cell signaling
C-term TSC2	14-3-3 protein theta	Mouse	3	Cell signaling
C-term TSC2	14-3-3 protein zeta	Mouse	1	Cell signaling
C-term TSC2	TSC2-BP1	Mouse	20	Phosphorylation
C-term TSC2	TSC2-BP2	Mouse	3	Proteosomal degradation
C-term TSC2	TSC2-BP3	Mouse	3	Cell signaling
Full length TSC2	14-3-3 protein theta	Mouse	2	Cell signaling
Full length TSC2	14-3-3 protein eta	Mouse	1	Cell signaling
C-term TSC2	14-3-3 protein epsilon	HeLa	1	Cell signaling
C-term TSC2	TSC2-BP4	HeLa	2	Cell adhesion
C-term TSC2	TSC2-BP5	HeLa	3	Proteosomal degradation
C-term TSC2	TSC2-BP6	HeLa	3	Not defined

In the last two years several groups have reported the interaction between tuberin and various isoforms of 14-3-3 proteins [29]. The 14-3-3 proteins form a family of abundant and widely expressed 28–33 kDa acidic polypeptides. They are expressed in all eukaryotic cells and are highly conserved in amino acid sequences within a wide range of organisms, including higher eukaryotes. Seven isoforms encoded by seven distinct genes are identified in mammals. Variants of 14-3-3 proteins assemble in homo- and heterodimers. They bind to phosphoserine-containing motifs in a sequence-specific manner and function as adaptor molecules modulating binding properties of key players in signal transduction and the cell cycle [30]. Localization of 14-3-3 proteins in cytoplasm, nucleus, various membranes, cytoskeletal and centrosome structures has been reported. Numerous studies provide evidence for a pathophysiological importance of changes in 14-3-3 expression and localization in conditions such as cancer and neurodegenerative diseases [30]. Which functions of the TSC proteins are affected by their interaction with 14-3-3 proteins and whether specific functions of 14-3-3 proteins might be controlled via the association with tuberin is still under investigation.

The binding experiments indicated that tuberin is able to interact with multiple 14-3-3 isoforms and that tuberin contains multiple 14-3-3-binding sites, consistent with Scansite prediction of eight potential consensus 14-3-3-binding motifs in the tuberin amino acid sequence [30, 31]. Furthermore, phosphorylation of Ser 1210 in TSC2 is required for its association with 14-3-3. It has been proposed that the interaction with 14-3-3 mediates the proteosomal degradation of TSC2. It is important to note that when the level of TSC2 decreases in cells, the aggregation of TSC1 occurs. However, the mechanism of TSC2 degradation is not well understood. The presence of multiple 14-3-3-binding sites in tuberin is consistent with the hypothesis that the tuberin-hamartin complex may integrate several different input signals [12].

The isolation of 13 clones encoding well-characterized interactors for TSC2 14-3-3 is a very good indicator of the validity of the screen. The other isolated clones were found to encode signaling molecules (36 clones, which encode 6 proteins), factors regulating protein folding and stability (6 clones, which encode 2 proteins), proteins regulating cell adhesion (2 clones encode 1 protein) and proteins with non-defined function (3 clones encoding 1 protein). We are particularly interested in further analysis of TSC2-BP1 and TSC2-BP5 clones, which have the potential to regulate the phosphorylation status and the stability of TSC1-2 complex. We are currently investigating the specificity of their interaction with TSC1-2 by co-immunoprecipitating transiently expressed and endogenous proteins. Once confirmed, the physiological relevance of identified interactions will be the subject of our further studies.

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ИДЕНТИФИКАЦИЯ НОВЫХ СВЯЗЫВАЮЩИХ БЕЛКОВ-ПАРТНЕРОВ ОПУХОЛЕВОГО СУПРЕССОРА TSC2 МЕТОДОМ ДВУГИБРИДНОЙ СИСТЕМЫ ДРОЖЖЕЙ

Цель: **идентификация новых белков-партнёров TSC2.** ***Методы:*** **в работе была использована DupLEX-A™ двугибридная система дрожжей, разработанная OriGene Technologies. Сконструированные «байт»-конструкты, содержащие полноразмерную форму и С-концевой участок TSC2, были проверены в тестах на экспрессию, проникновение в ядро и самоактивацию с использованием стандартного протокола DupLEX-A™ системы. Выполнен двугибридный скрининг кДНК библиотек клеток HeLa и эмбриона мыши с последующим отбором позитивных клонов методом полового слияния. Последовательности отобранных клонов идентифицированы с помощью сиквенс-анализа и поиска гомологии в базах данных.** ***Результаты:*** **скрининг кДНК библиотек с помощью полноразмерной формы TSC2 и его С-концевого домена позволили отобрать 102 положительных клона. Для 82 клонов методом полового слияния было подтверждено наличие взаимодействия. В результате проведенного анализа последовательностей были идентифицированы уже известные, а также новые белки-партнёры TSC2.** ***Выводы:*** **с помощью дрожжевой двугибридной системы были найдены новые партнеры TSC2, представленные белками цитоскелета, различными сигнальными молекулами, а также белками, регулирующими клеточную адгезию. Также нами было показано наличие взаимодействия между TSC2 и некоторыми изоформами белков семейства 14-3-3, что в свою очередь подтверждает достоверность обнаруженных взаимодействий TSC2 с вышеуказаными белками.**

Ключевые слова: **бугорчатый склероз, TSC1/2, дрожжевая двугибридная система, белок-белковые взаимодействия.**