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Pam (Protein associated with Myc) functions as an E3 Ubiquitin ligase and regulates TSC/mTOR signaling

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Abstract

The tumor suppressor tuberin, encoded by the Tuberous Sclerosis Complex (TSC) gene *TSC2*, negatively regulates the mammalian target of rapamycin (mTOR) pathway, which plays a key role in the control of cell growth and proliferation. In addition to naturally occurring mutations, several kinases including Akt, RSK1, and ERK are known to phosphorylate and inactivate tuberin. We demonstrate a novel mechanism of tuberin inactivation through ubiquitination by Pam, a putative RING finger-containing E3 ubiquitin (Ub) ligase in mammalian cells. We show that Pam associates with E2 ubiquitin-conjugating enzymes, and tuberin can be ubiquitinated by Pam through its RING finger domain. Tuberin ubiquitination is independent of its phosphorylation by Akt, RSK1, and ERK kinases. Pam is also self-ubiquitinated through its RING finger domain. Moreover, the TSC1 protein hamartin, which forms a heterodimer with tuberin, protects tuberin from ubiquitination by Pam. However, TSC1 fails to protect a disease-associated missense mutant of TSC2 from ubiquitination by Pam. Furthermore, Pam knockdown by RNA interference (RNAi) in rat primary neurons elevates the level of tuberin, and subsequently inhibits the mTOR pathway. Our results provide novel evidence that Pam can function as an E3 Ub ligase toward tuberin and regulate mTOR signaling, suggesting that Pam can in turn regulate cell growth and proliferation as well as neuronal function through the TSC/mTOR pathway in mammalian cells.

Keywords: TSC; Tuberin; Hamartin; Mammalian target of rapamycin; Ubiquitination; Pam; RING finger

1. Introduction

Inactivating mutations in either the *TSC1* or *TSC2* gene are responsible for tuberous sclerosis complex (TSC), an autosomal dominant disorder, which is characterized by formation of slow-growing, benign hamartomas in multiple organs [1]. The protein products of *TSC1* and *TSC2*, hamartin and tuberin (also referred to as TSC1 and TSC2, respectively), form a tight complex and function together in the cell. Tuberin has GTPase activating protein activity toward the small GTP-binding protein Rheb. The TSC protein complex restrains cell growth and proliferation by acting as a focal point integrating a diverse range of signaling pathways with the mTOR pathway (for reviews see [2,3]). A growing body of evidence suggests that abnormal regulation of the TSC-mTOR pathway may be a widespread molecular mechanism for the pathological development of several hamartomatous syndromes including TSC (for review see [3]).

We previously identified Pam (Protein associated with Myc) as a tuberin interactor and demonstrated a genetic interaction between the homologs of these proteins in *Drosophila* [4]. Pam

Abbreviations: TSC, tuberous sclerosis complex; mTOR, mammalian target of rapamycin; Ub, ubiquitin; RNAi, RNA interference; Pam, Protein-associated with Myc; HIW, highwire; RPM-1, regulator of presynaptic morphology; RHD, regulator of chromosome condensation-homolgy domain; RZF, RING finger; HEK293T, human embryonic kidney 293T cell; IP, immunoprecipitation; WB, western blotting; DIV, days *in vitro*; DPT, days post-transfection; PBS, phosphate-buffered saline; Ubc, ubiquitin-conjugating enzyme.

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is an extremely large protein which belongs to an evolutionally conserved family of proteins (PHR family) including Phr1 in mouse, Highwire (HIW) in *Drosophila*, Regulator of Presynaptic Morphology (RPM-1) in *C. elegans*, and Esrom in zebrafish. These proteins contain multiple domains including a Myc-binding region, regulator of chromosome condensation (RCC)-homology domains (RHD), and a C-terminal RING finger (RZF) domain which is present in a large family of E3 Ub ligases [5]. Several genetic studies demonstrate that Pam homologs regulate presynaptic growth [6–12]. Members of the PHR family have been suggested to control a variety of signaling pathways including JNK/p38 MAPK signaling in *Drosophila* and *C. elegans* [13,14], TGF-β/BMP signaling in *Drosophila* [15], and cAMP signaling pathway in mammalian cells [16]. In addition, a very recent report suggests that RPM-1 positively regulates a Rab GTPase pathway to promote vesicular trafficking via late endosomes, thereby regulating synapse formation and axon termination [17]. The results obtained from both *Drosophila* and *C. elegans* suggest that the highly conserved RZF domain is critical for E3 Ub ligase activity of Pam homologs, particularly in regulation of synapse development [13,14]. However, the function of Pam as an E3 Ub ligase in mammalian cells remains unknown.

In this study, we examine whether Pam targets tuberin for degradation through ubiquitination and thus can regulate TSC/mTOR signaling pathway. Our results demonstrate that Pam interacts with specific E2 enzymes and is capable of ubiquitinating tuberin. In addition, hamartin protects tuberin from ubiquitination by Pam. Furthermore, suppression of Pam in primary neurons results in stabilization of tuberin and down-regulation of mTOR signaling. In addition to cell proliferation and growth, in neuronal cells, TSC1/2 and mTOR are implicated in many processes which are critical for neuronal development and long-term modification of synaptic strength [18–20]. Furthermore, Ub and ubiquitination enzymes have emerged as key regulators of synaptic development, function and plasticity [21]. Therefore, our findings suggest that Pam, as an E3 Ub ligase and a regulator of TSC/mTOR signaling, could play an essential role in synaptic development and function in mammalian neurons.

2. Materials and methods

2.1. Cell culture, antibodies, and reagents

Human embryonic kidney 293T (HEK293T) cells were maintained in DMEM with high glucose (4.5 g/l glucose) (Gibco) containing 10% FBS (Gibco). Dissociated hippocampal or cortical neuronal cultures were prepared from E18 rats (Charles River Laboratories), plated either on coverslips coated with poly-D-lysine (PDL, 1 mg/ml, Sigma) for transfection or on PDL (0.1 mg/ml)-coated 60 mm dish for lentiviral infection. Neuronal cultures were maintained in growth media containing Neurobasal Media (Gibco) supplemented with 2% B27 Supplement, 2 mM L-glutamine, 50 U/ml penicillin, and 50 g/ml streptomycin, as described [20]. Primary antibodies used are anti-FLAG M2, anti-GAPDH (Sigma), anti-GST, anti-p53 (Santa Cruz), anti-myc 9E10 (Development Study Hybridoma Bank), anti-HA (Covance), anti-His (Qiagen), anti-phospho-S6 (S235/236), anti-S6, anti-phospho-S6K (T389) (Cell Signaling Technologies). Anti-Pam (PP1) and anti-TSC2 (TSDF) antibodies were described previously [4]. ALLN, MG132, and cycloheximide were obtained from Calbiochem.

2.2. Constructs

Generation of full-length Pam and truncated Pam fragments (Pam F1, Pam F2, and Pam F3) has been previously described [22]. Myc-tagged mutant Pam F3 (Pam F3-3A) and naturally occurring tuberin mutants R905Q and R611Q were generated using the QuickChange Site-Directed Mutagenesis kit (Stratagene). Myc-tagged Pam F3ΔRZF was generated by digestion of Pam F3 with Sap1 and Xho1 restriction enzymes to delete the C-terminus including the RZF domain. FLAG-tagged wild type TSC2 and mutant TSC2 (S939A/T1462A) were kind gifts from B. D. Manning, and Ub-HA was a gift from Y. Jin. FLAG-tagged TSC2 (S1798A) was generously provided by J. Blenis, and Xpress-tagged TSC2 (S664/S540A) was kindly given by P. P. Pandolfi.

2.3. RNAi

To knock down expression of endogenous Pam in rat neurons, pSuper-rPam RNAi constructs were designed as described [23]. The highest efficiency of Pam suppression was observed when targeting bp 671–689 of rat Pam (5′-GGAGCCTCCAAGCCCTGCT-3′). The target sequence was not homologous to any other genes using a BLAST database search. A scrambled sequence (5′-CAGTCGCGTTTGCGACTGG-3′) and a modified sequence of rat Pam bp 671–689 containing two point mutations (5′-GGAGCCTCCGGGCCCTGCT-3′) were used as controls. These sequences were also used to generate the control and Pam RNAi constructs in the lentiviral pLKOpuro.1 vector kindly provided by Dr. Sheila Stewart of Washington University [24].

2.4. Transfection and infection

HEK293T cells (80–90% confluent) were transfected using lipofectamine 2000 (Invitrogen), as recommended by manufacturer's instructions. For neuronal transfection, 4 days *in vitro* (DIV) rat hippocampal neuronal cells (8×10^4 cells/well of a 24-well plate) were transfected using lipofectamine 2000 (Invitrogen) with a 4:1 ratio of pSuper-rPam RNAi (1.6 μg) or pSuper vector control (1.6 μg) along with peGFP-N1 (0.4 μg, Clontech) as a reporter of transfection. Cerebral cortical neurons (2 or 5 DIV, 1.7×10^6 cells/60 mm dish) were also infected with the lentiviral constructs, and 4 days after infection cell lysates were prepared for western analysis. Lentiviral particles were produced by a NINDS funded Neuroscience Vector Core at the Massachusetts General Hospital.

2.5. Ubiquitination assays and immunoprecipitation

At 20 h-post transfection, HEK293T cells were harvested and lysed in 0.5% NP-40 lysis buffer containing 150 mM NaCl, 50 mM Tris (pH 7.5), 50 mM NaF, 1 mM Na orthovanadate, 2 mM EDTA, and 1× Complete protease inhibitor cocktail (Roche). HEK293T cells were treated with proteasome inhibitor MG132 (50 μM) or ALLN (10 nM) for 1 h prior to lysis. Lysates were then used for immunoprecipitation (IP). Briefly, protein lysates (1 mg) were incubated with 2–3 μg of anti-FLAG M2, anti-myc 9E10, anti-Xpress, anti-p53, or anti-TSC2 (TSDF) antibody for 15 h at 4 °C. Subsequently, either protein-G Sepharose or protein-A Sepharose (Amersham-Pharmacia) was added and incubated for an additional 3 h. Precipitates were washed and resuspended in 2× SDS sample loading buffer. Lysates and immunoprecipitates were separated by 4–15% gradient or 5% SDS-PAGE (Bio-Rad), and transferred to nitrocellulose (Bio-Rad). Membranes were then blocked, detected with the appropriate primary antibody, followed by horseradish peroxidase-conjugated secondary antibodies (Chemicon), and then visualized using the ECL system (Amersham-Pharmacia).

2.6. In vitro binding assay

At 20 h-post transfection with Pam F3-Myc, HEK293T cells were harvested and lysed in 0.5% NP-40 lysis buffer. Lysates (500 μg) were then incubated with 5 μg of recombinant GST, UbcH2-GST (Calbiochem), UbcH5a-GST (EMD), UbcH5b-GST (Calbiochem), UbcH5c-GST (BIOMOL), Ubc6-His (EMD), or Ubc7-His (BIOMOL) proteins for 2 h at 4°C. Anti-Myc antibody 9E10 (2 μg) was then added and incubated for 15 h at 4°C, followed by incubation with