Pam and Its Ortholog Highwire Interact with and May Negatively Regulate the TSC1-TSC2 Complex*

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Tuberous Sclerosis Complex (TSC) is an autosomal dominant disorder associated with mutations in TSC1, which codes for hamartin, or TSC2, which codes for tuberin. The brain is one of the most severely affected organs, and CNS lesions include cortical tubers and subependymal giant cell astrocytomas, resulting in mental retardation and seizures. Tuberin and hamartin function together as a complex in mammals and Drosophila.

We report here the association of Pam, a protein identified as an interactor of Myc, with the tuberin-hamartin complex in the brain. The C terminus of Pam containing the RING zinc finger motif binds to tuberin. Pam is expressed in embryonic and adult brain as well as in cultured neurons. Pam has two forms in the rat CNS, an ~450-kDa form expressed in early embryonic stages and an ~350-kDa form observed in the postnatal period. In cortical neurons, Pam co-localizes with tuberin and hamartin in neurites and growth cones. Although Pam function(s) are yet to be defined, the highly conserved Pam homologs, HIW (Drosophila) and RPM-1 (Caenorhabditis elegans), are neuron-specific proteins that regulate synaptic function. Here we show that HIW can genetically interact with the Tsc1-Tsc2 complex in Drosophila and could negatively regulate Tsc1-Tsc2 activity. Based on genetic studies, HIW has been implicated in ubiquitination, possibly functioning as an E3 ubiquitin ligase through the RING zinc finger domain. Therefore, we hypothesize that Pam, through its interaction with tuberin, could regulate the ubiquitination and proteasomal degradation of the tuberin-hamartin complex particularly in the CNS.

Tuberous sclerosis complex (TSC) is an autosomal dominant disorder characterized by benign hamartomas in brain, kidney, heart, lung, and skin. It is a developmental disorder with abnormalities in cell migration, differentiation, and proliferation. Neurological complications include seizures, mental retardation, and autism (1). The disease is caused by mutations in tumor suppressor genes TSC1 or TSC2 encoding hamartin and tuberin, respectively (2, 3). Hamartin and tuberin associate in vivo forming a complex with other proteins (4). Rodent models of Tsc1 and Tsc2 develop renal cyst adenomas with liver hemangiomatous observed in mouse models (5–8). Mutations in either Drosophila Tsc1 or Tsc2 show increase in cell and organ size, whereas co-expression of dTsc1 and dTsc2 inhibits growth and reduces cell size (9–12). Studies from fly also suggest that dS6 kinase may be an inhibitory target of dTsc1 and dTsc2 in growth control (10–12). Recent studies have confirmed that mammalian TSC1 and TSC2 inhibit S6 kinase (S6K). Cells harboring mutations in either TSC1 or TSC2 have constitutively high phosphorylation of both S6K and its substrate S6 (8, 13–18). Furthermore, tuberin and hamartin function together to inhibit target of rapamycin-mediated signaling to S6K in mammals as well as in fly (15, 16, 18, 19). Akt phosphorylates tuberin and inhibits tuberin-hamartin function (14, 16, 20, 21). Furthermore, it is evident from the most recent reports that the small GTPase Rheb is a direct target of tuberin and hamartin in both the Drosophila and mammalian systems (22–24). These studies demonstrate Rheb GTPase-activating protein activity for tuberin, and the loss of tuberin and/or hamartin lead to an increase in GTP-bound Rheb, which in turn leads to activation of the mammalian target of rapamycin/S6K/4E-binding protein signaling pathway. Thus, recent studies have placed tuberin and hamartin in growth signaling pathways; however, critical CNS functions of these proteins remain unknown. Our recent work demonstrates that unlike kidney lesions, CNS lesions such as cortical tubers do not display a second somatic mutation and other mechanisms might play a role during tumorigenesis in the CNS (25). Furthermore, we have shown that the tuberin-hamartin complex exists with neurofilament light chain and ezrin, radixin, and moesin (ERM) proteins in the growth cone and could play a role in neuronal migration (26). In our efforts to understand the tuberin-hamartin complex in the CNS, we have identified Pam (protein-associated with Myc) (27) as a physiologically relevant tuberin interactor and have shown that Pam exists in complex with tuberin-hamartin in the CNS. Our results also demonstrate that HIW, the Drosophila homolog of Pam, can genetically interact with dTsc1-dTsc2 in the fly. The evidence that the Pam homologs in worm (RPM-1) (28, 29) and in Drosophila (HIW) (30) are important regulators of synaptic growth suggests that the interaction of Pam with tuberin-hamartin will be highly relevant within the CNS.

EXPERIMENTAL PROCEDURES

cDNA Constructs—For the yeast two-hybrid screen, human tuberin (aa 449–1000) was amplified and cloned into the two-hybrid vector pEG202 (31). For domain mapping, tuberin deletion constructs were

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generated using the same strategy. Tuberin bait was also subcloned as a 5'-FLAG fusion protein into pcDNA3 vector. Pam spanning aa 4492–4641 in vector pJG4–5 was released and cloned into pGEX4T-1 (PF1). PF2 (Pam aa 4312–4641) in pGEX2TK was a kind gift from Dr. Q. Guo (National Institutes of Health, Bethesda, MD). PF2 was also cloned as a Myc fusion protein into pCMV3b vector (Stratagene). The integrity of all of the inserts was confirmed by sequencing.

Interaction Trap Screening—Yeast strain Egy48 was sequentially transformed with the tuberin bait, and human fetal frontal cortex library was fused to the activation domain of GAL4 in the plasmid pJG4–5 and selected on Ura, His, Trp plates (32). Positive clones which showed β-galactosidase activity within 2 days on plates and within 60 min by filter assay were isolated, sequenced, and analyzed for homology with sequences in the NCBI data base.

Antibodies—Primary antibodies utilized included: rabbit polyclonals tuberin antibody C20, and Mdm2 (Santa Cruz Biotechnology); hamartin antibodies HP6 and HF3 (33); monoclonals FLAG antibody M2 (Sigma); c-Myc antibody 9E10 (Developmental Studies, Hybridoma Bank, University of Iowa); ERM antibody 13H9 (a kind gift from Dr. F. Solomon, Massachusetts Institute of Technology); Vamp-2 (Synaptic Systems, Goettingen, Germany); and Neu-N (Chemicon). PP1, a polyclonal antibody against human Pam peptide (aa 4519–4534) was generated in rabbits (Research Genetics) and affinity-purified using the sulfotlink affinity purification kit (Pierce Laboratories).

Cell Culture, Transfection, and Expression of GST Fusion Proteins—PC12 and 293T cells were grown using standard conditions. Primary cortical neurons from embryonic day 19-old embryonic rat brain were cultured as described previously (26). For immunocytochemistry, cultured neurons were fixed with 4% paraformaldehyde in phosphate-buffered saline for 10 or 30 min at RT and stored in phosphate-buffered saline at 4 °C. FLAG fusion construct of tuberin bait in pcDNA3 and Myc-tagged PF2 was transfected into 293T cells with LipofectAMINE 2000 (Invitrogen) using standard protocols described with the reagent. PF1 and PF2 were expressed employing E. coli BL21pLysS and isopropyl-1-thio-D-galactopyranoside.

Immunoprecipitation, Pull-down Assays—For co-immunoprecipitation of endogenous Pam and tuberin, PC12 cells and embryonic rat brain were lysed in Nonidet P-40 lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 0.5% Nonidet P-40, 50 mM sodium fluoride, 1 mM orthovanadate, 2 mM EDTA) supplemented with Complete protease inhibitor mixture. Immunoprecipitations were done with either anti-tuberin antibody or anti-tuberin plus anti-hamartin antibodies, resolved on 6% SDS-PAGE, and transferred to nitrocellulose membranes. Immunoblots were detected with affinity-purified anti-Pam PP1. Affinity pull-down assays were performed as described earlier (26) using GST-Pam (PF1 and PF2) and lysates from 293T cells followed by immunoblots using antibodies for FLAG, tuberin, and hamartin.

Immunocytochemistry—Fixed neurons were permeabilized with 0.1% Triton X-100 (Sigma), blocked with 10% normal goat serum containing 1% bovine serum albumin, and incubated with 13H9, anti-Neu-N, or anti-Vamp2 followed by secondary antibody (26). Coverslips were then incubated with PP1, the respective secondary antibody, and then mounted in gelvatol-mounting media. For double staining with PP1 and C20 or HF3, PP1 was conjugated with Alexa Fluor 594 (Mo-
lecular Probe). Cells were incubated with C20 or HF3 followed by donkey anti-rabbit Cy2 and then conjugated PP1. Images were captured using the LSM5 Pascal software coupled to a Zeiss LSM Pascal Vario 2 RGB confocal system. Negative controls included omitting primary antibodies and blocking of PP1 or C20 with immunogenic peptide.

**Fly Stocks**—The fly line with double (UAS-Tsc1 and UAS-Tsc2) transgenes and an eye-specific GAL4 line, eyGAL4, were described previously (12). The hiw mutant lines (ND8, EP1305, ND42, ND69, and EMS) (30) were kindly provided by Dr. Hong Wan and Dr. Corey Goodman.

**RESULTS**

**Identification of Pam as a Binding Partner of Tuberin**—To identify proteins that associate with tuberin, we screened a fetal frontal cortex library with tuberin bait (aa 449–1000) using a LexA-based yeast two-hybrid system. Screening was performed on a total of $1 \times 10^6$ clones with two positive clones identified. Both clones matched the sequence of Pam, a large protein originally identified as a protein-associated with Myc, corresponding to aa 4492–4641 (27). The specificity of this interaction was verified in the two-hybrid assay by transcriptional activation of LacZ and Leu reporters with a panel of unrelated baits, which included Cdc2, bicoid, huntingtin, Fus3, and neurofibromin, none of which activated transcription.

The region of Pam isolated included the extreme C-terminal 223 amino acids (PF1). The last 324 amino acids of Pam are highly conserved with its homologs in Caenorhabditis elegans (RPM-1) and Drosophila (HIW). These family members are characterized by the presence of a RING zinc finger (RZF) motif (Fig. 1A). To determine whether the RZF motif influenced the interaction with tuberin, we carried out affinity pull-down assays using the mammalian expressed FLAG-tagged tuberin bait and GST fusion proteins of Pam aa 4419–4641 (PF1) and aa 4312–4641 (PF2) encompassing the entire RZF. In addition to confirming the interaction, our results indicated that the interaction between tuberin and PF2 was more efficient than with PF1 (Fig. 1B). GST alone was employed as a negative control (Fig. 1B). We generated a peptide antibody PP1 against human Pam peptide (aa 4519–4534), which recognized the Myc-tagged Pam fusion protein PF2 expressed in cells confirming the specificity of the antibody (Fig. 1C).

**Pam Interacts with the Tuberin-Hamartin Complex**—To examine the in vivo association of tuberin and Pam, we performed co-immunoprecipitation experiments in PC12 cells and rat embryonic brain. Anti-Pam polyclonal antibody PP1 was employed in immunoblots to detect endogenous Pam, which migrates at $\sim 450$ kDa. Pam cDNA has an open reading frame of 4,641 amino acids with a predicted mass of 510 kDa (27). Immunoprecipitation of tuberin using an anti-tuberin antibody followed by immunoblot analysis showed that endogenous Pam co-immunoprecipitates with tuberin in PC12 cells and embry-
onomic rat brain (Fig. 2, A and B). Interestingly, in rat brain a higher amount of Pam was seen in co-immunoprecipitations when tuberin and hamartin antibodies were utilized together (Fig. 2B). Equivalent amounts of lysates were used in these experiments, and pre-immune serum was employed as a negative control. In addition, Mdm2, another RING finger-containing protein, was not co-immunoprecipitated under the same conditions (Fig. 2C), confirming further the specificity of the interaction. Collectively, these results indicate that Pam interacts with tuberin in vitro and in vivo and may exist with the tuberin-hamartin complex in brain.

Mapping the Pam Binding Domain in Tuberin—To define the domain(s) responsible for the interaction of tuberin with Pam, deletion constructs within TSC2 were generated and employed in two-hybrid assays. The original tuberin bait (aa 449–1000) as well as a bait encompassing aa 895–1314 showed the strongest transcription activation, whereas tuberin spanning aa 795–1000 showed a lesser degree of transcription activation. Baits consisting of aa 449–914 and 394–549 were negative (Fig. 3). These results suggest that the tuberin domain encoding aa 914–1000 is necessary for the interaction between the two proteins.

Pam is Expressed in Developing Nervous System—We examined Pam expression by immunoblotting in cultured primary cortical neurons and in the developing rat brain. Pam antibody PP1 recognizes a predominant band of ~450 kDa in neurons cultured for 8–36 days in vitro (DIV). Pam expression was relatively unchanged in neurons from DIV8 until DIV22, after which it decreased gradually (Fig. 4A). In rat brain, Pam expression was abundant on embryonic day 16 but declined with increasing age. Interestingly, coupled with this decline in expression, the appearance of an ~350-kDa protein with gradual increasing expression was consistently observed in the postnatal period (Fig. 4B), the nature of which remains to be characterized.

Localization of Pam in Primary Cortical Neurons—We examined the localization of Pam in primary cortical neurons by indirect immunofluorescence. A strong nuclear localization of Pam was seen in the cell body, which is consistent with Myc binding. Additionally, punctate staining was observed along the neurites in axons and dendrites (Fig. 5, A, D, and G). No staining was detected when the PP1 antibody was preabsorbed with the corresponding peptide used as immunogen (Fig. 5B). Nuclear localization of Pam was further confirmed in neurons by double staining with Neu-N, a neuronal nuclear marker that completely co-localized with Pam (Fig. 5, D–F). The synaptic vesicle protein VAMP-2 revealed a partial co-localization with Pam in the cytoplasm of cell body and along the neurites (Fig. 5, G–I).

Co-localization of Pam with the Tuberin-Hamartin Complex in Neurites and Growth Cones—We next examined whether Pam and the tuberin-hamartin complex were present in similar subcellular locations within cultured neurons. Tuberin is predominantly cytoplasmic with a strong perinuclear localization in the cell body (Fig. 5C). However, evidence for tuberin translocation to the nucleus has been reported earlier through its association with steroid receptor family members (34, 35). Tuberin also showed punctate localization along the neurites. Pam co-localized with tuberin and hamartin along the neurites and in the growth cones (Fig. 6, A–F). It is noteworthy that Pam co-localized with hamartin in one of the filopodial extensions at the tip of a growth cone (Fig. 6F, arrowhead). We have recently reported that hamartin exists together with the ERM family of actin-binding proteins in growth cones (26). Here, double labeling experiments revealed that Pam partially overlaps with the ERM proteins in the growth cone (Fig. 6, G–I).
different genetic background (Fig. 7, D–H), strongly suggesting a genetic interaction between hiw and the dTsc1-Tsc2 complex. The enhancement of Tsc1+Tsc2 phenotype by the removal of the hiw gene indicates that hiw negatively regulates Tsc1-Tsc2 activity in Drosophila eye. hiw mutant flies alone appear similar to the wild type control shown (Fig. 7, A and B).

DISCUSSION

In this study, we have identified Pam as an interacting partner for tuberin and have demonstrated an association between the tuberin-hamartin complex and Pam in the CNS. Pam was identified as a binding partner for c-Myc (27). Northern blot analysis revealed Pam as a transcript of ~15 kb in many tissues with predominant expression in brain (27).2 In situ hybridization in rodent brain showed high expression of Pam mRNA in pyramidal cells of hippocampus and granule cells of dentate gyrus and cerebellum (36). Pam encodes a large protein with several interesting motifs (Fig. 1A). Among these motifs, the most notable include RCC1 (regulator of chromosome condensation) homology domains RHD1 (aa 498–740) and RHD2 (aa 874–1065), a Myc-binding region (aa 2413–2712), a RZF, and two zinc-finger motifs (Zn) at the extreme C-terminal domain (aa 4354–4583) (27). In another study, a

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2 V. Murthy and V. Ramesh, unpublished observations.
small region of Pam (aa 1028–1231) was isolated as an interactor of type V adenylyl cyclase and the RHD domain of Pam was shown to be a potent inhibitor of type V adenylyl cyclase activity (37). Here we show that the Pam domain encompassing the RZF and zinc motifs is necessary for its interaction with tuberin. Thus, it is evident that Pam, through different domains, could associate with a variety of proteins, ultimately participating in diverse cellular functions.

Our results demonstrate that Pam may have at least two forms in the rat CNS, an ~450-kDa form expressed in early embryonic stages and an ~350-kDa form observed in the postnatal period. An antibody raised against the extreme C-terminal region of the protein detects both forms. The two forms may arise because of alternative splicing or specific cleavage at the N terminus. Further studies are needed to define the nature of the two forms. In rat brain, we have observed the expression of tuberin and hamartin to be relatively stable during development with both proteins widely expressed in the nervous system (33). Although Pam is expressed abundantly in brain, its function in the mammalian CNS remains unknown. On the contrary, functions of Pam homologs in *Drosophila* and *C. elegans* are well understood employing the elegant genetic studies in these systems. HIW modulates synaptic growth at the larval neuromuscular junction. Presynaptic terminals in hiw mutants display large and more complex branches than in the wild type, and HIW is suggested to selectively affect the mechanism underlying synaptic growth (30). RPM-1, the worm homolog of Pam, is also shown to play a role during synaptogenesis. Loss of function mutations in the *rpm-1* gene reveal abnormally structured presynaptic terminals in neuromuscu-
lar junctions (28, 29). These results suggest that the function of these proteins as regulators of synapse formation and growth are conserved between fly and worm. Our results here demonstrate that HIW can genetically interact with and negatively regulate Tsc1-Tsc2 in Drosophila.

Mammalian Pam, Drosophila HIW, and C. elegans RPM-1 define a new family of large proteins that might play an essential role in the nervous system. Although the homology among these three proteins is distributed throughout the coding sequence, one of the most conserved regions is in the C terminus containing the zinc finger motifs and a RING-H2-type finger, which we identified as the region of Pam that interacts with the tuberin complex, one of the most conserved regions is in the C terminus (38). Furthermore, a genetic interaction has been identified between the deubiquitin ligase (38). Furthermore, a genetic interaction has been identified between the deubiquitin ligase activity of Mdm2, another RING finger-containing unknown. However, remarkably, Akt activates the E3 ubiquitin ligase.

A flurry of reports have appeared within the last year demonstrating that Akt phosphorylates and inactivates tuberin in a PI3-kinase-dependent manner in both mammals and fly (14, 16, 20, 21). Human TSC2 sequence has six potential Akt phosphorylation sites of which Ser-939 and Thr-1462 are conserved in dTsc2 (Ser-924 and Thr-1518). Mutations of these residues to non-phosphorylatable alanines in both systems show substantially reduced Akt-mediated phosphorylation (14, 21). Akt-mediated phosphorylation was also shown to promote degradation of the tuberin-hamartin complex particularly in the CNS (Fig. 8, model). Although the large size of Pam poses a challenge for identifying its function as an E3 ubiquitin ligase, studies aimed at this aspect will aid in the understanding of how the tuberin-hamartin complex is regulated and how the complex is inactivated by Akt-mediated phosphorylation.

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Pam Interaction with the Tuberin-Hamartin Complex

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