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Localization of the Diaphanous-related formin Daam1 to neuronal dendrites

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ABSTRACT

The Rho family of small GTPase proteins are involved in the formation and maintenance of neuronal dendrites. In this study, we show that Daam1, a member of the Diaphanous-related formin protein family and a downstream effector for RhoA, is localized to the dendrites of hippocampal neurons. Immunoblot analysis showed that Daam1 is enriched in the mouse hippocampus and co-fractionates in brain lysates with dendritic and synaptic proteins. Immunohistochemical analysis revealed that Daam1 protein distributes in a punctate pattern throughout the cell body and dendritic shafts of dissociated hippocampal neurons and organotypic hippocampal cultures. Although Daam1 is mostly expressed in the shaft of dendrites, co-stainings with SV2 or PSD95 revealed that Daam1 is also present at some synapses. In addition, viral directed expression of a fluorescently tagged Daam1 fusion protein in hippocampal slices resulted in targeted delivery to the dendrites of pyramidal neurons, leading to a reduction in the density of spines.

The Diaphanous-related formin (Drf) proteins are a novel class of actin nucleation and polymerizing factors [32]. The Drfs represent a subgroup of the formin protein family, that includes five members; Dia1, Dia2, Dia3, Daam1 and Daam2. Formin proteins are potent regulators of actin dynamics, but the defining property of the Drf-type formins is that they are regulated through interactions with the Rho-family GTPases. These proteins are activated when they interact with GTP-bound Rho proteins through their N-terminal binding domains, releasing the auto-inhibitory mechanism (reviewed by [32]). Once activated, Drf proteins potently stimulate the nucleation and elongation of actin fibers through the evolutionary conserved functional FH1 and FH2 domains [2,14]. Therefore, Drfs function as effectors for the Rho-GTPase signaling pathway, resulting in the elongation of actin filaments that are associated with changes in the morphology of many cell types.

An important characteristic of many neurons is the presence of an elongated and multi-branched dendritic tree. The formation and maintenance of these complex structures depends on an underlying cytoskeleton that consists of a core of microtubules and a cortex of actin microfilaments [26]. The Rho-family of small GTPase proteins are signaling factors that regulate many intracellular systems, including both the actin and microtubule cytoskeletons [11]. A number of studies have demonstrated the involvement of the Rho-family GTPase proteins in the regulation of dendrite development in mammalian, *Xenopus* and *Drosophila* neurons (reviewed in [17,22]). In general, the expression of activated RhoA opposes the development and elaboration of dendrites, while activated Rac1 and Cdc42 promote dendrite outgrowth. Furthermore, the overexpression of active RhoA causes a decrease in the density of spines along dendrites [21,23,30]. Thus, the development and maintenance of dendrites is regulated by the balance of positive and negative GTPase signaling cascades. In the RhoA pathway, some of the downstream signaling effects involve the activity of Rho kinase (ROCK) [21,28], a well-studied effector that mediates RhoA effects on actin organization [24]. However, the complex nature of the RhoA signaling pathway suggests that additional effectors are likely to contribute to signaling in dendrites, and one such class of candidate effectors are the Drf proteins.

The Drf Daam1 (Dishevelled-associated activator of morphogenesis 1) has been linked to actin dynamics [9], and whose activity can be regulated by RhoA [19]. However, its role in regulating neuronal structure and function is not known. In this study, we have localized Daam1 in the dendrites of hippocampal pyramidal neurons. The known effects of Daam1 on actin cytoskeleton remodeling [1,32], suggests that it may participate in controlling the morphology of dendrites. We have shown that over-expression of Daam1 decreases the density of dendritic protrusions, known as spines.

Daam1 (KIAA0666, kindly provided by T. Nagase [13]) was cloned into pCMVTAG3B (Stratagene). HEK293T cells were lysed in 1% TritonX-100 after 48 h transfection. Mouse brain tissue (postnatal day 32) was homogenized in 1% TritonX-100 and

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0.5% deoxycholate. Samples (50 μg of transfected HEK293T cell lysates, 100 μg of whole brain lysates, and 40 μg of brain tissue lysates (P60)) were run on a SDS-PAGE and transferred onto PVDF (Millipore). Membranes were blocked with 5% non-fat dry milk, and incubated with either anti-Daam1 (1:5000), anti-myc (9E10B, 1:1000), or anti-GAPDH (Abcam, 1:3000) antibodies. Membranes were washed and incubated with peroxidase-conjugated secondary anti-rabbit (1:5000) or anti-mouse (1:2000) antibodies (Jackson Laboratory). Subcellular fractionation of mouse brain was carried out by differential centrifugation as described previously [12] and analyzed by western blot using NR1 (BD), Kv3.1b (Chemicon), and GRIP1 (BD) antibodies.

Hippocampal primary neuronal cultures (E17) were prepared and fixed in 4% paraformaldehyde/4% sucrose after 3 weeks in culture. Fixed neurons were permeabilized with 0.5% TritonX-100, and incubated in blocking solution (10% goat serum and 0.2% TritonX-100). Neurons were then incubated with anti-Daam1 (1:500) and anti-SV2 (1:100, Developmental Studies Hybridomas Bank) antibodies in 5% goat serum and 0.2% TritonX-100, followed by Alexa-Fluor488 goat-anti-rabbit and Alexa-Fluor568 goat-anti-mouse (1:300, Molecular Probes). Pre-absorption control stain was performed by mixing the antibody with a C-terminal Daam1 antigen. Images were obtained using a Zeiss LSM510 confocal microscope (63× objective). Each image represents a Z-stack maximum projection. For PSD95, fixed neurons (after 2 weeks in culture) were permeabilized with 0.2% TritonX-100/10 mM glycine/PBS, and incubated in blocking solution (5% BSA/PBS). Neurons were incubated with anti-Daam1 (1:500) and anti-PSD95 (1:200, clone K28/43, Antibodies Inc.), followed by Alexa-Fluor488 goat-anti-rabbit and Alexa-Fluor568 goat-anti-mouse. Images were obtained using a spinning disk confocal system (PerkinElmer Corporation) attached to a Nikon Eclipse TE2000 (Nikon). Images are maximal projections using MetaMorph (Molecular Devices Inc.) from Z-series stacks of 0.2 μm in thickness, using a 60× objective and processed using Photoshop (Adobe Systems).

Semliki-Forest virus (SFV) constructs were generated by Gateway technology (Invitrogen) using pSca [4] incorporating two mutations [16]. N-terminal EGFP (Clontech) tagged Daam1 was cloned into pENTR2B vector (Invitrogen) containing a viral subgenomic promoter and farnesylated membrane targeted red fluorescent protein (RFPf). The RFPf sequence was generated as described previously [10]. Viral particles were produced by transfecting BHK cells with SFV constructs, and prepared as described previously [10].

Organotypic hippocampal slices were prepared as described previously [10,29]. P6 mice were sacrificed, and the hippocampus was carefully dissected from the brain. The hippocampus was cut in slices of 300 μm thickness using a McIllwain tissue chopper (Stoelting), and placed onto culture plate inserts (0.4-μm pore size, Millipore). Inserts were incubated at 37 °C for 9 days, with media changes every 2–3 days. Slices were injected using a Picospritzer (General Valve), and incubated at 37 °C for 20 h, before being fixed in 4% paraformaldehyde/0.1 M PO_4^{2-} buffer for 30 min. Slices were imaged, or placed in blocking solution (10% goat serum and 0.1% TritonX-100) and incubated with anti-Daam1, followed by Alexa-Fluor488 goat-anti-rabbit. Imaging was performed with a spinning disk confocal system attached to a Nikon Eclipse TE2000. Images are maximal projections using MetaMorph from Z-series stacks with each stack 0.3 μm in thickness, using a 60× objective (with 1.5× zoom) and processed using Photoshop. For Fig. 3A, Daam1 localization within dendrites was determined by masking Daam1 immunostaining with the three-dimensional rendering of the dendritic segment shown (using IMARIS 4.5.2, Bitplane), leaving only those puncta within the dendritic spines and shaft. For analysis of spine morphology, at least 1000 spines were counted using Reconstruct (developed by J.C. Fiala and K.M. Harris, www.synapses.bu.edu) from 20 to 23 neurons for each condition (control and Daam1) from three independent experiments ± S.E. Each image contains a Z-stack maximum projection of a primary apical dendrite from a CA1 pyramidal cell. For these studies, spines

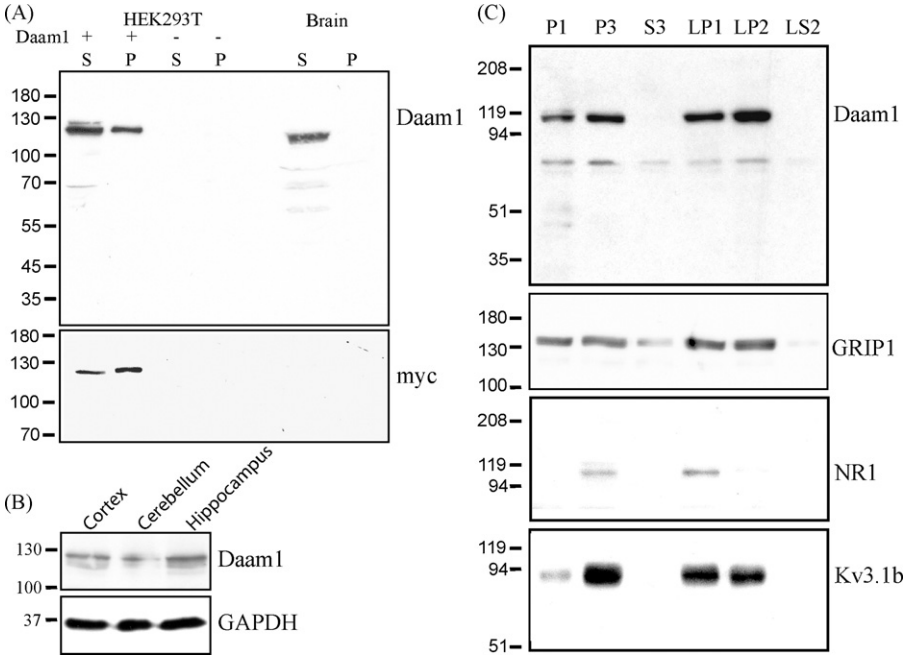

Fig. 1. Daam1 is expressed in mouse brain. (A) Immunoblot analysis of Daam1 on soluble (S) and insoluble pellet (P) extracts from transfected HEK293T cells and from a P32 mouse brain (top panel). Immunoblot analysis with a myc antibody confirms myc-Daam1 expression in transfected cells (bottom panel). (B) Expression of Daam1 in different brain regions. Protein extract from cortex, cerebellum, and hippocampus were immunoblotted for Daam1 and GAPDH. (C), Subcellular fractions of adult mouse brain reveal cofractionation of Daam1 with GRIP1, NR1, and Kv3.1b in the synaptic fractions (LP1 and LP2). P1, nuclear fraction; P3, microsomal membranes; S3, soluble protein fraction; LP1, synaptic membrane fraction; LP2, synaptic vesicle fraction; LS2, synaptosomal cytosolic fraction.