**Drosophila** Amphiphysin is implicated in protein localization and membrane morphogenesis but not in synaptic vesicle endocytosis

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**SUMMARY**

Amphiphysin family members are implicated in synaptic vesicle endocytosis, actin localization and one isoform is an autoantigen in neurological autoimmune disorder; however, there has been no genetic analysis of Amphiphysin function in higher eukaryotes. We show that *Drosophila* Amphiphysin is localized to actin-rich membrane domains in many cell types, including apical epithelial membranes, the intricately folded apical rhabdomere membranes of photoreceptor neurons and the postsynaptic density of glutamatergic neuromuscular junctions. Flies that lack all Amphiphysin function are viable, lack any observable endocytic defects, but have abnormal localization of the postsynaptic proteins Discs large, Lethal giant larvae and Scribble, altered synaptic physiology, and behavioral defects. Misexpression of Amphiphysin outside its normal membrane domain in photoreceptor neurons results in striking morphological defects. The strong misexpression phenotype coupled with the mild mutant and lack of phenotypes suggests that Amphiphysin acts redundantly with other proteins to organize specialized membrane domains within a diverse array of cell types.

Key words: Synapse, Postsynaptic density, Apical membrane, Membrane morphogenesis, *Drosophila*

**INTRODUCTION**

The creation of specialized membrane regions is important for establishing cell polarity and for the proper function of many differentiated cell types. Genetic and biochemical experiments have identified numerous proteins involved in the formation of distinct membrane domains; in neurons and yeast, one of these proteins is Amphiphysin (Amph; Amp – FlyBase). Vertebrate Amph I protein was discovered because of its enrichment in brain presynaptic terminal membrane preparations (Lichte et al., 1992), whereas Amph II is more widely expressed (Butler et al., 1997; Ramjaun et al., 1997). Both Amph I and II have an N-terminal coiled-coil domain, a central proline-rich domain and a C-terminal SH3 domain. Mutant analysis of vertebrate Amph I and II has not been described, but most evidence suggests that Amph proteins are involved in endocytosis, particularly in synaptic vesicle recycling (Wigge and McMahon, 1998). Amph I is concentrated at presynaptic terminals, and expression of the SH3 domain of Amph I can block endocytosis in neurons or fibroblasts, leading to concomitant accumulation of clathrin coated pits at the plasma membrane (Shupliakov et al., 1997; Wigge et al., 1997). In addition, the Amph I central region binds the endocytic proteins α-Adaptin and Clathrin, while the SH3 domain interacts with the endocytic proteins Dynamin and Synaptotagmin (David et al., 1996; Grabs et al., 1997; McPherson et al., 1996).

The role of Amphiphysin family members may not be limited to endocytosis. Vertebrate Amph II does localize to presynaptic sites, however, Amph II isoforms lacking the α-Adaptin-binding central domain are expressed in skeletal muscle (Butler et al., 1997). How, or if, these isoforms modulate endocytosis is not known. A *S. cerevisiae* Amph homolog, *rsv167*, shows conservation of the coiled-coil and SH3 domains, but the central domain is not well conserved nor is it needed for function (Sivadon et al., 1997).

We present the in vivo characterization of the single *Drosophila* amph (Amp – FlyBase) gene. We show that Amph protein is localized postsynaptically at neuromuscular junctions and is required for the localization of several postsynaptic proteins but not synaptic vesicle recycling. We also show that Amph is localized to specialized membrane domains in both epithelial and neural cell types, where it is
implicated in recruiting or stabilizing cortical proteins and organizing changes in membrane morphology.

MATERIALS AND METHODS

DNA constructs

The expressed sequence tags (ESTs) LD19810, LD02678, LD16210, HL01753, representing cDNAs of Amphiphysin were obtained from Berkeley Drosophila Genome Project (via Research Genetics). Each EST was sequenced. The RT-ISOC cDNA was identified and isolated via a reverse transcriptase and first strand synthesis (SuperScript II – Stratagene) with the following primer: 5'-GGACCACA-GAGGAATTACA-3'. The nested primer pair (5'-CCACAG-ACTGTTCCACA-3'/5'-CCCCAGTTTGTCGCTTCAGA-3') was subsequently used for amplification. The PCR products were cloned into the pCRII-TOPO vector (Invitrogen) and three independent clones for each were sequenced for confirmation of alternative splicing. The SalI/SpeI fragment representing the

Fig. 1. Amphiphysin gene organization and characterization of mutant alleles. (A) Amphiphysin consists of 10 exons extending over 18 kb of DNA, including a large 13 kb second intron that contains no detectable genes. The EP(2)2175 transposon is inserted 412 bp from the AUG encoding the first methionine of the cDNA; imprecise excision of the EP transposon produced the amph26 and amph54 null alleles. In amph26 there is a 3428 bp deletion (breakpoints –1925/1502) that removes the entire first exon; in amph54 there is a 1834 bp deletion (breakpoints –581/1253) that removes the entire first exon but does not remove the first exon of Sin3A. All phenotypes are observed with either mutant. The Sin3A and Gqα-3 genes are adjacent to the amph gene, but neither amph mutation affects the coding sequences of Sin3A or Gqα-3 genes, and the Sin3A and amph54 mutations complement each other (A. Razzaq, personal communication), consistent with amph54 having no effect on Sin3A function. (B) Western analysis of Drosophila extracts from Amphiphysin mutant and precise excisions (amph+) in each of the putative null mutants, amph26 and amph54 (data not shown), there is no immunoreactivity detected. (C) Schematic representations of the four Amph isoforms. In each case, the alternative splicing occurs in exon 8, reducing or eliminating the central domain of Amphiphysin (top); sequence analysis of the four isoforms from the end of exon 7 to the start of exon 9 (bottom). (D) Drosophila Amphiphysin does not interact with Drosophila Dynamin: western analysis and Coomassie staining of Dynamin binding to the SH3 domain of Amphiphysin. Drosophila extract was mixed with three different fusion proteins: GST, GST fused to the SH3 domain of Amph (GST-SH3) and GST fused to a mutated form of the SH3 domain (GST-SH3*). In each case, we could not detect any binding of Dynamin to the GST proteins. The asterisks indicate the GST proteins.
Characterization of Drosophila Amphiphysin

alternative spliced region of isoform C as well as the 3¢ end of Amphiphysin was substituted into the LD02678 EST via a SalI/NheI digest to create RT-ISOC, a full-length cDNA. UAS constructs: EcoRI/XhoI fragments of LD19810 (AmphA) and HL01753 (Amph B2) were cloned into PUAST (Brand and Perrimon, 1993) and transformed into flies.

Genomic analysis of amph26 and amph54
Genomic DNA was isolated from homozygous animals and subjected to a series of PCR reactions. The PCR reactions were designed to assay the entire genomic interval of amph for deleted segments. The following lists denotes the 5¢ position of the primer pairs used relative to alanine (+1) of the first methionine of the cDNA: –2846/2044; 1077/2372; 4669/6095; 12,799/13,483; 13,318/14,285; 13,734/15,410; 15,078/15982; 15,570/17,055; 16,633/17,764. Only the primer pair of 1077/2372 did not result in a band for either amph26 or amph54, but a subsequent PCR reaction using outside primers –2846/2372 did result in two PCR products ~1.6 kb and 3.4 kb (expected size 5.2 kb) for each of the above mutants, respectively. The PCR products were cloned and sequenced to identify the precise breakpoints of the deleted segment.

GST fusion proteins
The following regions of Amphiphysin were cloned into pGEX-4T1 (Pharmacia): amino acids 8-361, representing the coil-coiled domain, and 523-602, representing the SH3 domain. A third GST fusion protein was created that contained the SH3 domain but had these changes: G592R, P595L and A596G. The EcoRI/XhoI fragments of ESTs LD19810 and HL01753 were cloned into pGEX-4T1.

Immunohistochemistry and electron microscopy
Rabbit polyclonal antibodies to Amph were generated against the N-terminal (9907) and the SH3 (9906) GST fusion proteins. Embryos and larvae were stained as previously described (McDonald and Doe, 1997). Adult Drosophila heads were frozen in OCT and 14 µm sections were cut and fixed for 10 minutes in PEMFA (McDonald and Doe, 1997). The tissue was blocked in PBTB (phosphate-buffered saline (PBS) and 0.1% bovine serum albumin (BSA)) for 30 minutes, incubated in primary antibody (in PBTB) overnight at 4°C, washed in PBTB, incubated in secondary antibody for 2 hours at room temperature, washed in PBT (PBS and 0.1% Triton X), and mounted as previously described (Zelhof et al., 1997). The following primary antibodies were used: rabbit anti-Amph (9906 and 9907; 1:100), guinea pig anti-Dlg (1:1000), mouse anti-CSP (ab49; 1:50), mouse anti-Myc (Calbiochem; 1:100), sheep anti-Bifocal (1:1000), rabbit anti-Scribble (1:100), rabbit anti-LglC (1:100), mouse mAb2A12 (1:5), rhodamine-conjugated phalloidin (1:100-1000; Molecular Probes) and goat FITC-conjugated anti-horseradish peroxidase (HRP) (1:100). Histochemical detection was performed using the Vectastain Elite Kit (Vector Labs) in conjunction with metal enhanced DAB substrate (Pierce). Species-specific FITC-, Texas Red- and Cy5-conjugated secondary antibodies were obtained from Jackson Immunoresearch Labs (1:200). Electron microscopy of adult Drosophila eyes was as previously described (Baker et al., 1994).
Electrophysiology

Spontaneous miniature (minis or mEJP) and elicited synaptic potentials were recorded from bodywall muscles of third instar larvae in HL-3 solution using microelectrodes with an input resistance between 15 MΩ and 23 MΩ. Data were acquired and digitized using a PC computer with the use of pCLAMP 8 software (Axon Instruments). The analysis and presentation of figures were conducted on MiniAnalysis (Synaptosoft) and Origin (OriginLab). In all of the mini recordings, low extracellular Ca²⁺ (0.1 mM) and tetrodotoxin (TTX, 5 μM) were added to Ca²⁺-free HL-3 solutions to minimize multiple quantal events (Zhang et al., 1998). Samples used for final analysis were obtained from at least five different larvae. Total number of events (n) were pooled together for quantal analysis in histograms, cumulative probability plots and statistics. To study elicited transmitter release, a suction electrode was used to stimulate motor nerve axons that innervate muscles 6, 7, 12 and 13 in HL-3 solutions containing 1 mM Ca²⁺. At least three different larvae were used for data collection. All samples were recorded from muscles with resting potentials between –62 mV and –69 mV only. The resting potentials for amph²⁶; GluRIIB and control were –65.4±0.67 mV and –64.5±0.62 mV, respectively. There were no statistical differences between these resting potentials and the input resistances of the muscle cell. The amplitude of EJPs was analyzed for statistics using unpaired Student’s t-test, while the comparison of quantal size was conducted using Kolmogorov-Smirnov test. All of the statistical data are presented as mean±s.e.m. P<0.05 is considered significant.

Fly stocks

amph mutant alleles (amph²⁶, amph⁵⁴ and amph¹) (represents the
Characterization of Drosophila Amphiphysin

RESULTS

A single amphiphsin gene generates four protein isoforms

We identified amph in a misexpression screen (Rorth, 1996; Rorth et al., 1998) for genes that alter the branching pattern of sensory neuron axonal arbors. The transposon EP(2)2175 was inserted adjacent to the amph gene and produced a subtle disorganization of axon arborizations, which is due to the misexpression of amph. This amph gene is the only amph gene in Drosophila (Lloyd et al., 2000; Razzaq et al., 2000). We analyzed four amph ESTs and found that they represented three alternative splice isoforms encoding predicted proteins of 602 (AmphA), 522 (AmphB1) and 502 (AmphB2) amino acids that differ in the size of their central domain (Fig. 1). We generated antibodies to the Amph N- and C-terminal regions, and each antibody gave the predicted pattern on western blots, thus establishing the specificity of our antisera. In addition, both antisera detected a smaller 41 kDa band (AmphC) (Fig. 1B). We used RT-PCR from larval mRNA to confirm that all four proteins, including the 41 kDa AmphC isoform, are unique splice isoforms derived from the single amph gene. Each isoform contains the coiled-coil and SH3 domains (Fig. 1C).

Amph null mutants were created by imprecise excision of the EP(2)2175 transposon. Homozygous amph and amph flies were tested for protein expression using western blots and immunohistochemical staining of embryos, third instar neuromuscular junctions, and developing photoreceptor cells. We used antibodies that recognized the Amph N terminus, as well as antibodies recognizing the Amph C terminus, but we could not detect any protein expression (Fig. 1B). In addition, we mapped the lesions of amph and amph; both are deletions that remove the entire first exon and extend into the first intron (Fig. 1A). There is one predicted transcription start site in the 13 kb second intron (and no open reading frames), but we do not believe it is used because: (1) homozygous mutant embryos show no Amph protein; (2) using a primer representing the second putative start site, we could not detect any RT-PCR products (data not shown); and (3) all sequenced cDNAs contain the first exon and no alternative 5′ end was identified. As such, we conclude that both amph and amph are null alleles that produce no Amph protein product and do not affect the neighboring Sin3A gene.

precise excision of the EP(2) 2175 P-element) were provided by Dr C. O’Kane. The bifocalR38 and bifocalR47 alleles were provided by Dr W. Chia. The transgenic flies MHC-GluRIIB-Myc and MHC-GluRIIA-Myc were provided by Dr C. Goodman. Df(2R)vg-C was obtained from the Bloomington Stock Center.

Fig. 4. Amphiphysin is localized to the apical membrane domain of epithelial and neural cell types. (A-D) Lateral views of a developmental time series of a stage 5 embryo stained for Amph. At early stage 5, the syncitial nuclei have migrated to the periphery and the membrane has begun to invaginate between the nuclei. Amph is first localized to the apical surface and migrates basally as the membrane extends inwards during cellularization. (D) Upon the completion of cellularization at the end of stage 5, Amph is again localized apically. (E-H) Double labels of Amph and Bifocal protein localization during early cellularization (E,G) and after completion of cellularization (F,H). Amph protein (E,F) is enriched at the invaginating membrane during cellularization, whereas Bifocal protein (G,H) remains localized to the apical membrane domain. (I) Amph is apical in ectoderm and neuroblasts. Lateral view of a stage 9 embryo showing apical Amph in the ventral ectoderm and in neuroblasts (arrows). (J) Lateral view of a stage 16 embryo to emphasize that Amph is not detected in the embryonic CNS (bottom left panel) when compared with the staining in the epidermis and trachea (top panel) and hindgut (bottom right panel). (K,L) Amph is detected at the apical (luminal) membrane of tracheal tubules and of the esophagus. (K) Stage 15 embryo showing Amph expression at the apical surface in mature tracheal tubules. (L) Stage 15 embryo showing Amph expression in the esophagus. In addition, the non-epithelial secretory garland cells flanking the esophagus also express Amph. (M,N) Amph is localized to apical membrane and vesicles in internal tubular epithelia, such as the hindgut (M) and salivary glands (N). The arrow indicates Amph immunoreactive vesicles.
amphiphysin mutants lack obvious endocytic defects in synaptic vesicle recycling

Given the wide tissue distribution of Amph (see Fig. 2, Fig. 4, Fig. 5), and the existence of only one Drosophila amph gene, we expected amph mutants to have severe pleiotropic defects and in particular endocytic defects. To our surprise, amph null mutant animals survive to adulthood, although larvae move sluggishly and adults do not fly.

We tried several approaches to detect an endocytic defect in amph mutants. In vertebrates, Amph is implicated in endocytosis via its interaction with Dynamin, but we can not detect an interaction between the SH3 domain of Amph and Drosophila Dynamin (Fig. 1D), nor do we see protein colocalization with Dynamin in the embryo, neuromuscular junction or at any location that shows Amph expression. In addition, we have not been able to detect genetic interactions between amph and the endocytic mutant shibire (encoding Dynamin), although other endocytic mutants such as α-Adaptin show sensitive genetic interactions with shibire (Gonzalez-Gaitan and Jackle, 1997). Last, we find that amph mutants have normal presynaptic physiological properties, eliminating a role for Amph in synaptic vesicle recycling (Fig. 3).

Amphiphysin protein is enriched at the postsynaptic density of larval neuromuscular junctions

In order to explain the locomoter defects of both larvae and adults, we examined the larval neuromuscular junction (NMJ). First, Amph localization can be detected in the muscle and shows enrichment at the NMJ (Fig. 2). To determine whether it is presynaptic or postsynaptic, we did colocalization experiments with a postsynaptic marker, Discs large (Dlg; Dlg1 – FlyBase) (Lahey et al., 1994), and a presynaptic protein marker, Cysteine String Protein (Csp) (Umbach et al., 1994; Zinsmaier et al., 1994). We find that Amph and the postsynaptic marker Dlg are perfectly colocalized (Fig. 2A-D), whereas Amph protein surrounds but does not colocalize with the presynaptic marker Csp (Fig. 2E-H). Thus, the majority of the Amph protein is postsynaptic, although we cannot exclude the possibility that there are low levels of Amph at the presynaptic side of the NMJ. In addition, Amph appears to be specifically localized to the type I Dlg* glutamatergic synapses but absent from type II and III synapses (data not shown), which are thought to contain other neurotransmitters and modulators (Anderson et al., 1988; Cantera and Nassel, 1992; Gorczyca et al., 1993; Monastirioti et al., 1995).

Amphiphysin is required for postsynaptic protein localization and function

Given that Amph is localized to the NMJ, we next assayed for structural and functional defects at the neuromuscular junction (NMJ). amph mutants show no defects in the localization of presynaptic marker proteins (e.g. Dynamin, Cysteine String Protein and HRP) and there are no defects in bouton structure or number. By contrast, several postsynaptic proteins showed abnormal localization in amph mutants. In wild-type larvae, type I glutamatergic synapses show postsynaptic enrichment of Dlg (Fig. 3C) (Lahey et al., 1994). In addition, two other proteins, Scribble (Scrib) and Lethal giant larvae (L(2)gl – FlyBase), are colocalized with Dlg at the NMJ. Scrib, Lgl and Dlg are all tumor suppressor proteins that are colocalized in epithelia (Bilder et al., 2000). In amph mutants, there is a clear increase in Dlg and Scrib protein delocalized throughout
Characterization of *Drosophila* Amphiphysin

5011

... but we cannot detect a reduction at the synapse. *amph* mutants show a more severe Lgl phenotype: no Lgl protein can be detected at the synapse, although the localization of Lgl to the muscle M band is unaffected (Fig. 3C).

Dlg is considered to be a scaffold molecule for organizing the postsynaptic density, whereas the role of Lgl and Scrib at the synapse is not known. Changes in the localization of these proteins could potentially alter postsynaptic responsiveness to transmitter release. To test whether *amph* mutations alter synaptic physiology, we first measured the spontaneous release of transmitter by detecting the miniature excitatory junctional potentials (mEJPs). The amplitude of mEJPs (quantal size) is considered to be a measurement of the sensitivity of the muscle glutamate receptors to the spontaneous release of transmitter from single synaptic vesicles. We observed a small but statistically significant increase in mEJP amplitude in *amph* mutants (0.94±0.02 mV; n=2148) compared with wild-type controls (*amph*+/+, 0.81±0.01 mV; n=2804; P<0.05, Kolmogorov-Smirnov test) (Fig. 3A). Although quantal size in *amph* mutants is increased, elicited transmitter release measured by the amplitude of EJPs was found to be similar between *amph* mutant larvae and control larvae *amph*+/+: *amph* 26 was 35.5±1.29 mV (n=16) whereas *amph*+/+ was 34.9±1.22 mV (n=15) P>0.1 (Fig. 3B). The increase in quantal size suggests that the amount of transmitter per vesicle (i.e. quantum) is increased and/or that the postsynaptic receptors have become more sensitive to glutamate in the mutant. As Amph is primarily a postsynaptic protein, we reasoned that it is likely to play a direct role in regulating membrane organization or in selective protein targeting. To further investigate this possibility, we performed a detailed examination of Amph localization and expression patterns in embryonic, larval, pupal and adult tissues. We detected Amph in diverse cell types and in all...
Amph-expressing cells, Amph is restricted both spatially and temporally to a specific domain of the plasma membrane, typically a domain that is undergoing membrane remodeling or where a submembrane protein scaffold is being assembled.

During embryogenesis, Amph is first detected during cellularization, as membrane is being inserted between each nuclei of the syncitial blastoderm stage embryo. Initially, Amph is detected at the apical surface of the precellular embryo (Fig. 4A). As the membrane extends inward (basally) during cellularization, Amph decorates the leading edge (Fig. 4B,C,E-F); this differs from Bifocal (Bahri et al., 1997), which consistently labels apical membrane domain (Fig. 4G-H). Upon completion of cellularization, Amph returns to the apical surface of the newly formed cells (Fig. 4D), where it is colocalized with many apical membrane markers (Fig. 4G,H). Amph is subsequently localized to the apical membrane domain in the ventral ectoderm and in the neuroblasts that delaminate from this ectoderm (Fig. 4I), as well to the apical membrane of tubular internal tissues such as the esophagus, hindgut, trachea and salivary glands (Fig. 4J-N). In many of these tissues, Amph is also robustly detected in small punctate presumptive vesicles within the cytoplasm of the cell (Fig. 4N); the identity of these vesicles is unknown. Amph expression is not detected in embryonic postmitotic neurons or muscles (Fig. 4J and data not shown), although it is detected in larval muscles. Surprisingly, our analysis of amph mutants and misexpression studies did not reveal any pronounced defects in process of cellularization, trachea formation or asymmetric localization of cell fate determinants during embryonic neuroblast divisions; further indication that Amph function is either not necessary or redundant with other proteins.

**Amphiphysin aids in the organization of the rhabdomere membrane domain in photoreceptor neurons**

Adult *Drosophila* photoreceptor cells are specialized neurons that have an apical microvillar stack of intricately folded membranes, the rhabdomere, that contains the light-sensing rhodopsin proteins. Twenty-four hours after puparium formation (APF) – before the formation of the apical microvilli – each photoreceptor shows F-actin accumulation at the apical membrane, while Amph is distributed more diffusely (Fig. 5A-C). Forty-eight hours APF, Amph has localized into a dense crescent at the apical cortex of each photoreceptor neuron as the first actin-filled apical microvilli can be detected (Fig. 5D-F). By 55 hours APF, F-actin and Amph are tightly colocalized at the apical membrane domain of each photoreceptor neuron (Fig. 7D-F and data not shown), and it is this domain that will subsequently give rise to the rhabdomere. In the adult, Amph is no longer expressed in the photoreceptor cells, but can be detected in the lens-secreting cone cells (Fig. 5G-I).

Given the accessibility of *Drosophila* photoreceptors to genetic, immunohistochemical and electron microscopic examination, we investigated whether Amph is required for the biogenesis of the rhabdomere as a model for its general role in membrane morphogenesis. Homozygous amph mutant adults have superficially normal eyes that respond to light correctly. However, electron microscopy of amph mutant eyes reveals that the rhabdomere membranes are unusually closely packed and occasionally fused, thus creating very little inter-rhabdomere space (Fig. 7A).

To further explore the role of Amph in the elaboration and formation of the rhabdomeres, we used GMR-GAL4 to overexpress Amph in the developing photoreceptor neurons. Overexpression of GMR-GAL4 alone or with a control UAS-GFP transgene had no effect on rhabdomere morphology (Fig. 6A). By contrast, overexpression of the Amph can produce outwardly normal eye but with a loss of all recognizable internal cell structure by electron microscopy (data not shown). More informative weaker phenotypes can be generated by expressing Amph at a lower temperature (where GAL4 is less active and there is less Amph protein as confirmed by western blots; data not shown). Overexpression of intermediate levels of Amph produce two to three smaller
rhabdomeres per cell, split rhabdomeres or no rhabdomeres (Fig. 6B). Despite the severe morphological defects, these photoreceptor neurons are functional, and overexpression of Amph does not affect photoreceptor neuron cell fate decisions.

The Amph overexpression eye phenotype (split/ectopic rhabdomeres) is similar to the loss-of-function bifocal mutant phenotype (Bahri et al., 1997) (Fig. 7B), suggesting that amph and bifocal may act in the same genetic pathway. Bifocal is a novel protein that is colocalized with Amph at the apical membrane of newly formed embryonic cells (Fig. 4E-G), as well as at the apical membrane of photoreceptor neurons. In photoreceptor neurons, Bifocal expression precedes Amph and lasts into adulthood (data not shown). amph mutants show normal Bifocal localization (data not shown), but bifocal mutants show delocalization of Amph into a broad apical domain matching that of F-actin, rather than its normal tight apical crescent (Fig. 7D,E). The bifocal mutant phenotype can be suppressed by reducing Amph gene dosage by 50% (Fig. 7C). Thus, bifocalmediate localization of Amph to the future rhabdomere membrane domain is essential for normal eye development.

To determine the developmental origin of the amph overexpression phenotype, we assayed Amph, Bifocal and F-actin localization during rhabdomere development. Wild-type photoreceptors show an even distribution of Bifocal, Amph and F-actin at the apical surface of the cell (Fig. 6C,E). By contrast, photoreceptors overexpressing Amph have an abnormal punctate ‘ball’ of F-actin and Bifocal at the apical cortex (Fig. 6D,F) and Amph is delocalized from the apical membrane (Fig. 6G,H). We conclude that excess Amph protein leads to destabilization of the normal apical Amph localization, a mislocalization of Bifocal protein and F-actin, and the subsequent failure in rhabdomere morphogenesis.

These data lead to the following model for Amph function. Bifocal is localized to the apical membrane of photoreceptor neurons, where it recruits Amph and other proteins. This protein complex then aids in the morphogenesis of the intricately folded rhabdomere membrane. Loss of Amph results in a mild phenotype, perhaps because Bifocal and additional proteins are still apically localized and can promote rhabdomere morphogenesis. However, when Amph is mislocalized outside of the apical membrane domain (in Amph overexpression experiments or in bifocal mutants), it can induce the formation of ectopic rhabdomere membrane domains or inhibit rhabdomere morphogenesis by relocating or titrating away the necessary protein complex to form a rhabdomere.

DISCUSSION

Drosophila Amphiphysin is an ortholog of Amph II

All known Amph family proteins contain a N-terminal coiled-coil domain and a C-terminal SH3 domain with a variable central domain. In Amph I and Amph II, the central domain provides an additional link to endocytosis via the inclusion of binding sites for clathrin and α-Adaptin (Slepnev et al., 2000). Although sequence comparison does not clearly indicate whether Drosophila Amph is more related to either vertebrate Amph I or Amph II, examination of expression profiles, isoform organization and inferred functions suggest that Drosophila Amph is likely to be an Amph II ortholog.

First, both Drosophila Amph and vertebrate Amph II have a broad tissue distribution, including postsynaptic expression in skeletal muscle (Butler et al., 1997). Second, both Drosophila Amph and Amph II genes have isoforms that partially or completely remove the central domain (Fig. 1B) (Butler et al., 1997). Third, we show that Drosophila Amph is sufficient to organize membrane morphogenesis in photoreceptor neurons and necessary for protein localization at the postsynaptic NMJ. Similarly, the putative localization of Amph II to nodes of Ranvier and the localization of yeast Rvs167 to the protruding bud and shmoo membranes, suggests that Amph II and Rvs167 may be involved in regulating membrane morphogenesis, rather than endocytosis.

The absence of detectable endocytic defects in synaptic vesicle recycling in amph mutants is surprising, given the evidence linking Amph and synaptic vesicle endocytosis in vertebrate systems (Wigge and McMahon, 1998). Our results cannot completely eliminate a role of Amph in endocytosis. We find Amph protein on vesicles within cells known to be actively undergoing endo- and exocytosis, such as the hindgut, garland gland and salivary gland. A detailed characterization of the amph phenotype in these cells may ultimately reveal a role for Amph in regulating endocytosis.

Membrane morphogenesis and protein localization

There are two common features of Amph expression. First, Amph always localizes to a restricted subcellular membrane domain, typically the apical membrane in epithelial or neuronal cells. Second, Amph is detected at membranes that are undergoing ‘remodeling’ or morphogenesis. During cellularization, the addition of membrane occurs at defined sites and in a precise sequence (Lecuit and Wieschaus, 2000). Amph localization precisely correlates with the bi-phasic insertion of new membrane: first apical and then apical-lateral. In the tubular tracheal system, Amph is localized to the inner (apical) membrane domain. It has been hypothesized that tracheal tube size is controlled by regulating surface area of the apical membrane: during tracheal tube dilatation, the inner (apical) diameter of the tube increases dramatically, whereas the outer (basal) diameter shows little or no change (Beitel and Krasnow, 2000). Thus, Amph is present on the membrane domain that undergoes regulated alteration in curvature and surface area and as such might regulate the changes observed. Similarly, the Amph-related yeast Rvs167 protein is localized to the protruding bud or shmoo membrane (Balguerie et al., 1999), another actively ‘remodeled’ membrane domain.

Not only do we see Amph detected at membranes undergoing elongation or changes in surface area, we also detect Amph at highly folded membrane domains. For example, each photoreceptor rhabdomere is an interconnected stack of membranes that emerges from the apical neuronal membrane. The process of rhabdomere site selection, initiation, and elaboration is poorly understood. Our results indicate a role for Amph in organizing the intricately folded rhabdomere membrane. First, accumulation of Amph on the apical surface occurs together with an enrichment of F-actin at this site. Second, overexpression of Amph (by targeted misexpression or in bifocal mutants) results in the delocalization of endogenous Amph and the mislocalization of both F-actin, leading to either loss or ectopic rhabdomeres. Loss of rhabdomeres may be due to excess Amph titrating out...
factors necessary for the proper development of rhabdomeres; ectopic rhabdomeres may be due to ectopic Amph recruiting sufficient F-actin or other proteins to a second site in the cell and thus triggering formation of an extra rhabdomere. The development or maintenance of a cellular three-dimensional structure, like a rhabdomere or synapse, not only includes the rearrangement of the membrane and actin cytoskeleton but also involves the correct targeting and anchoring of proteins to these locations. Consistent with a role in protein targeting/anchoring, we find that in amph null mutants multiple postsynaptic proteins are mislocalized. Dlg and Scrib are partially delocalized, and Lgl is completely undetectable at the postsynaptic membrane (although Lgl staining at the muscle M line is unaffected). These defects are not due to a general delocalization of all postsynaptic proteins, as we observe normal localization of two different epitope-tagged glutamate receptors to the postsynaptic membrane.

How does the removal of Amph result in the delocalization of Lgl, Dlg and Scrib at the synapse? One possibility is that Amph may play a role in the establishment or maintenance of postsynaptic membrane structure. One characteristic of type I synapses is that they have an extensive subsynaptic reticulum, the highly folded membrane surrounding the synapse, that is not present in type II and type III synapses. amph mutants may have defects in the folded membrane that do not allow for proper retention of proteins at the synapse. Alternatively, Amph may act as a localized scaffold protein that recruits Lgl to the postsynaptic membrane, with Lgl being necessary for proper anchoring or targeting of Dlg and Scrib. This would be consistent with the known role of Lgl in mediating Dlg and Scrib localization in epithelia (Bilder et al., 2000). Lgl belongs to a family of proteins that regulate polarized exocytosis and protein targeting to specific membrane domains (Fujita et al., 1997; Lehman et al., 1999; Oshiro et al., 2000; Peng et al., 2000). In addition, deletion analysis of Dlg has identified a two-step process of synaptic targeting; Dlg is first targeted to the muscle plasma membrane and then to the subsynaptic reticulum (Thomas et al., 2000). Loss of Amph function gives a phenotype consistent with a failure in the second step of this process; however, we have been unable to detect direct interactions between Amph and Dlg proteins in vitro (data not shown). Further analysis of the physical interactions between Amph and Lgl, Dlg or Scrib may help illuminate the mechanism of postsynaptic protein localization, and the analysis of lgl and scrib synaptic phenotypes will be required to determine whether the functional defects at amph mutant synapses (increase in quantal size) are due to the mislocalization of Lgl, Dlg or Scrib.

Does Amphiphysin link the actin cytoskeleton with membrane morphogenesis?

How does Amph regulate membrane morphogenesis? Amph could directly regulate membrane morphogenesis, leading to changes in membrane topology. Alternatively, Amph could directly modify membrane structure, and only indirectly affect the actin cytoskeleton. These models are not mutually exclusive. The latter model (direct membrane binding) is supported by studies of mammalian Amph I, which show that Amph can directly bind lipid bilayers and distort them into high-curvature membranes; for example, Amph I can transform spherical liposomes into narrow tubules (Takei et al., 1999). The former model (actin regulation) is supported by work on yeast, Drosophila and vertebrate Amph proteins. First, there is the tight correlation between Amph expression and actin organization in all organisms tested: yeast bud and schmoo membrane extensions, and Drosophila cellularization and rhabdomere formation; and vertebrate nodes of Ranvier are all sites of F-actin enrichment and Amph localization. Second, our results show that the delocalization of Amph from the apical surface of photoreceptor cells, in bifocal mutants or amph overexpression mutants, results in the mislocalization of F-actin. Last, in yeast, this idea is supported by the identification of a protein interaction between Rvs167 SH3 domain and actin binding protein Abp1 (Lila and Drubin, 1997) as well as synthetic lethality between rvs167 and a subset of act1 alleles (Breton and Aigle, 1998). In Drosophila, it is currently unknown what protein(s) bind to the Amph SH3 domain; it will be interesting to determine if a similar actin-binding protein-SH3 domain interaction occurs in Drosophila. This type of biochemical analysis, as well as further genetic studies, will be necessary to understand the relationship between Amph, the actin cytoskeleton and membrane morphogenesis.

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