Internalization of LDL-receptor superfamily yolk-protein receptors during mosquito oogenesis involves transcriptional regulation of PTB-domain adaptors

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Accepted 28 January 2008
Journal of Cell Science 121, 1264-1274 Published by The Company of Biologists 2008
doi:10.1242/jcs.025833

Summary

In the anautogenous disease vector mosquitoes *Anopheles gambiae* and *Aedes aegypti*, egg development is nutritionally controlled. A blood meal permits further maturation of developmentally repressed previtellogenic egg chambers. This entails massive storage of extraovarian yolk precursors by the oocyte, which occurs through a burst of clathrin-mediated endocytosis. Yolk precursors are concentrated at clathrin-coated structures on the oolemma by two endocytic receptors, the vitellogenin and lipophorin receptors. Both these mosquito receptors are members of the low-density-lipoprotein-receptor superfamily that contain FxNPxY-type internalization signals. In mammals, this tyrosine-based signal is not decoded by the endocytic AP-2 adaptor complex directly. Instead, two functionally redundant phosphotyrosine-binding domain adaptors, Disabled 2 and the autosomal recessive hypercholesterolemia protein (ARH) manage the internalization of the FxNPxY sorting signal. Here, we report that a mosquito ARH-like protein, which we designate trephin, possess similar functional properties to the orthologous vertebrate proteins despite engaging AP-2 in an atypical manner, and that mRNA expression in the egg chamber is strongly upregulated shortly following a blood meal. Temporally regulated trephin transcription and translation suggests a mechanism for controlling yolk uptake when vitellogenin and lipophorin receptors are expressed and clathrin coats operate in previtellogenic ovaries.

Key words: LDL receptor, Adaptor, Clathrin-mediated endocytosis, Transcriptional regulation, Yolk protein

Introduction

The original description of clathrin-coated vesicles as intracellular transport shuttles was based upon ultrastructural studies of developing mosquito oocytes (Roth and Porter, 1964). Vesicular profiles encased within a 'bristle coat' were very prominent at the cortex of *Aedes aegypti* oocytes several hours following a blood meal (Roth and Porter, 1964). The dramatic appearance of this bristle border, which we now know is composed of clathrin and associated coat proteins, reflects the fact that both *A. aegypti* and *Anopheles gambiae* mosquitoes are anautogenous – adult females must feed before egg production (Raikhel and Dhadialla, 1992). Thus, unlike *Drosophila melanogaster*, where a succession of progressively more mature egg chambers develop continuously (Drummond-Barbosa and Spradling, 2001; Schonbaum et al., 2000), egg maturation in newly eclosed *A. aegypti* or *A. gambiae* females developmentally arrests at a previtellogenic stage (Raikhel and Dhadialla, 1992; Roth and Porter, 1964). A blood meal initiates the process of vitellogenesis – the uptake and storage of yolk. It is this anautogenous character that makes mosquitoes efficient disease vectors because reproduction obligatorily requires frequent contact with vertebrate hosts. In *A. aegypti* and *A. gambiae* females, a blood meal triggers a synchronous burst of vitellogenesis whereby primary follicles all increase in size ~300-fold over ~48 hours (Anderson and Spielman, 1971; Sappington et al., 1996), mainly because of accumulation of yolk granules within the oocyte.

The presence of clathrin-coated structures at the oolemma indicates that yolk precursors stored within membrane-bound yolk granules are not manufactured by the oocyte (Anderson and Spielman, 1971; Roth and Porter, 1964). Rather, the major site of yolk protein synthesis is the fat body, an arthropod organ that is a functional hybrid of the vertebrate liver and adipocyte combined (Rodenburg and Van der Horst, 2005). In adult female mosquitoes, the fat body synthesizes both vitellogenin (a lipophosphoglycoprotein) and lipophorin [a lipid-rich lipoprotein particle resembling mammalian low density lipoprotein (LDL)], which are secreted directly into the hemolymph. Specific receptors for both vitellogenin and lipophorin are expressed on the *A. aegypti* oocyte to concentrate and then relay the major circulating yolk components into the oocyte interior (Cheon et al., 2001; Snigirevskaya et al., 1997; Sun et al., 2000).

The structure of the mosquito vitellogenin and lipophorin receptors clearly indicates they are members of the LDL receptor superfamily (Cheon et al., 2001; Cho and Raikhel, 2001; Sappington et al., 1996). In most vertebrate members of the LDL receptor superfamily, such as the LDL receptor, the LDL-receptor-related protein 1 (LRP1) and megalin, one or more sorting signals of the FxNPxY type within the cytosolic domain specify clustering within clathrin-coated structures (Bonifacino and Traub, 2003; Davis et al., 1986). The mosquito lipophorin receptor has a canonical FxNPxY sequence in the cytosolic portion whereas both the *A.*

aegypti and *A. gambiae* vitellogenin receptors have an apparently degenerate FxNPxL sequence (Sappington et al., 1996). The *Caenorhabditis elegans* vitellogenin receptor, RME-2, is similarly an LDL receptor superfamily member and displays a standard [FY]xNPxY sorting signal (Grant and Hirsh, 1999).

Several lines of evidence indicate that, in chordates, the FxNPxY signal is not decoded by the classic AP-2 clathrin adaptor complex. First, the FxNPxY sequence has a strong propensity to adopt a type-1 β-turn conformation (Hutchinson and Thornton, 1994) that is structurally incompatible with engagement of the μ2 subunit, which binds to YxxØ-type sorting signals (Owen and Evans, 1998; Stolt et al., 2003; Yun et al., 2003). Accordingly, extinguishing HeLa cell AP-2 with small-interfering RNA (siRNA) oligonucleotides halts efficient transferrin receptor (YxxØ-type signal) but not LDL receptor (FxNPxY) internalization (Keyel et al., 2006; Maurer and Cooper, 2006; Motley et al., 2003). Two phosphotyrosine-binding (PTB)-domain proteins, termed Disabled 2 (Dab2) and the autosomal recessive hypercholesterolemia (ARH) protein, are instead responsible for LDL receptor endocytosis from the cell surface (Maldonado-Baez and Wendland, 2006; Robinson, 2004; Sorkin, 2004). Gene silencing of both Dab2 and ARH with siRNAs leads to prominent accumulation of the LDL receptor but not the transferrin receptor at the cell surface (Keyel et al., 2006; Maurer and Cooper, 2006). Consistent with the idea of AP-2-independent uptake of the FxNPxY signal, the operation of nematode RME-2 during yolk storage, although dependent on both clathrin and dynamin, is not inhibited by AP-2 μ2 or σ2 subunit RNA interference (RNAi), either alone or in combination (Grant and Hirsh, 1999). This suggests that RME-2 uptake does not depend on the heterotetrameric AP-2 complex, because the recognition of YxxØ signals requires the μ2 subunit (Bonifacino and Traub, 2003; Owen and Evans, 1998) and engagement of another sorting motif decoded by AP-2 – the [DE]xxxL[LI] signal (Bonifacino and Traub, 2003) – requires a hemicomplex of the α and σ2 subunits (Chaudhuri et al., 2007; Coleman et al., 2005; Doray et al., 2007; Janvier et al., 2003). Because of the demonstrated role of PTB-domain proteins in the recognition of the vertebrate FxNPxY signal, we have assessed the role of related PTB-domain proteins in mosquito yolk accumulation, a process vital for reproductive success. Striking transcriptional upregulation of a functionally equivalent ARH-like protein occurs within mosquito oocytes after blood feeding, revealing a novel form of regulation of clathrin-mediated endocytosis.

Results

LDL receptor superfamily members are widely represented in insect genomes (Chen et al., 2004; Ciudad et al., 2006; Gopalapillai et al., 2006; Rodenburg et al., 2006; Schonbaum et al., 2000; Tufail and Takeda, 2005; Tufail and Takeda, 2007). Within the cytosolic domain of many of these transmembrane receptors, a typical FxNPxY sorting signal is positioned analogously to the chief sorting signal in the mammalian LDL receptor (Table 1). In non-polarized mammalian cells, this FxNPxY signal is entirely responsible for the rapid internalization of the LDL receptor (Chen et al., 1990; Davis et al., 1986). In most instances the function of this putative endocytic sorting signal has not been directly verified in insects, yet both the vitellogenin and lipophorin receptors internalize bound ligands into the developing oocyte and recycle back to the cell surface (Atella et al., 2006; Rodenburg and Van der Horst, 2005; Seo et al., 2003; Snigirevskaya et al., 1997; Sun et al., 2000). The significant conservation of the key specificity-determining residues and positioning relative to the membrane surface therefore suggests that, as in chordates, invertebrate PTB-domain clathrin-associated sorting proteins (CLASPs) might decode this sorting signal to ensure efficient receptor endocytosis in insect cells.

A presumptive ARH-like CLASP in mosquitoes

ARH orthologues occur in mammals, amphibians and bony fish (Jones et al., 2003; Zhou et al., 2003). Putative ARH-like orthologues also exist in the disease vector mosquitoes *A. gambiae* (ENSANGP00000011117; GenBank accession number: XM_316919) (Zhou et al., 2003) and *A. aegypti* (DFCI Aedes aegypti Gene Index TC47196; VectorBase Gene ID: AAEL012518). The insect proteins have a similar size and domain organization as vertebrate ARH isoforms (Fig. 1A) and the N-terminal PTB domains of all these ARH proteins display substantial primary sequence homology. The human and *Xenopus* PTB domains are 91% identical, whereas these domains are both about 41% identical and 59% similar to the *Anopheles* PTB domain. This degree of sequence relatedness is similar to that of the *A. gambiae* LRP1 orthologue to the

Table 1. Putative invertebrate endocytic sorting signals

Species	Receptor	Accession No.	Sequence*
Homo sapiens	LDL	L00352	Tm-KNWRLKNISNIN**F**D**NP**V**Y**QKTTE-27
Xenopus laevis	Vitellogenin	AB006906	Tm-RNWQRKNMKSMN**F**D**NP**V**Y**LKTTE-31
Leucophaea mederae	Vitellogenin	AB255883	Tm-KYGNFKDSKFGFSMH**F**S**NP**T**F**GVQSP-84
Drosophila melanogaster	Yolkless	U13637	Tm-RQRGHTDLNINMH**F**Q**NP**LATLGGT-137
Apis mellifera	Vitellogenin	XM_001121707	Tm-QIKPNFSKKNNLSIH**F**Q**NP**S**Y**DQRNE-34
Solenopsis invicta	Vitellogenin	AY262832	Tm-KNKLKSKPASNLSCSSIH**F**Q**NP**S**Y**DRSDE-64
Blattella germanica	Vitellogenin	AM050637	Tm-KYRGKKNSKFGFSMH**F**Q**NP**T**F**GVRNS-86
Aedes aegypti	Vitellogenin	L77800	Tm-RRRYQHKFDIGMH**F**H**NP**ELSTADA-79
Periplaneta americana	Vitellogenin	AB077047	Tm-KRFGYKGPKLNFSLH**F**K**NP**T**F**GIKES-67
Leucophaea mederae	Lipophorin	AB218869	Tm-RHYLHRNVTSMN**F**D**NP**V**Y**RKTPE-37
Apis mellifera	Lipophorin	XM_395858	Tm-RHYLHRNVTSMN**F**D**NP**V**Y**RKTTE-34
Galleria mellonella	Lipophorin	DQ482581	Tm-RHYLHRNVTSMN**F**D**NP**V**Y**RKTTE-38
Bombyx mori	Lipophorin	AB201473	Tm-RHYLHRNVTSMN**F**D**NP**V**Y**RKTTE-37
Tribolium castaneum	Lipophorin	XM_962851	Tm-RKYVKRNMTSMN**F**D**NP**V**Y**RKTTE-38
Aedes aegypti	Lipophorin	AF355595	Tm-KHHVHRNSTSMN**F**D**NP**V**Y**RKTTE-35
Drosophila melanogaster	Lipophorin 2	AE014297	Tm-RRCTRAVNSMN**F**E**NP**V**Y**HKTTE-33
Caenorhabditis elegans	RME-2	NM_06814	Tm-60-KRIRRMDSSPTS**Y**G**NP**M**Y**DEVPE-24

*Residues are in single letter amino acid notation. Tm, transmembrane domain. Numbers indicate residues preceding or following the regions shown to the extreme C terminus of each protein.

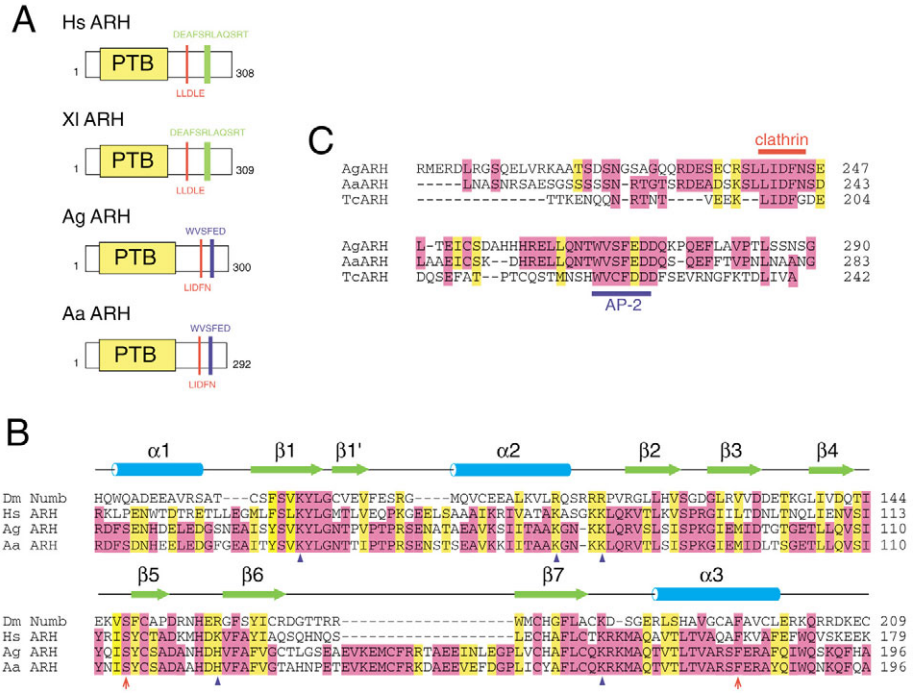

Fig. 1. Mosquito ARH-like orthologues. (A) Schematic of the domain organization of human (Hs), *Xenopus laevis* (Xl), *A. gambiae* (Ag) and *A. aegypti* (Aa) ARH and ARH-like proteins. The location of clathrin- (red) and AP-2 β2- (green) or putative α-appendage-binding (blue) interaction motifs is shown. (B) Sequence alignment of the PTB-domain regions of *D. melanogaster* (Dm) Numb (FlyBase Gene ID CG3779; PBD accession number: 1DDM), human ARH and *Anopheles* and *Aedes* ARH-like proteins. Numbered secondary structure elements (α helix, blue; β-strand, green) of the Numb PTB domain are indicated above. Identical residues (magenta) and chemically conservative substitutions (yellow) are boxed. Positionally conserved basic residues that, based on the structures of the Dab1 and Dab2 PTB domains, are probably involved in phosphoinositide binding (vertical arrowheads) and in FXNPXY-sequence engagement (vertical arrows) are indicated. (C) Sequence alignment of the C-terminal portions of the *Anopheles*, *Aedes* and *Tribolium castaneum* (Tc) ARH-like proteins. Conservation colored as in B and the location of the putative clathrin (red) and AP-2 α-appendage (blue) interaction motifs is indicated.

mammalian receptor (Moita et al., 2005). The two mosquito PTB domains are ~80% identical to one another and roughly 43% identical (60% similar) to a presumptive ARH orthologue (XM_962297) from the red flour beetle, *Tribolium castaneum*. In addition, two overlapping partial *Apis mellifera* ESTs (XM_624673 and XM_001122880) are about 53% identical and 63% similar to the two mosquito ARH-like PTB domains. Alignments clearly reveal that the vertebrate ARH/Dab2/numb/CED-6 PTB domain is the most closely related branch of the various PTB-domain proteins and dendrograms (TreeFam accession number: TF501786) illustrating the phylogenetic relationship of these domains indicate that they all fall within the Dab-like branch (Smith et al., 2006; Uhlik et al., 2005). This is significant because, in contrast to Dab-like PTB domains, members of the IRS- and Shc-like PTB-domain protein families require a phosphorylated NPx(*P*)Y ligand (Uhlik et al., 2005) and there is currently no evidence for phosphorylation of FxNPxY signals promoting endocytosis.

Structure-based sequence alignments additionally revealed that numerous core residues necessary for attaining the overall PTB-domain β-sandwich fold, and residues required for binding FxNPxY ligands, are conserved (Fig. 1B). The basic side chains, which, based on the crystal structures of the Dab1 and Dab2 PTB domains (Stolt et al., 2003; Yun et al., 2003), probably confer phosphoinositide binding, have also been retained through evolution. Indeed, liposome sedimentation assays validated that mosquito ARH-like proteins bound selectively to phosphatidylinositol-4,5-bisphosphate [PtdIns(4,5)P_2], similarly to mammalian ARH and Dab2 (Mishra et al., 2002a; Mishra et al., 2002b). Compared with glutathione *S*-transferase (GST)-fused human ARH, a GST-*A. aegypti* ARH (*Aa*ARH) bound to PtdIns(4,5)P_2-containing liposomes considerably better than to liposomes lacking this phospholipid (Fig. 2A, lane h compared with lane f). That this interaction is due to the PTB domain was supported by the strong association of residues 21-192 of the *Aa*ARH fused to GST with PtdIns(4,5)P_2 liposomes (Fig. 2A, lane l compared with lane j).

The *Anopheles* and *Aedes* ARH-like proteins differ from the vertebrate forms in two notable respects. First, there is a 19-amino-acid insertion within the PTB domain, which, based on the available PTB-domain structures of *Drosophila* Numb (Zwahlen et al., 2000), and mammalian Dab1 and Dab2 (Stolt et al., 2003; Yun et al., 2003), probably extends the loop between strands β6 and β7 of the PTB-domain β-sandwich considerably (Fig. 1B). The location of this insertion may modulate the recognition of potential FxNPxY-bearing ligands, because residues projecting off this loop are involved in accommodating the Tyr residue of the FxNPxY motif and discriminating between tyrosine and phosphotyrosine (Yan et al., 2002). The insert is absent from the related *T. castaneum* and *A. mellifera* proteins, suggesting it might be a dipteran feature.

To directly assess whether the mosquito ARH-like PTB domain associates physically with domain-engaging FxNxPY-type sequences we utilized the yeast two-hybrid interaction assay. *Aa*ARH (21-292) fused to the Gal4 DNA-binding domain indeed interacts with the cytosolic domain (residues 1099-1156) of the *A. aegypti* lipophorin receptor or with the cytosolic sector (residues 1824-1937) of the *D. melanogaster* vitellogenin receptor, Yolkless, fused to the Gal4 activation domain (Fig. 3). Only in the presence of both partners did the transformed AH109 yeast cells grow on plates lacking His, Leu and Trp. Introduction of two amino acid substitutions (S95T and F162V; see Fig. 1B) into the PTB domain of *Aa*ARH, which did not alter PtdIns(4,5)P_2 binding of the mammalian PTB fold (Stolt et al., 2005), abolished the association between the ARH and either receptor (Fig. 3). Deletion of the C-terminal 36 residues of the lipophorin receptor did not impede the interaction with the *Aa*ARH, because the truncated fusion protein still contained the FxNPxY sequence. However, substitution of the anchor Tyr for Cys (Y1116C) – mirroring a hypercholesterolemic disease mutation in humans (Davis et al., 1986) – abolished the interaction. The truncated wild-type protein also did not associate with the *Aa*ARH S95T and F162V PTB-domain double mutant to

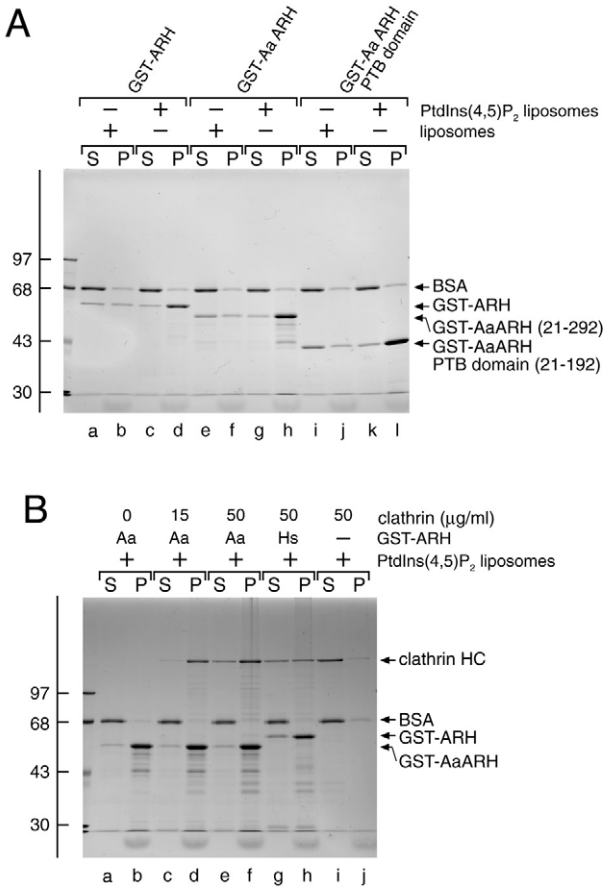

Fig. 2. Phosphoinositide binding of the *A. aegypti* ARH-like protein. (A) Human GST-ARH (60 μg/ml, residues 1-308; lanes a-d), GST-*Aa*ARH (residues 21-292; lanes e-h) or GST-*Aa*ARH PTB domain (residues 21-192, lanes i,j,k,l) were incubated with synthetic liposomes without (lanes a,b,e,f,i,j) or with PtdIns(4,5)P_2 (lanes c,d,g,h,k,l) on ice in the presence of 100 μg/ml BSA. After centrifugation, aliquots of 3% of each supernatant (S) and 25% of each pellet (P) were resolved by SDS-PAGE and stained with Coomassie Blue. The position of the molecular mass standards (in kDa) is indicated. (B) GST-*Aa*ARH (60 μg/ml, residues 21-292, lanes a-f) or GST-ARH (residues 1-308, lanes g,h) were incubated with PtdIns(4,5)P_2 liposomes in presence of 100 μg/ml BSA and either no (lanes a and b) or increasing concentrations (3 μg, lanes c,d or 6 μg, lanes e-j) of clathrin. After centrifugation, aliquots of 3% of each supernatant (S) and 25% of pellets (P) were resolved by SDS-PAGE and stained. The position of the clathrin heavy chain (HC) is indicated.

permit growth on triple-dropout plates. As these various mutations impede FxNPxY sequence recognition by the PTB domain (Howell et al., 1999; Stolt et al., 2005; Stolt et al., 2004), we conclude that the mosquito PTB domain is functionally homologous to the chordate ARH PTB domain.

The second striking difference between insect and chordate ARH proteins is that the sequence of the carboxyl ~100 residues of the mosquito ARH-like protein bears little homology to the vertebrate proteins. Pairwise sequence alignments suggest that this C-terminal segment of the mosquito ARH-like proteins probably display limited secondary structure. This is because there is only 55% identity (67% similarity) between the C-terminal 108 residues of the *A. aegypti* and *A. gambiae* proteins opposed to 81% (90% similarity) for the N-terminal 193 residues. Since unstructured or surface-exposed residues generally mutate with double the frequency of side chains within folded modules (Brown et al., 2002; Valdar and Thornton, 2001), one interpretation of the low sequence identity between the C-terminal portions of the *A. aegypti* and *A. gambiae* proteins is that this region is intrinsically disordered, similarly to numerous vertebrate CLASPs (Brett et al., 2002; Kalthoff et al., 2002; Owen et al., 2004). Another independent indication of a general propensity for disordered conformations is the high frequency (between 7 and 19%) of serine, glutamic acid, aspartic acid and leucine residues within the C-terminal segments of the mosquito ARH-like proteins; these side chains are typically more abundant in intrinsically unstructured regions of proteins (Zhang et al., 2007).

Remarkably, however, both a putative clathrin-box sequence (^{242}LIDFN) and a potential AP-2-adaptor-binding Wxx[FW]x[DE] motif are present in both *A. gambiae* and *A. aegypti* proteins (Fig. 1C). In fact, these two sequence motifs are essentially the largest major blocks of extended conserved sequence within the C-terminal segments of the *A. gambiae* and *A. aegypti* proteins (Fig. 1C). Moreover, the C-terminal segment of the presumptive ARH orthologue from *T. castaneum* displays little homology to the sequences of the *A. aegypti* and *A. gambiae* proteins except for core clathrin and AP-2-binding motifs (Fig. 1C). The same is true for the *A. mellifera* orthologue. These putative endocytic interaction sequences could impart the same functional capacities to the insect ARH-like proteins. Indeed, when fused to GST, the C-terminal 105 amino acids of *A. gambiae* ARH (*Ag*ARHC1) affinity isolated both AP-2 and clathrin from rat brain cytosol (Fig. 4A, lane d) whereas GST alone does not (lane b). The *A. aegypti* clathrin heavy chain is 81% identical (90% similar) to the mammalian heavy chain (Kokoza et al., 1997) and the extent of *A. aegypti* GST-ARH binding to mammalian clathrin is similar to that of the human GST-ARH counterpart. Binding of AP-2 by the mosquito ARH-like protein was less robust (Fig. 4A, lane f compared with lane d), although the presence of the α and β large chains and the μ2 subunit confirmed that the heterotetrameric adaptor complex was recognized. This is in agreement with the Wxx[FW]x[DE] motif engaging AP-2 more weakly than the $[DE]_nx_{1\text{-}2}$Fxx[FL]xxxR motif

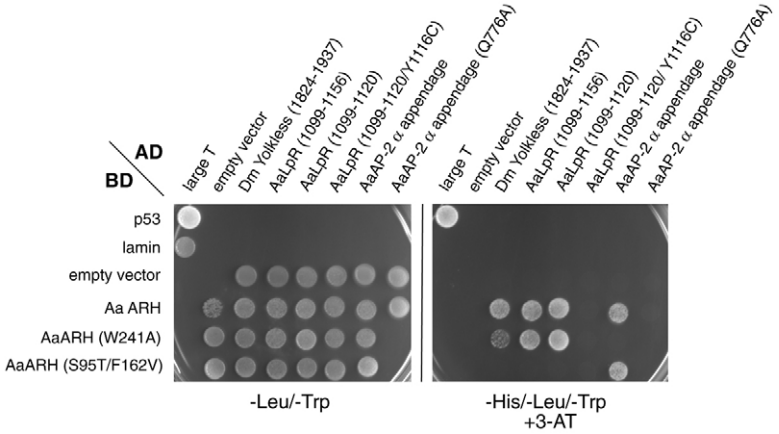

Fig. 3. Protein interactions in a yeast two-hybrid assay. *Saccharomyces cerevisiae* strain AH109 were transformed with the indicated plasmid combinations containing the pGBKT7-binding domain (BD) and the pGADT7-activation domain (AD), and spotted onto SD minimal medium plates lacking either Leu and Trp or His, Leu and Trp supplemented with 0.75 mM 3-amino-1,2,4-triazole (3-AT) and grown at 30°C.

found in vertebrate ARH proteins (Edeling et al., 2006; Mishra et al., 2005; Schmid et al., 2006).

The ability to engage clathrin was retained when the GST-*Aa*ARH (21-292) was associated with the surface of PtdIns(4,5)P_2-containing liposomes (Fig. 2B). The *A. aegypti* protein recruited clathrin onto sedimentable liposomes in a dose-dependent fashion (Fig. 2B, lanes b,d,f). Similarly, a human GST-ARH fusion bound clathrin (lane h), although in the absence of an ARH-type protein, clathrin remained soluble and was recovered in the supernatant (lane i compared with lane j). The direct participation of ^{242}LIDFN clathrin box and the adjacent ^{267}WVSFEDD sequences in binding to clathrin and AP-2 was verified by site-directed mutagenesis. Alteration of the LIDFN to AAAFN (LID→AAA) significantly attenuated clathrin binding by the GST-*Ag*ARHC1 protein while still preserving the AP-2 interaction (Fig. 4B, lane h). Likewise, alteration of WVSFEDD to AVSFEDD (W268A) essentially prevented the association of the GST-*Ag*ARHC1 with soluble mammalian AP-2 (lane j), but left clathrin binding intact.

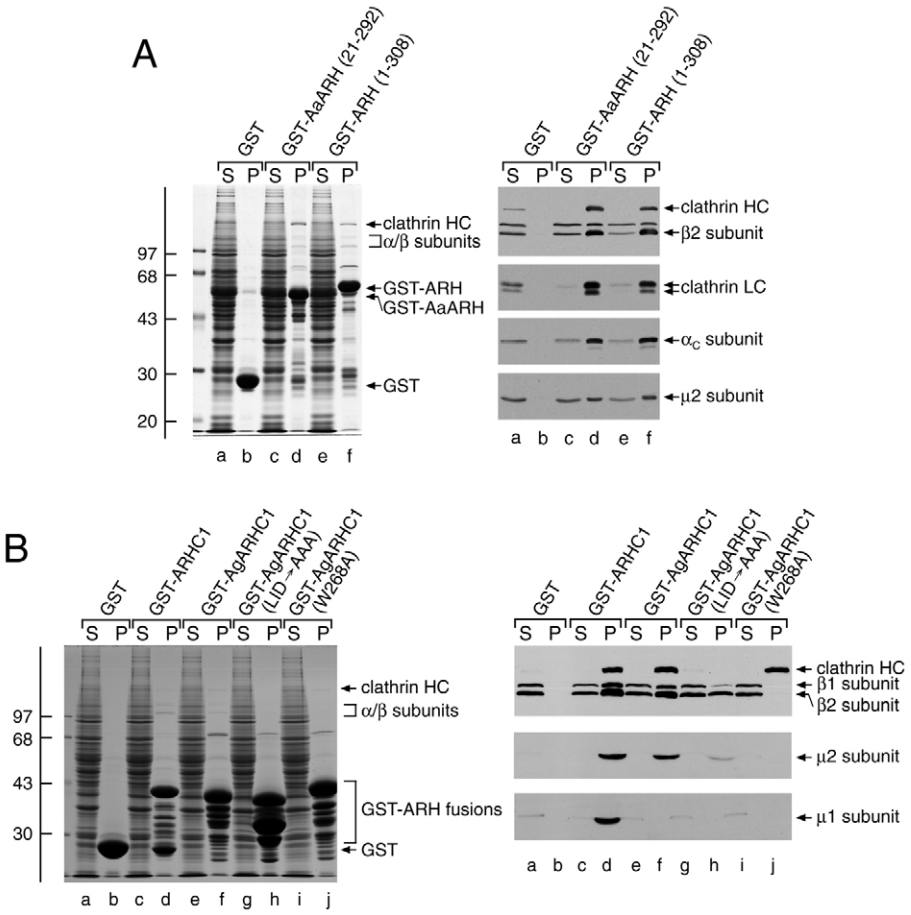

Fig. 4. Coat-binding properties of mosquito ARH-like proteins. (A) GST (100 μg, lanes a and b) or GST-*Aa*ARH (residues 21-292; lanes c and d) or human GST-ARH (residues 1-308; lanes e and f) on glutathione-Sepharose was incubated with rat brain cytosol. After centrifugation, aliquots of 2% of each supernatant (S) and 12.5% of each washed pellet (P) were resolved by SDS-PAGE and stained or transferred to nitrocellulose. Portions of the blots were probed with the anti-clathrin heavy chain (HC) mAb TD.1 and AP-1/2 β subunit antibody GD/1, or anti-μ2 subunit serum, or anti-α subunit mAb clone 8, or anti-clathrin light chain (LC) mAb Cl57.3. Only the relevant portion of each blot is shown. (B) GST (~250 μg, lanes a and b), GST-ARHC1 (lanes c and d), GST-*Ag*ARHC1 (residues 196-300; lanes e and f), GST-*Ag*ARHC1 (LID→AAA) (lanes g and h), or AgARHC1 (W268A) (lanes i and j) on glutathione-Sepharose was incubated with rat brain cytosol. After centrifugation, aliquots of 2.5% of each supernatant (S) and 12.5% of washed pellets (P) were resolved by SDS-PAGE and stained or transferred to nitrocellulose. Portions of the blots were probed with the anti-clathrin HC mAb TD.1 and affinity purified anti-AP-1/2 β subunit antibody GD/1, or anti-μ2 subunit serum or affinity purified anti-μ1 antibody RY/1. Note that only the human ARHC1-fusion protein binds to the μ1 subunit (AP-1) as a consequence of the selectivity for the β-subunit appendage. The mammalian AP-1 β1 and AP-2 β2 subunits are ~85% identical and the appendages share binding partners.

Altered AP-2 appendage selectivity in insects

The AP-2 adaptor complex is a roughly bilaterally symmetrical heterotetramer composed of two hemicomplexes: an α·σ2 subunit pair and a β2·μ2 subunit pair (Owen et al., 2004). The core of the complex is formed by the N-terminal portions of the α and β2 chains bound to their respective σ2 and μ2 subunits. The C-terminal regions of the α and β2 subunits are independently folded domains, termed the appendages, that project off the adaptor core on flexible polypeptide hinges (Owen et al., 2004). AP-2 contacts over 20 endocytic components, including CLASPs and so-called 'accessory' factors, many of which have orthologues in arthropods and nematodes. The α- and β2-subunit appendages represent the principal AP-2 interaction hubs and are structurally and functionally homologous (Schmid et al., 2006; Traub, 2005). Each appendage has two known contact surfaces, one on the N-terminal sandwich subdomain and a second on C-terminal platform subdomain (Edeling et al., 2006; Owen et al., 2004; Schmid et al., 2006). The Wxx[FW]x[DE] interaction motif binds selectively to the AP-2 α appendage sandwich site (Jha et al., 2004; Ritter et al., 2003; Walther et al., 2004) and, in vitro, the *Ag*ARHC1 bound the mammalian α appendage but not the β2 appendage (Fig. 5A, lane d compared with lane h). A GST-*Aa*ARH (21-292) fusion protein also associated with the monomeric α_C-subunit appendage in pull-down assays (Fig. 5B, lane d). The strong inhibitory effect of a Q782A substitution (lane h compared with lane d), which is known to alter the surface chemistry of the Wxx[FW]x[DE] recognition site (Mishra et al., 2004; Praefcke et al., 2004; Ritter et al., 2004), indicates that the mosquito Wxx[FW]x[DE] motif also engages the sandwich subdomain of the α appendage. By contrast, the equivalent region of the human ARH protein (residues 180-308, ARHC1) binds only the AP-2 β2 appendage and not the α appendage (Edeling et al., 2006; He et al., 2002; Mishra et al., 2005; Mishra et al., 2002b; Schmid et al., 2006). One possible interpretation of these results is that both the *Anopheles*/*Aedes* and vertebrate ARH-like proteins bind to AP-2 and clathrin during vesicle formation but that a switch in appendage selectivity has occurred during evolution. Comparison of the genomic organization of the mosquito ARH-like loci with

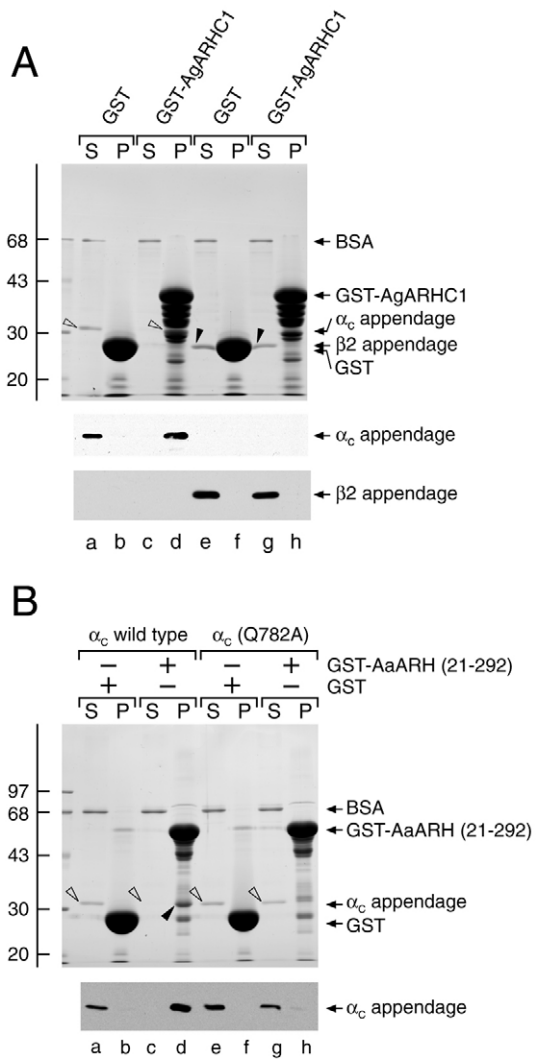

Fig. 5. Appendage selectivity of the mosquito ARH-like proteins. (A) GST (~400 μg, lanes a,b,e,f) or GST-*Ag*ARHC1 (residues 196-300; lanes c,d,g,h) immobilized on glutathione-Sepharose was incubated with 100 μg/ml of either thrombin-cleaved α_C appendage (open arrowheads) or β2 appendage (arrowheads) in the presence of 25 μM PPACK and 100 μg/ml BSA. After centrifugation, 2.5% of each supernatant (S) and 12.5% of each washed pellet (P) were resolved by SDS-PAGE for staining or fivefold less of each supernatant and pellet were transferred onto nitrocellulose. Blots were probed with either anti-AP-2 α subunit mAb 100/2 or affinity-purified rabbit anti-AP-2 β2 subunit antibodies. (B) GST (~250 μg, lanes a,b,e,f) or GST-*Aa*ARH (residues 20-292) on glutathione-Sepharose was incubated with either 50 μg/ml thrombin-cleaved α_C wild type (lanes a-d) or α_C (Q782A) mutant (lanes e-h) in the presence of 100 μg/ml BSA. After centrifugation, 2.5% of each supernatant (S) and 12.5% of washed pellets (P) were resolved on SDS-PAGE for staining or fivefold less of each supernatant and pellet transferred onto nitrocellulose. The blot was probed with the anti-AP-2 α subunit mAb 100/2. Stained α appendage in supernatant (open arrowheads) and pellet (arrowhead) fractions is indicated.

the human *ARH* suggests how this might have arisen. The gene encoding ARH in *A. gambiae* covers 7.2 kb on chromosome 3R, whereas in *A. aegypti* it spans 31.6 kb. Both are composed of four exons encoding very similar portions of the polypeptide, producing spliced transcripts of 1330 bp (*A. gambiae*) and 1789 bp (*A. aegypti*). The PTB domain is encoded by sequences within exons 2 and 3 in both mosquito species, but exon 3 terminates at the start of the WxxF motif, ~65 residues after the end of the modular PTB fold. By contrast, the human *ARH* gene spans 25.2 kb on chromosome 1 and contains nine exons. The PTB domain is encoded entirely by exons 2, 3, 4 and 5. This segmentation of the vertebrate ARH gene could have facilitated the evolution of a distinct C-terminal sequence with alternative adaptor-binding priorities.

The independently folded appendage domain of the *A. aegypti* AP-2 α subunit (AAEL004469) is 56% identical to the mammalian protein. To ensure that a binary interaction between the *A. aegypti* ARH and the *A. aegypti* AP-2 α appendage also occurs, we used the yeast two-hybrid assay. AH109 yeast cells transformed with *A. aegypti* pGBKT7BD-ARH and pGADT7-AP-2 α appendage indeed grew on triple-dropout plates, whereas cells transformed with one partner and a corresponding empty vector grew only on double-dropout plates (Fig. 3). Importantly, site-directed mutagenesis of either the Wxx[FW]x[DE] sequence in the *A. aegypti* ARH (W261A) or the cognate interaction surface on the AP-2 α appendage [Q776A; equivalent to mammalian α_C subunit residue Q782A substitution (Mishra et al., 2004; Praefcke et al., 2004; Ritter et al., 2004)] prevented growth on plates lacking His, Leu and Trp. Yet the PTB-domain mutations that prevented the *A. aegypti* ARH binding to the FxNPxY sequences had no affect on the *A. aegypti* ARH–AP-2 α appendage interaction, as expected.

The intracellular localization of a GFP-tagged *A. gambiae* ARH-like protein verifies the interaction properties identified by sequence analysis and in vitro biochemical assays. The punctate distribution of the ectopically expressed fusion protein in HeLa cells revealed that the mosquito ARH-like proteins did target to AP-2/clathrin-containing structures at the cell surface (Fig. 6A-D"). Although there was a sizable soluble population of the fusion protein, it still concentrated at surface puncta that were positive for AP-2 (Fig. 6D, arrows). The degree of colocalization was highest in substrate-adherent ventral plasma membrane sheets derived from cells that inadvertently sheared off the coverslip (Fig. 6E). The same phenomenon occurs when staining for human ARH (Mishra et al., 2002b). HeLa cells ectopically expressing very high levels of the *A. gambiae* GFP-ARH-like fusion sometimes contained large intracellular aggregates that were also positive for AP-2 (Fig. 6G,H, arrows). This phenotype is frequently observed upon gross overexpression of AP-2- (and clathrin-) binding segments of endocytic proteins (Edeling et al., 2006; Krauss et al., 2006; Martina et al., 2001).

Simultaneously inactivating both the clathrin- and AP-2-binding information (a LID→AAA/W267A double mutant) altered the distribution of the GFP-tagged protein: the protein generally failed to concentrate in puncta and colocalization with AP-2 is not obvious. Instead, the protein was more diffusely distributed within the transfected cells (Fig. 6I-K), and is also seen in filopodial extensions of the plasma membrane. Taken together, these experiments show that the mosquito proteins display the four critical functional attributes of mammalian CLASPs: they bind via structurally distinct sites to cargo (the FxNPxY sorting signal), PtdIns(4,5)P_2, clathrin and AP-2.

Expression profiles in mosquito ovaries

To explore whether the ARH-like CLASP plays a role in concentrating vitellogenin and lipophorin receptors at clathrin-coated buds on the oocyte surface, we first used RT-PCR to evaluate whether transcripts for AaARH are present in *Aedes* ovaries. An expected 0.8 kb ARH PCR product was found in both

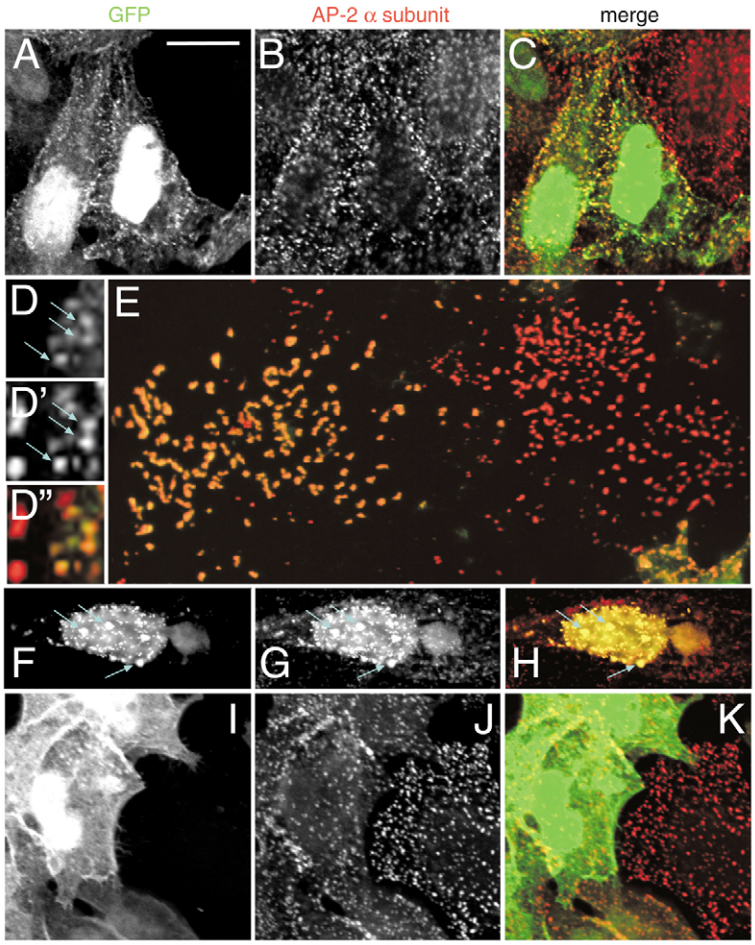

Fig. 6. Subcellular localization of a tagged *Ag*ARH-like protein. HeLa cells, transiently transfected with the GFP-*Ag*ARH-like protein, were fixed and stained with mAb AP.6 directed against the α subunit of the AP-2. GFP (green, A,D,F,I) and AP-2 (red, B,D',G,J) signals were collected sequentially by confocal microscopy and representative single optical sections are shown. Scale bar: 20 μm. An enlarged region illustrating the degree of colocalization (D"; arrows) of GFP-*Ag*ARH-like fusion (D) with AP-2 (D') is shown, as well as an example of large intracellular aggregates of the fusion protein below the nucleus (F) also containing AP-2 (G,H; arrows). A region where several adjacent cells mechanically detached from the glass surface leaving only adherent ventral plasma membrane (E) shows a high degree of colocalization of the GFP-AgARH-like protein (yellow) compared with the non-transfected cells (red). The generally diffuse intracellular distribution of the doubly mutated GFP-*Ag*ARH-like (LID→AAA/W268A) mutant is shown (panels I-K).

previtellogenic ovaries and ovaries from mosquitoes 16 hours after a blood meal (Fig. 7). For comparison, both vitellogenin receptor and AP-2 α subunit transcripts were present in previtellogenic and post-blood-meal ovaries. The expression of the vitellogenin receptor was in good agreement with previous studies (Sappington et al., 1996). The presence of the *A. aegypti* AP-2 transcript is in agreement with the ultrastructural identification of assembled clathrin structures devoid of an internal membrane in previtellogenic *A. aegypti* oocytes (Raikhel, 1984). Importantly, the vitellogenin receptor is only expressed in the female germline (Sappington et al., 1996) and no message for the receptor is found in Aag-2 cells – an *A. aegypti*-derived cell line (Lan and Fallon, 1990). The 0.8 kb *A. aegypti* ARH-like PCR product was similarly absent in Aag-2 cells, although, as expected, the 0.5 kb *A. aegypti* AP-2 α subunit PCR product was generated, reiterating the ubiquitous expression pattern and activity of AP-2.

The presence of transcripts for the *A. gambiae* ARH-like protein (ENSANGP00000011117) in ovaries (and the fat body) 24 hours after blood feeding is confirmed by using the *Anopheles gambiae* gene expression profile (www.angaged.bio.uci.edu). The microarray analysis also revealed that similarly to the vitellogenin receptor transcript, the ARH-like mRNA level is roughly double in pre-blood-meal females than in males. This is in contrast to the relative abundance of transcripts for the clathrin heavy chain (ENSANGP00000018215), clathrin light chain (ENSANGP00000026839) and the AP-2 α subunit (ENSANGP00000019991), which are roughly equivalent in males and pre-blood-fed females. Between 48 and 96 hours after a blood meal, increases in the transcript abundance of the *Ag*ARH-like protein, clathrin, AP-2 and, as expected, the vitellogenin receptor were observed. Although these are whole-body transcript levels, the data do suggest that induction of ARH-like gene expression occurs in blood-fed *A. gambiae*.

Expression patterns within the *Aedes* oocyte

The *A. aegypti* egg chamber is composed of eight germline-derived cells (seven nurse cells and an oocyte) encapsulated by a single epithelial monolayer, termed somatic follicle cells (Roth and Porter, 1964). To evaluate whether the ARH-like transcript is found in the germline, we used whole-mount in situ hybridization. In previtellogenic egg chambers, low levels of mRNA were detected with a digoxigenin-labeled antisense riboprobe, distributed diffusely in both the nurse cell and future oocyte cytoplasm (Fig. 8A). Little message was evident in the follicle cells. Six hours after a blood meal, the oocyte, positioned at the posterior end relative to the germarium and secondary follicle (see Fig. 8D), shows a striking increase in transcript abundance (Fig. 8B). Although the ARH-like message was also present in the adjacent nurse cells, it was generally concentrated in the vitellogenic oocyte. After 20 hours, the oocytes were rapidly expanding because of yolk accumulation (Fig. 8F) and the ARH-like protein mRNA was more strongly expressed in the oocyte and also in the attendant nurse cell cytoplasm (Fig. 8C,D), to which the oocyte is physically linked by ring canals (de Cuevas and Spradling, 1998). At this stage, the expression pattern of the ARH protein was similar to that of the vitellogenin receptor (Cho and Raikhel, 2001; Sappington et al., 1996) (Fig. 8E), although little increase in the relative expression of the mRNA in the secondary follicles was evident. This indicates that induction of ARH-like transcripts following a blood meal occurs selectively in the primary vitellogenic egg chamber and not in the next follicle, which will develop after egg laying and a subsequent blood meal.

Discussion

We have found that functionally homologous monomeric CLASPs exist in insects to mesh the FxNPxY-type sorting signal with clathrin-coated structures forming at the cell surface. Yet, the designation ARH is clearly a misnomer for this protein in mosquitoes and the alternative name posted in GenBank, LDL receptor adaptor protein 1 (LDLRAP1), is also inaccurate. The latter

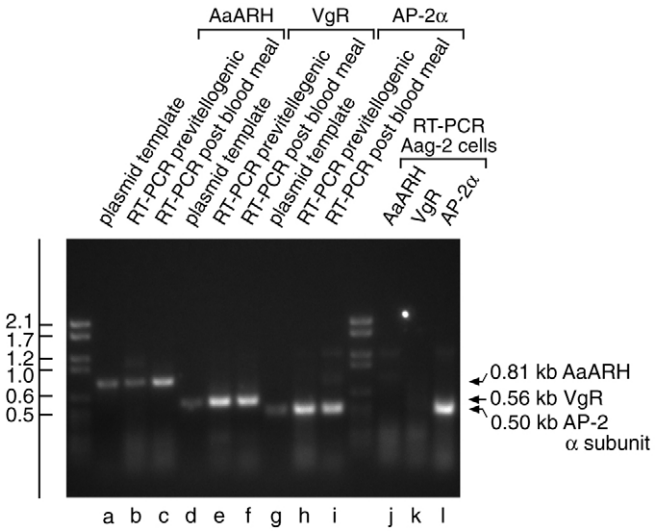

Fig. 7. mRNA expression in *Aedes* ovaries. RT-PCR using RNA isolated from either previtellogenic (lanes b,e,h) or 16 hour post-blood-meal ovaries or with RNA derived from cultured Aag2 cells and primer pairs designed to amplify a 0.81 kb *Aa*ARH, 0.56 kb vitellogenin receptor (VgR) or a 0.5 kb AP-2 α subunit product. As controls, PCR with the identical primer sets and DNA plasmid template (lanes a,d,g) was performed. The position of the DNA standards (in kb) is indicated.

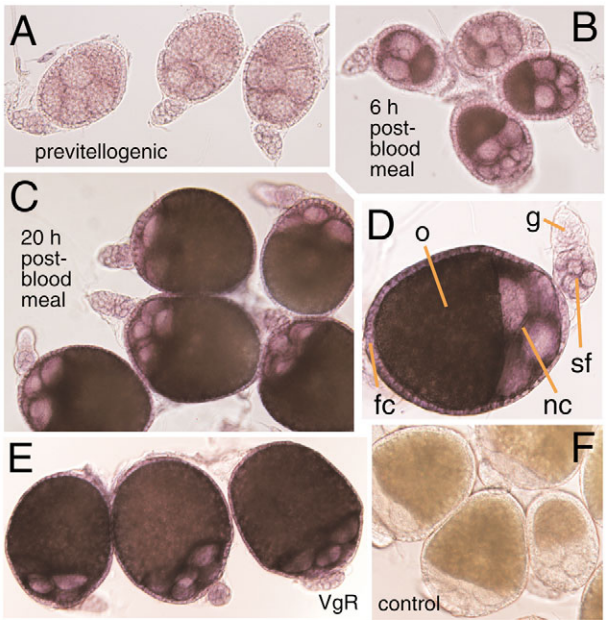

Fig. 8. Changes in the ARH-like transcript during *A. aegypti* egg chamber development. Whole-mount in situ hybridization analysis of ovaries dissected from previtellogenic (A), 6 hour (B) or 20 hour post-blood-meal (C-F) females. Fixed ovaries were hybridized with antisense *Aa*ARH-like (A-D), vitellogenin receptor (VgR; E) or no (F) riboprobe. The purple staining indicates the positioning of mRNA transcripts. Samples in A-D were treated identically and the images obtained under the same conditions. fc, follicle cells; g, germarium; nc, nurse cells; o, oocyte; sf, secondary follicle.

can easily be confused with RAP (receptor-associated protein), an LDL receptor family-specific chaperone that is present within the endoplasmic reticulum lumen of vertebrate (Herz, 2006) and invertebrate (Culi and Mann, 2003) cells. Based on our studies, we suggest the designation trephin for the mosquito protein, from the Greek word trephô, which means to nourish or fatten.

An intriguing difference in the insect trephin protein is that a Wxx[FW]x[DE] motif governs interactions with the AP-2 adaptor α-subunit appendage opposed to the single $[DE]_nx_{1\text{-}2}$Fxx[FL]xxxR motif found in all vertebrate ARH isoforms. The $[DE]_nx_{1\text{-}2}$Fxx[FL]xxxR motif is also present as a single AP-2-binding determinant in another CLASP, β-arrestin, which sorts G-protein-coupled receptors into clathrin-coated vesicles (Edeling et al., 2006; Schmid et al., 2006). Intriguingly, the non-visual arrestin Kurtz in *D. melanogaster* harbors the canonical $[DE]_nx_{1\text{-}2}$Fxx[FL]xxxR sequence ^{451}**DD**II**F**ED**F**ARL**R** (Roman et al., 2000). Moreover, presumptive *A. aegypti* (AAEL003116) and *A. gambiae* (*ARRK*, AGAP011360) β-arrestins have the identical sequence (Merrill et al., 2003). This indicates that the AP-2 β-appendage specific $[DE]_nx_{1\text{-}2}$Fxx[FL]xxxR motif already existed in insects but that over the course of evolution a switch in the selectivity of the vertebrate ARH counterparts occurred. Again, inspection of the genomic structure of the β-arrestin gene suggests a possible mode for reorienting the interaction of ARH with the AP-2 heterotetramer. Non-visual arrestin genes from mosquitoes to mammals all have the $[DE]_nx_{1\text{-}2}$Fxx[FL]xxxR sequence encoded by the last exon. This final exon encodes the C-terminal 48 residues of the *Aedes* β-arrestin and the last 27 amino acids of the *Anopheles* β-arrestin. The distance from the proximal splice site to the first Asp of the $[DE]_nx_{1\text{-}2}$Fxx[FL]xxxR ranges from 30 residues in *A. aegypti* to only two in human β-arrestin 1. The general modularity of the AP-2 β2-appendage binding sequence in the arrestins that is preserved through evolution could have facilitated, by duplication and shuffling, incorporation of this new adaptor binding information in chordates as the ARH gene divided into more exons. However, we cannot exclude the possibility that the $[DE]_nx_{1\text{-}2}$Fxx[FL]xxxR sequence in vertebrate ARH arose by convergent evolution of a sequence unrelated to β-arrestin.

An operational feature of the $[DE]_nx_{1\text{-}2}$Fxx[FL]xxxR motif allows us to propose why this sequence has been selected in the vertebrate ARH orthologues. Both β-arrestins and ARH use the motif to access a relatively privileged surface on the AP-2 β2 subunit appendage (Edeling et al., 2006; He et al., 2002; Mishra et al., 2005; Schmid et al., 2006). This allows both of these CLASPs to translocate to preassembled clathrin-coated structures, synchronizing selective cargo clustering with pre-existing endocytic structures (Hamdan et al., 2007; Santini et al., 2002; Scott et al., 2002). Perhaps, by engaging a different contact surface on the other AP-2 appendage, the trephin Wxx[FW]x[DE] motif facilitates cooperative assembly of clathrin-coated vesicles with AP-2, or biases the forming coats toward a larger size distribution to allow more receptor and coupled ligand to be internalized within each vesicular carrier. The ~140 nm clathrin-coated vesicles in vitellogenic *A. aegypti* oocytes (Roth and Porter, 1964) are indeed larger than the typical ~90-100-nm coated vesicles normally observed in mammalian cells.

We find low comparative expression of trephin transcript in previtellogenic egg chambers and a substantial induction of trephin message within 6 hours of a blood meal. Little trephin mRNA induction is observed in secondary follicles. Our interpretation of these data is that blood feeding triggers a rapid and sizable increase in trephin protein levels in the oocyte. There could be several reasons for temporally regulated expression of this CLASP. Vitellogenin

and lipophorin receptor expression (Cheon et al., 2001; Sappington et al., 1995; Sappington et al., 1996), and clathrin mRNA (Kokoza et al., 1997), is clearly evident shortly following eclosion. Immunocytochemical studies indicate that the vitellogenin receptor is positioned cortically in previtellogenic ovaries, suggesting that it accesses the oolemma (Sappington et al., 1995). Previtellogenic follicles also clearly have clathrin-coated buds and vesicles as well as microvilli at the oocyte surface membrane 48-72 hours after eclosion (Raikhel and Lea, 1985; Roth and Porter, 1964). There is a 15-fold increase in clathrin-coated structures to approximately 300,000/oocyte 7 hours after a blood meal (Roth and Porter, 1964). It is also known that *A. aegypti* ovarioles are patent from an early, previtellogenic stage (Anderson and Spielman, 1971; Raikhel and Lea, 1985), allowing ligands access to surface-positioned receptors. Although vitellogenin levels are low before a blood meal, lipophorin levels must be normal, because lipophorin is an energy carrier required for flight as well as basic cell metabolism (Rodenburg and Van der Horst, 2005). Nutritionally regulated trephin gene transcription may allow selective uncoupling of LDL receptor gene family members from clathrin-mediated endocytosis in previtellogenic ovaries, thus preventing precocious yolk granule accumulation that might be deleterious.

Our proposal for selective transcriptional regulation of yolk uptake by trephin is in general agreement with recent computational modeling of the governing features of receptor-mediated endocytosis (Shankaran et al., 2007). Regulation of ligand transport is suggested to occur in either an avidity- or a consumption-controlled manner. Because of the high density of vitellogenin (and lipophorin) receptors in oocytes, capture of ligand (yolk precursors) is highly efficient and largely independent of ligand concentration (avidity). The system is thus predicted to be modulated by a consumption parameter, β – a partition coefficient related to the rate of ligand internalization or dissociation off a receptor before uptake can occur (Shankaran et al., 2007). The model suggests that increases in the extent of intracellular delivery by receptors of this type occurs through elevation of the internalization rate. The quantitative values for the vitellogenin receptor in this study were from *Xenopus* oocytes but, presuming similar general properties for the mosquito vitellogenin (and lipophorin) receptor, CLASPs would be critical regulators of clathrin-mediated endocytosis, because they directly determine the rate and extent of receptor capture and packaging into transport vesicles.

Other related CLASPs could also increase the efficiency of yolk-precursor internalization in mosquitoes. Three additional members of the Dab-like PTB-domain family have been directly or indirectly implicated in endocytosis: Dab2 (Keyel et al., 2006; Maurer and Cooper, 2006; Morris and Cooper, 2001), numb (Berdnik et al., 2002; Santolini et al., 2000) and CED-6/GULP (Martins-Silva et al., 2006). Both the *A. aegypti* and *A. gambiae* genomes encode apparent Disabled (AAEL011241/AAEL008229 and ENSANGP00000004338, respectively), numb (AAEL001476 and ENSANGP00000006159, respectively) and CED-6 (AAEL012821/AAEL012967 and ENSANGP00000021640, respectively) orthologues. Oddly, despite also being Dipteran insects, *Drosophila* do not encode a direct trephin orthologue. However, the FNxPxY-type sorting signals in Yolkless and the lipophorin receptor (Table 1) clearly suggest the involvement of other PTB-domain CLASPs in vitellogenesis. Very recently, it has been shown that the *C. elegans* Dab2 orthologue, DAB-1, is required for yolk deposition in developing oocytes (Holmes et al., 2007). However, *dab-1* mutant adults are viable and produce near-normal numbers of eggs, suggesting some degree of functional redundancy; there is clear functional overlap between Dab2 and ARH in certain mammalian cells (Keyel et al., 2006; Maurer and Cooper, 2006). Future work should clarify whether all, some, or none of these other PTB-domain proteins participate in yolk granule accumulation in blood-fed mosquitoes. Irrespective of this, the fact that ectopic overexpression of an AP-2- and clathrin-binding defective form of the *X. laevis* ARH orthologue strongly interferes with oocyte yolk accumulation (Zhou et al., 2003) indicates that a primary ancestral function of the trephin protein could have been to promote egg maturation.

Materials and Methods

Plasmids

The plasmids encoding GST-ARH, GST-AP-2 α_C appendage and GST-AP-2 β2 appendage have been described (Edeling et al., 2006; Mishra et al., 2005; Mishra et al., 2002b). A C-terminal segment (residues 196-300) of the *A. gambiae* ARH-like protein was amplified by PCR from EST 4A3B-AAD-D-05-R (supplied by George Christophides and Fotis Kafatos, EMBL, Heidelberg, Germany) and ligated into pGEX-4T-1. The full-length *A. gambiae* ARH-like protein (residues 1-300) was fused to the C-terminus of green fluorescent protein in pEGFP-C1 by inserting a PCR product from the same EST. The *A. aegypti* ARH-like protein (residues 21-292) was similarly amplified from EST clone NABR733 (supplied by David Severson, University of Notre Dame, IN) and ligated into pGEX-4T-1 and the yeast two-hybrid vector pGBKT7. The *A. aegypti* lipophorin receptor cytosolic domain (residue 1099-1156) was amplified by PCR and cloned into pGADT7. The *A. aegypti* AP-2 α_C appendage (residue 690-933) was amplified from EST clone TC50458 by PCR, first using primer extension to incorporate missing 5′ sequence, followed by PCR and ligation into pGBKT7. Site-directed mutagenesis was done using the QuikChange system (Stratagene) and clones and mutations were confirmed by double-stranded dideoxynucleotide sequencing.

Materials, reagents and cell culture

Cholesterol, PtdCho, PtdEth, PtdIns and PtdSer were all from Sigma whereas the PtdIns(4,5)P_2 was purchased from Roche. Stocks of synthetic multilamellar liposomes composed of 35% (w/w) PtdCho, 35% PtdEth, 10% PtdSer, 10% cholesterol and either 10% PtdIns (control) or 10% PtdIns(4,5)P_2 were prepared at a concentration of 4 mg/ml in 10 mM HEPES-KOH, pH 7.6, 1 mM EDTA by three freeze-thaw cycles. To prepare cytosol, frozen rat brains (PelFreez) were homogenized at 4°C in 25 mM HEPES-KOH, pH 7.2, 250 mM sucrose, 2 mM EDTA and 2 mM EGTA supplemented with 1 mM PMSF and Complete (Roche) protease inhibitor cocktail using a Waring blender (Traub et al., 1996; Traub et al., 1993). To prepare recombinant proteins, plasmids encoding GST or the different GST-fusions were transformed into *E. coli* BL21. For protein production, bacteria were induced with 100 μM isopropyl-1-thio-β-D-galactopyranoside and recovered after incubation for 3-5 hours at room temperature. Lysates were prepared on ice in 50 mM Tris-HCl, pH 7.5, 300 mM sodium chloride, 0.2% (w/v) Triton X-100, 10 mM β-mercaptoethanol by sonication. After centrifugation to remove insoluble material, the GST fusions were collected on glutathione-Sepharose, washed, eluted with 10 mM glutathione and dialyzed into PBS, 1 mM DTT. For some experiments, proteins were cleaved from GST with thrombin and then D-Phe-Pro-Arg chloromethyl ketone (Calbiochem), an irreversible thrombin inhibitor, added to 25 μM final concentration.

Primary mouse antibodies used include the anti-clathrin heavy chain mAb TD.1, anti-clathrin light chain mAb Cl57.3, and the anti-AP-2 α-subunit mAbs 100/2, AP.6 and clone 8. We have described the affinity-purified anti-AP-1/2 β subunit GD/1 and anti-AP-1 μ1 subunit RY/1 antibodies (Traub et al., 1996). The rabbit R11-29 anti-μ2 serum was provided by Juan Bonifacino (NICHD, NIH, Bethesda, MA) whereas rabbit anti-β2 appendage antiserum was affinity purified on nitrocellulose-immobilized His_6-tagged β2 subunit hinge+appendage from serum provided by Harvey McMahon (MRC LMB, Cambridge, UK).

HeLa SS6 cells were cultured in DMEM supplemented with 10% fetal calf serum and 2 mM L-glutamine and plated onto round glass coverslips prior to transfection with Lipofectamine 2000 as described previously (Keyel et al., 2006; Mishra et al., 2005). Aag2 cells were generously provided by Anne Fallon (Lan and Fallon, 1990). The cells were maintained at 28°C in Eagle's MEM supplemented with 5% heat-inactivated fetal calf serum, 2 mM L-glutamine, 1× non-essential amino acids, 1× MEM vitamin solution and 25 mM HEPES.

Yeast two-hybrid assay

The Matchmaker GAL4 system was used as we have described (Edeling et al., 2006; Mishra et al., 2005). Appropriately transformed *S. cerevisiae* strain AH109 was first selected at 30°C on SD minimal medium plates lacking Leu and Trp. Individual clones were selected and then streaked or spotted onto SD medium lacking Leu and Trp or plates without His, Leu, and Trp and supplemented with 0.75 mM 3-amino-1,2,4-triazole (3-AT).

Protein-binding assays

Liposome-binding assays (200 μl) contained 0.4 mg/ml control or PtdIns(4,5)P_2 liposomes, 100 μg/ml BSA and 60 μg/ml GST-fusion protein in 25 mM HEPES-KOH, pH 7.2, 125 mM potassium acetate, 5 mM magnesium acetate, 2 mM EGTA, 2 mM EDTA and 1 mM DTT (assay buffer) (Mishra et al., 2002b). When clathrin was added, purified trimers were added after ultracentrifugation to remove aggregates. Clathrin was purified from rat brain coated vesicles (Mishra et al., 2002a). GST-based pull-down assays, electrophoresis and immunoblotting were performed exactly as described (Edeling et al., 2006; Jha et al., 2004; Mishra et al., 2004; Mishra et al., 2005; Mishra et al., 2002a; Mishra et al., 2002b).

Reverse-transcription PCR

RNA was purified from previtellogenic and 24 hour post-blood-meal *A. aegypti* ovaries dissected in PBS. The RNA was extracted from ovaries or Aag-2 cells with Trizol (Invitrogen). Aliquots of 800 ng total RNA were used for the first strand reaction with AMV reverse transcriptase and an oligo-dT primer. After incubation at 42°C for 60 minutes, 6% of each reaction was used for the second-stage PCR reaction using Taq polymerase with the following primer pairs: *Aedes* ARH-like protein forward, 5′-ATGGCACTCGCCAACAACATCCG-3′ and reverse, 5′-GTTAGAG-CAAATTGCAGTCCTG-3′; *Aedes* vitellogenin receptor forward, 5′-GTCACG-TGTGTGTTCCC-3′ and reverse, 5′-TCAGAGGATCAACCGCTGC-3′; and *Aedes* AP-2 α subunit forward, 5′-GGAAGCCGGAGCACAAATC-3′ and reverse, 5′-GGTCTACCAGTAGATTGCA-3′. Control reactions contained 10 pmol of a DNA plasmid containing the appropriate insert.

Whole-mount in situ hybridization

The general method (Kokoza et al., 1997; Sappington et al., 1996) was adapted from a *Drosophila* protocol (Tautz and Pfeifle, 1989), and the following gene-specific oligos were used to first generate a DNA template: *Aedes* ARH-like protein forward primer, 5′-GCAGCGCACGATCACGTGTTCGC-3′ and reverse primer, 5′-GCG**TAA-TACGAC TCACTATAGGGAGA**AGTCCTGCTCCATCCATTGGC-3′; *Aedes* vitellogenin receptor forward primer, 5′-GTCACGTGTGTGTTCCC-3′ and reverse primer, 5′-GCG**TAATACGACTCACTATAGGGAGA**TCAGAGGATCAACCG-CTGC-3′. The reverse primers include the T7 polymerase promoter sequence (bold) required to generate the riboprobes. Approximately 500 ng of purified PCR template and T7 RNA polymerase to generate digoxigenin-labeled probes using a digoxigenin-11 UTP RNA labeling mix (Roche). The RNA probes were heated to 65°C for 10 minutes in 120 mM sodium carbonate, 80 mM sodium bicarbonate, pH 10.2 to generate smaller fragments to better penetrate fixed samples. Labeled RNA was ethanol precipitated and resuspended in 50% formamide, 5× SSC, 100 μg/ml sonicated salmon sperm DNA, 50 μg/ml heparin, 0.1% Tween 20 in DEPC-treated water (hybridization buffer). The concentration of probes was confirmed by dot blot analysis.

Ovaries from previtellogenic and 6 hour or 20 hour post-blood-meal *Aedes* females were dissected and fixed in 4% paraformadehyde-DMSO (5:1) for 30 minutes at room temperature. After washing in PBS containing 0.01% Tween 20 (PBST), the ovaries were treated with 8 μg/ml proteinase K in PBST for 10 minutes, washed and refixed in 4% paraformaldehyde-DMSO (5:1) for 20 minutes. Fixed ovaries were prehybridized in hybridization buffer for 60 minutes at 55°C followed by 12 hours at 55°C in the presence or absence of the specified digoxigenin-labeled RNA probe. After hybridization, ovaries were washed in hybridization buffer over ~5 hours with four changes of hybridization buffer. The hybridized ovaries were blocked in 1% normal goat serum in PBST for 10 minutes and then incubated for 60 minutes with an alkaline phosphatase-conjugated anti-digoxigenin mAb (Roche) in 1% goat serum in PBST. After washing, the color reaction was performed in the presence of 5-bromo-4-chloro-3′-indolyphosphate *p*-toluidine (BCIP) and nitro-blue tetrazolium (NBT).

We thank George Christophides, Fotis Kafatos and David Severson for generously providing mosquito ESTs, Anne Fallon for kindly supplying Aag-2 cells, Juan Bonifacino for anti-μ2 serum and Harvey McMahon for the anti-β2 appendage serum. Supported in part by NIH grant R01 DK53249 and AHA Established Investigator Award (0540007N) to L.M.T.

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