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Polarized Dendritic Transport and the AP-1 μ1 Clathrin Adaptor UNC-101 Localize Odorant Receptors to Olfactory Cilia

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Summary

Odorant receptors and signaling proteins are localized to sensory cilia on olfactory dendrites. Using a GFP-tagged odorant receptor protein, *Caenorhabditis elegans* ODR-10, we characterized protein sorting and transport in olfactory neurons in vivo. ODR-10 is transported in rapidly moving dendritic vesicles that shuttle between the cell body and the cilia. Anterograde and retrograde vesicles move at different speeds, suggesting that dendrites have polarized transport mechanisms. Residues immediately after the seventh membrane-spanning domain of ODR-10 are required for localization; these residues are conserved in many G protein-coupled receptors. UNC-101 encodes a μ1 subunit of the AP-1 clathrin adaptor complex. In *unc-101* mutants, dendritic vesicles are absent, ODR-10 receptor is evenly distributed over the plasma membrane, and other cilia membrane proteins are also mislocalized, implicating AP-1 in protein sorting to olfactory cilia.

Introduction

Neurons are polarized cells with distinct proteins in their axons and dendrites. Dendrites in turn contain specialized subdomains, such as synaptic spines and the cilia of sensory neurons, that are enriched in signaling molecules and specific membrane proteins. The sensory transduction molecules of olfaction, vision, and mechanosensation reside within cilia or modified cilia, but relatively little is known about protein sorting into cilia (Deretic, 1998; Tam et al., 2000).

A variety of mechanisms direct plasma membrane proteins to specific domains of neurons, including local translation, polarized transport, targeted secretion to domains of the plasma membrane, and protein clustering by PDZ domain proteins. Studies of viral membrane protein sorting in cultured neurons suggest that the neuronal somatodendritic compartment may be analogous to the epithelial basolateral domain (Dotti and Simons, 1990). However, some of the proteins implicated in basolateral epithelial sorting are not expressed in neurons (Folsch et al., 1999), suggesting that these systems are similar but not identical. Sorting of membrane proteins to the basolateral epithelial plasma membrane also has similarities with sorting to the intracellular endosome compartment. Both processes are initiated at the *trans*-Golgi network (TGN), where proteins are packaged into transport vesicles that are coated with clathrin and the heterotetrameric AP-1 adaptor complex (Traub et al., 1993, 1995; Traub and Kornfeld, 1997; Schmid, 1997). AP-1 provides a membrane binding site for clathrin and also interacts with membrane proteins to select specific cargo for the vesicle (Marks et al., 1997; Robinson, 1997). AP-1 consists of four subunits: two large chains, β′ and γ; one medium chain, μ1 or AP47; and one small chain, σ1 or AP19. The μ1 subunits interact with transmembrane cargo proteins bearing a YxxØ sorting motif, and other subunits may also interact with cargo proteins (Ohno et al., 1995; Rapoport et al., 1998; Orzech et al., 1999). The YxxØ sorting motif and other short peptide sequences in the cytoplasmic tails of membrane proteins are implicated in sorting to basolateral domains of epithelial cells and in sorting to endosomes (Casanova et al., 1991; Hunziker et al., 1991; Matter et al., 1992). There appear to be several forms of AP-1 with different functions. For example, epithelial cells express both a generic μ1A subunit that mediates sorting to endosomes and an epithelial-specific μ1B subunit of AP-1 that mediates sorting of some but not all basolateral plasma membrane proteins (Folsch et al., 1999).

Most studies of protein sorting are conducted in cultured cells, which lose some of their differentiated properties. However, the transparent nematode *Caenorhabditis elegans* can be used to examine neuronal protein sorting and transport in vivo. We are studying protein sorting in chemosensory neurons of the amphid sensory organs. Each chemosensory neuron is bipolar, with an axon that joins the nerve ring neuropil and a dendrite that extends about 100 μm and ends in a sensory cilium at the tip of the nose. Many olfactory signaling molecules, including odorant receptors, G proteins, and ion channels, are enriched in chemosensory cilia (Coburn and Bargmann, 1996; Sengupta et al., 1996; Colbert et al., 1997; Roayaie et al., 1998). The G protein-coupled odorant receptor ODR-10 and other *C. elegans* odorant receptors are highly concentrated in sensory cilia. Localization of receptors does not depend on their extreme C termini, since cilia sorting is observed when the green fluorescent protein (GFP) is appended to the C terminus of the receptor proteins. The novel membrane protein ODR-4 is required for folding or sorting of odorant receptors, including ODR-10; in its absence, receptor proteins are retained in the cell body (Dwyer et al., 1998).

Here, we show that the odorant receptor ODR-10-GFP is rapidly transported in vesicles in the dendrite of a chemosensory neuron, identify residues in ODR-10 that are required for localization, and identify a protein essential for sorting of ODR-10 and other proteins to sensory cilia. *unc-101* encodes one of two *C. elegans* μ1 subunits of the AP-1 clathrin adaptor complex (Lee

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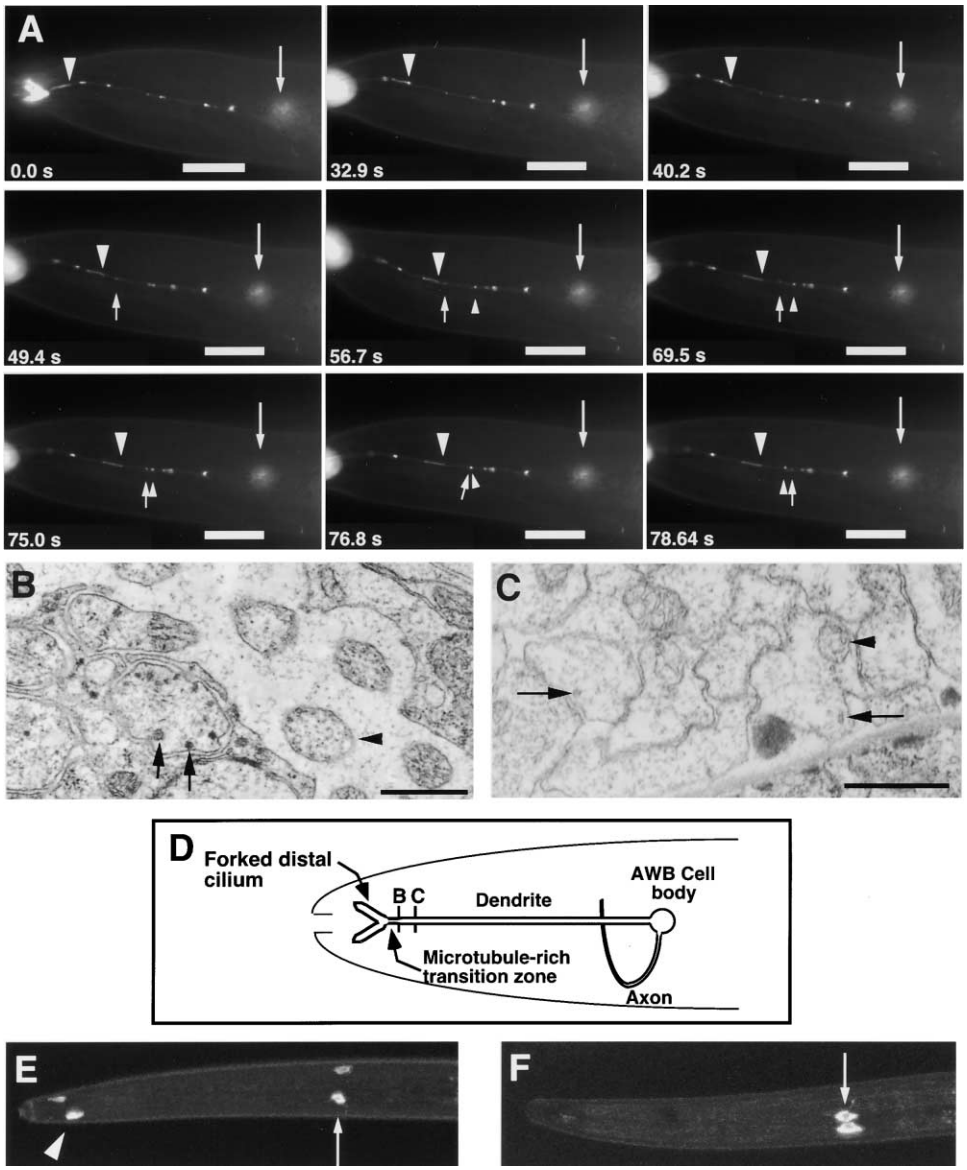

Figure 1. Transport Vesicles in Sensory Dendrites

(A) CCD time-lapse microscopy of ODR-10-GFP vesicles in the dendrite of the AWB neuron. A long tubule emerged from the cilium and moved retrogradely (large arrowhead). Two smaller vesicles passed one another from opposite directions (small arrow and small arrowhead). Scale bar represents 20 μm. Large arrow points to the cell body as a reference. Anterior is at left, and dorsal is up.

(B) Electron micrograph of sensory cilia and transition zones. One sensory dendrite is sectioned immediately under the transition zone of the cilia; arrows indicate two coated vesicles (about ten are present in this image). Arrowhead indicates the microtubule-rich distal cilium of an adjacent neuron. Scale bar = 500 nm.

(C) Electron micrograph of sensory dendrites, approximately 10 μm from the cilia. Arrows indicate dendritic vesicles in two dendrites. Arrowhead indicates a mitochondrion in one of the dendrites. Scale bar = 500 nm.

(D) Schematic diagram of AWB neuron with cell body, dendrite (~100 μm long), proximal cilium with transition zone, and forked distal cilium (~5 μm long). Approximate locations of sections in (B) and (C) are indicated. Anterior is at left.

(E) ODR-10-GFP is able to localize to the abnormal AWB cilia (arrowhead) in a *daf-19* mutant animal, despite the absence of the microtubule-rich transition zone of the cilium.

(F) STR-1-GFP fails to localize to the AWB cilia in a *daf-19* mutant and is instead retained in the cell body (arrow).

(E and F) Anterior is at left, and dauer larvae are shown. Wild-type dauer larvae exhibit normal cilia localization of ODR-10-GFP and STR-1-GFP in AWB (data not shown). (E) and (F) are confocal Z series projected into a single plane.

et al., 1994). In *unc-101* mutants, ODR-10-GFP is delocalized over the entire plasma membrane, suggesting a role for AP-1 in receptor localization to cilia. Our results suggest that AP-1 and particularly the μ1 subunit may contribute to sorting of plasma membrane proteins to cilia.

Results

ODR-10-GFP Is Present in Rapidly Moving Vesicles in the Dendrites of AWB Neurons

The odorant receptor ODR-10 is expressed in the cilia of the two AWA olfactory neurons (Sengupta et al., 1996).

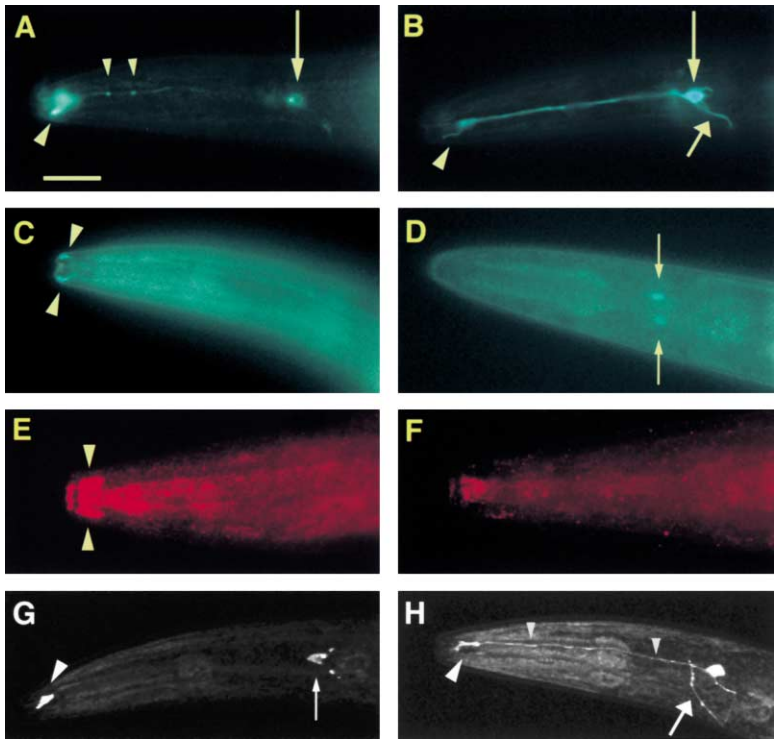

Figure 2. Sorting to Cilia Requires the AP-1 Clathrin Adaptor UNC-101

(A) The AWB::ODR-10-GFP protein is localized strongly in the AWB cilium (large arrowhead) in wild-type animals and also in vesicles in the dendrite (small arrowheads) and cell body (arrow). Bar represents 30 μm for all panels. (B) In an AP-1 *unc-101*(*m1*) mutant animal, AWB::ODR-10-GFP is localized uniformly over the entire plasma membrane of the AWB neuron, including the cilium (arrowhead), dendrite, cell body (thin arrow), and axon (wide arrow). This *unc-101* animal has altered cilium morphology; a single branch is present instead of the normal forked cilium. (C and D) The TRP-related channel OSM-9-GFP requires AP-1 UNC-101 for localization to the OLQ mechanosensory cilia. (C) In a wild-type animal, OSM-9-GFP localizes to the OLQ cilia (arrowheads). (D) In an AP-1 *unc-101*(*m1*) mutant animal, GFP-tagged OSM-9 is delocalized: it is present in the OLQ neuronal cell bodies (arrows) and faintly in the dendrites and cilia (not visible in this photograph). (E and F) The endogenous transmembrane guanylyl cyclase ODR-1 is absent from the AWC cilia in an *unc-101* mutant. (E) ODR-1 protein is seen in the two AWC cilia (arrowheads) in a wild-type animal. (F) In an AP-1 *unc-101*(*m1*) mutant animal, ODR-1 protein is not detectable in the AWC cilia (nonspecific staining is visible in the pharynx). Animals were fixed and stained with a rabbit polyclonal antibody to ODR-1 protein (L'Etoile and Bargmann, 2000) and Cy3-conjugated secondary antibodies. (G) The candidate odorant receptor STR-1-GFP is localized to the forked AWB cilium (arrowhead) in wild-type animals, with a low level present in the cell body (arrow). (H) STR-1-GFP is partially delocalized to the dendrite (small arrowheads) and axon (wide arrow) in the AP-1 mutant *unc-101*(*m1*) but is still enriched in the AWB cilium (large arrowhead). Anterior is at left in all panels. (G) and (H) are confocal Z series projected into a single plane.

ODR-10-GFP is also localized to cilia when expressed in the two AWB olfactory neurons under the strong promoter of the candidate receptor gene *str-1* (Troemel et al., 1997). The strong AWB expression of ODR-10-GFP allowed us to see both cilia and discrete puncta in the dendrite and cell body of the AWB neuron (Figures 1A and 2A). The punctate structures were heterogeneous in size and shape; some appeared to be $\leq$0.5 μm in diameter, whereas others were tubular and $\geq$2 μm in length. They will be called dendritic vesicles, but, due to the limits of light microscopy and the small diameter of the AWB dendrite, we could not resolve whether these particles are vesicles inside the dendrites or protein clusters on the dendritic plasma membrane.

The ODR-10-GFP-containing dendritic vesicles were capable of rapid movement both toward the cilia (anterograde movement) and toward the cell body (retrograde movement) (see Figure 1A and supplemental movie S1 at http://www.neuron.org/cgi/content/full/31/2/277/DC1). We recorded and analyzed vesicle movements in 14 adult animals, in which a total of 50 moving vesicles were observed for periods of 30 s to several minutes. The movement was saltatory, involving rapid steps, rests, and resumption of movement. In four instances, a vesicle changed direction during observation. Vesicles entered both the cilia and the cell body and emerged from these compartments as well. The speed of vesicle movement ranged from 0.3 to 2.1 μm/s. During active periods, anterograde vesicles moved at a mean rate of 1.42 $\pm$ 0.09 μm/s (n = 19), whereas retrograde vesicles moved at a mean rate of 0.71 $\pm$ 0.04 μm/s (n = 31). The rates of anterograde and retrograde movement were significantly different from one another ($p < 0.001$), suggesting that two different transport mechanisms are used. The duration of individual vesicle movements ranged from 3 to 52 s, with a mean duration of anterograde movement of 7.4 $\pm$ 0.9 s and a mean duration of retrograde movement of 12.4 $\pm$ 2.1 s.

The dendritic vesicles moved at rates comparable to motor protein-driven transport (0.5–5.0 μm/s) (Hirokawa, 1998). To test the hypothesis that the vesicles were carried by ATP-dependent motor proteins, animals were treated with sodium azide, which uncouples oxidative phosphorylation in mitochondria, thereby disrupting ATP production. In *C. elegans*, this causes paralysis that recovers rapidly after azide is removed. When animals were anaesthetized in 5 mM sodium azide, no vesicle movement was observed, suggesting an ATP requirement for vesicle movement.

To ask whether dendritic vesicles were present in nontransgenic animals, we examined electron micrographs of the sensory dendrites of wild-type adults. Immediately proximal to the transition zone at the base of the sensory cilia we observed an accumulation of coated vesicles similar to those previously described by Perkins et al. (1986), with as many as 10 to 15 vesicles in a single dendrite (Figure 1B). The sub-cilia compartment could be a locus of protein sorting or transport. At other regions of the dendrite, vesicles were present but more sparse (Figure 1C); coated vesicles were present at a

frequency of at least one vesicle per 3–5 μm of dendrite. This density of vesicles is consistent with that observed in the ODR-10-GFP transgenic animals and indicates that vesicles are present in normal dendrites and are not an artifact of overexpressing a membrane protein.

Vesicle movement and ODR-10 localization to cilia appeared normal in mutants for three *C. elegans* kinesin genes, *unc-104*, *unc-116*, and *osm-3*, and in mutants for the cytoplasmic dynein *che-3* (Hall and Hedgecock, 1991; Patel et al., 1993; Shakir et al., 1993; Wicks et al., 2000; data not shown). Many additional candidate kinesin, myosin, and dynein motors are encoded by the *C. elegans* genome but are not represented by existing mutants. ODR-10 was also present in the cilia of *unc-33* and *unc-44*/ankyrin mutants, which have widespread defects in neuronal polarity (Li et al., 1992; Otsuka et al., 1995; data not shown).

The distinct anterograde and retrograde vesicles observed in AWB dendrites suggest that the dendrite has a polarized cytoskeleton. In frog olfactory neurons, the basal body at the base of the cilia has been suggested to orient dendritic microtubules (Burton, 1985). The AWB cilia have two domains: a proximal microtubule-rich domain that corresponds to a modified basal body or transition zone and a forked distal domain that lacks highly organized microtubules (Perkins et al., 1986; Figure 1D). In *daf-19* mutants, all sensory neurons lack the proximal transition zone and organized microtubules, and these mutants have been described as lacking all sensory cilia (Perkins et al., 1986). *daf-19* encodes a transcription factor required for expression of many cilium proteins (Swoboda et al., 2000). We examined *daf-19* mutants to test the hypothesis that the basal body/transition zone is required for dendritic polarity. Surprisingly, ODR-10 protein was still localized to the distal AWB dendrite in *daf-19* mutants, though there was also protein accumulation in the cell body (Figure 1E). This result shows that the microtubule-rich transition zone is not essential for ODR-10 sorting to the end of the dendrite. A different candidate receptor, STR-1, was trapped in the cell body in *daf-19* mutants, suggesting that different receptors have different requirements for protein sorting (Figure 1F). We conclude that dendrites have a polarized transport mechanism that localizes ODR-10 receptor protein to cilia even in the absence of a transition zone or basal body.

The Clathrin Adaptor UNC-101 Is Required for Localization of the Odorant Receptor ODR-10 and Other Membrane Proteins to Cilia

The localization and transport of ODR-10-GFP protein were examined in mutants for genes that might affect membrane traffic in neurons. One gene, *unc-101*, was found to be essential for normal ODR-10-GFP localization to cilia. UNC-101 is related to the μ1 (or AP47) subunit of AP-1 found on clathrin-coated vesicle buds at the TGN (Lee et al., 1994). In *unc-101(m1)* null mutants, ODR-10-GFP was uniformly distributed on the entire cell surface (Figure 2B). The defective localization was not caused by increased ODR-10-GFP expression in *unc-101* mutants, since it was observed across a range of protein levels. When expressed at low levels, ODR-10-GFP was localized to the cilia of wild-type animals but only detectable in the cell body and not the cilia of *unc-101* mutants. Thus, UNC-101/AP-1 is required to restrict ODR-10-GFP to cilia.

The AWB cilia often appeared truncated in *unc-101* mutants (Figure 2B, arrowhead). When examined using the STR-1-GFP marker, 51% of *unc-101* mutants had unbranched AWB cilia, whereas 49% had the characteristic forked cilia observed in wild-type animals (n = 137). We considered the possibility that truncated cilia indirectly cause a sorting defect, by examining ODR-10 localization in other mutants with defects in cilium morphogenesis. No defect in ODR-10 localization was observed in *osm-6,* which encodes a component of transport rafts for cytoplasmic cilia proteins (Collet et al., 1998; Cole et al., 1998), *che-3*, which encodes a ciliary cytoplasmic dynein (Wicks et al., 2000), and *daf-19* (Figure 1; Dwyer et al., 1998; data not shown). In all of these mutants, ODR-10 is localized to severely misshapen cilia. These results suggest that *unc-101* has a specific role in protein sorting.

To extend the analysis of *unc-101* and define its functions, we examined the localization of several cilia proteins in different types of sensory cilia. *C. elegans* sensory neurons can have many cilia morphologies: branched (AWB), dendritic (AWA), fanlike (AWC), or simple cilia (ASI, OLQ, and others). Cilia membrane proteins include receptors, ion channels, and other signaling molecules. *unc-101* was required to localize ODR-10-GFP receptor to cilia of the AWA neurons, the AWB neurons, or the ASI neurons (Figure 2, data not shown, see below). *unc-101* was also required to localize the predicted channel protein OSM-9-GFP in the OLQ mechanosensory cilia (Figures 2C and 2D). To ensure that *unc-101* affects endogenous cilia proteins and not just GFP fusion proteins, we examined localization of the transmembrane guanylyl cyclase ODR-1 (L'Etoile and Bargmann, 2000). ODR-1 protein is localized to the AWC olfactory cilia in wild-type animals but was not detectable in AWC cilia in *unc-101* mutants (Figures 2E and 2F). Because of the limited sensitivity of the antibody, it was not possible to determine whether ODR-1 was mislocalized or absent. We conclude that *unc-101* affects the localization of several different cilia membrane proteins and acts in multiple classes of ciliated neurons.

Two candidate olfactory receptor proteins, STR-1 and STR-2, were partially delocalized in *unc-101* mutants. STR-1 is related to ODR-10 and expressed in the AWB olfactory neurons, and a STR-1-GFP fusion protein is localized to the AWB cilia (Troemel et al., 1997) (Figure 2G). STR-1-GFP was enriched in the AWB cilia in *unc-101* mutant animals but was also present at increased levels in dendrites and axons (Figure 2H). A similar partial defect was observed for STR-2-GFP in the AWC olfactory neurons (data not shown). Thus, some receptors are enriched in cilia in the absence of *unc-101*, indicating that there may be multiple pathways of receptor targeting or retention.

Two Different Clathrin Adaptors Have Axonal and Dendritic Sorting Functions within a Single Neuron

Ciliated sensory neurons in *C. elegans* are bipolar, with an axon that contains pre- and postsynaptic specializations and a dendrite that terminates in the sensory cilia.

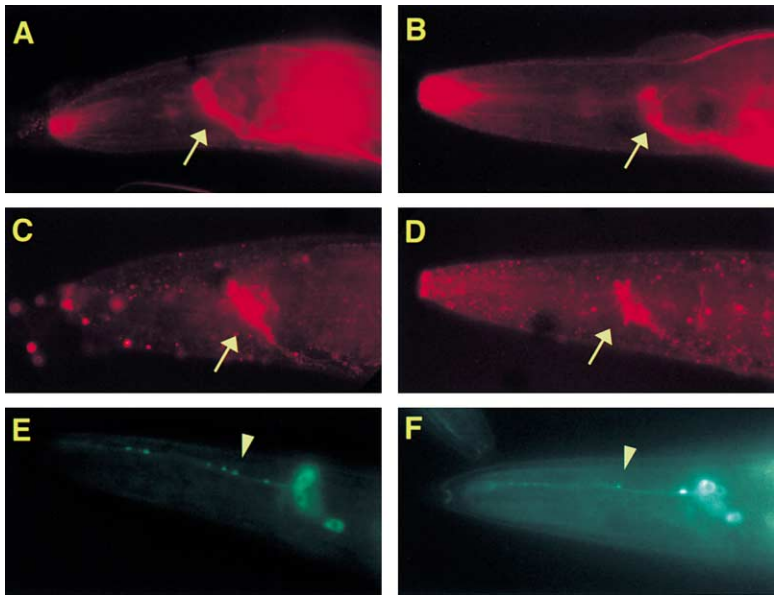

Figure 3. UNC-101 Does Not Disrupt the Axonal Localization of Syntaxin and GLR-1 or Intracellular Localization of ODR-4

(A and B) The t-SNARE syntaxin localizes to axons of the nerve ring (arrows) in both wild-type (A) and AP-1 *unc-101*(*m1*) mutant animals (B). Animals were fixed and stained with rabbit antibodies to *C. elegans* syntaxin (Saifee et al., 1998) and Cy3-conjugated secondary antibodies.

(C and D) The glutamate receptor GLR-1 localizes to axons of the nerve ring (arrows) in both wild-type (C) and AP-1 *unc-101*(*m1*) mutant animals (D). Animals were fixed and stained with rabbit IgG to GLR-1 (Maricq et al., 1995) and Cy3-conjugated secondary antibodies.

(E and F) The localization of the type II membrane protein ODR-4-GFP is punctate in the dendrite (arrowheads) and reticular in the cell body of chemosensory neurons in both wild-type (E) and *unc-101* mutant (F) animals.

To ask whether *unc-101* mutants exhibit a general defect in protein sorting, the localization of other membrane proteins was examined. All *C. elegans* neurons express syntaxin, which is mostly localized to axons (Saifee et al., 1998). In whole-mount immunostaining, syntaxin is concentrated in the nerve ring and ventral nerve cord and mostly excluded from cell bodies (Figure 3A). This staining pattern was unchanged in *unc-101* mutants (Figure 3B). If syntaxin were as delocalized in *unc-101* mutants as ODR-10, we would expect either reduced staining in the axon or additional staining in the cell bodies; neither was observed.

A subset of sensory neurons, interneurons, and motor neurons express the glutamate receptor *glr-1* (Hart et al., 1995; Maricq et al., 1995). In whole-mount immunostaining, GLR-1 has a staining pattern consistent with GLR-1 localization to synapses on axons (Rongo et al., 1998) (Figure 3C). GLR-1 is concentrated in axons and not cell bodies in *unc-101* mutants (Figure 3D). Thus, two axonal proteins maintain their localization in *unc-101* mutants.

ODR-4 is a transmembrane protein that is required for the function and localization of olfactory receptors, including ODR-10 (Dwyer et al., 1998). ODR-4-GFP is present in internal membranes in the cell body, dendrites, and axons of sensory neurons but not in cilia (Figure 3E and Dwyer et al., 1998). ODR-4-GFP does not require *unc-101* for its localization (Figure 3F).

The *C. elegans unc-11* gene encodes the clathrin adaptor protein AP180 and is required for localization of the synaptic vesicle protein synaptobrevin (Nonet et al., 1999). To further investigate the specificity of the *unc-101* phenotype, we compared the effects of the clathrin adaptors *unc-11* and *unc-101* on the localization of two different GFP-tagged proteins in the same sensory neuron.

The ASI chemosensory neurons are bipolar neurons with simple, unbranched cilia, a long dendrite, and axons with about seven synapses each in the adult nerve ring (White et al., 1986). Both ODR-10 and the axonal synaptic vesicle protein synaptobrevin (SNB-1) were separately expressed under the ASI-specific *str-3* promoter and examined in wild-type and mutant animals. ODR-10 protein was localized to the ASI cilia in wild-type animals but was delocalized on the plasma membrane of the cell body, axon, and dendrite in *unc-101* mutants (Figures 4A and 4B). Thus, *unc-101* is required for localization of ODR-10 to the ASI cilia. By contrast, ODR-10-GFP was localized normally to the sensory cilia of *unc-11* mutants (data not shown).

When expressed in ASI, the synaptic vesicle protein SNB-1-GFP localizes to about seven vesicle clusters per axon, suggesting that it labels ASI synapses (Figure 4C). SNB-1-GFP localization in ASI was normal in *unc-101* mutants (Figure 4D). However, in the ASI neurons of *unc-11* mutants SNB-1-GFP was delocalized to the entire plasma membrane, including both axons and dendrites (Figure 4E). These results indicate that UNC-101 and UNC-11 clathrin adaptors can act in the same sensory neuron to target proteins to cilia or axonal synaptic vesicles, respectively.

unc-101 Affects the Formation of ODR-10-Containing Dendritic Vesicles

unc-101 could affect protein localization by creating specific transport vesicles at the TGN, mediating resorting or exocytosis when the dendritic vesicles arrive at the cilia, or stimulating endocytosis of excess protein at the cilia. To distinguish among these possibilities, we asked whether dendritic vesicles were present in *unc-101* mutants. Dendritic ODR-10-GFP fluorescence in *unc-101* mutants was observed following photobleaching of the ubiquitous plasma membrane fluorescence. Following control photobleaching experiments, moving dendritic vesicles were observed in 22/28 wild-type animals but in only 1/20 *unc-101* animals (Figures 5A–5H). In the remaining 19 *unc-101* animals, neither anterograde nor retrograde moving vesicles were observed. These results indicate that *unc-101* is required for the

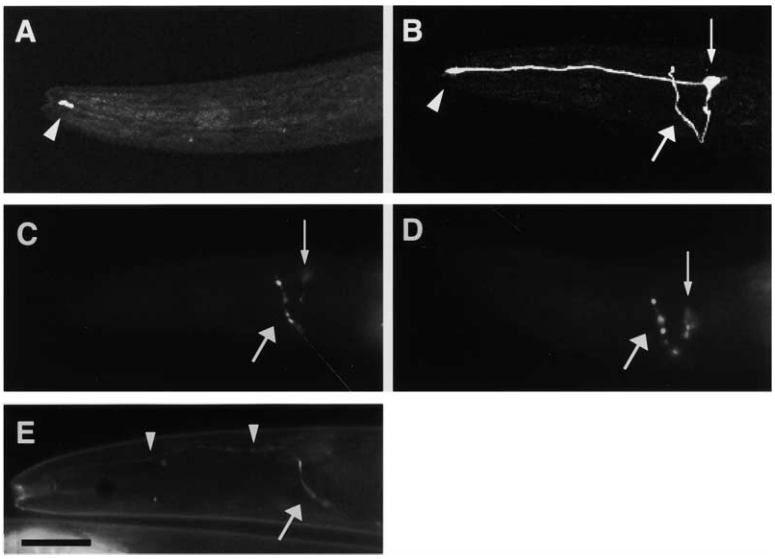

Figure 4. The μ1 Clathrin Adaptor Protein UNC-101 and the AP180 Clathrin Adaptor Protein UNC-11 Have Different Functions in the Same Neuron

(A and B) UNC-101 is required in the ASI neuron for ODR-10-GFP localization. When expressed in the ASI neuron, ODR-10-GFP is restricted to the ASI cilium in a wild-type animal ([A] arrowhead) but not in an *unc-101*(*m1*) mutant animal (B). Thin arrow indicates the cell body, wide arrow indicates the axon, and arrowhead denotes the cilium. (C and D) The *unc-101*(*m1*) mutation does not disrupt localization of the synaptic vesicle protein synaptobrevin in ASI. When expressed using the *str-3* promoter, SNB-1-GFP is localized to clusters in the ASI axon in both wild-type (C) and *unc-101* mutant (D) animals. Wide arrow indicates axon, and thin arrow points to the cell body. (E) SNB-1-GFP is delocalized in the axon (wide arrow) and dendrite (arrowheads) in the AP180 mutant *unc-11*(*ky280*). Bar represents 30 μm for all panels. Anterior is at left and dorsal is up. (A) and (B) are confocal Z series projected into a single plane.

formation of dendritic vesicles or the sorting of ODR-10 into the vesicles.

By sequence, UNC-101 is most similar to the μ1 adaptors that act in the TGN, but it has not been tested directly to see whether it behaves as a μ1-like TGN adaptor or a μ2-like plasma membrane endocytosis adaptor. Some localized epithelial plasma membrane proteins are sorted directly from the TGN, whereas oth-

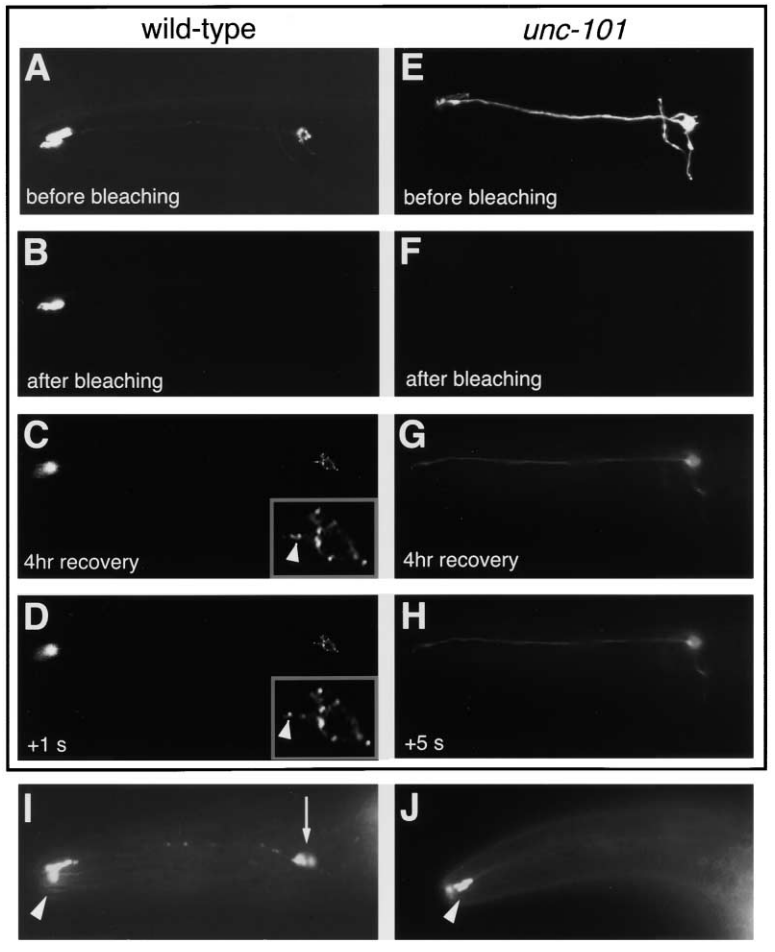

Figure 5. ODR-10 Dendritic Vesicles Are Not Detected in *unc-101* Mutants

(A–D) Wild-type animal (A) before photobleaching, (B) after photobleaching, and (C and D) after recovery. (C) and (D) were taken 1 s apart. A vesicle is moving in an anterograde direction near the cell body (arrowheads, insets). (E–H) *unc-101* animal (E) before photobleaching, (F) after photobleaching, and (G and H) after recovery. (G) and (H) were taken 5 s apart. Although fluorescence recovered slowly, no moving vesicles were observed. (I) AWB::ODR-10-GFP is localized properly to the cilia in the AP-2 mutant *dpy-23*(*e840*) (arrowhead). Arrow denotes cell body. (J) The dynamin mutant *dyn-1*(*ky51ts*) localizes AWB::ODR-10-GFP properly to the AWB cilia (arrowhead). Anterior is at left in all panels.

ers are secreted nonspecifically to the cell surface and sorted following endocytosis (Mostov and Cardone, 1995). If endocytosis is an essential component of sorting to cilia, mutations that affect endocytosis should disrupt ODR-10 localization. DYN-1 encodes a *C. elegans* dynamin that acts in endocytosis (Clark et al., 1997; Grant and Hirsh, 1999); ODR-10-GFP localization was normal in a *dyn-1*(*ts*) mutant at the nonpermissive temperature (Figure 5I). Localization was also unaffected in mutants for the *C. elegans* DPY-23 protein (Figure 5J), which is related to the μ2 (or AP50) subunit of AP-2 required for endocytosis (G. Garriga, personal communication; Schmid, 1997). Although neither of these mutants is a null mutant, the results show that endocytosis can be partly defective without disrupting ODR-10 localization, and they support the model that *unc-101* acts as a μ1 AP-1 subunit at the TGN.

Carboxy-Terminal Sequences of ODR-10 and STR-1 Receptors Affect Their Localization

Many proteins, including serpentine receptors and rhodopsin, have sorting signals in their carboxyl termini (Tam et al., 2000). When the carboxy-terminal residues that followed the last transmembrane domains of ODR-10 and STR-1 were deleted, the truncated, GFP-tagged proteins were trapped in the cell body on reticular structures, suggesting that the carboxyl terminus was required either for sorting or for protein folding (Figure 6A and data not shown). However, a STR-1-ODR-10 chimera in which the C terminus of ODR-10 replaced the C terminus of STR-1 was sorted to the olfactory cilia, suggesting that the ODR-10 C terminus could support the folding or sorting of STR-1 (Figure 6B).

As noted above, the localization of STR-1 was less affected by *unc-101* mutations than ODR-10. The STR-1-ODR-10 chimera bearing the ODR-10 carboxyl terminus behaved significantly more like ODR-10 than did full-length STR-1. In *unc-101* mutants, the STR-1-ODR-10 chimera had less protein localized to cilia than STR-1 (Figure 6C) and an increased percentage of animals without any localization to cilia (Figure 6D) (for STR-1, 59% of *unc-101* animals had some GFP enrichment in cilia, versus 15% for ODR-10 and 27% for STR-1-ODR-10; $p < 0.05$). This result suggests that the carboxyl terminus of the ODR-10 receptor influences its requirement for *unc-101*.

The carboxyl termini of STR-1 and ODR-10 are largely dissimilar, except for a hydrophobic/basic residue combination immediately after the seventh transmembrane domain (FR in ODR-10, YR in STR-1). This pair of residues is highly conserved throughout the G protein-coupled receptor (GPCR) superfamily and is part of a short cytoplasmic helix in rhodopsin that lies perpendicular to the transmembrane helices (Palczewski et al., 2000). The same putative helix includes an FxF motif conserved in STR-1, ODR-10, and other *C. elegans* odorant receptors. We mutated both the FR and the FxF residues to alanines in ODR-10-GFP and examined localization of the altered proteins. When expressed in AWB using the *str-1* promoter, the FR to AA mutant ODR-10-GFP was not enriched in the cilia. Instead, the mutant protein was present in a few large vesicles or a reticular pattern in the cell body and was frequently delocalized evenly across the cell membrane (Figure 6E). In these animals, 27% of neurons examined had GFP detectable only in the cell body, 42% had GFP in the cell body and proximal axon and dendrite, and 31% had GFP visible on the membrane of the entire cell, including cilia ($n = 78$ AWB neurons). The latter class was reminiscent of the even delocalization phenotype of *unc-101* mutants. The FNF to ANA mutant was localized to cilia in a pattern indistinguishable from wild-type ODR-10-GFP (data not shown). These results show that the FR residues in the ODR-10 C terminus are required for localization of ODR-10 to cilia. Localization of the FR to AA ODR-10 protein was identical in wild-type and *unc-101* mutants, suggesting that the FR mutation disrupts a step at or before the step affected by UNC-101.

Discussion

Active Transport of Odorant Receptor Protein in Dendrites

The odorant receptor ODR-10 is localized to the cilia of *C. elegans* chemosensory neurons, where it functions in olfactory transduction. Our results suggest that localization involves specific sequences in ODR-10, an *unc-101*-dependent vesicle sorting pathway, and the transport of ODR-10-containing vesicles through dendrites to cilia.

GFP-tagged ODR-10 protein appears to be transported to the sensory cilia in fast-moving vesicles that travel along the dendrite. The rapid speed of vesicle movement and its requirement for ATP indicate that the ODR-10-GFP vesicles undergo active transport in the dendrite. The ODR-10-GFP dendritic vesicles move at different speeds in the anterograde and retrograde directions, suggesting that at least two motors mediate their transport. Three kinesin genes and a dynein gene were not required for ODR-10 transport, but the *C. elegans* genome encodes at least 21 additional kinesin-related genes, 6 dynein-related genes, and 17 myosin-related genes (The *C. elegans* Sequencing Consortium, 1998). These include a gene related to a vertebrate kinesin that is localized to dendrites (Marszalek et al., 1999).

Dendrites are often described as having randomly oriented microtubules, but the different anterograde and retrograde speeds of vesicle movement suggest that *C. elegans* sensory dendrites have an intrinsic cytoskeletal polarity. In frog olfactory neurons, dendritic microtubules are polarized with the + ends directed toward the cell body (Burton, 1985), and microtubules in the distal half of hippocampal dendrites are also polarized (Baas et al., 1988). One potential source of polarity information in sensory dendrites is the transition zone of the cilium, which is a modified basal body and therefore a potential microtubule organizing center. *daf-19* mutants, which lack this structure, are still able to localize ODR-10 protein, suggesting that there is another source of dendritic polarity in the absence of the transition zone.

The transport of ODR-10 to olfactory cilia is reminiscent of opsin sorting to rod photoreceptor outer segments, which are modified cilia. Vesicles containing opsin are transported in a polarized fashion from the photoreceptor cell body through the rod inner segment

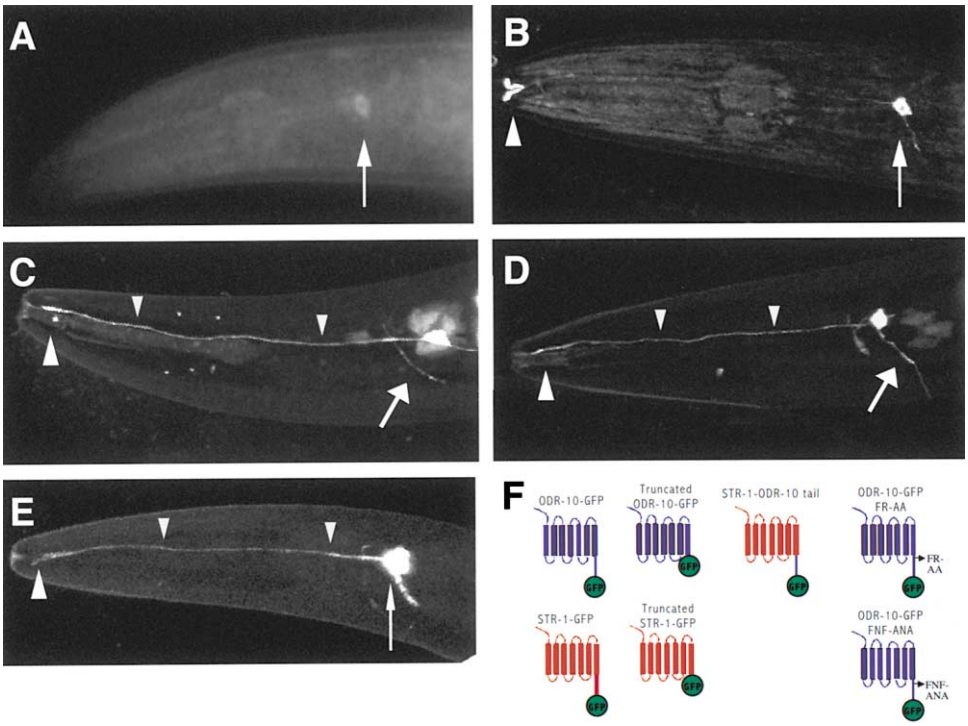

Figure 6. Cytoplasmic Residues of STR-1 and ODR-10 Affect Localization to Cilia

(A) The C-terminal tail of ODR-10 is required for folding or sorting to the cilia. ODR-10 truncated just after the seventh transmembrane domain and tagged with GFP was retained in the cell body (arrow) in wild-type animals. STR-1 was similarly affected (data not shown). (B) A STR-1-ODR-10 tail chimera tagged with GFP localizes normally to the AWB cilia (large arrowhead) in a wild-type animal. (C and D) The STR-1-ODR-10 tail chimera displays more delocalization in an *unc-101*(*m1*) mutant than STR-1 does. Two examples are shown: (C) partly localized and (D) delocalized. Compare the cilia-enriched localization of full-length STR-1 in Figures 2G and 2H. Large arrowhead denotes cilia, small arrowheads denote dendrites, and arrows denote cell bodies. (E) An ODR-10 FR→AA mutant protein is delocalized to the cell body, axons, and dendrites. Although reminiscent of *unc-101* mutants (Figure 2B), a larger fraction of the protein is trapped in intracellular compartments than is observed in *unc-101* mutants. (F) Diagram of GFP fusion proteins and control proteins (see text). (B)–(E) are confocal Z series projected into a single plane. All animals are adults. Anterior is at left, and dorsal is up.

by an unknown mechanism (Deretic and Papermaster, 1991). There, they fuse with the plasma membrane at the basal body. Specific C-terminal sequences as well as dynein, kinesin II, and myosin VII motors are all required for opsin's ultimate delivery to the rod outer segment (Tai et al., 1999; Liu et al., 1999; Marszalek et al., 2000; Tam et al., 2000). The initial polarized movement of vesicles in both photoreceptors and *C. elegans* olfactory neurons suggests the existence of a common cilia sorting pathway that could be used in a variety of sensory neurons.

UNC-101/AP1 Targets ODR-10 and Other Membrane Proteins to Cilia

UNC-101, a predicted μ1 subunit of the AP-1 clathrin adaptor complex, is essential for membrane protein targeting to cilia. *unc-101* affects localization of ODR-10 in several classes of chemosensory neurons, including those with simple cilia (ASI) and those with complex cilia (AWA, AWB). It also affects the localization of the OSM-9 channel in OLQ mechanosensory cilia and the localization of the transmembrane guanylyl cyclase ODR-1 in AWC chemosensory cilia. *unc-101* may be a general *trans*-acting sorting factor for many cilia membrane proteins.

We propose that UNC-101 acts at the TGN to sort ODR-10 into the dendritic transport vesicles, which may target ODR-10 to the cilia plasma membrane. This model is consistent with the absence of ODR-10-containing dendritic vesicles in *unc-101* mutants. Mammalian AP-1 can interact directly with the kinesin KIF13A to transport plasma membrane proteins (Nakagawa et al., 2000); thus, protein sorting and transport could be directly coupled at the level of the AP-1 complex. The ubiquitous plasma membrane localization of ODR-10 in the *unc-101* mutants suggests that the loss of AP-1/μ1 causes ODR-10 to enter a different class of vesicles that can fuse with any plasma membrane. In a second model, UNC-101's effect on ODR-10 could be indirect: UNC-101 may be required for the localization of another protein that mediates targeting of ODR-10 and other cilium proteins. Most AP-1-coated vesicles from the *trans*-Golgi network fuse with the endosome, where their protein contents may be resorted to another compartment (Traub and Kornfeld, 1997). Our results do not distinguish whether ODR-10 uses an endosome as an intermediate step to the cilia, but one candidate for an endosome-like compartment in sensory neurons is the accumulation of coated vesicles observed at the base of the cilia.

The STR-1 and STR-2 candidate receptors showed

some enrichment in sensory cilia in the absence of *unc-101*. These membrane proteins may use other cilia-specific sorting pathways, or they may be captured and retained by cytoplasmic proteins in the cilia. STR-1 localization was abnormal in *daf-19* mutants, which lack the microtubule specializations of cilia (Perkins et al., 1986; Swoboda et al., 2000), but some ODR-10 protein localized to the ends of dendrites in this mutant. Thus, the genetic requirements for sorting can differ for two related receptor proteins expressed in the same neuron.

Although *unc-101* was previously shown to encode a clathrin adaptor, its targets had not been identified (Lee et al., 1994). *unc-101* has an uncoordinated phenotype, suggesting a role in motor neurons or muscles, and also acts as a negative regulator of the epithelial *C. elegans* EGF receptor/vulval signaling pathway (Lee et al., 1994). One potential target of *unc-101* in vulval signaling is the EGF receptor, which has a dynamic subcellular localization in epithelial cells with a temporary basolateral location that is essential for signaling (Simske et al., 1996; Whitfield et al., 1999). The *unc-101*(*m1*) allele used in this study behaves as a null allele (Lee et al., 1994), but a second putative μ1 AP-1 chain, *apm-1,* is encoded in the *C. elegans* genome. *apm-1* has overlapping but not identical functions with *unc-101* (Shim et al., 2000). Since this protein cannot fully compensate for a loss of *unc-101* function, the two μ1 proteins probably have different cargo specificities or cell-type specificities.

Mutating the FR residues after the seventh transmembrane domain of ODR-10 affects receptor localization, causing a delocalized distribution that is similar to ODR-10 distribution in *unc-101* mutants. This delocalized distribution is different from the cell body retention of carboxy-terminal truncated receptors; it suggests that the ODR-10 FR mutant is folded and at least partly released from the endoplasmic reticulum but that it is not recognized by the cilia sorting machinery. An FR or YR motif in this position is present in most G protein-coupled receptors, both in *C. elegans* and in other animals. In the crystal structure of rhodopsin, the corresponding phenylalanine residue points toward the hub of the transmembrane domains, potentially stabilizing other regions of the receptor, including the short cytoplasmic helix that begins at the FR residues (Palczewski et al., 2000). The rhodopsin structure suggests that the FR residues' effect on protein sorting could be indirect and mediated through other intracellular domains of the receptor. Alternatively, a different conformation of the receptor could allow the conserved FR residues to interact directly with AP-1 or other proteins of the cellular sorting machinery.

Our results suggest an analogy between sorting of plasma membrane proteins to the basolateral epithelial surface and sorting to sensory cilia. An epithelial-specific AP-1 μ1B protein affects basolateral localization of the LDL receptor and transferrin receptor in polarized epithelial cells (Folsch et al., 1999). Basolaterally directed vesicles with transferrin receptor appear to be clathrin coated (Futter et al., 1998). Like *unc-101* mutants, epithelial cells lacking μ1B have defects in cell morphology and sorting defects in a subset of transmembrane proteins (Folsch et al., 1999). However, the analogy between UNC-101 and μ1B is not exact, because *unc-101* and *apm-1* are both equally similar in sequence to the mammalian μ1A and μ1B proteins, and mammalian μ1B is not expressed in neurons. The analogous functions of *unc-101* and μ1B suggest that specific AP-1 protein complexes may contribute to a variety of subcellular sorting events. In *C. elegans,* most neurons do not have well-defined axons and dendrites; sensory neurons are unique in having one axon and one dendrite with distinct polarities (White et al., 1986). It will be interesting to determine whether specific AP-1 complexes localize proteins to other dendrite-like postsynaptic processes in *C. elegans* or to cilia or dendrites in other animals.

Experimental Procedures

Strains and Genetics

Wild-type nematodes were *C. elegans* variety Bristol, strain N2. Strains were maintained at 20°C using standard methods (Brenner, 1974). Many strains were provided by the Caenorhabditis Genetics Center. The following strains were used or created in this work: CX3877 *kyIs156 X*, CX3878 *unc-116*(*e2310*) *III; kyIs156 X*, CX 4059 *unc-101*(*m1*) *I*; *kyIs156 X*, PR802 *osm-3*(*p802*) *IV*, CX3882 *unc-104*(*e1265*) *II*; *kyIs156 X*, CX3875 *che-3*(*e1124*) *I*; *kyIs156 X*, CB3253 *dpy-23*(*e840*) *lon-2*(*e678*) *X*, CX3876 *osm-6*(*p811*) *V*; *kyIs156 X*, JT 5531 *daf-19*(*m86ts*); *daf-12*(*m20*)*X*, CX280 *unc-11*(*ky280*) *I*; *kyIs105 V*, and CX3572 *kyIs105 V*. *kyIs105*=*ASI::VAMP-GFP*, *kyIs156*=*AWB::ODR-10-GFP. ASI::ODR-10-GFP* was examined in extrachromosomal arrays.

Paralytic agents used in agarose pads on slides were either 5 mM sodium azide or 1–7.5 mM levamisole. Levamisole causes hypercontraction of the animals' muscles. To control for the possibility that the levamisole was causing an artifactual movement of the ODR-10-GFP-containing vesicles, animals were examined using two other immobilization procedures. Rapid vesicle movement was observed when piperazine, a $GABA_A$ receptor agonist, was used as an anesthetic and when paralyzed animals (such as animals mutant for the muscle myosin *unc-54*) were examined without anesthetic (data not shown).

Microscopy

Photographs were taken on a Zeiss Axioplan II fluorescence microscope or a BioRad MRC 1024 confocal microscope. Time-lapse images were captured using a cooled CCD camera based on Kodak 768 × 512 CCD (Princeton Instruments), a Princeton Instruments Micromax Controller, and Metamorph software (Universal Imaging). The signal was increased by 2 × 2 binning of pixels. Exposure times were 100 ms, and images were captured every 0.9 s. Sections (30 nm) for electron microscopy were prepared and processed as described (Jorgensen et al., 1995). Antibody staining was conducted as described (L'Etoile and Bargmann, 2000).

For photobleaching experiments, animals anaesthetized with levamisole were bleached at 100% power for 45 min using a BioRad MRC 1024 confocal microscope. This protocol bleached the cilia, cell bodies, and dendrites of *unc-101* mutants and the cell bodies and dendrites of wild-type animals (wild-type cilia were too intense to bleach). Animals were recovered for 3–4 hr on food to allow new protein synthesis, remounted onto slides with levamisole, and examined on a compound microscope.

Quantitative Analysis of Vesicle Speeds

Vesicle speeds were estimated by the following method. Frames in a Metamorph time-lapse CCD sequence were stacked. Speeds were only measured for movements in which the vesicle was in focus and was in continuous movement. Speeds were not measured for vesicles less than about half a micron long, because they were not well-resolved. Distances were calibrated in Metamorph by imaging a hemocytometer (VWR). Time was measured using the time-stamp feature of Metamorph.

ODR-10/STR-1 Mutations and Chimeras

The seventh transmembrane domain of ODR-10 ends with the residues LII and is followed by the predicted cytoplasmic tail sequence

RDFRRTIFNFLCGKKNSVDESRSTTRANLSQVPT(stop). In ODR-10-GFP, the V at −3 is followed by a polylinker sequence (GSGIPDPL VLAVEK) and GFP. A carboxy-terminal deletion was made using a BclI site in ODR-10 such that the LI residues were followed by the polylinker and GFP. A similar truncation of STR-1 was made at the analogous position. STR-1-GFP has the predicted cytoplasmic tail residues KCYRKAVIKYLNILFSFCSNQPTIV followed by polylinker sequence and GFP. These residues were replaced with the predicted ODR-10 tail residues, polylinker, and GFP for the STR-1-ODR-10 chimera. The FR→AA and FNF→ANA mutant ODR-10-GFP clones were made using the Quickchange mutagenesis kit (Stratagene). All clones were sequenced through the carboxy-terminal regions and the fusion site with GFP. Sequences are available upon request.

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