A stomatin and a degenerin interact in lipid rafts of the nervous system of Caenorhabditis elegans


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Sedensky, M. M., J. M. Siefker, J. Y. Koh, D. M. Miller III, and P. G. Morgan. A stomatin and a degenerin interact in lipid rafts of the nervous system of Caenorhabditis elegans. Am J Physiol Cell Physiol 287: C468–C474, 2004. First published April 21, 2004; 10.1152/ajpcell.00182.2003.—In Caenorhabditis elegans, the gene unc-1 controls anesthetic sensitivity and normal locomotion. The protein UNC-1 is a close homolog of the mammalian protein stomatin and is expressed primarily in the nervous system. Genetic studies in C. elegans have shown that the UNC-1 protein interacts with a sodium channel subunit, UNC-8. In humans, absence of stomatin is associated with abnormal sodium and potassium levels in red blood cells. Stomatin also has been postulated to participate in the formation of lipid rafts, which are membrane microdomains associated with protein complexes, cholesterol, and sphingolipids. In this study, we isolated a low-density, detergent-resistant fraction from cell membranes of C. elegans. This fraction contains cholesterol, sphingolipids, and protein consistent with their identification as lipid rafts. We then probed Western blots of protein from the rafts and found that the UNC-1 protein is almost totally restricted to this fraction. The UNC-8 protein is also found in rafts and coimmunoprecipitates UNC-1. A second stomatin-like protein, UNC-24, also affects anesthetic sensitivity, is found in lipid rafts, and regulates UNC-1 distribution. Mutations in the unc-24 gene alter the distribution of UNC-1 in lipid rafts. Each of these mutations alters anesthetic sensitivity in C. elegans. Because lipid rafts contain many of the putative targets of volatile anesthetics, they may represent a novel class of targets for volatile anesthetics.

Multiple lines of evidence suggest that unc-1 and unc-8 affect the same physiological function in controlling both locomotion and anesthetic sensitivity. Genetic data indicate that unc-1 and unc-8 interact with each other to control movement and behavior in anesthetics (31). Loss-of-function alleles of unc-1 are able to suppress a neomorphic allele of unc-8, and dominant alleles of each interact to produce a novel phenotype. Antibodies to UNC-1 stain major nerve tracts (35); this finding confirmed a similar distribution seen with green fluorescent protein (GFP)-UNC-1 fusion constructs that rescued the null phenotype (31). Tavernarakis et al. (43) originally identified unc-8 as a degenerin in specific neurons of C. elegans. They localized UNC-8 to a small subset of ventral cord neurons and postulated that UNC-8 is part of a sodium channel that senses stretch/curvature during the sinusoidal movement of the nematode. By responding to this stretch, the sodium channel is thought to control the amplitude of the wave of muscle contractions that propagate along the body of the animal. Chalfie and colleagues (11, 15) showed that the stomatin-like protein (SLP) MEC-2 directly interacts with ENaC subunits in controlling the nematode’s response to light touch. They provided corroborating electrophysiological data by expressing the proteins in frog oocytes (11). We have shown that UNC-24, another SLP in C. elegans, affects the function of UNC-1. Mutations in unc-24, which phenocopy unc-1(0) alleles, block the movement of UNC-1 from the perinuclear region to the peripheral cell membrane (35). Together, these data indicate that SLPs interact with each other as well as with ENaCs.

In mammals, stomatin and ENaCs are also found in a wide array of cell types, including those of the central nervous system (5, 10, 22, 34, 36). Work by Fricke et al. (10) localized both stomatin and ENaC subunits to the same cells in the dorsal root ganglion (DRG) of rats. Because direct mechanical stimulation is unlikely to involve the DRG, the stomatin-ENaC interaction may have a function other than directly transducing mechanical input to the nervous system. One possibility is the transduction of painful signals to the central nervous system. Consistent with a broader role in the nervous system, mammalian stomatin has been shown to interact with other proteins, including G protein-coupled receptors and soluble N-ethylmaleimide-sensitive factor attachment protein target receptor (SNARE) complex proteins (19, 23, 39). Finally, channels closely related to ENaCs, the acid-sensitive ion channels (ASICs), have been shown to be important in nociception (5, 17).

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Stomatins were originally identified in the membranes of mammalian red blood cells (RBCs); it is absent in RBC membranes from patients with the hemolytic anemia stomatocytosis (6, 40). RBCs from patients with stomatocytosis have abnormal gradients of sodium and potassium across their cell membranes. The original model of stomatin’s structure postulated a long intracellular domain that was likened to a “ball and chain” anchored in the plasma membrane by the molecule’s transmembrane segment (40). In this manner, stomatin was thought to regulate associated membrane channels. However, new data outlining the role of lipid rafts have modified theories concerning the function of stomatin in the cell membrane.

It is now well established that cellular membranes in mammals do not consist of uniformly distributed lipid and cholesterol components. Instead, microdomains exist within the cell membrane (14, 24). These domains are thought to serve as scaffolds to arrange protein complexes and partially regulate their function. The mammalian protein caveolin is a scaffolding protein in one type of membrane domain known as a cave. Stomatins now have been known to share structural similarities (14, 24, 42). Similarly to caveolin, stomatin has been associated with cell membrane microdomains; these domains are known as lipid rafts (21, 33, 38). Lipid rafts are detergent-resistant, low-density regions of the membrane that are thought to be important in sequestering protein complexes (14, 24). In addition, these membrane fractions are relatively rich in cholesterol and sphingolipids. Stomatins are postulated to play a role similar to that of caveolin, i.e., to regulate the formation and maintenance of membrane domains.

Originally isolated from mammalian endothelial cells in culture (14), lipid rafts have been identified in three invertebrate species: Drosophila, Dictyostelium, and Strongylocentrotus (2, 13, 32). The proposed functions for UNC-1 have led us to study whether lipid rafts exist in C. elegans and to determine whether UNC-1 or UNC-8 is present in lipid rafts. We hypothesized that 1) lipid rafts exist in C. elegans and that UNC-1 and UNC-8 are sequestered to rafts; 2) in the absence of UNC-24 or UNC-1, lipid rafts will not form; and 3) if rafts form in the absence of UNC-24, the proteins UNC-1 and UNC-8 will not segregate normally to the rafts; because 4) rafts have been shown to form in the endoplasmic reticulum, UNC-24 will be found in the raft fraction; and, finally, because 5) ENaCs and stomatins genetically interact and are found in the same cells, UNC-1 and UNC-8 will physically interact.

We have demonstrated that lipid rafts, when isolated under conditions similar to those in other species, do exist in C. elegans. UNC-1, UNC-24, and UNC-8 are all found in lipid rafts. Null mutations in unc-24 and unc-8 affect the distribution of UNC-1 in rafts, whereas unc-1(0) eliminates UNC-8 from lipid rafts. However, none of these mutations eliminate the formation of a discernible raft fraction in C. elegans. The importance of lipid rafts as a potential target of the lipid-soluble volatile anesthetics is discussed.

MATERIALS AND METHODS

Nematodes. Nematodes were cultured as previously described (4, 26). The wild-type nematode is N2 in all experiments and was obtained from the Caenorhabditis Genetics Center, as was the null allele, unc-24(eDfQ8), unc-1(e580) was obtained from Carl Johnson (Axys Pharmaceuticals, San Francisco, CA). unc-1(e58)) was isolated in our laboratory. unc-8(e151b145) was kindly supplied by Monica Driscoll (Rutgers University). All experiments were performed at 20–22°C.

Antibodies. The cDNA for UNC-1 was isolated as described by Rajaram et al. (30). Monoclonal antibodies against the entire UNC-1 protein were prepared in the laboratory of Man Sun Sy as previously described (35). In the UNC-1 studies presented in this article, we used a single monoclonal anti-UNC-1 antibody; the precise epitope of this antibody is not known. The partial unc-8 cDNA was supplied by Monica Driscoll. Polyclonal antibodies against the expressed partial UNC-8 protein were isolated from chickens and affinity purified by Pocono Rabbit Farm & Laboratory (Canadensis, PA). Rabbit polyclonal antibodies against UNC-24 were raised against the COOH terminus of the protein (amino acids 400–415) and affinity purified. Western blotting and antibody probing were done according to standard methods (7). Immunohistochemical techniques were followed as previously described (35).

Lipid rafts. Lipid rafts were isolated using a modification of the technique of Moffett et al. (24). Nematodes were grown in 500 ml of liquid culture, spun down in the cold, and separated from Escherichia coli and nematodes by centrifugation through a sucrose cushion (8, 46). Animals were sonicated on ice with 50 μl of protease inhibitor cocktail (leupeptin, pepstatin A, and 50 μl of PMSF inhibitor, 10 μg/ml each) in homogenization buffer (20 mM Tris, 20 mM HEPES, pH 7.4, 30 mM mannitol, and 10 mM CaCl₂) and centrifuged at 1,000 g to remove nuclei. The supernatant was removed and then spun at 60,000 g at 4°C for 30 min. The membrane pellets were suspended in 2 ml of ice-cold TNE (50 mM Tris-Cl, pH 8.0, 130 mM NaCl, and 5 mM EDTA) containing 1% Triton X-100. The resulting suspension was placed on either a 12-ml (for cholesterol and sphingolipids determination, see Fig. 1, C and D) or 8-ml (for Western blot analysis) sucrose step gradient and centrifuged at 100,000 g for 16–20 h at 4°C. Sucrose step gradients consisted of 2 ml of 5%, 4 ml of 35%, and 2 ml of 40% sucrose (8-ml gradient) or 2 ml of 5%, 6 ml of 35%, and 4 ml of 40% sucrose (12-ml gradient) containing Triton-X 100. The density of gradient fractions was determined by weighing known volumes on an analytical balance. Fractions (1 ml) of the gradient were extracted with methanol-chloroform (20, 24), run on 10–12% SDS-PAGE gels, and stained with Coomassie blue per standard techniques. Western blots were probed with UNC-1, UNC-8, or UNC-24 antibodies.

Phospholipids (lecithin, lysolecithin, and sphingomyelin) were analyzed spectrophotometrically using the phospholipids B reagent (Wako Chemicals, Richmond, VA). Phospholipids are first hydrolyzed by phospholipase D. Choline is released by choline oxidase, which causes the quantitative release of hydrogen peroxide. Hydrogen peroxide production is determined at 505 nm by the peroxidase-catalyzed production of quinone pigment from 4-aminoantipyrine and phenol. Cholesterol content was determined using a similar protocol from Synchron Systems (Beckman Coulter, Fullerton, CA). Free cholesterol is released by cholesterol esterase and oxidized by cholesterol oxidase. This reaction also releases hydrogen peroxide quantitatively, which is analyzed as described above for phospholipids (20).

Coimmunoprecipitation. Total protein was isolated from wild-type worms. Worms were homogenized in NET buffer (150 mM NaCl, 5 mM EDTA, and 50 mM Tris, pH 7.5) plus 1% Triton X-100, PMSF, and 250 μg of protease inhibitor. The resulting homogenate was spun in a clinical centrifuge. The resulting supernatant was exposed to antibodies to UNC-8 bound to Sepharose beads for 1–2 h. The beads were washed with sample buffer and boiled with 60 μl of SDS sample buffer to elute the bound protein. The resulting eluate was run on a 10% PAGE gel, transferred to a Western blot, and probed using established methods (7).

RESULTS

Lipid rafts. Adapting a protocol previously used to isolate lipid rafts in mammals (24), we detected a detergent-insoluble
fraction in membrane extracts of *C. elegans*. This was usually seen as a conspicuous white ring floating in the middle-density portion (1.08–1.12 g/ml) of the sucrose step gradient (Fig. 1A), generally fractions 3–8 of a 12-ml gradient or fractions 3–6 of an 8-ml gradient (in Fig. 1, each lane represents a 1-ml fraction). Early studies were performed with 12-ml gradients and later ones with 8-ml gradients (see MATERIALS AND METHODS). Although the absolute fractions in which the raft was most conspicuous were not identical in all preparations, the raft was always within the middle step of the gradient; for each preparation, its position was noted relative to subsequent gradient fractionation. The middle-density fractions contained protein (Fig. 1B) as well as cholesterol and sphingolipid (Fig. 1, C and D). These characteristics are the same as those that define lipid rafts in mammals (14, 24). The density of the middle fractions progressed from 1.08 to 1.12 from the top to the bottom of this step; densities of 1.08–1.12 g/ml are reported for rafts isolated from mammals (14). Second peaks of cholesterol and sphingolipids, as well as protein, were also found in the higher density fractions.

**UNC-1.** Probing a Western blot of membrane fractions with anti-UNC-1 indicated that UNC-1 localized to those fractions of the membrane that contained lipid rafts (Fig. 2A). In rafts, UNC-1 segregates at ~26 kDa, indicating that it is processed from its predicted size of 32 kDa; the larger form is readily detected in whole worm preparations of N2 (35). Essentially no UNC-1 was seen in the membrane pellet fraction in the N2 animal. We subsequently used UNC-1, when present, as a marker for rafts (see below). Rafts do exist in unc-1(0) animals, however. A pronounced white band was seen in the middle-density region of the sucrose gradients. Protein, cholesterol, and sphingolipids were present in the same fractions from *unc-1*(e580) or *unc-1*(fc53) animals as in wild-type animals (data not shown). As expected, no UNC-1 could be detected in the rafts of *unc-1*(0) animals (Fig. 2B).

**UNC-8.** The UNC-8 protein was detected in lipid rafts (Fig. 3A) in wild-type animals. However, UNC-8, unlike UNC-1, also were localized to the higher density membrane pellet (not shown). In *unc-8*(0) animals, no UNC-8 was detectable in the fractions that contained rafts (data not shown), though rafts were macroscopically visible on sucrose gradients and were localized by the presence of protein, cholesterol, and lipid in the middle-density, detergent-resistant fractions (data not shown). In addition, UNC-8 protein was not detected in lipid raft preparations from *unc-1*(0) animals (Fig. 3B). The distribution of UNC-1 in a sucrose gradient was the same in both N2 and *unc-8*(0) animals. However, the predominant form of UNC-1 protein was ~32 kDa in *unc-8*(0) (compared with 26 kDa in N2, Fig. 3C); in addition, a band of ~30 kDa also appears in *unc-8*(0) animals, as well as in itself in *unc-1*(0) animals.

In two independent experiments, the position of the UNC-1 band was noted relative to subsequent gradient fractionation. The middle-density fractions contained protein (Fig. 1B) as well as cholesterol and sphingolipid (Fig. 1, C and D). These characteristics are the same as those that define lipid rafts in mammals (14, 24). The density of the middle fractions progressed from 1.08 to 1.12 from the top to the bottom of this step; densities of 1.08–1.12 g/ml are reported for rafts isolated from mammals (14). Second peaks of cholesterol and sphingolipids, as well as protein, were also found in the higher density fractions.

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LIPID RAFTS IN C. ELEGANS

Our results indicate that lipid rafts exist in C. elegans with physical characteristics similar to those seen in other organisms. These rafts are of a similar density to those found in vertebrates and are enriched in both cholesterol and sphingolipids (14, 24). However, cholesterol and sphingolipids are also found in the high-density fractions of the sucrose gradients. Nusrat et al. (28) also found that sphingolipids were enriched in the high-density fractions of the detergent-insoluble glycolipid. However, they did not find a second peak for cholesterol.

DISCUSSION

Our results indicate that lipid rafts exist in C. elegans with physical characteristics similar to those seen in other organisms. These rafts are of a similar density to those found in vertebrates and are enriched in both cholesterol and sphingolipids.
It is not clear whether these fractions identify rafts with different physical properties or whether significant amounts of cholesterol are present in other cellular locations. It is possible that nematodes differ from mammals in their cellular distribution of cholesterol.

It is unknown whether stomatins or similar proteins are necessary for raft formation or maintenance. In the wild-type strain N2, essentially all UNC-1 is associated with rafts. However, UNC-1 expression is not required for all raft formation, since rafts are also present in unc-1(0) alleles. Though UNC-1 appears to be essentially a pan-neuronal stomatin and is therefore widely expressed, a total of 10 SLPs are potentially encoded by the C. elegans genome. It is therefore likely that other SLPs are also associated with rafts in C. elegans and may serve to support raft formation.

It is important to note that the UNC-1 protein is found in the raft isolates even when it is sequestered to the perinuclear region. UNC-24, which is primarily restricted to the perinuclear region (Koh JY, Meir J, and Miller DM, unpublished data), is also found in rafts. These findings indicate that we have identified both mature rafts in the plasma membrane and probable immature rafts in the endoplasmic reticulum. At present, we cannot separate these populations.

Our previous immunohistochemical studies of UNC-1 in unc-24 mutants indicate that UNC-1 may move from the perinuclear region of neurons to axonal membranes (35). Data presented here show that UNC-1 associates with lipid rafts in both wild-type and unc-24 animals, although more of the 32-kDa form is seen in unc-24 animals. In total protein preparations from unc-24(0) animals, the 32-kDa form is virtually the only identifiable species of the UNC-1 protein (35). In addition, in isolates from unc-24 animals, some UNC-1 is found in the higher density fractions of the step gradient (not shown). We interpret these data to indicate that lipid rafts are formed in the endoplasmic reticulum and contain an incompletely processed UNC-1, which is seen as the predominant 32-kDa form of UNC-1 in raft fractions. UNC-24 is necessary for moving UNC-1 as part of a raft to the cell membrane, and UNC-24 has both a stomatin domain and a lipid transfer domain (1). We postulate that UNC-24 binds both lipids and stomatins and facilitates the processing of UNC-1 into its usual 26-kDa form in lipid rafts in neuronal membranes. Lack of functional UNC-24 also results in UNC-1 accumulation in a higher density membrane fraction than a raft, which may represent immature rafts located in the endoplasmic reticulum.

The protein UNC-8 is also associated with the lipid raft fraction on sucrose gradients. Interestingly, previous studies have noted that ENaCs are not associated with rafts in mammalian kidney cells (12). However, since UNC-1 strongly associates with rafts and communoprecipitates UNC-8, it is not surprising that UNC-8 also associates with rafts in C. elegans. Its presence in rafts is dependent on the presence of UNC-1, based on its absence from rafts in the unc-1(0) animal. We have previously reported genetic data to link the functions of UNC-1 and UNC-8 (31). Coupled with the communoprecipitation results and their colocalization to lipid rafts in nematodes, as well as the association of their homologs in other systems, these proteins appear to physically interact in vivo.

In unc-8(0), the size of the predominant UNC-1 in lipid rafts is increased; it may be that binding to UNC-8 facilitates final processing of the UNC-1 protein in a raft. As would be expected, both the 32- and 26-kDa forms of the UNC-1 protein are seen on Western blots of total worm protein in wild-type animals (35). However, it appears that the predominant form of UNC-1 found in the mature raft, as well as in total protein, is the 26-kDa form.

As noted in the Introduction, stomatin was originally identified as a protein missing from the membranes of RBCs in patients with a hereditary hemolytic anemia. These changes were presumed to be the result of mutations in the stomatin gene that resulted in loss of the protein. However, no mutations were found in stomatin in patients with stomatocytosis (6). In addition, a mouse knockout of this gene does not have hemolytic anemia (6, 47). This has led to reconsideration of what might be the precise role of stomatin.

Chalfie and colleagues (11, 15) elucidated the physiological relationship of the SLP MEC-2 with two ENaCs, MEC-4 and MEC-10. Mutations in these proteins disrupt the nematode’s response to light touch; thus they are postulated to be necessary for the transduction of a mechanosensory signal in C. elegans. Chalfie et al. (15) further showed that the presence of MEC-2 is necessary for maximal sodium current conduction by MEC-4 homomeric channels in Xenopus oocytes. It remains to be seen whether all ENaCs in C. elegans associate with rafts or with SLPs. However, our results, coupled with those showing that MEC-2 interacts with MEC-4, indicate that this association is likely of a general nature. It remains unclear whether stomatin plays a primary role in raft homeostasis or functions primarily as an interacting partner with other raft proteins.

We previously showed (25, 31) that mutations in unc-1, unc-8, and unc-24 each alter sensitivity to volatile anesthetics. In addition, ENaCs are now known to be crucial to neuronal function in the response to pain (3, 9). One class of the ENaC family is the ASIC. ASICs are proton-gated channels found in both the peripheral and central nervous systems in mammals. These channels have been shown to be responsible for the transduction of pain and mechanosensation. These data, coupled with the colocalization of ENaCs and stomatins in the DRG of mammals (10), demonstrate the ubiquitous nature of the association of these two families of proteins across very different phyla and may have important implications for understanding neuronal functions crucial to processing of sensory information.

For the past two decades, considerable debate has centered on the molecular sites of action of volatile anesthetics and how these agents cause loss of consciousness and alleviate pain (18). These unique compounds are very lipophilic; their potency is a direct function of their oil/gas partition coefficient (18, 27). However, they also can affect protein function in a lipid-free environment (27). As a result, both proteins and lipids have been postulated to be primary target of these unique agents. The list of proteins associated with lipid rafts includes ligand-gated channels, G protein-coupled receptors, and members of the SNARE complex (24, 41, 44). Each of these has been postulated to be a target of volatile anesthetics (16, 18, 45). Because rafts form a complex containing both lipids and proteins, it is possible that volatile anesthetics can perturb specific protein complexes embedded in this unique lipid milieu. These data may eventually serve as a unifying model for understanding how volatile anesthetics work at the molecular level.
In summary, we found that rafts do exist in nematodes and that UNC-1, UNC-8, and UNC-24 are present in lipid rafts in *C. elegans*. Loss of UNC-1 alters the distribution of UNC-8 into rafts, but does not alter the formation of rafts. Mutations in unc-24 and unc-8 alter the processing of UNC-1 found in lipid rafts. Mutations in unc-24 do not alter the distribution of UNC-8 into lipid rafts. Because these proteins are known to play a role in anesthetic response, we propose that rafts are possible targets for volatile anesthetics.

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