Cholera Toxin Is Found in Detergent-insoluble Rafts/Domains at the Cell Surface of Hippocampal Neurons but Is Internalized via a Raft-independent Mechanism*

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A number of studies have demonstrated that cholera toxin (CT) is found in detergent-insoluble, cholesterol-enriched domains (rafts) in various cells, including neurons. We now demonstrate that even though CT is associated with these domains at the cell surface of cultured hippocampal neurons, it is internalized via a raft-independent mechanism, at both early and late stages of neuronal development. CT transport to the Golgi apparatus, and its subsequent degradation, is inhibited by hypertonic medium (sucrose), and by chlorpromazine; the former blocks clathrin recruitment, and the latter causes aberrant endosomal accumulation of clathrin. Moreover, both internalization of the transferrin receptor (Tf-R), which occurs via a clathrin-dependent mechanism, and CT internalization, are inhibited to a similar extent by sucrose. In contrast, the cholesterol-binding agents filipin and methyl-β-cyclodextrin have no effect on the rate of CT or Tf-R internalization. Finally, once internalized, CT becomes more detergent-soluble, and chlorpromazine treatment renders internalized CT completely detergent-soluble. We propose two models to explain how, despite being detergent-insoluble at the cell surface, CT is nevertheless internalized via a raft-independent mechanism in hippocampal neurons.

Cholera toxin (CT) consists of a pentameric B subunit that binds with high affinity to ganglioside GM1 and an A subunit comprising two peptides, A1 and A2, linked by a disulfide bond. The A1 subunit is responsible for activation of adenylate cyclase via the stimulatory G protein, Gs. Electron microscopy analysis in A431 cells, and in cultured liver cells, demonstrated that CT does not bind uniformly over the plasma membrane but is rather concentrated in membrane invaginations (1) identified as caveolae (2). Caveolae contain the coat protein, caveolin (3), and are enriched in glycosphingolipids (GSLs) and cholesterol (4). Biochemical analysis has shown that the GSLs and cholesterol found in caveolae are insoluble in nonionic detergents at low temperature (4). However, not all cells contain caveolin or morphologically distinct caveolae. Smooth muscle cells, fibroblasts, adipocytes, endothelial cells, and epithelial cells express caveolin/caveoleae, but lymphocytes and neurons do not (3). Even in cells lacking caveolae, a significant fraction of cellular cholesterol and GSLs are found in detergent-insoluble complexes (5–7), sometime known as rafts (8); and these complexes are indistinguishable, using the criteria of detergent insolubility, from those associated with caveolae (9).

Due to its association with caveolae and/or detergent-insoluble domains, it is normally assumed that CT is internalized by the pinching off of caveolae from the plasma membrane (10), followed by transport to the Golgi apparatus and endoplasmic reticulum (11, 12). Recent studies in A431 cells, which express high levels of caveolin, in CaCo-2 cells, which express low levels of caveolin, and in Jurkat cells, which express no caveolin, demonstrated that the cholesterol-binding agent filipin disrupted CT internalization and the subsequent generation of cAMP in all three cell types (13), presumably due to extraction of cholesterol from caveolae or detergent-insoluble domains.

In contrast to the cell types mentioned above, less is known about how CT is internalized in neurons; neurons contain high levels of GSLs, and particularly of GM1 (14). In cultured hippocampal neurons (15), high levels of GM1 can be detected after about 2 days in culture (16), and consequently CT binding increases as neurons mature (17). CT is internalized by an energy- and temperature-dependent mechanism to the Golgi apparatus (18), and at least in CT containing the A subunit (rather than holo-CT which only contains the B subunit, but still binds GM1), to the endoplasmic reticulum (17). Transport to the Golgi apparatus and cAMP elevation are inhibited by cationic amphiphilic drugs (i.e. chlorpromazine, imipramine, and sphingosine) (18), which block receptor recycling by disrupting the assembly-disassembly of clathrin from coated pits and endosomes (19). These data are consistent with the rapid appearance of CT in the same endosomal compartment as that labeled by α2-macroglobulin, a ligand that enters cells via clathrin-coated pits (20), and data showing that CT is not excluded from clathrin-coated pits, even though it is enriched in caveolae in cells that contain these structures (1, 2).

To ascertain the mechanism of CT internalization in neurons, and the relationship of detergent insolubility to the mode of internalization, we now examine the effect of various drugs that inhibit clathrin-mediated endocytosis or bind cholesterol and hence disrupt detergent-insoluble domains. In addition, we compare CT internalization with that of the transferrin receptor (Tf-R), which is internalized exclusively via a clathrin-mediated mechanism (21). We confirm that CT is found in detergent-insoluble membrane domains at the cell surface of 8–9-day-old hippocampal neurons (22, 23), as is the GPI-anchored protein Thy-1 (24), but CT is nevertheless internalized via a raft-independent mechanism.
**EXPERIMENTAL PROCEDURES**

**Materials—**CT was purchased from Calbiochem. Horseradish peroxidase-conjugated CT was from List Biological Laboratories, Inc., Campbell, CA. Bodipy succinimydyl ester was from Molecular Probes Inc., Eugene, OR. Na\textsuperscript{125}I (carrier-free) was from Amersham Pharmacia Biotech. IODO-BEADS were from Pierce. Chloroprome (CPZ), filipin, methyl-β-cyclodextrin (MβCD), and Tf were from Sigma.

**Incubation of Hippocampal Neurons with Bodipy-CT—**Bodipy-CT was prepared by conjugation of CT with Bodipy succinimydyl ester according to the manufacturer’s instructions. Hippocampal neurons cultured at low density (6,000–12,000 cells/13-mm glass coverslip) (15, 18) were incubated with 10 nM Bodipy-CT for 30 min at 13–16 °C in Hank’s balanced salt solution (HBSS) containing 10 mM HEPES, pH 7.4, and 0.1% (w/v) bovine serum albumin (BSA). After washing, neurons were incubated in N2.1 medium (serum-free medium) (15, 16) in the continuous presence of the drugs.

**CT Degradation—**CT was iodinated as described (25) to a specific activity of 57 Ci/mmol. Neurons (25,000 cells/13-mm glass coverslip) were incubated with 5 nM \textsuperscript{125}I-CT for 30 min at 13–16 °C in HBSS containing 10 mM HEPES, pH 7.4, and 0.1% (w/v) BSA. After washing with HBSS, neurons were incubated in N2.1 medium without glutathione at 37 °C for 8 h and then cooled on ice. Coverslips were removed from the culture dishes, and the radioactivity of coverslips (cell-associated CT) and trichloroacetic acid-soluble material in the culture medium (degraded CT) was determined. The amount of degraded CT was expressed as a percentage of total cell-associated radioactivity. To examine the effect of drugs on CT degradation, neurons were preincubated in N2.1 medium without glutathione for 30 min at 37 °C. Neurons were incubated with Bodipy-CT as described above in the continuous presence of the drugs.

**CT Endocytosis—**Neurons (50,000 cells/13-mm glass coverslip) were incubated with 5 nM \textsuperscript{125}I-CT for 30 min at 13–16 °C in HBSS containing 10 mM HEPES, pH 7.4, and 0.1% (w/v) BSA. After washing with HBSS, neurons were incubated in N2.1 medium without glutathione at 37 °C for 8 h and then cooled on ice. Coverslips were removed from the culture dishes, and the radioactivity of coverslips (cell-associated CT) and trichloroacetic acid-soluble material in the culture medium (degraded CT) was determined. The amount of degraded CT was expressed as a percentage of total cell-associated radioactivity. To examine the effect of drugs on CT degradation, neurons were preincubated in N2.1 medium without glutathione for 30 min at 37 °C. The CT degradation assay was performed as described above in the presence of drugs.

**CT Endocytosis—**Neurons (50,000 cells/13-mm glass coverslip) were incubated with 5 nM \textsuperscript{125}I-CT for 30 min at 13–16 °C in HBSS containing 10 mM HEPES, pH 7.4, and 0.1% (w/v) BSA. After washing with HBSS, neurons were incubated in N2.1 medium without glutathione at 37 °C for the indicated period and then placed on ice for 5 min to inhibit endocytosis. To remove cell-surface \textsuperscript{125}I-CT, neurons on coverslips were incubated on ice for 5 min with acid buffer (0.5 M NaCl and 0.2 M acetic acid, pH 2.5), followed by two rapid washes with ice-cold HBSS. The radioactivity of coverslips (internal CT) and of the acid wash was measured. After acid washing, >96% of cell-surface CT was removed.

**Tf Endocytosis—**Iron loading, iodination, and measurement of Tf endocytosis was performed according to McGraw and Subtil (26). The effect of drugs on CT and Tf endocytosis was measured in the presence of drugs after preincubation of neurons with the drugs for 30 min at 37 °C.

**Detergent Insolubility and Flotation on Sucrose Gradients—**Neurons at high density (180,000 cells/24-mm coverslip) (16) were labeled with \textsuperscript{125}I-CT or \textsuperscript{125}I-Tf as above and removed by scraping with a rubber policeman into ice-cold TNE buffer (25 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, pH 7.5). The cells were lysed for 20 min at 4 °C with Triton X-100 (1% v/v). Lysates were centrifuged for 10 min at 16,000 × g at 4 °C, and radioactivity in the supernatant (detergent-soluble fraction) and in the pellet (detergent-insoluble fraction) was counted.

**Sucrose Gradient Analysis of Lysates—**Sucrose gradient analysis of lysates was performed as described with slight modifications (27). The cell lysate was brought to 40% sucrose in TNE buffer without Triton X-100, and then incubated with 5 mM \textsuperscript{125}I-CT at 13–16 °C for 30 min. Neurons were removed from the coverslips by scraping and treated with Triton X-100 (1% v/v, 4 °C, 20 min). Detergent extracts were centrifuged at 16,000 × g for 10 min, and radioactivity in the supernatant (detergent-soluble fraction) and pellet (detergent-insoluble fraction) was counted. Data are means ± S.D., n = 2. B, sucrose gradient analysis of cell-surface CT. Eight-day-old (closed squares) and 2-day-old neurons (open squares) were labeled with \textsuperscript{125}I-CT (5 nM, 13–16 °C, 30 min) and then lysed with detergent. After centrifugation of the detergent extracts on sucrose gradients, fractions (400 μl) were collected from the top (fraction 1) of the gradients. Fraction 13 is the pellet, C, distribution of TF-R (closed circles) and endogenous GM1 (open circles) in 8-day-old neurons. TF receptors were labeled with \textsuperscript{125}I-Tf for 30 min at 4 °C, and detergent extracts were analyzed on a sucrose gradient as in B. Endogenous GM1 ganglioside was detected by TLC immunoblotting of lipid extracts from each fraction, using horseradish peroxidase-CT; the inset shows a typical immunoblot.

**RESULTS**

**CT and Ganglioside GM1 Are Detergent-insoluble in Hippocampal Neurons—**When the neuronal cell surface of 8-day-old neurons was labeled with \textsuperscript{125}I-CT and extracted with 1% Triton X-100 at low temperature, 67% of the CT was detergent-insoluble (Fig. 1A); in contrast, all of the Tf-R was detergent-insoluble (not shown). After depletion of cholesterol using MβCD (28), the detergent solubility of CT increased in a dose-dependent manner (Fig. 1A), demonstrating that cell-surface CT is associated with detergent-insoluble, cholesterol-enriched domains in hippocampal neurons.

We further analyzed detergent extracts by flotation on sucrose gradients. In young neurons (i.e. 2-day-old), 27% of cell-
surface CT was found in low density fractions (fractions 2–4, Fig. 1B), but in older neurons (i.e. 8–9 day-old), 50% of CT was found in the low density fractions (Fig. 1B), as was Thy-1 (not shown) (23, 24). Similarly, ~65% of endogenous GM1 was obtained in low density fractions (Fig. 1C), which also contained neutral lipids (presumably cholesterol), demonstrating that binding of CT to GM1 does not alter the distribution of endogenous GM1 on sucrose gradients. The Tf-R was found to quantify CT transport to the Golgi apparatus, CT degradation (32). Thus, in hippocampal neurons, BFA inhibited CT degradation by ~60% (Fig. 4). Sucrose, CPZ, and MβCD also inhibited CT degradation in a dose-dependent manner (not shown), with maximal inhibition of degradation of ~50–70% (Fig. 4); MβCD inhibited CT degradation to a lower extent in older neurons. The inhibition of CT degradation by MβCD can be explained by a reduction in the rate of clathrin-mediated endocytosis (33, 34). In contrast, filipin did not inhibit degradation to any significant extent (Fig. 4). These data on CT

**FIG. 2. Effect of drugs on Golgi apparatus labeling.** 2- (A–E) and 9-day-old neurons (F–J) were incubated with or without drugs at 37 °C for 30 min. Neurons were subsequently incubated with Bodipy-CT (10 mM, 13–16 °C, 30 min) and then incubated for a further 1 (2-day-old) or 2 h (9-day-old) with or without drugs. A and F, control; B and G, 0.3 M sucrose; C and H, CPZ (20 μg/ml); D and I, MβCD (1 mM); E and J, filipin (1 μg/ml). Arrows indicate the Golgi apparatus. Note that the morphology of the Golgi apparatus changes as neurons mature (25). Bar = 10 μm.

**FIG. 3. Quantification of Golgi apparatus labeling.** Neurons were incubated with drugs and Bodipy-CT as in Fig. 2. The number of neurons in which the Golgi apparatus was clearly labeled was counted. Data are means ± S.D. of 2–4 independent experiments. After some treatments (indicated by n.d., not determined) it was not possible to quantitatively analyze Golgi labeling as cells became detached from the coverslips (see text). Statistically significant differences (Student’s t test) from control cells are indicated by asterisks (p < 0.01).

Cholesterol-binding Agents Have No Effect on CT Transport to the Golgi Apparatus or on CT Degradation—To determine the relationship between detergent insolubility and the mechanism of internalization, we performed a series of experiments to quantify CT transport to the Golgi apparatus, CT degradation, and the rate of CT internalization.

CT was transported to the Golgi apparatus in control neurons (Fig. 2, A and F), with ~90% of cells labeled after 1 h in 2-day-old neurons (Fig. 3) and ~60% of cells labeled after 2 h in 8–9-day-old neurons (Fig. 3); the differences in the rate of Golgi apparatus labeling are similar to those reported previously (17).

Hypertonic medium (i.e. sucrose), which renders clathrin unavailable for assembly into normal coated pits (29), signifi-

![Image](https://example.com/image.png)
transport to the Golgi apparatus, and its degradation, support the idea that CT internalization does not occur via detergent-insoluble rafts/domains in hippocampal neurons.

The Rate of CT and Tf Internalization Are Modified by Sucrose and CPZ but Not by MβCD or Filipin—We next compared the effects of the drugs on CT and Tf internalization by kinetic analysis. The rate of CT internalization was calculated using Equation 1,

\[
[\text{CT}] = [\text{CT}]_{\text{ss}} \times [1 - \exp(-kt)] (\text{Eq. 1})
\]

where \([\text{CT}]_{\text{ss}}\) is internalized CT; \([\text{CT}]_{\text{ss}}\) is internalized CT at steady state; \(k\) is the rate constant (which is the sum of the internalization rate constant and the recycling rate constant), and \(t\) is time.

Data points were fitted to a curve (shown in Equation 2)

\[
y = 2.58 + 8.41x[1 - \exp(-0.110x)] (r = 0.861) \quad (\text{Eq. 2})
\]

for 2-day-old neurons (Fig. 5A) and (Equation 3)

\[
y = 2.50 + 9.03x[1 - \exp(-0.050x)] (r = 0.964) \quad (\text{Eq. 3})
\]

for 8-day-old neurons (Fig. 5B).

Due to the relatively slow binding of CT to the plasma membrane, we could not directly measure an internalization rate constant for CT; however, the initial rate of internalization is an approximation of the internalization rate constant, which was faster in 2-day-old (0.0238/min; Fig. 5A) than in 9-day-old neurons (0.0051/min; Fig. 5B). We had previously shown that the rate of CT transport to the Golgi apparatus (17), and its rate of degradation (see above), are slower in older neurons, but the current kinetic analysis directly demonstrates that the rate of CT internalization is a rate-limiting step in regulating CT transport. In both 2- and 8–9-day-old neurons, CT endocytosis reached steady state after 60 min.

The kinetics of internalization of Tf differed from that of CT, as Tf binding to the cell surface is very fast (26). Thus, the amount of cell-surface Tf is constant at all time points, and the internalization rate of Tf is determined as a ratio of internal Tf to cell-surface Tf (0.0129/min in 9-day-old neurons) (Fig. 5C). No analysis of Tf binding or internalization could be performed in 2-day-old neurons since they do not express high enough levels of the Tf-R (36, 37).

Even though sucrose and CPZ inhibited CT transport to the Golgi apparatus (Figs. 2 and 3) and its degradation (Fig. 4), they had opposite effects on the rate of CT internalization. Sucrose completely inhibited CT internalization in 2- (Fig. 6A) and 8–9-day-old (Fig. 6B) neurons, whereas CPZ increased the rate of CT internalization (Fig. 6A and B). The effect of sucrose on internalization is consistent with it affecting clathrin availability for coated pit assembly (29), and the effect of CPZ is consistent with it affecting a more downstream step in the endosomal pathway (19), possibly receptor recycling. In contrast, neither MβCD nor filipin had any effect on CT internalization. Since we were unable to determine directly a kinetic rate constant for MβCD- and filipin-treated neurons, as many MβCD- and filipin-treated cells detached from the coverslips, we could not directly measure an internalization rate constant for CT; however, the initial rate of internalization was calculated using the effects of the drugs on CT and Tf internalization by kinetic analysis. The initial rate of internalization was calculated by linear regression analysis. The data are from a representative experiment using 2-4 coverslips for each time point and are expressed as means ± S.D. C, eight-day-old neurons were incubated with [125I]-Tf at 37 °C. At indicated times, neurons were washed with acid buffer to remove cell-surface [125I]-Tf. The ratio of radioactivity in the acid-buffer wash (cell-surface Tf) to the amount on the coverslips (internalized Tf) was calculated, and is shown as means for three coverslips at each time point ± S.D.

Fig. 4. Effect of drugs on CT degradation. Neurons were treated with or without drugs (30 min, 37 °C), labeled with [125I]-CT (30 min, 13–16 °C), washed, and then incubated for 8 h at 37 °C in the presence or absence of drugs (sucrose, 0.3 M; CPZ, 20 μg/ml; MβCD, 1 mM; filipin, 1 μg/ml; BFA 4 μg/ml). CT degradation was linear versus time of incubation (not shown), and CT degradation was slower in mature neurons than in immature neurons (2.3 and 12% of cell-associated radioactivity in day 8) (Ref. 17). Data are means ± S.D., n = 3. Statistically significant differences (Student’s test) from control cells are indicated by asterisks (p < 0.0001 in day 2; p < 0.02 in day 8).

Fig. 5. Rate of internalization of CT and Tf. Two-day-old (A) and 8-day-old neurons (B) were incubated with [125I]-CT (30 min, 13–16 °C), washed, and then incubated at 37 °C for the indicated times. Cell-surface [125I]-CT was removed by acid washing, and acid-resistant [125I]-CT (internalized [125I]-CT) was quantified. The initial rate of internalization was calculated by linear regression analysis. The data are from a representative experiment using 2–4 coverslips for each time point and are expressed as means ± S.D. C, eight-day-old neurons were incubated with [125I]-Tf at 37 °C. At indicated times, neurons were washed with acid buffer to remove cell-surface [125I]-Tf. The ratio of radioactivity in the acid-buffer wash (cell-surface Tf) to the amount on the coverslips (internalized Tf) was calculated, and is shown as means for three coverslips at each time point ± S.D.
we analyzed their effects at steady state (i.e., after 60 min of incubation). Neither drug affected internalization in 2- (Fig. 6, A and B), 6 and 8-day-old (B) neurons, treated with sucrose (closed squares) or CPZ (open squares), and compared with control neurons (closed circles). Data are from a representative experiment (repeated three times) using two coverslips for each point time, and expressed as means ± S.D. Two- (C and D) and 8-day-old (E and F) neurons were treated with increasing concentrations of MβCD (C and E) or filipin (D and F) (37 °C, 30 min) and then incubated with 125I-CT (30 min, 13–16 °C) in the presence of drugs. Neurons were further incubated at 37 °C for 1 h and washed with acid buffer to remove cell-surface 125I-CT. Radioactivity on the coverslips (internal 125I-CT, closed circles) and in the acid buffer wash (cell-surface 125I-CT, closed squares) was counted. The open squares show the amount of internalized CT binding to cell surface of 2-day-old neurons at low temperature; note that filipin treatment caused an increase in the amount of cell-surface CT (C and D), probably because ganglioside GM1 was more accessible to exogenously added CT. Data are from a representative experiment (repeated three times) using 2–3 coverslips for each point and are expressed as means ± S.D.

To compare directly CT and Tf internalization, we examined the effects of the drugs on steady state levels of CT internalization and on the rate of Tf internalization. Sucrose inhibited CT and Tf internalization, whereas CPZ increased the amount of internalized CT and increased the rate of Tf internalization (Fig. 7); the effect of CPZ is probably due to inhibition of receptor recycling. Neither MβCD nor filipin had any effect on the amount of internalized CT or on the rate of Tf internalization (Fig. 7). Based on these data, and that presented above, we suggest that CT is internalized by a similar mechanism to that of Tf-R internalization, namely via clathrin-dependent endocytosis, although we cannot exclude other clathrin- or raft-independent mechanisms.

**DISCUSSION**

In neurons, CT could potentially be internalized via either a clathrin-dependent mechanism, together with receptor-bound ligands such as Tf, or via a mechanism that depends on the integrity of detergent-insoluble domains/rafts. Our previous studies (18) could not distinguish between these two possibilities but did demonstrate that CT passes through the same endosomal compartment as receptor-bound ligands internalized via clathrin-mediated endocytosis (18). In the current study, kinetic analysis of the initial rates of internalization and direct comparison with Tf-R internalization strongly suggest that CT is internalized by a similar mechanism to that of Tf-R. Since Tf-R is internalized exclusively via clathrin-mediated endocytosis (21), our data suggest that CT is also internalized via a clathrin-mediated mechanism, although we cannot exclude the possibility that additional nonclathrin-mediated pathways are involved.

There are at least two models that could explain the apparent paradox between the association of CT with detergent-insoluble domains at the cell surface and its internalization via a clathrin-dependent mechanism in neurons (Fig. 9). In the first model, CT escapes the domains prior to internalization...
The distribution of internalized 125I-CT in CPZ-treated neurons is shown as follows: 107,000 for cell-surface 125I-CT, 13,000 for internal 125I-CT, and 19,000 for internal 125I-CT in CPZ-treated neurons. The total number of counts/min loaded onto the gradient after further 60 min at 37 °C. Internalized 125I-CT (distinguished from cell-surface 125I-CT) was internalized after 30 min of incubation in CaCo-2 cells (13). Shiga toxin is found in caveolae. The microdomain is internalized via clathrin-coated pits (2). This model also implies that the rate of diffusion out of the domains, and the rate of diffusion into clathrin-coated pits, might be a means to regulate the rate of endocytosis. This is consistent with observations in primary neurons that the prion protein, a GPI-anchored protein associated with detergent-insoluble domains, is more detergent-soluble than another GPI-anchored protein, Thy-1, and is also internalized much more rapidly than Thy-1 (24). In hippocampal neurons, CT is enriched in detergent-insoluble domains in the neuronal cell surface (25, 36, 39). Interestingly, in hippocampal neurons, only 13% of cell-associated CT is internalized at steady state in 8-day-old neurons, but ~70% of CT is internalized after 30 min of incubation in CaCo-2 cells (13). These different rates of internalization might be due to tighter association of CT with domains in neurons than in CaCo-2 cells. In addition, internalization from caveolae and also clathrin-coated pits in CaCo-2 cells may increase the rate of CT internalization compared with neurons in which internalization does not occur via caveolae.

In the second model (Fig. 9), the raft itself diffuses into the coated pit and is internalized together with raft components, including CT, cholesterol, and GSLs. There is ongoing debate about the size of membrane rafts, but recent measurements suggest that they might be small enough to be internalized via clathrin-coated pits. As in the first model, CT association with domains is transient, since CT is less detergent-insoluble once internalized, but in contrast to the first model, CT exits the domains in early endosomes rather than at the plasma membrane. Rafts exist in recycling endosomes and the sorting of membrane components out of the rafts depends on their preference for association with the domains and can be regulated by cholesterol and GSL levels. The reason that CPZ alters the detergent solubility of CT may be a result of the fact that other endocytic compartments, such as late endosomes, do not contain rafts. We suggest that CPZ renders CT detergent-soluble due to recruitment of clathrin to an endosomal population (involved in transport to the Golgi apparatus) that does not contain rafts (see Fig. 9).

CT is not the only example of a molecule that is detergent-insoluble at the cell surface but is internalized via a clathrin-dependent mechanism. For instance, the epidermal growth factor receptor is detergent-insoluble at the plasma membrane but is internalized via a clathrin-dependent mechanism (reviewed in Ref. 3). Likewise, the GSL-binding toxin, Shiga toxin, is also found in detergent-insoluble domains but is internalized via both clathrin-dependent and -independent pathways (47). Similar to our findings, Shiga toxin internalization cannot be inhibited by cholesterol-binding agents. Shiga toxin and verotoxin both bind to the neutral GSL, Gb3 globoside, whereas CT binds to the acidic GSL, GM1. These two GSLs may be differentially distributed over the cell surface in different cell types, since GM1 is enriched in caveolae in cells that contain these structures (2), whereas there is no evidence that Shiga toxin is found in caveolae.

Do membrane domains play a role in regulating neuronal development, similar to their proposed roles in the development of polarity in epithelial cells? There is some evidence to support this possibility, based on analysis of the effect of deleting cholesterol and GSLs on axonal sorting (22), and on the effect of manipulating lipid levels on detergent insolubility of GPI-anchored proteins (23). However, it should be considered...
that rafts play no specific role in the targeting of GPI-anchored proteins to axons (48) but rather act to cluster proteins together into functionally important complexes (i.e., signaling complexes). In support of this are data showing that some raft components (i.e., GPI-anchored proteins (24) and CT (49)) are distributed uniformly over both the axonal and dendritic surfaces in polarized neurons, whereas they are nonuniformly distributed (i.e., polarized) in some epithelial cells (reviewed in Ref. 50); the lack of polarized distribution of raft components would not negate the possibility that they function to cluster proteins at the cell surface. An extension of the “clustering” hypothesis is the idea that a function of rafts is to regulate the rate of endocytosis of membrane components. This would not necessitate the polarized distribution of raft components but would rather depend solely on raft composition, which would in turn impinge upon the rate of diffusion out of the raft and hence the rate of endocytosis. If this hypothesis is correct, then the rate of internalization to early endosomes would regul-

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