Distribution and Transport of Cholesterol-rich Membrane Domains Monitored by a Membrane-impermeant Fluorescent Polyethylene Glycol-derivatized Cholesterol*

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Cholesterol-rich membrane domains function in various membrane events as diverse as signal transduction and membrane traffic. We studied the interaction of a fluorescein ester of polyethylene glycol-derivatized cholesterol (fPEG-Chol) with cholesterol-rich membranes both in cells and in model membranes. Unlike filipin and other cholesterol probes, this molecule could be applied as an aqueous dispersion to various samples. When added to live cells, fPEG-Chol distributed exclusively in the outer plasma membrane leaflet and was enriched in microdomains that dynamically clustered by the activation of receptor signaling. The surface-bound fPEG-Chol was slowly internalized via clathrin-independent pathway into endosomes together with lipid raft markers. Noteworthy, fPEG-Chol could be microinjected in the living cells in which we found Golgi apparatus as the sole major organelle to be labeled. PEG-Chol, thus, provides a novel, sensitive probe for unraveling the dynamics of cholesterol-rich microdomains in living cells.

The content and distribution of cholesterol is regulated dynamically by complex mechanisms. Inside the mammalian cells a graded level of free cholesterol distributes in various membrane organelles, with the highest accumulation as domains in the post-Golgi membranes (1). In the plasma membrane cholesterol is also accumulated in microdomains with specific phospholipids such as sphingomyelin. These domains, often referred to lipid rafts, ubiquitously distribute from yeast to mammals, playing important roles in cellular functions (2–5).

Removal of cell surface cholesterol by methyl-β-cyclodextrin (MβCD) results in disintegration of these domains, affecting diverse activities such as signaling, adhesion, motility, and membrane trafficking (6, 7). Little is known, however, about the membrane trafficking of cholesterol in live cells. This is apparently due to the lack of sensitive probes. In addition to the specificity as a precondition, use of a small amount of highly sensitive probe is necessary since probing the dynamics relies on the lowest impact of the function of the cholesterol-containing domains. Moreover, cholesterol is very unique in that its level in various membranes is tightly linked even to the transcriptional or secretory activity (8). Therefore, when using a small molecule as a probe, its insertion at a high numbered residue in functioning membranes should be avoided to reduce unexpected impact on the cellular activities. In this respect, filipin, which can be successfully used to detect cholesterol in fixed cells, is not suitable for live cells studies since, in addition to the poor fluorescence property, it yields a cytotoxic side effect by sequestering cholesterol. Similarly, using cholesterol-binding proteins as probes, which accumulate cholesterol and even form pores, often becomes problematic.

Polyethylene glycol cholesterol ethers (PEG-Chols) are a unique group of non-ionic amphiphatic cholesterol derivatives (Fig. 1) (9). Because of low toxicity, various PEG-Chols were initially used in vivo to disperse otherwise water-insoluble antibiotics (10). Recently it was reported that PEG(50)-Chol (Mf ~ 2600; 50 is average number of PEG repeats) was singly dispersed in aqueous media and distributed on the surface of cultured cells (9, 11). Without affecting the clathrin-dependent endocytosis, PEG(50)-Chol could inhibit caveolae-like endocytosis in A431 cells, although the required amount was as high as 3 × 10⁸ molecules/cell (11). This specificity suggests that PEG-Chols may interact with specific membrane components at much lower dose.

In the present study we showed that a fluorescein-tagged
PEG-Chol (fPEG-Chol) partitioned from an aqueous media into cholesterol-rich membranes both in cells and in model membranes. These properties of fPEG-Chol made it possible to apply this compound to follow the re-organization of cell surface cholesterol as well as intracellular cholesterol dynamics. Moreover, because fPEG-Chol is water soluble and does not transverse lipid bilayer, we could localize cholesterol-rich domains in the cytoplasmic leaflet of intracellular organelle by microinjection.

**MATERIALS AND METHODS**

**Cells and Reagents**—Cultured skin fibroblasts from patients with Niemann-Pick type C (NPC) and from healthy subjects were established and maintained as described (12). Mouse melanoma cell line MEB4 and its glycosphingolipid-deficient mutant GM95 (13) were generous gifts of Dr. Yoshio Hirabayashi (Brain Science Institute, RIKEN). The cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. COS7 cells were grown in RPMI 1640 medium containing 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Chinese hamster ovary (CHO) cells were grown as described (14). Cholesterol was purchased from Sigma. 2-(4,4-Difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoyl)-1-hexadecanoyl-sn-glycero-3-phosphocholine (BODIPY-C12-PC), Alexa Fluor 546-labeled cholera toxin B subunit (CTxB), Alexa Fluor 594-labeled CTxB, Texas Red transferrin, and rhodamine dextran were purchased from Molecular Probes (Eugene, OR). All other lipids were from Avanti polar lipids (Alabaster, AL). Anti-TGN46 antibody was from Serotec (Oxford, UK). Phycoerythrin-conjugated anti-CD59 monoclonal antibody was purchased from Pharmingen (San Diego, CA). Randomly methylated-β-cyclodextrin was from Cyclolab (Budapest, Hungary).

**Binding of fPEG-Chol to Cholesterol-containing Liposomes**—1 mM sphingomyelin (SM) vesicles containing various amount of cholesterol were analyzed the binding of fPEG-Chol. Vesicles were prepared as described (15) and incubated with 2 μM fPEG-Chol for 30 min at room temperature. Unbound fPEG-Chol was washed with centrifugation at 15,000 × g for 15 min. Fluorescence of the pellet was measured and normalized by phosphorous of SM.

**Transfer of fPEG-Chol between Membranes**—250 μM (final concentration of phospholipids) acceptor liposomes were added to 50 μM donor palmitoyloleoylphosphatidylcholine (POPC) liposomes containing 0.5 μM fPEG-Chol and 0.5 μM N-rhodamine dipalmitoylphosphatidylethanolamine. The release of fluorescence resonance energy transfer was measured at 30 °C by monitoring the time course of fluorescence emission spectrum at 535 nm with excitation at 488 nm.
Loading Dehydroergosterol (DHE) on MβCD—DHE-MβCD complex was prepared as described (16). In brief, DHE in ethanol was dried under argon and subsequently dissolved in MβCD in Medium 1 (150 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, and 20 mM Heps, pH 7.4), making the initial ratio of MβCD to DHE 8:1 (mol/mol). The resulting suspension was vortexed and sonicated until it clarified. It was then incubated in a rocking water bath overnight at 37 °C and centrifuged to remove insoluble DHE aggregates.

Microscopy—For immunofluorescence of fixed and permeabilized fibroblasts, cells were incubated with 3% paraformaldehyde followed by the treatment with 50 μg/ml digitonin for 5 min at room temperature before the addition of the antibody. The specimens were observed using Zeiss LSM 510 confocal microscope. In Fig. 4, the specimens were observed under an Olympus BX51 microscope equipped with a Hamamatsu C-4742-98 cooled CCD camera, which was controlled by a MetaView imaging software. In Fig. 6, fluorescence microscopy and digital image acquisition were carried out using an Olympus IX71 microscope equipped with UPFlan Apo 100× objective. Images were acquired using a Hamamatsu Image Intensifier Unit C8600 connected with a C-2400 CCD camera. DHE was imaged using a filter cube obtained from Chroma Technology Corp. (Brattleboro, VT) (335-nm [20-nm bandpass] excitation filter, 365-nm longpass dichromatic filter, and 405-nm [40 nm bandpass] emission filter).

RESULTS

PEG-Chol and Filipin Similarly Localized Cholesterol-rich Membranes in Fixed and Permeabilized Cells—We prepared a fluorescein ester of PEG-Chol that contains a fluorescein on the distal end of PEG chain (iPEG-Chol, (9)). iPEG-Chol was added to fixed and digitonin-permeabilized normal human skin fibroblasts (Fig. 2, A–D). After washing by a conventional immunofluorescence protocol, the Golgi apparatus and intracellular small vesicles became fluorescent (Fig. 2A). The pattern of iPEG-Chol fluorescence was very similar to that of filipin (Fig. 2B).

As described previously (17), we defined partial colocalization of iPEG-Chol and the immunofluorescence for a trans-Golgi network (TGN) marker, TGN46, in the Golgi (Fig. 2, C and D) (18). NPC is an autosomal recessive, neurovisceral disease. The hallmark of the NPC syndrome is the intracellular accumulation of unesterified cholesterol (12, 19, 20). In contrast to normal fibroblasts, iPEG-Chol brightly stained numerous perinuclear compartments that were previously identified as lyso-bisphosphatidic acid-rich late endosomes (12). In contrast, the appearance of the Golgi apparatus was normal in the NPC cells (Fig. 2G). Again, the fluorescence co-localized with filipin fluorescence (Fig. 2, E and F). Co-localization of iPEG-Chol and filipin were observed in other cell types such as melanoma cells (Fig. 2, I and J), and their derivatives, which are deficient in glycolipid synthesis (Fig. 2, K and L) (13) as well as COS7 cells (Fig. 2, M and N). Both iPEG-Chol and filipin staining were abolished when cells were pretreated with MβCD (Fig. 2, O and P), which removes cholesterol from cells (21). iPEG-Chol labeling was reduced when iPEG-Chol was preincubated with SM/Chol (1:1) liposomes (Fig. 2R). A similar result was obtained with PC/Chol (1:1) liposomes. chol-free SM or PC liposomes were much less effective to decrease iPEG-Chol labeling (Fig. 2Q).

PEG-Chol Is Preferentially Distributed to Cholesterol-rich Membranes in Model Membranes—The results in fixed-permeabilized cells suggested the preference of iPEG-Chol to cholesterol-containing membranes. When SM liposomes were mixed with iPEG-Chol, the addition of cholesterol increased the fluorescence recovered with the membrane (Fig. 3A). In Fig. 3, B and C, we incubated iPEG-Chol pre-embedded in POPC liposomes with various liposomes. The fluorescence of iPEG-Chol was initially quenched by including an acceptor molecule of fluorescence resonance energy transfer, N-rhodamine phosphatidyethanolamine (22, 23). These liposomes were mixed with acceptor liposomes that contained no probe. Because rhodamine phosphatidyethanolamine is a non-exchangeable phospholipid, once iPEG-Chol moved into the acceptor liposomes, its fluorescence would be de-quenched. Remarkably, fluorescence was dequenched depending on the cholesterol content of liposomes. In contrast, cholesterol-containing liposomes did not release the quenching of non-exchangeable N-NBD-phosphatidylethanolamine fluorescence in rhodamine-phosphatidylethanolamine/phosphatidylcholine/PEG-Chol liposomes (data not shown). These results indicated that iPEG-Chol moved from phosphatidylethanolamine liposomes to cholesterol-containing liposomes via aqueous transfer but not by membrane fusion. Our results suggest that, even once embedded in cholesterol-free membranes, iPEG-Chol will move to cholesterol-rich membranes.

PEG-Chol Reveals Re-organization of Cell Surface Cholesterol-rich Domains—Unlike filipin and DHE, iPEG-Chol is membrane non-permeable (see below). This characteristic made it possible to examine the detailed distribution of cell surface cholesterol by iPEG-Chol. In Fig. 4 we examined the distribu-
distribution of fPEG-Chol on the cell surface by wide-angle video-enhanced microscopy. After a brief incubation with fPEG-Chol and subsequent wash and fixation (the whole procedure was completed within 2 min), we could detect uneven distribution of fluorescence (Fig. 4, A and C). Many of fPEG-positive area were co-localized with CTxB (Fig. 4, B and D). CTxB binds to GM1, which is non-randomly distributed on the plasma membranes, and accumulates in rafts/caveolae (24). This surface staining was abolished when cells were treated with MβCD (Fig. 4, E and F). We also measured the distribution of fPEG-Chol in cells shortly after the stimulation with epidermal growth factor (EGF). It is suggested that EGF receptor localizes cholesterol-rich plasma membrane domains and that binding of EGF to the receptor is dependent on cell surface cholesterol (25–27). fPEG-Chol fluorescence was co-localized with the distribution of biotin-labeled EGF when EGF was added at 4°C (Fig. 4, G and H). When EGF was added at 37°C, clustering of the EGF receptors was observed (Fig. 4J). These clusters were labeled with fPEG-Chol (Fig. 4J). The cell surface distribution of GM1 was also examined under these conditions. GM1 was also enriched in these clusters and co-localized with fPEG-Chol (Fig. 4K and L). These results indicate that EGF induced the redistribution of both fPEG-Chol and GM1 to the same clusters where EGF receptors were enriched.

fPEG-Chol in the Outer Plasma Membrane Leaflet Is Slowly Internalized Together with Lipid Raft Markers—Previously it was shown that PEG-Chol specifically inhibits clathrin-independent endocytosis (9, 11). Endocytosis was significantly reduced when cells were preincubated with 2.5 μM or higher concentrations of PEG-Chol for 30 min at 37°C. When human skin fibroblasts were incubated with 2.5 μM PEG-Chol for 30 min at 37°C, cells internalize 37 ± 8 pmol of PEG-Chol/mg of protein. Endocytosis was not affected when cells were briefly incubated with low concentrations of PEG-Chol. Therefore, it is possible to follow the fate of cell surface PEG-Chol in living cells under these conditions. We treated cells with 1 μM fPEG-Chol for 5 min at room temperature. Under these conditions cells incorporated 1.8 ± 0.3 pmol of PEG-Chol/mg of protein. This amount of PEG-Chol was sufficient to follow the fate of fluorescence. However, this concentration of fPEG-Chol did not affect the endocytosis of dextran and CTxB. After labeling cells with fPEG-Chol, we chased the internalization of fluorescence at 37°C in the presence of 1 mg/ml rhodamine dextran. Most of the fPEG-Chol fluorescence stayed on the plasma membrane after 10 min of chase (Fig. 5A and B). After 60 min, fluorescent compartments surrounded the nucleus (Fig. 5D). fPEG-Chol could further stain intracellular vesicles. Most of these vesicles were not co-localized with internalized rhodamine dextran (Fig. 5E). After 180 min, the Golgi apparatus was prominently labeled with fPEG-Chol whereas rhodamine fluorescence was distributed in endosomes/lysosomes (Fig. 5C and F). fPEG-Chol fluorescence in intracellular vesicles was highly enhanced after neutralizing the vesicular pH by ammonium chloride (Fig. 5G and H). We then compared the internalization of fPEG-Chol with those of known raft markers. Cells were preincubated with the PE-conjugated monoclonal antibody against glycosylphosphatidylinositol-anchored protein, CD59 (Fig. 5I and J) or Texas Red-labeled transferrin (Fig. 5, O–Q) together with fPEG-Chol. After washing, cells were incubated for 20 min at 37°C, treated with ammonium chloride at room temperature, and then immediately observed. fPEG-Chol well co-localized with lipid raft markers CD59 and GM1. In contrast, fPEG-Chol was not co-localized with internalized transferrin. Transferrin is internalized via clathrin-dependent endocytosis, whereas glycosylphosphatidylinositol-anchored proteins and cholera toxin are
endocytosed by clathrin-independent mechanisms (28). Our results suggest that the raft domains were internalized via clathrin-independent pathway into acidic organelle. During prolonged incubation, fPEG-Chol was further transported to the Golgi apparatus.

Recently intracellular transport of cell surface cholesterol in CHO cells was studied by using DHE (16). DHE was enriched in the endocytic recycling compartment. We then compared the internalization of fPEG-Chol with that of DHE in CHO cells. As reported, DHE was detected in the juxta-nuclear region (Fig. 5).
minated the Golgi apparatus (Fig. 7). Surprisingly, the pat-
CfPEG-Chol in living normal skin fibroblasts, fluorescence illu-
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tion obtained by other probes but also uncovered the yet un-
known trafficking of cholesterol-containing membranes in live

Little is known about the transbilayer membrane distribu-
tion of cholesterol in live cell organelles. Because fPEG-Chol is
membrane-impermeable, we microinjected this molecule in live
cells to monitor the distribution of cholesterol in the cytoplas-
mic side of intracellular organelles. When we microinjected
fPEG-Chol in living normal skin fibroblasts, fluorescence illu-
minated the Golgi apparatus (Fig. 7C). Surprisingly, the pat-
tern was similar in NPC cells. The numerous cholesterol-rich
late endosomes, which could be detected in fixed and perme-
abilized cells (Fig. 2E), were not labeled at all (Fig. 7D). These
results suggest that the Golgi apparatus solely exposes choles-
terol-rich domains to the cytoplasm of both the normal and
NPC fibroblasts. Microinjection of fPEG-Chol did not inhibit
the endocytosis of rhodamine dextran. After prolonged incuba-
tion, fluorescence was dispersed throughout the cytoplasm
data not shown).

**DISCUSSION**

**PEG-Chol, a Unique Water-soluble Probe for Cholesterol-rich Domains**—Several probes have been used to localize choles-
terol-rich domains. Filipin has spread widely (31) despite the
limitation due to the high photosensitivity and toxicity (Ref. 32
and reference therein). Naturally occurring fluorescent choles-
terol analog DHE has been used to follow the fate of unesteri-
fied sterol in living cells (16, 33). Because DHE itself is water-
insoluble and has a lower fluorescence quantum yield than
conventional fluorophores, a substantial amount of this mole-
cule has to be added to live cell studies in a form complexed
with MβCD. These conditions may become an obstacle in some
experiments since chronic treatment of cells with MβCD-cho-
lesterol alters cellular lipid metabolism (34). Recently a novel
protein probe (BCθ) derived from bacterial toxin, perfringolysin
O, has been shown to selectively bind to cholesterol-rich mem-
brane domains (32, 35–37). However, because BCθ is a bulky
protein (Mθ ~ 57 kDa), it may induce an alteration of the
membrane organization.

Although liposome studies suggested that fPEG-Chol has
background distribution into the hydrophobic membrane mi-
lieu as other hydrophobic molecules do, it showed the following
unique characteristics: 1) preferential partition to cholesterol-
rich membranes such as SM/Chol, 2) higher stability and quan-
tum efficiency of the fluorophore than DHE or filipin, 3) single
dispersion in aqueous phase at ~μM concentrations, 4) low cell
toxicity, 5) unlikely to permeate or flip-flop membranes, 6)
relatively low molecular weight (~2,600). We stress that these
properties of fPEG-Chol not only complemented the informa-
tion obtained by other probes but also uncovered the yet un-
known trafficking of cholesterol-containing membranes in live

**Cellular Distribution of Cholesterol-rich Domains—An in
vitro study indicates that fPEG-Chol is useful in documenting

**FIG. 6.** fPEG-Chol and DHE are differently internalized. A, CHO cells were pulsed with DHE-loaded MβCD for 5 min at 37°C and chased
for 90 min in Medium 1 (150 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, and 20 mM Hepes, pH 7.4) containing 2 g/liter glucose (M1 glucose).
B, CHO cells were labeled with 1 μM fPEG-Chol for 5 min at room temperature. Cells were then washed and incubated in M1 glucose for 90 min.
Cells were treated with ammonium chloride before imaging. For both panels cells were imaged at room temperature. Cells were not doubly labeled
in MβCD because fPEG-Chol binds DHE. Bar, 20 μm.

**FIG. 7.** Microinjected fPEG-Chol is accumulated in the Golgi apparatus. Human skin fibroblasts were incubated with 1 μM fPEG-Chol
(A) or 1 μM C6-NBD-phosphatidylserine (B) for 30 min at 10°C. Cells were washed, and fluorescence images were immediately recorded
under confocal microscope. C and D, 100 μM fPEG-Chol in 25 mM Hepes-KOH, pH 7.0, containing 25 mM KCl, 2.5 mM magnesium acetate,
0.25 M sucrose (23, 42) was microinjected to normal (C) and NPC (D) human skin fibroblasts at room temperature. Fluorescence images were
observed under confocal microscope. Bar, 20 μm.
dynamics of raft-like structures inside the cells as well as on the surface. Although the presumably high mobility on the surface may not allow complete visualization in the living cells, short exposure to iPEG-Chol revealed numerous spots on the cell surface. This probe followed the reorganization of these domains after activation of EGF signaling, in which iPEG-Chol aggregated with EGF and GM1.

With iPEG-Chol, late endosomes were stained in fixed normal and Niemann-Pick type C cells. Interestingly, even in NPC fibroblasts, microinjection of iPEG-Chol illuminated for the first time the Golgi apparatus, but not late endosomes, as the sole major organelle that exposes cholesterol-rich domain cytoplasmically. Late endosomes were previously shown to be highly enriched with a specific phospholipid, lysobisphosphatidic acid (12, 38–40). Enrichment of several antibodies to this lipid interferes with its function in accumulation and distribution of cholesterol. Our present results suggest that accumulated cholesterol and lysobisphosphatidic acid reside in the same luminal leaflet, raising the possibility of direct interaction between these molecules in NPC.

**Fate of Cell Surface Cholesterol**—Recently intracellular transport of cell surface cholesterol in CHO cells was studied by using a DHE/MβCD (16). DHE was enriched in the endocytic recycling compartment localized by transferrin. The delivery of DHE to the endocytic recycling compartment from the plasma membrane was only slightly affected by energy depletion, suggesting the involvement of a non-vesicular pathway. Our results indicate that the routes of internalization of DHE and PEG-Chol are different. Because DHE undergoes rapid transbilayer movement (41), such a mechanism may involve spontaneous flip-flop of the molecule from the outer to inner plasma membrane leaflets followed by the accumulation to an endocytic recycling compartment (16). In contrast, iPEG-Chol restricted in the outer leaflet cholesterol-rich domain likely monitored the slow clathrin-independent traffic to the Golgi region. We suggest here that the trafficking of the cholesterol in the outer and inner plasma membrane monolayers occur independently.

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