Endocytic Sorting of Lipid Analogues Differing Solely in the Chemistry of Their Hydrophobic Tails

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Abstract. To understand the mechanisms for endocytic sorting of lipids, we investigated the trafficking of three lipid-mimetic dialkylindocarbocyanine (DiI) derivatives, DiIC₁₆(3) (1,1'-dihexadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate), DiIC₁₂(3) (1,1'-didodecyl-3,3',3'-tetramethylindocarbocyanine perchlorate), and FAST DiI (1,1'-dilinoleyl-3,3',3'-tetramethylindocarbocyanine perchlorate), in CHO cells by quantitative fluorescence microscopy. All three DiIs have the same head group, but differ in their alkyl tail length or unsaturation; these differences are expected to affect their distribution in membrane domains of varying fluidity or curvature. All three DiIs initially enter sorting endosomes containing endocytosed transferrin. DiIC₁₆(3), with two long 16-carbon saturated tails is then delivered to late endosomes, whereas FAST DiI, with two cis double bonds in each tail, and DiIC₁₂(3), with saturated but shorter (12-carbon) tails, are mainly found in the endocytic recycling compartment. We also find that DiOC₁₆(3) (3,3'-dihexadecyloxocarbocyanine perchlorate) and FAST Dio (3,3'-dilinoleyoxyocarbocyanine perchlorate) behave similarly to their DiI counterparts. Furthermore, whereas a phosphatidylcholine analogue with a BODIPY (4,4-difluoro-4-bora-3a,4a-diaza-s-indacene) fluorophore attached at the end of a 5-carbon acyl chain is delivered efficiently to the endocytic recycling compartment, a significant fraction of another derivative with BODIPY attached to a 12-carbon acyl chain entered late endosomes. Our results thus suggest that endocytic organelles can sort membrane components efficiently based on their preference for association with domains of varying characteristics.

Key words: endocytosis • lipid • dialkylindocarbocyanine • membrane domain • membrane curvature

Mammalian cells internalize membrane components and fluid by an assortment of mechanisms that deliver contents to peripherally distributed tubulovesicular sorting endosomes. From the sorting endosomes, most membrane-bound molecules enter the endocytic recycling pathway, while most of the volume gets delivered to late endosomes/lysosomes (Mukherjee et al., 1997). It has been shown that efficient recycling of most membrane-bound molecules does not require any specific signal(s) (Dunn et al., 1989; Mayor et al., 1993). Although most membrane proteins are efficiently recycled back to the cell surface, some are preferentially retained in the sorting endosomes and are thereby targeted to the late endosomal pathway. Specific amino acid sequences in the cytoplasmic domains of cation-independent mannose-6-phosphate receptor (Johnson and Kornfeld, 1992) and signaling receptors such as the epidermal growth factor receptor (Opresko et al., 1995) have been proposed to mediate their late endosomal targeting. While several lipid analogues with one very short acyl chain (e.g., C₆-NBD-SM [N-(6-((7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoyl)sphingosyl phosphocholine]) are recycled with an efficiency of 95% or higher (Koval and Pagano, 1989; Mayor et al., 1993), others containing long saturated chains including rhodamine-labeled phosphatidylethanolamine and some glycosphingolipids are delivered predominantly to the late endocytic structures (Kok et al., 1990, 1991; Sandhoff and Klein, 1994). However, the sites of lipid sorting in the endocytic pathway have not been identified, and the efficiency of these sorting processes has not been investigated.

Targeted trafficking of lipids might be due to variations in intrinsic physical properties such as the length and degree of unsaturation of their acyl chains. A consequence of such variation would be differential partitioning preference of these lipids or lipid analogues in coexisting lateral membrane domains of varying composition and motional characteristics (“fluidity” differences). While the lipids with long and saturated acyl chains (such that the length of the acyl chains would roughly match the hydrophobic thick-
ness of the surrounding bilayer) will preferentially partition into more rigid or ordered domains, those with short or unsaturated tails would prefer to enter more fluid regions of the bilayer.

Recent studies strongly suggest that the plasma membrane and the membranes of internal organelles of mammalian cells are not in a homogeneous "fluid" state (Brown and London, 1997). While the size, shape, and precise motional properties of the lateral domains are not yet clear, the presence of these domains is likely (Friedrichson and Kurzchalia, 1998; Varma and Mayor, 1998). Glycolipid- and cholesterol-enriched domains have also been proposed to play a role in biosynthetic protein and lipid sorting (Simons and Ikonen, 1997). Furthermore, recent experiments with BODIPY (4,4-difluoro-4-bora-3a,4a-diaza-s-indacene)-labeled lipid analogues indicate that a redistribution of lipids occurs at the plasma membrane or in the forming endosomes within seconds after the initiation of endocytosis (Chen et al., 1997).

The shape of lipids is another characteristic that could cause partitioning into membrane regions of varying curvatures and thereby result in differential trafficking. Indeed, several long saturated chain glycoinositolphospholipids have been found to concentrate in the inner involutions of multivesicular bodies by electron microscopy (Sandhoff and Klein, 1994). Recently, such inward invaginations have been shown to be enriched in a unique lipid, lysobisphosphatidic acid (Kobayashi et al., 1998). It is thus possible that such invaginations, with a curvature opposite to that of the emanating tubules, may represent a specialized domain that enhances the segregation of some membrane components from the recycling pathway.

**Rationale for Choosing the Model Lipids for the Present Study**

To determine whether properties of the hydrocarbon tail could efficiently target lipids after internalization, we used the dialkylindocarbocyanine (DiI) series of lipid analogues. These analogues have varying propensities to partition into coexisting lateral membrane domains of varying fluidity and have different relative head group to tail cross-sectional areas resulting in varying overall shapes (approximated as cone, cylinder, or inverted cone). The DiI analogues are composed of an indocarbocyanine head group and two hydrophobic alkyl chains (Fig. 1), which impart to them an overall amphiphilic character (Simms et al., 1974; Haugland, 1996) and allow them to insert into the membrane with their head groups roughly normal to the plane of the bilayer (Axelrod, 1979) in a manner analogous to naturally occurring lipids. The relatively long hydrophobic alkyl chains result in their strong association with the host plasma membrane such that once inserted, they traffic as an integral part of it. Since the DiI derivatives are not naturally occurring lipids, they are not subject to intracellular metabolic turnover.

As shown in Fig. 1, two of the analogues we used contain saturated alkyl chains. DiIC_{15}(3) (1,1'-dihexadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate) has tails with 16 carbons each, whereas DiIC_{16}(3) (1,1'-didodecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate) has 12 carbon chains. The differential partitioning preferences of C_{16}DiI s into domains of varying motional characteristics have been investigated in model membrane systems. In systems with coexisting gel and fluid phases, an approximate match of the probe alkyl chain length with those of the host lipid acyl chains led to a preferential partitioning of the probe into gel phases (Klausner and Wolf, 1980; Spink et al., 1990). The alkyl chain length of DiIC_{16}(3) approximately matches those most prevalent in the lipids of various CHO cell lines (Callaghan et al., 1992; Ackinnon et al., 1992). Thus, in CHO cell membranes, DiIC_{16}(3) would be expected to preferentially partition into more rigid (or highly ordered) domains, whereas DiIC_{15}(3) would enter more fluid domains. The other lipid analogue used in this study, FAST DiI (1,1'-dilinoleyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate), has two 18-carbon chains with two cis double bonds in each chain. Lipids with unsaturated tails preferentially enter fluid domains in model membranes containing coexisting gel and fluid phases (Muir et al., 1995).

In rat basophil leukemia (RBL) cells, preferential segregation of DiIC_{16}(3) into specific lateral domains containing aggregated immunoglobulin E receptors has been observed (Thomas et al., 1994). Furthermore, during phagocytosis of 6-µm beads, RBL cells specifically exclude DiIC_{15}(3) but not FAST DiI from the forming phagosomes (Pierini et al., 1996).

In addition, the overall shapes of the different DiI derivatives used in this study are quite different, which, in turn, would mean that they would have differential partitioning preferences into membrane regions of varying curvatures (Cullis and de Kruijff, 1979; Genois, 1989). Both DiIC_{15}(3) and DiIC_{16}(3) have a head group cross-sectional area that is larger than the alkyl chains (provided the chains are stretched out all-trans). They would thus exhibit an inverted cone shape and preferentially partition into membrane regions with a convex curvature. FAST DiI has a cylindrical or cone shape by virtue of the cis double bonds and would be preferentially accommodated in membranes of concave curvature. Thus, the set of DiI probes chosen for the present study are ideally suited to address the role of membrane domains in intracellular sorting since they vary in both their fluidity and curvature preferences.

As seen in Fig. 1, the DiO derivatives are identical to their DiI counterparts except that the head groups of DiO contain an oxygen atom as part of the heterocyclic ring system, instead of a carbon atom attached to two methyl groups in DiI (Haugland, 1996). It is thus likely that the DiI and DiO head groups would interact differently with neighboring lipids and/or proteins. Thus, if both DiI and DiO derivatives with the same alkyl chain chemistry traffic identically in CHO cells, it would reinforce the argument that the alkyl chain properties are important in trafficking. To test a larger variation in head groups, we used phosphatidylcholine derivatives (Fig. 1) that have a zwitterionic head group, unlike the anionic DiI and DiO head groups (Haugland, 1996). One long 16-carbon acyl chain ensured stable incorporation into the membrane bilayer. We used BODIPY FL lipid analogues since the fluoro-
The fluoro- phore has been reported to localize to the membrane inte- rior in a manner roughly normal to the plane of the bilayer (Johnson et al., 1991; Haugland, 1996), whereas in NBD lipid analogues the fluorophore loops back toward the hydrophilic interface (Chattopadhyay and London, 1987). Furthermore, given the size of the BODIPY FL fluoro- phore, it is expected that incorporating it at the end of a 12-carbon acyl chain would result in a derivatized acyl chain that would roughly span the thickness of one membrane leaflet in a CHO cell. BODIPY FL C\textsubscript{12}-HPC \[\text{[2-(BODIPY-3-dodecanoyl)-1-hexadecanoyl-sn-glycero-3-phosphocholine]}\] would thus be somewhat similar to the DiIC\textsubscript{16}(3) or DiOC\textsubscript{16}(3) in terms of the properties of the hydrophobic tail. BODIPY FL C\textsubscript{5}-HPC \[\text{[2-(BODIPY-3-pentanoyl)-1-hexadecanoyl-sn-glycero-3-phosphocholine]}\] would, on the other hand, be expected to more closely mimic DiIC\textsubscript{12}(3).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{chemical_structures.png}
\caption{Chemical structures of the lipid analogues used in this study: (a) DiIC\textsubscript{16}(3), (b) DiIC\textsubscript{12}(3), (c) FAST DiI, (d) DiOC\textsubscript{16}(3), (e) FAST DiO, (f) BODIPY FL C\textsubscript{5}-HPC, and (g) BODIPY FL C\textsubscript{12}-HPC. In the nomenclature, the subscripted numbers refer to the length of the alkyl chains, while the numbers in the parentheses refer to the number of carbons bridging the two indocarbocyanine rings that constitute the head group.}
\end{figure}

**Materials and Methods**

**Materials**

All fluorescence probes were obtained from Molecular Probes Inc. The purity of the lipid analogues was checked by thin layer chromatography using chloroform/methanol/water (65:35:5 vol/vol) as the solvent system. Labeled dextran was dissolved in PBS, pH \textasciitilde7.4, and was extensively dia- lyzed before use to remove any unconjugated dye. Cy3 was obtained as a protein conjugation kit from Amersham Life Sciences.

Human transferrin (Tf) was obtained from Sigma Chemical Co. It was then iron loaded and passed through a Sephacryl S-300 gel filtration system as previously described (Yamashiro et al., 1984). Succinimidyl ester of Oregon green, Alexa 488, and Cy3 were then separately conjugated to the iron-loaded Tf following the manufacturer’s instructions. Labeled transferrin was dialyzed thoroughly to remove the unbound dye. DiIC\textsubscript{18}(3)-labeled low density lipoprotein (DiI-LDL) was a gift from Dr. R.N. Ghosh (Cornell University Medical College, NY).

All tissue culture supplies were from GIBCO BRL. All other chemicals were from Sigma Chemical Co.
Cells and Cell Culture

CHO cell lines expressing the human Tf receptor (TRV b3; McGraw et al., 1987) were grown in bicarbonate-buffered Ham's F-12 medium supplemented with 5% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 200 μg/ml geneticin. Genetin was used as a selection for the transfected Tf receptors. A ll cells were grown in a 5% CO2 environment in humidified incubation set at 37°C. The cells for microscopy were grown on plastic tissue culture dishes whose bottoms were replaced with poly- o-lysine-coated coverslips, as described previously (Salzman and Maxfield, 1989). All experimental manipulations as well as microscopy were carried out in these dishes.

Labeling Cells with the Lipid Analogues

Stock solutions of the DiI derivatives were made in ethanol and stored at −80°C under argon. For making the labeling solutions, 750 nmol of DiIC16(3) or 75 nmol of DiIC3(3) or FAST DiI were dissolved in 400 μl ethanol. The ethanolic solutions were then injected, while vortexing, into an equimolar amount of fatty acid-depleted BSA in 1 ml PBS at pH 7.4 (final ethanol concentration 40% vol/vol). This mixture was then dialyzed thoroughly against several changes of PBS. As the ethanol was slowly exchanged during dialysis, some of the DiI got transferred to the hydrophobic fatty acid binding sites on the BSA, which can at some points aggregate. The solutions were then filtered through a 0.2 μm syringe filters and stored at −80°C under argon.

Cells on coverslips-bottom dishes were taken out of the CO2 incubator, rinsed several times with isotonic Medium 1 (150 mM NaCl, 5 mM KCl, 1 mM CaCl2, 1 mM MgCl2, and 20 mM Hepes, pH 7.4; supplemented with 2 g/liter glucose), and then labeled with an appropriate dilution of a DiI labeling solution (2 μM DiIC3(3), 31 nm DiIC3(3), and 75 nm FAST DiI). The concentrations of different DiI derivatives were optimized such that the signal was not saturated. All the dilutions were in the cells (as assessed by integrated fluorescence power per cell) were roughly matched. We used the concentration of each sample that would give us a useful fluorescence signal. A ll labeling reagents were ultracentrifuged at 100,000 g for 20 min just before an experiment and equilibrated to 37°C. When the supernatant from this procedure was run on a Sephacryl S-300 gel filtration column, the DiI derivatives were usually more than 95% fully labeled with the different DiI derivatives as described above, rinsed, and further incubated with 5% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 200 μg/ml geneticin. This solution was used as a stock solution for the labeling of the cells. The labeling solutions were prepared by diluting the stock solution with Medium 1 and then incubated with the cells for 2 min at 37°C. When cells were labeled using these labeling solutions at 0.2 g/liter glucose), and then labeled with an appropriate dilution of a DiI labeling solution. The cells were fixed and counterstained with 10 μg/ml of TO-PRO-3 and then mounted with Fluoromount-G.

Endocytosis Assays

Endocytic fates of the DiI derivatives were determined by comparing their intracellular distributions with those of the endocytosed Tf at various time points after the initial loading of the DiI derivatives on to the plasma membrane of TRV b1 cells. Tf transferrin, bound to its receptor, was used as a marker for the endocytic recycling route (Dunn et al., 1988; Muckermann et al., 1997). A comparison of the trafficking of various DiI derivatives with Tf thus allowed us to determine the degree of overlap of the endocytic trafficking routes of these DiI derivatives with that of Tf.

The general experimental methods used to analyze the endocytic behavior of various DiI derivatives were as follows. Cells equilibrated to 37°C were labeled for 2 min at 37°C with the appropriate dilution of a DiI labeling solution, rinsed several times with Medium 1, and then incubated with pre-warmed Oregen green-labeled Tf (OG-Tf; or a leuca 488-conjugated Tf in the confocal experiments) for either 5 or 30 min. A t the end of the incubation period, the cells were rinsed with ice-cold Medium 1 and fixed. To confirm the identity of the punctate structures labeled by some DiI derivatives as late endosomes/lysosomes, TRV b1 cells were labeled with different DiI derivatives as described above, rinsed, and further incubated with 1 mg/ml fluorescein-labeled dextran for 60 min. After the incubation, the cells were rinsed and fixed as described above.

Wide-Field Fluorescence Microscopy and Image Analysis

Fluorescence microscopy and digital image acquisition were carried out using a Leica DM IRB microscope (Leica Mikroskopie und Systeme G mBH) equipped with a cooled CCD camera (Frame Transfer Pentax camera with a S12 × S12 back-thinned E E V chip, No. S122 FTB; Prince- ton Instruments) driven by Image-1/M etamorph Imaging System software (U niversal Imaging Corp.). A ll images were acquired using a high magnification (63×, 1.4 NA) oil immersion objective. DiI derivatives were imaged using a standard rhodamine filter set, while Oregon green was imaged using a fluorescein filter set. The fields to be imaged were chosen on the basis of cell morphology and were then chosen to have the structure of interest (e.g., the endocytic recycling compart- ment [E R C]) in focus in the green (OG-Tf) channel. Choosing the areas for imaging in the non-DI channel was especially important for the quantita- tive analyses, since the labeling pattern of the DiI derivatives varied significantly from cell to cell, and this distribution could be potentially skewed by observer bias if the focal plane was chosen in the DiI-labeled fields. We chose Oregon green over fluorescein to label Tf for quantitative microscopy since Oregon green has spectrosopic properties similar to fluorescein, but it has significantly higher photostability and a lower photobleaching rate than fluorescein (Haugland, 1996).

A ll image analysis was carried out using the Image-1/M etamorph Imaging System software. For quantitative analyses, the images of cells double labeled with OG-Tf (green) and one of the DiI derivatives (red) and a corresponding differential interference contrast image were sequentially acquired using the CCD camera (12 bit format) and were processed as follows. The fluorescence images were first background corrected by applying a median filter using a 64 × 64 pixel area (0.24 μm/pixel), and the background image was subtracted from the acquired image. The degree of crosstalk from one channel to the other was determined using cells labeled singly with each of the fluorophores. These images of singly labeled cells were background corrected, followed by measurement of integrated fluorescence intensity in the whole field in both channels. Since autofluorescence was negligible at the exposures used for these experiments, the fluorescence intensity observed in the unlabeled channel (after background subtraction) was taken to represent signal crossover. Cross- over measurements were made for four different fields for each fluorophore and averaged to obtain a representative crossover fraction. This crossover fraction of each image was then subtracted from the corre- sponding image in a double-labeled set before further analysis. The cross- over intensity was <2% of the true fluorescence intensity in all cases.

The cell outlines for each set of double-labeled fields were placed manually in the corresponding differential interference contrast image, and then copied on to the Tf- and DiI-labeled fields. In each cell, the whole cell green (Tf) and red (DiI) fluorescence intensities were measured. Simultaneously in each cell, the relative intensity in the E R C was measured by placing a small box (4 × 4 pixel area) in several locations within the morphologically defined perinuclear recycling compartment. In case of the DiI-labeled fields, precaution was taken not to include any punctate structures that may lie within the rather large area occupied by the E R C. The ratio of fluorescence intensities sampled within the E R Cs for the DiI derivative to Tf was then normalized by the whole cell fluorescence intensity ratio for that cell to correct for cell to cell variation in Tf receptor expression and Tf uptake. For the results presented in Figs. 7-9, the measurements were made for ∼50 cells in each data set, and the data were collected from experiments carried out on two different days. The validity as well as the limits of this method were first tested using control experiments (see Results).

Confocal Microscopy

Confocal microscopy was performed using an A xiovert 100M inverted microscope equipped with an LSM 510 laser scanning unit and a 63× 1.4 NA Plan Apo 1.4 NA oil immersion objective. Cells were excited with a 505-530-nm band pass filter to collect green (A lexa 488) emission and a 585-nm long-pass filter to collect red (D iI) emission. Confocal slices were obtained in 0.2-μm increments. The images were collected in eight-bit format, exported from the LSM 510 software, and each confocal slice was
the data obtained from MetaMorph Imaging System were routinely trans-
formed confoocal slices were also produced using the MetaMorph software.
For visual output purposes, the digital images (both wide-field and con-
final) were clipped to the relevant eight bits, transferred to a Macintosh
Power PC, and intensity mapped through logarithmic look up tables (luts)
using a dobe Photoshop software and printed on a dye sublimation printer
(SpectraStar Dsx; General Parametrics Corp.). For quantitative analyses,
the data obtained from MetaMorph Imaging System were routinely trans-
ferred to Microsoft Excel spreadsheet software for further calculations.

Results

All DiI Derivatives Show Uniform Labeling of the
Plasma Membrane

For our studies, we needed to develop a method of incorporation of different DiI derivatives in the plasma membr-
anes of TRVb-1 cells that would not produce particu-
lates containing DiI. Such particulates could potentially produce artifacts in our experiments, since they could ei-
ther be pinocytosed by cells or stick nonspecifically to the
membrane) labeling with no gross heterogeneity in distri-
bution. There is some variation in intensity that is consis-
tent with surface projections or a small amount of endocytosis during the 2-min incubation. To ensure that the DiI
labeling the cell surface had been released from albumin,
we did a control experiment in which cells were incubated
with a labeling solution containing BSA covalently conju-
gated to fluorescein (data not shown). No detectable fluo-
rescein signal was left after the labeled BSA was rinsed
away.

All DiI Derivatives Enter the Same Sorting Endosomes
as Transferrin

Since the Dil derivatives used in this study label the
plasma membrane uniformly, they would be expected, a
priori, to enter the cells through all available endocytic
routes. While these pathways appear to merge at the level of the peripherally distributed tubulovesicular sorting
endosomes in most cell types (Tran et al., 1987; Raub et al.,
1990), in some cases, such as ruffling A431 cells, they have
been reported to remain segregated from each other (Hew-
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DiI was internalized in 1 min, most vesicles with detect-
able DiI contain Tf. In some Tf-labeled vesicles, it is diffi-
cult to see the Dil because of the high plasma membrane
background. We conclude that both Tf and the DiI deriva-
tives enter the cell surface and slowly diffuse from there. We found that many previously published labeling protocols produced
high levels of aggregated DiI in the aqueous loading buff-
ers. It was difficult to remove these particles by centrifuga-
since most of these protocols required the use of or-
gean solvents, and at the high speeds necessary to remove
all the particles there was a separation of the organic
phase with most of the Dil probes dissolved in it. As de-
scribed in Materials and Methods, we developed a labeling
protocol in which Dil derivatives were transferred from an
ethanolic stock solution to fatty acid–free BSA in aqueous
solution. Ethanol was then removed by dialysis, and the
particulates were removed by ultracentrifugation. This
method produced a labeling solution with no DiI particles
detectable by fluorescence microscopy or gel filtration chro-
matography. Fig. 2 shows TRVb-1 cells labeled using these
Dil/BSA labeling solutions after appropriate dilution at
37°C for 2 min. All three Dil derivatives exhibited plasma
membrane labeling with no gross heterogeneity in distri-
Fig. 2. Cell surface (plasma membrane) labeling by (a) DiIC
d(3), (b) DiIC
12(3), and (c) FAST DiI. The cells were la-
culated for 2 min at 37°C with a fi-
cial concentration of 2 μM
DiIC
12(3), 31 nM DiIC
16(3), and
75 nM FAST Dil. They were then washed several times with
ice-cold Medium 1 supple-
mented with 2 g/liter glucose and
75 mm fast Dil. They were
were washed several times with
ice-cold Medium 1 supple-
menced with 2 g/liter glucose and
fixed with 2% paraformalde-
hyde for 10 min on ice. The ex-
cess paraformaldehyde was rinsed
thoroughly and the cells were
warmed to room temperature
before microscopy. Bar, 10 μm.
that organize near the microtubule organizing center, and when labeled with a fluorescent marker the ERC appears as a large perinuclear fluorescent spot (Yamashiro et al., 1984). Since exit from the ERC is the slowest step in the endocytic recycling itinerary of the Tf receptor, the ERC is the most brightly labeled structure at steady state (Yamashiro et al., 1984).

The following experiments were designed so that the cell surface Tf receptors would always be saturated with Tf, and the internalization of plasma membrane-associated DII derivatives could be compared with Tf. Fig. 4 shows cells that were labeled with 2 μM DII C16(3) for 2 min, washed, and then incubated with 10 μg/ml OG-Tf for 5 (a and b) or 30 (c and d) min. We observe that after 5 min of internalization, OG-Tf is mainly in the ERC, which appears as a single area of fluorescence near the center of each cell (Fig. 4 a). In contrast, a significant fraction of the DII C16(3) appears in punctate, vesicular structures (Fig. 4 b). A small fraction of DII C16(3) does appear to codistribute with the Tf at early times. By 30 min, nearly all internalized DII C16(3) is found to segregate away from Tf, and appears in discrete punctate structures that are distributed throughout the cell (Fig. 4 c and d). In similar experiments (data not shown), we find that even at earlier times after endocytosis (1–5 min) there is never a significant concentration of DII C16(3) in the pericentriolar area. Most of the DII C16(3) remains in punctate structures that remain discrete and increase in brightness over this period. This suggests that most DII C16(3) is retained in the sorting endosome and does not exit along with transferrin receptors that are delivered to the endocytic recycling compartment.

To examine whether the small fraction of DII C16(3) that appears to colocalize with OG-Tf at 5 min by wide-field microscopy is indeed in the same morphological structures as Tf, we carried out confocal microscopy of cells double labeled with DII C16(3) and A lexa 488-labeled Tf after a
5-min chase. Fig. 4, e and f, shows a single optical section through these cells, while Fig. 4, g and h, shows a summation projection through all the optical sections through the cell. As is clear from Fig. 4, e and f, a fraction of DilC₁₆(3) at this time does localize to structures that overlap with Tf-containing ERC and cannot be accounted for by out-of-focus fluorescence from late endosomes/lysosomes containing DilC₁₆(3). However, the summation projection images (Fig. 4, g and h) show that this population represents a small fraction of total intracellular DilC₁₆(3) fluorescence.

Fig. 5 shows the results from a similar experiment in which the cells were double labeled with OG-Tf (a and c) and DilC₁₂(3) (b and d), and Fig. 6 shows cells that were double labeled with OG-Tf (a and c) and FAST Dil (b and d). Figs. 5 and 6 show the relative distributions of OG-Tf and the Dil derivative after 5 (a and b) and 30 (c and d) min of endocytosis. The Dil derivatives (Figs. 5 and 6, b and d) seem to traffic in a fashion qualitatively similar to Tf (a and c), at least until 30 min after initiation of endocytosis. This was in contrast to the trafficking of DilC₁₆(3), where after 30 min of endocytosis nearly all DilC₁₆(3) was found in punctate structures devoid of Tf.

To better understand the trafficking of the Dil derivatives in relatively large cell populations, we carried out a quantitative analysis of the colocalization of various Dil derivatives and Tf in the ERC. The objective was to obtain an estimate of how similar the trafficking of each of the Dil derivatives was to that of Tf, without explicitly defining the alternate destinations of the Dil derivatives. Such an estimate could be obtained from the ratio of the fluorescence intensities of a Dil derivative to that of Tf in the region of the ERC after varying periods of endocytosis. In this analysis, a ratio closer to 1 indicates a trafficking behavior more similar to Tf. To account for differences among cells in the total amount of each fluorophore per cell, we normalized the ratio of fluorescence intensities in the ERC region of every cell to the ratio of the total fluorescence of the two fluorophores in that cell. The details of the image analysis protocol are discussed in Materials and Methods.

We tested the validity of this image analysis protocol and determined the upper and lower limits on the ratios in the ERC that could be reliably measured. In the first test case, the cells were labeled with a mixture of OG-Tf (green fluorescence) and Cy3-Tf (red fluorescence) continuously for 30 min. Fig. 7 a shows a frequency histogram (n = 50) comparing the distribution of the two Tfs. As expected for molecules that traffic identically, we obtained a narrow distribution of ratios in the ERC that is centered around 1. To test the case where two probes should sort efficiently from each other, we double-labeled cells with a mixture of Dil-LDL (which is trafficked to the late endosomes) and OG-Tf (which recycles efficiently). The cells were labeled for 5 min with a mixture of both probes at 37°C, rinsed, and then incubated further at 37°C in the presence of OG-Tf for 5 (a and b) or 30 (c and d) min. The cells were then washed and fixed as described in Fig. 2. Bar, 10 μm.
pected to show a nonzero ratio to Tf in the ERC region using this image analysis protocol.

Fig. 8 shows a quantitative analysis of the ratios of various DiI derivatives to Tf in the ERC regions of TRVb-1 cells (n = 50 for each data set). Fig. 8, a and d, shows the distribution of the ratios in the ERC for DiIC16(3), b and e for DiIC12(3), and c and f for FAST DiI. Fig. 9 shows the mean ratio of each DiI derivative to Tf in the ERC at each time point for easier comparison. The analysis shows that even at early times (5 min of endocytosis), the distributions for the three DiI derivatives are significantly different from each other. While FAST DiI shows the highest degree of colocalization with Tf in the ERC (ratio centered around 0.8), DiIC16(3) shows the lowest ratio (centered around 0.4) with DiIC12(3) showing an intermediate behavior. This means that FAST DiI and DiIC12(3) traffic in a manner more like Tf, compared with DiIC16(3). These results show that sorting of DiIC16(3) occurs relatively quickly after endocytosis, with a significant difference in the ratio in the ERC by 5 min. By 30 min, the differences among various DiI derivatives becomes more pronounced. They all sort away from the Tf recycling pathway to different degrees, with the difference being most pronounced for DiIC16(3) (Fig. 8, a and d). In fact, the distribution of the ratio values for DiIC16(3) to Tf is similar to the distribution for LDL to Tf (Fig. 7 b).

From the results presented above, we find that DiIC16(3) separates from Tf very efficiently following endocytosis into sorting endosomes, and by 30 min its overlap with Tf in the ERC region is similar to the overlap seen for LDL. DiIC12(3) and FAST DiI show greater overlap with Tf. However, in none of these cases does the ratio of a DiI derivative to Tf reach 1 (as seen when two differently tagged Tfs are used to label the cells). This indicates that the DiI derivatives exhibit a range of partitioning preferences that lead to differential trafficking, and the sorting is not all-or-none. We also note that the distributions of the ratios of various DiI derivatives to Tf in the ERCs of CHO cells (Fig. 8) are significantly wider than a similar distribution for the cells double labeled with Cy3-Tf/OG-Tf or LDL/Tf (Fig. 7). This is indicative of cell-to-cell variability in the trafficking of the DiI derivatives. The basis for this heterogeneity is unknown.

DiIC16(3) Preferentially Enters Late Endosomes/Lysosomes

We used colocalization with high molecular weight fluorescein-labeled dextrans as an assay to test whether the punctate structures that contained the DiI derivatives at later times were late endosomes/lysosomes. In Fig. 10, we show a colocalization of the various DiI derivatives with fluorescein dextran after a 60-min chase. The cells were labeled for 2 min at 37°C, washed, and then incubated for 60 min in the presence of 1 mg/ml fluorescein dextran. Fig. 10, a, c, and e, shows the distribution of DiIC16(3) to Tf (Fig. 7 b). In fact, the distribution of the ratio values for DiIC16(3) to Tf is similar to the distribution for LDL to Tf (Fig. 7 b).

Figure 7. Control experiments to assign limits on the ratios in the ERC that can be reliably measured by our image analysis protocol. (a) A case where both probes traffic identically. TRVb-1 cells were coincubated with 10 μg/ml OG-Tf and 5 μg/ml Cy3-Tf for 30 min at 37°C, and washed and fixed as discussed in Fig. 2. The analysis regime is discussed in detail in Materials and Methods. This figure shows a frequency histogram (n = 50) where the number of cells having ratios of Cy3-Tf/OG-Tf in different ratio intervals are shown. (b) A case where the two probes completely segregate from each other. In this case, cells were coincubated with 1 μg/ml DiI-LDL and 10 μg/ml OG-Tf for 30 min at 37°C, rinsed, chased for another 5 min at 37°C, and fixed as described in Fig. 2. The figure shows a frequency histogram of DiI-LDL/OG-Tf (n = 50).
Endocytic Sorting of Lipid Analogue Based on the Chemistry of Their Tails Is Not Unique to the Dil Derivatives

To ensure that the endocytic sorting of the Dil derivatives reported in this paper is not unique to these fluorophores, we conducted a similar experiment with two other sets of lipid analogues. One set, DiOC16(3) and FAST Dil, were identical to the Dil analogues except that they contained an oxygen atom instead of a carbon atom attached to two methyl groups in their head groups (see Fig. 1). Both Dil derivatives contained long chains, but in the former case they were both saturated, while in the latter they contained two double bonds each. The other set consisted of two analogues of phosphatidylcholine, which both had one saturated 16-carbon tail, whereas the second tail contained a BODIPY FL fluorophore at the end of either a 5- or a 12-carbon acyl chain (BODIPY FL C5-HPC and BODIPY FL C12-HPC, respectively; see Fig. 1).

Fig. 11 shows the distribution of these lipid analogues in TRVb-1 cells singly labeled with each fluorophore. Cells were labeled with 150 nM FAST Dil (Fig. 11 a), 2 μM DiOC16(3) (Fig. 11 b), 30 nM BODIPY FL C5-HPC (Fig. 11 c), and 1 μM BODIPY FL C12-HPC (Fig. 11 d). For each type of lipid analogue, the concentrations of the labeling solutions were adjusted to provide approximately the same level of incorporation in the cells. The cells were labeled for 2 min at 37°C with each labeling solution, rinsed, incubated further at 37°C in Medium 1 for 30 min, and then fixed lightly with paraformaldehyde. The results show that while the lipid analogues with unsaturations in their tails (e.g., FAST Dil; Fig. 11 a) or containing one short tail (e.g., BODIPY FL C5-HPC; Fig. 11 c) predominantly entered a central fluorescent compartment, lipid analogues containing long saturated tails (e.g., DiOC16(3); Fig. 11 b and BODIPY FL C12-HPC; Fig. 11 d) entered punctate structures distributed throughout the cells. Double-labeling studies (data not shown) confirm the identities of the central fluorescent compartment as the ERC and the punctate structures as late endosomes/lysosomes. We do observe some differences in the degrees to which

Figure 8. Quantitative analysis of the results presented in Figs. 4–6. The results are shown as frequency histograms of the ratio of the various Dil derivatives to Tf in the ERC at various time points. a–c show the ratios in the ERC after 5 min, while d–f show them after 30 min. DiIC16(3) (a and d), DiIC12(3) (b and e), and FAST Dil (c and f).
DiOC$_{16}$(3) and BODIPY FL C$_{12}$-HPC are directed to the late endocytic pathway. While DiOC$_{16}$(3) appears to be as efficient as DiIC$_{16}$(3) in being delivered to the late endosomes in 30 min, we see a larger fraction of BODIPY FL C$_{12}$-HPC in the ERC at this time. However, as seen in Fig. 11, the difference between these analogues and the corresponding analogues with unsaturated and short chains is very clear. These results are similar to our observations with the DiI analogues in which lipid analogues with short or unsaturated tails recycle efficiently, but those with long and saturated tails are preferentially directed to the late endocytic pathway.

**Discussion**

The results presented here show that different DiI derivatives, varying solely in the composition of their alkyl chains, exhibit differential trafficking after their internalization from the cell surface into sorting endosomes. While the DiI derivative with long and saturated tails (i.e., DiIC$_{16}$(3)) preferentially enters the late endocytic pathway, the derivatives with shorter (DiIC$_{12}$(3)) or unsaturated (FAST DiI) tails are efficiently delivered to the ERC. These observations are reinforced by two other sets of lipid analogues.

There are several steps along the endocytic pathway where sorting of different DiI analogues could have occurred. First, some of the segregation might have occurred at the plasma membrane. Domains enriched in DiIC$_{16}$(3) have been observed on the plasma membrane of rat basophil leukemia cells when IgE receptors are cross-linked (Thomas et al., 1994). Although we did not detect any inhomogeneity in the distribution of the DiI derivatives on the cell surface, we cannot rule out submicroscopic segregation and/or variations in the rates of internalization of different DiI derivatives. However, even if cell surface heterogeneity is present, it could not account for the sorting we observe between DiIC$_{16}$(3) and the other two DiI derivatives, since we report that all DiI derivatives initially enter sorting endosomes that also contain endocytosed Tf. Thus, most of the sorting must take place at a step after the internalization of DiIC$_{16}$(3) and Tf into common sorting endosomes.

The sorting endosomes serve as the branch point for entry into either the recycling route or the late endocytic route. We propose that this is the major site where DiIC$_{16}$(3) sorts out of the endocytic recycling route. This site of sorting is consistent with the fact that the sorting occurs as early as 5 min after endocytosis. DiIC$_{16}$(3) is retained in punctate endosomes while FAST DiI begins to
The retention of DiIC\textsubscript{16}(3) in the sorting endosomes and its subsequent delivery to the late endocytic structures does not appear to be determined, in any significant way, by specific lipid–protein interaction with some transmembrane protein. In control experiments (data not shown), we have seen that DiIC\textsubscript{16}(3) is delivered to late endosomes even when the cells are labeled with 10-fold more DiIC\textsubscript{16}(3) than used in the experiments reported in this study. It has been estimated in the case of rat basophilic leukemia cells that optimal labeling of the cell surface with DiIC\textsubscript{16}(3) results in \(10^7\)–\(10^8\) DiI molecules per cell (Thomas et al., 1994). On the contrary, even a highly expressed protein on the cell surface has \(10^9\)–\(10^{10}\) copies (R yan et al., 1988; Gennis, 1989). Considering that such a protein would also interact with native lipids, it seems unlikely that stoichiometric binding to any transmembrane protein or even a group of proteins would redirect all DiIC\textsubscript{16}(3) to the late endosomes. In contrast, if lateral membrane domains of varying fluidity and/or curvatures did exist in the membranes of the sorting endosomes, DiIC\textsubscript{16}(3), by virtue of its long saturated alkyl chains, would partition preferentially into the more rigid domains or those with a convex curvature. We show several variations of these possibilities in Fig. 12. We suggest that if any specific lipid–protein interaction has to occur, it would be feasible only within the context of fluidity- or curvature-dependent cosegregation of the lipids and proteins in question, so that high enough effective concentrations can be achieved.

**Domain-based Segregation in the Sorting Endosomes: A Working Hypothesis**

The differential trafficking of the lipid analogues with long saturated hydrophobic tails relative to those with short or unsaturated tails can be rationalized if the vesicular region of the sorting endosomes are considered to represent membrane domains that are more rigid than those in the attached tubules that bud from the sorting endosomes [see Fig. 12, (1)]. There is evidence that narrow tubules in model membrane systems are enriched in more fluid domains. For example, when either gel or fluid phase liposomes were subjected to suction by a micropipette, it was found to be easier to pull fluid membranes into long narrow tubules in response to suction as compared with membranes in the gel phase (E vans and N eedham, 1987). Further, a study that measured diffusion coefficients of transmembrane proteins in red blood cell “tethers” (hollow membranous cylinders, up to 36 \(\mu\)m long and \(\sim 100\) nm in diameter) found a dramatic increase in the diffusion coefficients of these proteins in the tether membrane (B erk and H ochmuth, 1992). In an alternate model, the bilayer fluidity in both the vesicular and the tubular parts of the sorting endosome could be roughly similar, except that the necks joining the tubules to the vesicle, which are under high curvature stress, would be specifically enriched in lipids that can be accommodated in regions of high curvature [Fig. 12, (2)]. In this case, any lipid analogue that cannot easily traverse this small but highly curved fluid (disordered) domain at the neck of the tubule would be preferentially retained in the sorting endosome. Both alternatives are feasible and have been proposed previously in biophysical treatments of bud formation in model membranes (Lipowsky, 1993; Sackmann and F eder, 1995).

In addition, the shape of an amphiphile can be related to its propensity to induce curvature in model membranes, as well as to partition preferentially into preexisting membrane domains of varying curvatures (Cullis and de K ruijff, 1979; Gennis, 1989). More precisely, if a molecule has approximately equal cross-sectional areas in its head group and tail region, the molecule can be considered as a cylinder that does not induce any curvature in a bilayer and would preferentially partition into planar regions of the membrane (no curvature preference). On the other hand, amphiphiles with larger (an inverted cone) or smaller (a cone) head group cross-sectional areas would induce opposite curvatures or partition into preexisting regions of opposite curvature. Since DiIC\textsubscript{16}(3), with its alkyl tails stretched out all-trans, would approximate an inverted cone, it would be expected to preferentially enter intraluminal invaginations or involutions in the sorting endosome membrane and hence segregate from the recycling pool of membrane-bound molecules [Fig. 12, (3)]. FAST DiI, on the other hand, would look more like a cylinder or a cone (depending on the number of gauche conformations in the alkyl tails in addition to the two cis double bonds) and hence would have either no curvature preference or a preference for concave curvatures that would result in its enhanced entry into the tubules of the sorting en-
dosomes. This difference in shape might partly explain why DiIC$_{12}$ simply recycles less efficiently than FAST DiI even though both have similar fluidity preferences.

From the sorting endosomes, most membrane-bound components enter the ERC and are recycled out to the cell surface with a half time of 10–12 min. The ERC has been shown to have sorting functions of its own (Johnson et al., 1993, 1998; Marsh et al., 1995). Recent studies show that glycosylphosphatidylinositol (GPI)–anchored proteins are recycled approximately three times more slowly than C$_6$-NBD-SM or Tf receptor (Mayor et al., 1998). The recycling of GPI-anchored proteins can be restored to the same rate as Tf receptor when the cells are grown in media that lower cellular cholesterol by $\sim$40% (Mayor et al., 1998). Cholesterol is known to be a major modulator of bilayer motional characteristics and to be involved in the formation of ordered domains in model membrane systems (Yeagle, 1985; Schroeder et al., 1995). The cholesterol dependence suggests the involvement of ordered lipid domains in the slow recycling of GPI-anchored proteins. The ERC does not, however, appear to be a major site for the sorting of DiIC$_{16}$ from other recycling markers, since at no time during its endocytic itinerary do we see a significant concentration of DiIC$_{16}$ in the ERC. As seen in Fig. 4, there is a minor fraction of DiIC$_{16}$ that appears to localize at the ERC by optical microscopy. Even though some DiIC$_{16}$ appears to enter the ERC, its efficiency of sorting to late endosomes would be comparable with that found for ligands that bind to receptors. For example, $\sim$20% of all internalized $\alpha_2$-macroglobulin (Yamashiro et al., 1989) and $\sim$40% of insulin (Levy and Olefsky, 1987) are recycled undegraded during each endocytic passage through the cell.

Several previously reported examples of lipid sorting can be understood in terms of our working hypothesis based on lipid shapes and fluidity preferences. For example, C$_6$-NBD-SM and related lipid analogues that recycle efficiently contain one very short (6-carbon) acyl chain to which the reporter group is attached. The polar NBD group would make this tail loop up to the membrane inter-

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**Figure 12.** A schematic representation of the endocytic sorting of lipid analogues occurring at the sorting endosomes. Molecules internalized from the cell surface enter the tubulovesicular sorting endosomes. Here, most of the surface area (including most of the membrane-bound molecules) are in the long tubular projections that repeatedly pinch off and deliver their contents to the recycling route, while some membrane components are specifically retained in this organelle and are eventually delivered to late endosomes. Such sorting out of the recycling route would require that these molecules do not enter the tubules that contain material destined to recycle. Here, we propose mechanisms that could act to constrain a fraction of membrane-bound molecules in the membrane surrounding the spherical part of the sorting endosome. (1) The tubules could be enriched in more fluid domains so that lipids that preferentially partition into such domains [e.g., DiIC$_{12}$ and FAST DiI] would be enriched there, while rigid domain–preferring lipids such as DiIC$_{16}$ could be depleted. (2) The necks of the tubules could represent extremely fluid domains (due to excessive curvature stress), and only those membrane components that can traverse these domains would be able to enter the tubules in significant proportions. (3) Certain lipids such as DiIC$_{16}$ may be enriched in the invaginations of the vesicular region of the sorting endosome by virtue of their inverted cone shape. See text for more detailed discussion. The above possibilities are not mutually exclusive.
face (Chattopadhyay and London, 1987), so that the total cross-sectional area occupied by this molecule would be quite large. These factors would be expected to result in preferential partitioning of this lipid analogue to more fluid regions of the bilayer, and thus would be predicted by our model to predominantly enter the endocytic recycling pathway. On the other hand, some lipids that have been reported to be targeted to late endocytic compartments contain long, saturated acyl chains (e.g., rhodamine-labeled phosphatidylethanolamine and some glycosphingolipids; Kok et al., 1990, 1991) that would result in their preferential partitioning to the more rigid parts of the bilayer. In fact, Sandhoff and Klein (1994) have suggested that the retention of certain lipid components such as glycosphingolipids in the inner involutions of the endosomes may play a role in their eventual delivery to the degradative pathway.

Our observations are also consistent with the recent observations of Chen et al. (1997). This study used fibroblasts whose plasma membranes were labeled with a BODIPY FL-labeled lipid analogue that fluoresced green at low concentrations (monomer fluorescence) and red at high concentrations (excimer fluorescence). The plasma membranes for these experiments were labeled such that all the cell surface fluorescence was only monomer (green) fluorescence. Interestingly, when these cells were allowed to endocytose for as short as 7 s, it was found that, while some newly formed endosomes still fluoresced green, others in the same cell exhibited red (excimer) fluorescence. A II plasma membrane fluorescence still remained green. These observations could either mean that the lipid analogue segregated into lateral domains more or less enriched in this analogue just before being endocytosed, or that all endosomes contain a roughly similar number of copies of the fluorescent lipid analogue, but that in a subset of those endosomes the analogue segregated into subdomains, thereby increasing their effective concentration and giving rise to excimer fluorescence. Both these possibilities would be consistent with our working hypothesis for lipid sorting in fibroblasts.

Sorting of lipids occurs at many membrane trafficking steps. For example, the sorting of DiIC$_{16}$(3) out of the recycling pathway occurs primarily at the sorting endosomes, while GPI-anchored proteins are delayed in their export from the E.R. Similarly, the TGN has been reported by several groups to be the primary site along the biosynthetic pathway for sorting of apically and basolaterally destined lipids (Simons and van Meer, 1993). It is conceivable that lipid sorting occurs to some extent at every step where a vesicle or tubule buds from an organelle. We emphasize that the possibilities presented in our model are neither exhaustive nor mutually exclusive. On the contrary, they are presented solely as pointers toward the various possibilities that are conceivable. In general, the principles involved in this type of sorting include the chemistry of the individual membrane component and the composition of the host bilayer. The process we observe in endosomes may very well reflect a more ubiquitous mechanism in several intracellular sorting processes. This would offer an energetically inexpensive mechanism for a relatively efficient sorting of certain classes of molecules from others (Parsegian, 1995). In the cellular milieu, such mechanisms would act in concert with other, more specific, protein–protein or protein–lipid interactions that could modulate or "fine-tune" this basal level sorting.

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