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, DiIC\textsubscript{16}(3) (1,1'-dihexadecyl-3,3',3',3'-tetramethylindocarbocyanine perchlorate), DiIC\textsubscript{12}(3) (1,1'-didodecyl-3,3',3',3'-tetramethylindocarbocyanine perchlorate), and FAST-DiI (1,1'-dilinoleyl-3,3',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. DiIC\textsubscript{16}(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 DiIC\textsubscript{12}(3), with saturated but shorter (12-carbon) tails, are mainly found in the endocytic recycling compartment. We also find that DO\textsubscript{C16}(3) (3,3'-dihexadecyloxycarbocyanine perchlorate) and FAST-DiO (3,3'-dilinoleyloxycarbocyanine 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., C\textsubscript{6}-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-
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 glycosphingolipids have been found to concentrate in the inner involutes 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 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 (Sims et al., 1974; Haulgand, 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<sub>16</sub>(3) (1,1'-dihexadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate) has tails with 16 carbons each, whereas DiIC<sub>9</sub>(3) (1,1'-didodecyl-3,3',3'-tetramethylindocarbocyanine perchlorate) has 12 carbon chains. The differential partitioning preferences of C<sub>16</sub>DiIs 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<sub>4</sub>(3) approximately matches those most prevalent in the lipids of various CHO cell lines (Callaghan et al., 1992; Mackinnon et al., 1992). Thus, in CHO cell membranes, DiIC<sub>4</sub>(3) would be expected to preferentially partition into more rigid (or highly ordered) domains, whereas DiIC<sub>16</sub>(3) would enter more fluid domains. The other lipid analogue used in this study, FAST DiI (1,1'-dilinoleyl-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 (Mouritsen and Jorgensen, 1995).

In rat basophil leukemia (RBL) cells, preferential segregation of DiIC<sub>9</sub>(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<sub>16</sub>(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; Gennis, 1989). Both DiIC<sub>16</sub>(3) and DiIC<sub>9</sub>(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 (Haulgand, 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 (Haulgand, 1996). One long 16-carbon acyl chain ensures stable incorporation into the membrane bilayer. We used BODIPY FL lipid analogues since the fluorophore 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 Kurzchal, 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-borato-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).

Abbreviations used in this paper: DiI, dialkylindocarbocyanine; ERC, endocytic recycling compartment; GPI, glycosylphosphatidylinositol; OG, Oregon green–labeled transferrin; Tf, transferrin.

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phore has been reported to localize to the membrane interior 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 fluorophore, 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 C₁₂-HPC [2-(BODIPY-3-dodecanoyl)-1-hexadecanoyl-sn-glycero-3-phosphocholine] would thus be somewhat similar to the DiIC₁₆(3) or DiOC₁₆(3) in terms of the properties of the hydrophobic tail. BODIPY FL C₅-HPC [2-(BODIPY-3-pentanoyl)-1-hexadecanoyl-sn-glycero-3-phosphocholine] would, on the other hand, be expected to more closely mimic DiIC₁₂(3).

**Materials and Methods**

**Materials**

A II 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 7.4, and was extensively dialyzed 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 Sephracyl 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. DiIC₁₆(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 (TR v b1; 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. Geneticin was used as a selection for the transfected Tf receptors. All cells were grown in a 5% CO₂ environment in humidified incubators set at 37°C. The cells for microscopy were grown on plastic tissue culture dishes whose bottoms were replaced with poly- 

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 Dil derivatives were made in ethanol and stored at –80°C under argon. For making the labeling solutions, 750 nmol of DiIC₈(3) or 75 nmol of DiIC₁₂(3) or FAST Dil 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 Dil got transferred to the hydrophobic fatty acid binding sites on the BSA, while much of it self-aggregated, and remained suspended in the labeling solution. The dialysate was then centrifuged at 100,000 × g for 20 min at 4°C. When the supernatant from this procedure was run on a Sephacryl S-300 gel filtration column, the Dil eluted as a single peak associated with the BSA. A scanning photop- 

tometric analysis showed a loading efficiency (DiI/BSA; mol/mol) of ~0.1 for DiIC₈(3), 0.3 for DiIC₁₂(3), and 0.25 for FAST Dil. The Dil-loaded BSA solutions were then sterilized by passing through 0.2 μm syringe filters and stored at 4°C under argon.

Cells on coverslip-bottom dishes were taken out of the CO₂ incubator, rinsed several times with isotonic M edium 1 (150 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, and 20 mM HEPES, pH 7.4; supplemented with 2 g/l glucose), and then labeled with an appropriate dilution of a Dil labeling solution (2 μM DiIC₈(3), 31 nM DiIC₁₂(3), and 75 nM FAST Dil). The concentrations of different Dil derivatives were optimized such that the signal-to-noise ratio (of all analogues in the cells (as assessed by in- 

tegrated fluorescence power per cell) were roughly matched. We used the minimum concentration in each case that would give us a useful fluores- 

cence signal. A Dil labeling reagent were ultracentrifuged at 100,000 × g for 20 min just before an experiment and equilibrated to 37°C. When cells were labeled using these labeling solutions at 0°C, we obtained very good labeling of the plasma membrane. However, there was also extensive la-

beling of the background (predominantly the extracellular matrix). This background was dramatically reduced when labeling was instead carried out for a very short time (2 min) at 37°C. Thus, the latter approach was used for all the experiments presented here. After labeling, the cells were rinsed with ice-cold M edium 1 and fixed ligand with 2% paraformalde- 

yde for 10 min at 0°C.

Fatty-acid free BSA was loaded with the other lipid analogues used in this study using identical procedures. Final concentrations of the different fluorophores used to label cells were 2 μM DiOC₈(3), 150 nM FAST DIO, 30 nM BODIPY FL C₁₂H₂PC, and 1 μM BODIPY FL C₁₂H₃PC.

Endocytosis Assays

Endocytic fates of the Dil derivatives were determined by comparing their intracellular distributions with those of the endocytosed Tf at various time points after the initial loading of the Dil derivatives on to the plasma membranes of TR v B-1 cells. Transferin, bound to its receptor, was used as a marker for the endocytic recycling route (Dunn et al., 1989; Muhker-

jee et al., 1997). A comparison of the trafficking of various Dil derivatives with Tf thus allowed us to determine the degree of overlap of the en-

docytic trafficking routes of these Dil derivatives with that of Tf.

The general experimental methods used to analyze the endocytic be-

havior of various Dil derivatives were as follows. Cells equilibrated to 37°C were labeled for 2 min at 37°C with the appropriate dilution of a Dil labeling solution, rinsed several times with Medium 1, and then incubated with pre-warmed 0.01% digitonin–labeled Tf (OG-Tf; or a lexa 488–conju- 
gated Tf in the confocal experiments) for either 5 or 30 min. At 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 

Dil derivatives as late endosomes/lysosomes, TR v B-1 cells were labeled with different Dil derivatives as described above, rinsed, and further incu-

bated at 37°C with 1 mg/ml fluorescent-labeled dextrans 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 DMIRB microscope (Leica Mikroskopie und Systeme G mBH) equipped with a cooled CCD camera (Frame Transfer Pentamax camera with a 512 × 512 back-thinned EEV chip, No. 512EFTB; Princeton Instruments) driven by Image-1/M etamorph Imaging System software (U niversal Imaging Corp.). All images were acquired using a high magnification (63×; 1.4 NA) oil immersion objective. Dil derivatives were im-

aged using a standard rhodamine filter set, while Oregon green was im-

aged using a fluorescein filter set. The fields to be imaged were chosen on the wide spread of cell morphology and the different Dil derivatives were chosen to have the structure of interest (e.g., the endocytic recycling compart-
ment [ERC]) in focus in the green (OG-Tf) channel. Choosing the areas for imaging in the non-Dil channel was especially important for the quan-
titative analyses, since the labeling pattern of the Dil 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 Dil-labeled fields. We chose Oregon green over fluorescein to label Tf for quanita-
tive microscopy since Oregon green has spectroscopic properties similar to 

fluorescein, but it has significantly higher photostability and a lower pKa and higher fluorescence yield than fluorescein (Haugland, 1996).

A Dil image analysis was carried out using the Image-1/M etamorph Im-
aging System software. For quantitative analyses, the images of cells dou-

bled labeled with OG-Tf (green) and one of the Dil derivatives (red) and a 

corresponding differential interference contrast image were sequentially 

acquired using the CCD camera (12 bit format) and were processed as fol-

ows. The fluorescence images were first background corrected by applying 

a median filter using a 64 × 64 pixel area (0.2 μm/pixel), and the 

background image was subtracted from the acquired image. The degree of 

crossover of signal 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 in-

tegrated fluorescence intensity in the whole field in both channels. Since autofluorescence was negligible at the exposures used for these experi-

ments, 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 fluo-

rochrome 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-

overs intensity was ~2% of the true fluorescence intensity in all cases.

The cell outlines for each of the double-labeled set of fields were placed manually in the corresponding differential interference contrast image, and then copied on to the Tg- and Dil-labeled fields. In each cell, the whole cell green (Tf) and red (Dil) fluorescence intensities were mea-

sured. Simultaneously in each cell, the relative intensity in the ERC 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 Dil-labeled fields, precaution was taken not to include any 

punctate structures that may lie within the rather large area occupied by the 

ERC. The ratio of fluorescence intensities sampled within the ERCs for the Dil 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 Dil 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 R esults).

Confocal Microscopy

Confocal microscopy was performed using an A xiophot 100M inverted mi-

roscope equipped with an LSM 510 laser scanning unit and a 63× 1.4 NA 

plan apochromat objective (all from Carl Zeiss, Inc.). Samples were ex-

cited with a 25-mW argon laser emitting at 458 and 488 nm and a 0.5 mW helium/argon laser emitting at 543 nm; emissions were collected using a 505–530-nm band pass filter to collect green (Alexa 488) emission and a 585-nm long-pass filter to collect red (DiI) emission. Confocal slices were obtained in 0.2-μm increments. The images were collected in eight-bit for-

mat, exported from the LSM 510 software, and each confocal slice was

Published March 22, 1999

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The Journal of Cell Biology, Volume 144, 1999
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 membranes of TRVb-1 cells that would not produce particulates containing DiI. Such particulates could potentially produce artifacts in our experiments, since they could either be pinocytosed by cells or stick nonspecifically to 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 buffers. It was difficult to remove these particles by centrifugation since most of these protocols required the use of organic solvents, and at the high speeds necessary to remove all the particles there was a separation of the organic phase with most of the DiI probes dissolved in it. As described in Materials and Methods, we developed a labeling protocol in which DiI 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 chromatography. Fig. 2 shows TRV b-1 cells labeled using these DiI/BSA labeling solutions after appropriate dilution at 37°C for 2 min. All three DiI derivatives exhibited plasma membrane labeling with no gross heterogeneity in distribution. There is some variation in intensity that is consistent 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 conjugated to fluorescein (data not shown). No detectable fluorescein signal was left after the labeled BSA was rinsed away.

All DiI Derivatives Enter the Same Sorting Endosomes as Transferrin

Since the DiI 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 (Hewlett et al., 1994). It was thus important to document that all the DiI analogues used in this study predominantly entered sorting endosomes, which were characterized by the presence of fluorescent Tf at early times after the initiation of endocytosis of the fluorescent markers. We achieved this by double-labeling TRV b-1 cells for very short times (1 min) with a mixture of 15 μg/ml OG-Tf and each of the DiI derivatives, followed by immediate fixation and looking for colocalization of OG-Tf and the DiI analogues (Fig. 3). Although only a small fraction of the DiI was internalized in 1 min, most vesicles with detectable DiI contain Tf. In some Tf-labeled vesicles, it is difficult to see the DiI because of the high plasma membrane background. We conclude that both Tf and the DiI derivatives enter the same sorting endosomes.

DiI<sub>C<sub>16</sub>(3) Gets Sorted Away from Both FAST DiI and DiI<sub>C<sub>18</sub>(3) after Endocytosis

Intracellular fates of the DiI derivatives were followed by comparing their trafficking to that of receptor-bound OG-Tf, which follows the endocytic recycling pathway. In TRV b-1 cells, Tf exits the sorting endosome with a <t>1/2</t> of ~2 min and is delivered to the ERC (Dunn et al., 1989; Mayor et al., 1993). Exit from the ERC and delivery to the cell surface occurs with a <t>1/2</t> of 10–12 min (McGraw et al., 1987; Dunn et al., 1989). In TRV b-1 and other CHO cell lines, the ERC is a collection of narrow tubular elements.
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 D il derivatives could be compared with Tf. Fig. 4 shows cells that were labeled with 2 μM D ilC 16(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 D ilC 16(3) appears in punctate, vesicular structures (Fig. 4 b). A small fraction of D ilC 16(3) does appear to codistribute with the Tf at early times. By 30 min, nearly all internalized D ilC 16(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 D ilC 16(3) in the pericentriolar area. Most of the D ilC 16(3) remains in punctate structures that remain discrete and increase in brightness over this period. This suggests that most D ilC 16(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 D ilC 16(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 D ilC 16(3) and A lex 488–labeled Tf after a 5 min chase, imaged using laser scanning confocal microscopy. The cells were then washed and fixed as described in Fig. 2, while a–d show cells double labeled with 10 μg/ml Alexa 488–Tf and 2 μM D ilC 16(3) after 5 min chase, imaged using wide-field epifluorescence microscopy. The cells were double labeled with 10 μg/ml Alexa 488–Tf and 2 μM D ilC 16(3), imaged using wide-field epifluorescence microscopy. The cells were then washed and fixed as described in Fig. 2. a–d show cells double labeled with 10 μg/ml Alexa 488–Tf and 2 μM D ilC 16(3) after 5 min chase, imaged using laser scanning confocal microscopy. e and f show the distributions of Alexa 488–Tf, while g and h show summation projection of all the optical slices from the same cell. Bars, 10 μm.
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.
expected 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 \( (n = 50 \text{ for each data set}) \). Fig. 8, a and d, shows the distribution of the ratios in the ERC for DiIC_{16}(3), b and e for DiIC_{12}(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), DiIC_{16}(3) shows the lowest ratio (centered around 0.4) with DiIC_{12}(3) showing an intermediate behavior. This means that FAST DiI and DiIC_{12}(3) traffic in a manner more like Tf, compared with DiIC_{16}(3). These results show that sorting of DiI derivatives becomes more pronounced. They all sort away from the Tf recycling pathway to different extents, with the difference being most pronounced for DiIC_{16}(3) (Fig. 8, a and d). In fact, the distribution of the ratio values for DiIC_{16}(3) to Tf is similar to the distribution for LDL to Tf (Fig. 7b).

From the results presented above, we find that DiIC_{16}(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. DiIC_{12}(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/O-G-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.

DiIC_{16}(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 with each DiI derivative, washed, and then incubated for another 5 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/O-G-Tf \( (n = 50) \).

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 \( \mu \)g/ml O-G-Tf and 5 \( \mu \)g/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/O-G-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 \( \mu \)g/ml DiI-LDL and 10 \( \mu \)g/ml O-G-Tf for 5 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/O-G-Tf \( (n = 50) \).
Endocytic Sorting of Lipid Analogues 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 similar experiments with two other sets of lipid analogues. One set, DiOC16(3) and FAST DiO, 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 DiO 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 constituted 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 DiO (Fig. 11a), 2 μM DiOC16(3) (Fig. 11b), 30 nM BODIPY FL C5-HPC (Fig. 11c), and 1 μM BODIPY FL C12-HPC (Fig. 11d). 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 DiO; Fig. 11a) or containing one short tail (e.g., BODIPY FL C5-HPC; Fig. 11c) predominantly entered a central fluorescent compartment; lipid analogues containing long saturated tails (e.g., DiOC16(3); Fig. 11b and BODIPY FL C12-HPC; Fig. 11d) 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...
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
enter the ERC within 5 min. As expected for a molecule that is retained in the sorting endosomes, nonrecycled DiIC$_{16}(3)$ is subsequently delivered to the late endosomes (Fig. 10).

The retention of DiIC$_{16}(3)$ 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$_{16}(3)$ is delivered to late endosomes even when the cells are labeled with 10-fold more DiIC$_{16}(3)$ 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$_{16}(3)$ results in $\sim 10^7$–$10^8$ DiI molecules per cell (Thomas et al., 1994). On the contrary, even a highly expressed protein on the cell surface has $\sim 10^5$–$10^6$ 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$_{16}(3)$ 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$_{16}(3)$, 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 (Berk and Hochmuth, 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 Feder, 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 Krujff, 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$_{16}(3)$, 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-
The difference in shape might partly explain why DiIC16(3) 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; Presley et al., 1993; Marsh et al., 1995). Recent studies show that glycosylphosphatidylinositol (GPI)-anchored proteins are recycled approximately three times more slowly than C6-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 \( \approx 40\% \) (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 DiIC16(3) from other recycling markers, since at no time during its endocytic itinerary do we see a significant concentration of DiIC16(3) in the ERC. As seen in Fig. 4, there is a minor fraction of DiIC16(3) that appears to localize at the ERC by optical microscopy. Even though some DiIC16(3) 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, \( \approx 20\% \) of all internalized \( \alpha_2 \)-macroglobulin (Yamashiro et al., 1989) and \( \approx 40\% \) 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, C6-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-
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 invaginations 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. All 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 Dil-16:0/3 out of the recycling pathway occurs primarily at the sorting endosomes, while GPI-anchored proteins are delayed in their export from the ERC. 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, 1988). 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.

We thank Drs. Tim McGraw, Bill Mallet, Lynda Pierini, and Richik Ghosh for critical reading of the manuscript and for helpful discussions. S. Mukherjee acknowledges a postdoctoral fellowship from the Norman and Rosita Winston Foundation for Biomedical Research. The study was supported by grant DK27083 from the National Institutes of Health and by a grant from the Human Frontier Science Program.

Received for publication 3 March 1998 and in revised form 10 February 1999.

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