Characterization of Rapid Membrane Internalization and Recycling*

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Lipids and other membrane constituents recycle between the plasma membrane and intracellular endocytic compartments. In CHO cells, approximately half of the internalized C₆-NBD-SM, a fluorescent lipid analogue widely used as a membrane marker, recycles via the endocytic recycling compartment with a $t_{1/2}$ of $\sim 12$ min (Mayor, S., Presley, J. F., and Maxfield, F. R. (1993) J. Cell Biol. 121, 1257–1269). Surprisingly, the rest returns to the plasma membrane very quickly. A detailed kinetic study presented in this paper indicates that after a brief internalization pulse, 42–62% of the internalized C₆-NBD-SM returns to the plasma membrane with a $t_{1/2}$ of $\sim 1–2$ min. Similar results are obtained using HEP2 and nonpolarized Madin-Darby canine kidney cells. Using FM dyes of different hydrophobicity, we show that rapid recycling involves passage through an endocytic organelle that was subsequently identified as the sorting endosome by co-localization with internalized transferrin and low density lipoprotein. These results imply that the membrane internalization rate is much higher than previously estimated, with a $t_{1/2}$ as short as 5–10 min. Rapid internalization and recycling would facilitate processes such as nutrient uptake and cholesterol efflux.

Endocytic recycling is essential for regulation of surface expression of proteins and for the uptake of nutrients. After internalization, endocytosed molecules are delivered rapidly to sorting endosomes (1, 2), which consist of vesicles with tubular extensions that are involved in transport of recycling material (e.g. transferrin receptor) out of the sorting endosomes. A major recycling pathway involves subsequent passage through the endocytic recycling compartment (ERC), which in CHO cells is a collection of tubules concentrated near the centriole, from which molecules recycle back to the plasma membrane ($t_{1/2}$ $\sim 9–12$ min) (3–5).

Fluorescent lipid probes are very well suited for kinetic studies of endocytic recycling. Very bright signals can be obtained, and after internalization pulses, efficient desorption of certain lipid analogs from the plasma membrane allows accurate measurements of recycling with minimal interference from probe molecules left in the plasma membrane (6). After nonselective internalization, a fluorescent lipid analog, C₆-NBD-sphingomyelin (C₆-NBD-SM), exits sorting endosomes, enters the ERC, and then returns to the plasma membrane with kinetics indistinguishable from transferrin (Tf) in CHO cells (4). After a 10-min internalization pulse with C₆-NBD-SM, the efflux kinetics from CHO cells suggested the existence of a second, faster recycling pathway, in addition to the pathway through the ERC with a $t_{1/2}$ of $\sim 12$ min (7). Taking full advantage of the properties of lipid analogs, we have now characterized rapid kinetics of membrane recycling in various cell types, and we found that nearly half of the internalized membrane recycles with a $t_{1/2}$ of about 1.5 min. This surprisingly rapid recycling requires internalization of the lipids with a $t_{1/2}$ of 5–10 min in order to maintain membrane balance. These results imply that in most cells the exchange of membrane between the plasma membrane and the endosomes is much more extensive than formerly estimated. Rapid recycling was previously thought to be unique to specialized cells, and lipid analogs (e.g. C₆-NBD-SM) have been used to study rapid recycling in cells such as erythroblasts, showing a recycling $t_{1/2}$ of 2 min (8). Our findings, however, indicate that rapid recycling exists in many cell types. This has important consequences for processes such as nutrient uptake, cholesterol efflux, and regulation of surface expression of receptors.

We confirmed the rapid recycling using another series of fluorescent lipophilic dyes, the FM dyes (Fig. 1), having a dicaticonic head group that prevents flip-flop across the bilayers (9). The partition of the FM dyes between the aqueous phase and the lipid is determined by the tail length, and the quantum yield increases up to 2 orders of magnitude when FM dyes are transferred from water into membranes (9). Taking advantage of these properties, we could determine whether rapid recycling involved fusion with a larger endosomal structure (e.g. a sorting endosome), followed by budding of recycling membrane (see Fig. 7). When the amphiphilic FM dyes enter an endocytic organelle, they distribute between membrane and aqueous volume according to their partition coefficients. As a consequence, a higher fraction of the less hydrophobic probes would be left behind when recycling membrane buds out from a parent organelle such as a sorting endosome. On the other hand, if rapid recycling involves direct return of vesicles that pinch from the plasma membrane, there should be no difference in recycling efficiency of various probes, because all internalized contents are released upon fusion.

Using three FM dyes of different hydrophobicity, we show that the rapid recycling pathway involved an organelle that was subsequently identified as the sorting endosome by double labeling with LDL.
Rapid Membrane Internalization and Recycling

EXPERIMENTAL PROCEDURES

Materials—C6-NBD-SM, FM 2–10, FM 1–43, and FM 1–84 were purchased from Molecular Probes, Inc. (Eugene, OR). The Cy5 labeling kit was obtained from Amersham Pharmacia Biotech. Human Tf was from Sigma. Iron-loaded Tf was passed through a Sephacryl S-300 gel filtration system as described previously. Cy5 was then conjugated to iron-loaded Tf following the manufacturer’s instructions. Unbound dye was removed first by passage through a sizing column and then by overnight dialysis in phosphate-buffered saline. Dil-LDL (LDL labeled with 3,3-dioctadecylinodocarbocyanine) was a gift from Dr. Ira Tabas (Columbia University, New York). All tissue culture supplies were from Life Technologies, Inc. Dioleoylphosphatidylcholine was from Avanti Polar lipids, Inc. (Birmingham, AL). All other chemicals were from Sigma.

Cells—TRVb-1 is a modified CHO cell line that lacks endogenous Tf receptor and expresses the human Tf receptor (10). MDCK type II and HEp2 cells were from ATCC (Manassas, VA). All cells were grown at 37 °C in a 5% CO2 humidified incubator. TRVb-1 cells were grown in bicarbonate-buffered Ham’s F-12 medium supplemented with 5% fetal bovine serum, 200 mg/ml Geneticin as a selection for the transfected Tf receptors, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2/μliter glucose; MDCK and HEp2 cells were grown in bicarbonate-buffered Dulbecco’s modified Eagle’s medium supplemented with 5% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells to be labeled with Dil-LDL were transferred to a similar Ham’s F-12 medium but with 5% lipoprotein-deficient serum in place of fetal bovine serum to up-regulate the cells’ LDL receptors. Cells for microscopy were plated 24 h before the experiment in 35-mm plastic tissue culture dishes with a 7-mm hole in the bottom covered by poly-n-lysine-coated coverslips.

Biochemical Study of C6-NBD-SM Efflux Kinetics—To create the C6-NBD-SM labeling solution, a mixture (2:3 mol/mol) of C6-NBD-SM and dioleoylphosphatidylcholine (both stocks in ethanol) was dried down under argon and redissolved in ethanol (20 μl total lipid concentration). This ethanol solution was injected into medium 1 (150 mM NaCl, 5 mM KC1, 1 mM CaCl2, 1 mM MgCl2, and 20 mM Hepes, pH 7.4) while vortexing and dialyzed at 4 °C overnight against excess phosphate-buffered saline to remove ethanol present in the solution (initially <8%). It was then diluted in medium 1 (M1) to a final total lipid concentration of 50 μM to make the labeling solution.

Cells were plated in 26-cm2 tissue culture dishes 24 h before the experiment. Kinetic measurements were made as described previously (1) with a few exceptions. In brief, cells were incubated in M1-glucose (medium 1 with 2/μliter glucose) for 5 min and pulsed with C6-NBD-SM labeling solution for 2–10 min at 37 °C. The cells were then immediately washed with ice-cold M1-glucose and incubated with back-exchange medium (5% fatty acid-free bovine serum albumin in M1-glucose) on ice for 1 h, during which six washes of ice cold back-exchange medium with 10-min intervals were applied. After the back-exchange process, cells were washed with 37 °C M1-glucose, and 6 ml of 37 °C chase medium (1% fatty acid-free bovine serum albumin in M1-glucose) was added to the cell dish. This was defined as chase time 0. Cells were chased in the chase medium at 37 °C for up to 60 min. At each time point, 600 μl of chase medium was taken from the cell dish, and 600 μl of fresh pre-warmed chase medium was added back to the dish. At the end of the 60-min chase, the remaining chase medium was removed, and cells were incubated in 2.5 ml EDTA on ice for 15 min. Cells were then harvested with a cell scraper to determine cell-associated fluorescence.

Chase medium aliquots taken at each time point, and the remaining chase medium, EDTA wash, and harvested cells were all extracted with butanol. Fluorescence of butanol solutions was measured with a spectrofluorometer (Fluorolog 2, Spex Industries Inc., Edison, NJ). Excitation wavelength was 465 nm, and fluorescence was quantified by integrating peak area from 518 to 558 nm. Unlabeled chase medium was used for background correction.

To obtain the kinetic curves in Figs. 2 and 3, the following calculations were made: (a) total lipid = cell-associated fluorescence + fluorescence remaining in chase medium + fluorescence in chase aliquots; (b) fraction of total lipid in the chase medium at each time point $f = \frac{(lipid in chase aliquot)}{total lipid}$; (c) fraction of lipid that was cell-associated $f = 1 - f$.

To measure the release rate of C6-NBD-SM by the chase medium, cells were labeled on ice for 5 min, washed with ice-cold M1-glucose, and incubated in chase medium at 37 °C. Aliquots of chase medium were taken at different time points, and the percentage of cell-associated fluorescence was calculated for each time point (see “Experimental Procedures”). A computer fitting program was used to give the parameters that defined the best fit to the data points. Data points are presented by open circles, and double exponential decay fits are plotted as solid lines. The dotted line in A shows the best fit to a single exponential decay. Kinetic parameters obtained from the fitting program are listed in Table I. Data points for all kinetic curves in this paper were derived from an average of triplicate experiments.

To test back-exchange efficiency, two methods were used. The overall strategy was to measure the amount of surface-bound fluorescence at the end of the back-exchange procedure, without interference from exocytosis. This was accomplished by either keeping cells on ice at all times, thereby preventing endocytosis and exocytosis, or fixing cells to prevent exocytosis. In the first assay for measuring back-exchange efficiency, cells were labeled on ice for 30 min to ensure incorporation of C6-NBD-SM into the plasma membrane and back-exchanged on ice for 1 h, saving all of the washes. Cells were then incubated in EDTA and harvested, as described above, to determine cell-associated fluorescence. To test whether labeling at 37 °C had any effect on the incorporation of C6-NBD-SM that could have altered the back-exchange efficiency, we pulsed the cells at 37 °C for 2 min, fixed them with 2% paraformaldehyde, and then applied the normal back-exchange procedure. At the end of this procedure, 37 °C chase medium was added to the fixed cells, and aliquots of chase medium were taken for the first 10 min afterwards.

To measure the release rate of C6-NBD-SM by the chase medium, cells were labeled on ice for 30 min, washed with ice-cold M1-glucose, and incubated in chase medium at 37 °C. Aliquots of chase medium were taken at times from 10 s to 5 min. Fluorescence from these aliquots and cell-associated fluorescence were obtained to calculate fractions of C6-NBD-SM removed at different chase times.

Kinetic Measurements of FM Dye Trafficking Using Wide Field Flu-
Microscopy was carried out using a Leica DMIRB microscope (Leica stage maintained at 33–34 °C throughout acquisition. Fluorescence microscopy was carried out using an Axiosvert 100M inverted microscope equipped with an LSM 510 laser-scanning unit and a 63 × 1.4 NA plan Apochromat objective (Carl Zeiss, Inc.). Since FM dyes have broad emission spectra, filter sets were selected carefully to maximize true signal while minimizing crossover of signal from one channel to the other. Samples double-labeled with Dil-LDL and FM dyes were excited with a 25-milliwatt argon laser emitting at 488 nm for FM dyes and a 1.0-milliwatt helium/neon laser emitting at 543 nm for Dil. A 585–615-nm band pass filter was used for collecting Dil emissions, and a 650-nm long pass filter was used for FM emissions. Samples double-labeled with Cy5-Tf and FM dyes were excited with the argon laser emitting at 488 nm for FM dyes and a helium/neon laser emitting at 532 nm for Cy5. A 530–600-nm band pass filter was used for collecting FM emissions, and a 650-nm long pass filter was used for Cy5 emissions. The two channels were scanned alternately in a line-by-line fashion, having only one laser line and one detector channel on at each time. Fraction of cross-over was measured to be less than 10% using single-labeled samples of each probe. Images were corrected for background (as described above for FM dyes) and crossover (14).

Partition Coefficient Measurements of the FM Dyes—Diocetylphosphatidylcholine was purchased as a chloroform solution. It was dried in vacuo and then in vacuo overnight and dissolved in M1, forming multimellar vesicles (final lipid concentration was 3.5 mM). This suspension was sonicated for 30 min to break up large multilamellar vesicles. The solution cleared after sonication by forming unilamellar vesicles. The method for measuring partition coefficients was adapted from Huang and Haugland (15). The solution containing unilamellar vesicles was diluted in M1 to make solutions containing different concentrations of liposomes. For each experiment, fluorescence resulting from titration of liposomes against a constant dye concentration was measured with a spectrofluorometer. Two concentrations of each dye were used for two parallel runs: FM 2–10 (0.2 mM, 0.15 mM), FM 1–43 (0.5 μM, 0.4 μM), and FM 1–84 (4 μM, 2 μM). The excitation wavelength for FM 2–10, FM 1–43, and FM 1–84 were 465, 468, and 468 nm, respectively, and the emission wavelengths were 608, 593, and 590 nm, respectively. Inner filter effect was corrected for the case of FM 2–10, where the correction was meaningful, using the equation,

\[
F_{\text{corrected}} = F_{\text{measured}} \cdot \frac{A_{\text{ex}}}{A_{\text{em}}} 
\]

where \(A_{\text{ex}}\) and \(A_{\text{em}}\) are absorption readings at excitation and emission wavelength, respectively (16).

Equations used to obtain partition coefficients were described by Huang and Haugland (15). The partition coefficient of a membrane probe, \(K_p\), was defined as follows,

\[
K_p = \frac{P_f/M}{P/W} \quad \text{(Eq. 2)}
\]

where \((P_f/M)\) and \((P/W)\) refer to molar ratios of membrane-bound probe \(P_f\) to membrane lipids \(M\) and of fluid phase probe \(P\) to water \(W\), respectively.

RESULTS

\(C_{6\text{-NBD-SM}}\) Efflux Kinetics

Back-exchange Efficiency and Rate of Exchange by Chase Medium—The experiments reported here required highly efficient removal of fluorescent lipids from the plasma membrane by back-exchange. To determine the back-exchange efficiency,
two methods were employed. When cells were labeled and back-exchanged on ice, less than 2% of total fluorescence was found to be cell-associated at the end of the back-exchange. This was consistent with previous findings (7, 17). We also tested whether labeling at 37 °C had any effect that might give a different back-exchange efficiency. We fixed cells after incubation with C6-NBD-SM at 37 °C, back-exchanged on ice, and then monitored the release of C6-NBD-SM from cell surface into prewarmed chase medium. Since the fixed cells were incapable of undergoing exocytosis, what was released into the chase medium was presumably membrane-bound probes left at the end of the back-exchange procedure. Aliquots taken from the chase medium did not show fluorescence above background. These results indicated that the release of C6-NBD-SM measured in our kinetic studies after back-exchange was due to exit of recycled C6-NBD-SM from cells, rather than release of uninternalized probes from the cell surface.

The validity of our C6-NBD-SM efflux measurements also depended on the release rate of C6-NBD-SM from the cell surface into the chase medium. Our control experiments showed that 50% of recycled C6-NBD-SM reaching the plasma membrane was removed by the chase medium within 40 s, and more than 90% was removed within 2 min. This desorption from the membrane is faster than the fastest recycling process we measure, but the exocytic rate constants measured with C6-NBD-SM may be slightly underestimated, since we ignored the rate of extraction from the plasma membrane. Because the release process was kinetically complex, we decided that it would only introduce more errors if we were to correct the rate of rapid recycling with a calculated release rate.

Existence of a Second Component in C6-NBD-SM Recycling—
The exit kinetics of C6-NBD-SM were determined from the amount of C6-NBD-SM recycled back to the plasma membrane and released into the chase medium. With a pulse time shorter than 10 min, there was very little hydrolysis of C6-NBD-SM to C6-NBD-Cer (7), which could subsequently be transported to the cell surface via nonvesicular transport. Plots of fraction of cell-associated fluorescence versus chase time are given in Fig. 2. The fraction of lipid that was cell-associated at each chase time point (open circles) was fit with a double exponential decay \( l = a e^{-bt} + c e^{-dt} + e \). Theoretical fits were then plotted as solid lines in Fig. 2. If C6-NBD-SM efflux occurred by a single component, first-order kinetic process, a single exponential decay would be expected to fit the data points. However, this was not the case. To illustrate this point, a single exponential best fit was added to Fig. 2A. Represented by a dotted line, the single exponential decay could not account for the rapid decrease of fluorescence during the first 10 min of chase time. It could, however, provide a good fit for the slower process dominating the second half of the chase time. A double exponential decay, on the other hand, very well accounted for all of the data.

A double exponential decay fit to data points obtained from 2-min pulse, 60-min chase experiments of C6-NBD-SM gave a rapid recycling component with a half-time of 1–2 min and a slow recycling component with a half-time of ~12 min (Fig. 2A, Table I). The rapid recycling component was found to account for 42% of the recycling population. As the length of pulse time increased to 5 and 10 min, the relative percentage of rapid recycling component decreased to 28 and 23%, respectively (Fig. 2, B and C; Table I). The best fit rate constant for each component, however, remained approximately constant regardless of the pulse length.

To see whether a similar rapid recycling pathway existed in other cell types, we measured C6-NBD-SM efflux kinetics in HEp2 human carcinoma cells (Fig. 3A, Table I) and nonpolarized MDCK cells (Fig. 3B, Table I). Like CHO cells, these two cell lines contained a fast recycling component with a half-time of t2/3 of ~1 min. When clathrin-mediated endocytosis was blocked in CHO cells by preincubation in K– -depleted buffer (18), both the slow and fast recycling populations were still detectable (Fig. 3C, Table I). In this case, Tf internalization was reduced by 74%, and C6-NBD-SM internalization was reduced by 45%.

**Efflux Kinetics of the FM Dyes**—To determine whether an endocytic organelle compartment was involved in the rapid recycling pathway, FM dyes of different hydrophobicity were used. The three FM dyes used in this study have very similar chemical structures, with the only difference being the number of carbon atoms in their tails (Fig. 1). This difference has the largest impact on each dye’s degree of hydrophobicity, quantified by its partition coefficient.

Using the method described under “Experimental Procedures,” the partition coefficients for FM 2–10, FM 1–43, and FM 1–84 were calculated to be 2.35 \( \times \) 10^4, 2.78 \( \times \) 10^5, and 7.78 \( \times \) 10^5, respectively. The partition coefficients are useful in representing relative differences in hydrophobicity among the three FM dyes. The relative differences in partition coefficients are consistent with differences in release times measured in cultured neurons (15).

The dye dissociation times from plasma membrane for FM 2–10, FM 1–43, and FM 1–84 have been measured in synaptotagmin boutons to be 0.7, 3, and 6 s at 24 °C, respectively (13). Because recycled FM dyes could readily be released into aqueous medium in which they are nonfluorescent, we used continuous live cell imaging to study efflux kinetics of the FM dyes. This enabled us to directly measure cell-associated fluorescence by wide field microscopy on a temperature-controlled microscope stage.

Fig. 4 shows efflux kinetics of the FM dyes after a 30-s pulse. Open circles represent data points from experiments in which a
A similar chilling procedure was applied to remove the membrane-bound probes, and results from experiments lacking this process are represented by *solid triangles*. The two methods gave similar results. FM 1–43 and FM 1–84 showed a biphasic kinetic profile similar to that of C6-NBD-SM (Fig. 4, A and C). Data points from these hydrophobic FM dyes fit well to a double exponential decay, giving a half-time of 2 min for the fast component and 11 min for the slow component. Kinetic parameters from Fig. 4 are listed in Table I.

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FM 2–10 data points were fit with a single exponential decay with a half-time of 12 min (Table I). These data show that all probes share a common recycling kinetic process with a half-time of 8–14 min depending on the cell type, and hydrophobic probes have a second, more rapid recycling pathway with a half-time of 1–2 min. The rapid recycling population becomes less detectable as the pulse length increases, as expected as a consequence of equilibration of the fast process during the loading pulse.

Fig. 4. Efflux kinetics of FM dyes after 30-s pulse periods. Cells were labeled with one of the FM dyes for 30 s at 37 °C, either rinsed in 37 °C M1-glucose (open circles) or rinsed with ice-cold M1-glucose, kept on ice while further washes of M1-glucose were applied to ensure complete removal of surface-bound dye molecules, warmed up with 37 °C M1-glucose (open circles), and imaged on a 37 °C temperature-equilibrated microscope stage for 1 h. Efflux kinetic curves of FM 2–10 (A), FM 1–43 (B), or FM 1–84 (C) following a 30-s pulse were obtained by quantification of wide field microscopy images. Fluorescence power was normalized to that of the first reading at the beginning of the chase. FM 2–10 data points were fit with a single exponential decay with a half-time of 12 min. Data points from FM 1–43 and FM 1–84 fit well to a double exponential decay, which generated a half-time of 2 min for the fast component and 11 min for the slow component. Kinetic parameters from Fig. 4 are listed in Table I.

The data show that all probes share a common recycling kinetic process with a half-time of 8–14 min depending on the cell type, and hydrophobic probes have a second, more rapid recycling pathway with a half-time of 1–2 min. The rapid recycling population becomes less detectable as the pulse length increases, as expected as a consequence of equilibration of the fast process during the loading pulse.

Copolarization of FM Dyes with DiI-LDL in Sorting Endosomes and with Cy5-Tf in the ERC—Double label experiments were performed to see if FM dyes were entering the same endosomes as LDL and Tf. To visualize the locations of FM dyes at the beginning of chase experiments after a 30-s pulse, double label experiments using DiI-LDL and FM dyes were performed. Cells were first incubated briefly with DiI-LDL to label endosomes and then pulsed with one of the FM dyes. Cells were pulsed with the two probes separately to prevent FM dyes from binding to DiI-LDL molecules on the cell surface or in solution and subsequently co-internalizing with DiI-LDL. When imaged by confocal microscopy, endosomes in CHO cells appear as punctate dots located under the plasma membrane. Fig. 5, A–C, shows a cell double-labeled with DiI-LDL and FM 2–10. The difference in the trafficking kinetics of the two probes might have contributed to the fact that the FM dyes did not co-localize entirely with DiI-LDL. At the time these live cell images were taken (1–2 min after pulsing), some FM molecules had already trafficked to a pericentriolar ERC, while a portion of sorting

Fig. 5. Co-polarization of FM dyes with DiI-LDL. Cells were labeled with DiI-LDL for 4 min at 37 °C and subsequently with FM 2–10 (B) or FM 1–84 (E) at 37 °C for 30 s in the absence of the labeled LDL. Cells were then washed with ice-cold medium 1 to remove surface-bound dye molecules. Cells were imaged 1–2 min after being warmed up by adding 37 °C M1-glucose and kept on a 37 °C temperature-equilibrated microscope stage during image acquisition. A and D show DiI-LDL staining, B shows FM 2–10 staining, E shows FM 1–84 staining, and C and F are overlays of the double staining. The images are single plane confocal microscopy images. The full width at half-height for the optical sections is 0.8 μm. Bar, 10 μm.
endosomes containing Dil-LDL had become fusion-incapable (19).

Membrane tubules bud off from sorting endosomes and deliver recycling membrane constituents to a pericentriolar region in CHO cells. The ERC, consisting of a collection of these tubules, appears as a bright juxtanuclear spot when imaged by fluorescence microscopy. To see whether FM dyes recycle through the same compartments as other recycling molecules (e.g. Tf), double label experiments of FM dyes with Cy5-Tf were performed. Both probes started to accumulate in the perinuclear region as soon as 5 min after pulsing and remained extensively co-localized in the ERC for at least 15 min. Fig. 6, A–D, shows a cell double-labeled with Cy5-Tf and FM 2–10; and Fig. 6, E–H, shows a cell double-labeled with Cy5-Tf and FM 1–84. Based on the efflux kinetics, the fraction of the two dyes remaining is expected to be different after a 15-min chase, but the purpose of this experiment is to see if some of the dye remaining in the cells is in the ERC. Passage through the ERC would indicate that dyes leaving the cell with a remaining in the cells is in the ERC. The “direct return” model predicts that the fraction of FM 2–10 could be transported from the ERC back to sorting endosomes via an intracellular route (20), and this may be partially responsible for the high retention of FM 2–10 by cells.

**DISCUSSION**

**“Direct” Versus “Indirect” Model**—Two hypothetical models for rapid recycling are presented in Fig. 7. In the direct return model (A), vesicles bud off from the plasma membrane and subsequently fuse back with the plasma membrane, such that there is no mixing of membrane or contents with other organelles. If this model is correct, the fraction of all FM dyes passing through the rapid recycling pathway should be the same, because any FM dye in such a vesicle would stay trapped until released when vesicles fuse back with the plasma membrane.

Alternatively, the indirect return model (B) proposes that the internalized molecules traffic through a larger compartment where they redistribute between membrane and aqueous volume according to their partition coefficients. The redistribution process results in a higher recycling efficiency for the more hydrophobic probes. This occurs because the more hydrophobic molecules tend to distribute in the tubular portion of a tubulovesicular compartment where the membrane/volume ratio is higher. The more hydrophilic molecules, on the other hand, are distributed more in the luminal volume of such a compartment and recycle with lower efficiency. This model predicts a difference in recycling efficiency among different FM dyes through the rapid recycling pathway. As shown in Fig. 4, the more hydrophobic probes, FM 1–43 and FM 1–84, are much more

**FIG. 6.** Colocalization of FM dyes with Cy5-Tf. Cells were labeled with Cy5-Tf for 2 min at 37 °C and subsequently with one of the FM dyes at 37 °C for 30 s in the absence of the labeled protein. Cells were then washed with ice cold M1-glucose to remove surface-bound dye molecules. Cells were chased for 15 min at 37 °C before imaging on a temperature-controlled confocal microscope stage. A and E are single-plane DIC images. B and F show Cy5-Tf staining, C shows FM 2–10 staining, G shows FM 1–84 staining, and D and H are overlays of the double staining. Fluorescence images are projections of confocal microscopy images. The full width at half-height for the optical sections is 0.8 μm. Bar, 10 μm.

**FIG. 7.** Schematic diagrams of two models of the rapid recycling pathway. The “direct return model” is shown at the top, and the “indirect return model” is shown at the bottom. In A, internalized vesicles fuse back with the plasma membrane without going through sorting endosomes. Therefore, the number of molecules recycled back to the plasma membrane should be exactly the same as the number internalized, independent of partition between the membrane and the aqueous content. In contrast, in B, internalized vesicles fuse with sorting endosomes, and recycling membrane subsequently buds from sorting endosomes to return to the plasma membrane.
previously was, in fact, a rate describing a combination of inter-model below, what was taken as the internalization rate necessary to reconsider the internalization rate of lipid analogs of the internalized membrane recycles rapidly makes it necessary to reconsider the internalization rate of lipid analogs.

Membrane Turnover Rate—

The fact that approximately half of the internalized membrane recycles rapidly makes it necessary to reconsider the internalization rate of lipid analogs measured previously (21). As shown in Fig. 8 and in the kinetic model below, what was taken as the internalization rate previously was, in fact, a rate describing a combination of internalization and rapid recycling processes. PM, SE, and RC (Scheme 1) represent the probe concentration in the plasma membrane, sorting endosomes, and ERC, respectively. Rate constants $k_1$, $k_2$, and $k_3$ are included in Fig. 8.

A simple kinetic modeling of the rate constants leads to a relationship between the internalization rate $k_1$ and $k_1'$, the net internalization rate constant after the rapid recycling has come to steady state.

$$k_1' = \frac{k_2 + k_3}{k_3}$$

$k_1'$ was measured to be $0.035-0.046 \text{ min}^{-1}$ (21). If we take $k_2$ as $0.35-0.7 \text{ min}^{-1}$ and $k_3$ as $0.35 \text{ min}^{-1}$ (4, 22, 23), the true internalization rate, $k_1$, is calculated to be $0.069-0.138 \text{ min}^{-1}$. This result, in turn, suggests that the half-time for membrane turnover could be as short as 5–10 min, as opposed to 15–20 min (21). Therefore, the observation of a high fraction of rapid recycling reveals the plasma membrane system as being extremely dynamic with a very rapid turnover rate.

**Biological Role of Rapid Recycling**—It has been recognized previously that specialized cells may recycle molecules rapidly to carry out specific functions. In reticulocytes, a high rate of iron uptake is maintained by recycling Tf receptors with a half-time of ~90 s (24). The kinetics of endocytosis in renal proximal tubule showed that membrane recycling near the apical surface takes place with a $t_{1/2}$ of 1.5 min, and this process is used to recover small molecules such as vitamins from the urine (25). We now suggest that many cell types are capable of rapid recycling, and this process may become dominant in some specialized cells.

The rapid recycling pathway also provides an explanation for a discrepancy between the uptake kinetics of iron and that of transferrin. In HepG2 hepatoma cells (26) or in phorbol 12-myristate 13-acetate-treated K562 cells (27), the rate of iron uptake is almost twice as fast as that measured for transferrin. We propose that rapid recycling of transferrin through acidic sorting endosomes could account for the additional iron delivered to the cells, since a rapid recycling would have caused an underestimation of the rate of transferrin uptake. Rapid membrane turnover may also be essential for uptake of folate, which requires passage of folate bound to its glycosylphosphatidylinositol-anchored receptor through an acidic compartment (28). The folate receptor is delivered to sorting endosomes (29). The rapid recycling that we observe could also dramatically increase the rate of cholesterol efflux, which is mediated by high density lipoprotein endocytosis and recycling (30, 31). Rapid recycling might be interpreted as direct efflux at the plasma membrane in most experimental designs.

A kinetic model of Tf recycling and transcytosis in polarized MDCK cells was described by Sheff et al. (32). The slow recycling component had a half-time of ~12 min for exit to the basolateral plasma membrane, and the fast component had a half-time of ~6 min, which was interpreted to be direct recycling from early (sorting) endosomes. However, in light of our finding that rapid recycling from sorting endosomes has a $t_{1/2}$ of 1.4 min, it seems likely that the exit with a $t_{1/2}$ of 6 min is from another endosomal compartment. We suggest that the 6- and 12-min half-times described by Sheff et al. may reflect exit from some of the various Tf-containing endosomal compartments found in polarized MDCK cells (33) rather than the rapid recycling from sorting endosomes described in this paper.

In summary, we have detected and characterized a rapid recycling pathway in various nonpolarized mammalian cells using fluorescent lipid analogs. This suggests that rapid recy-
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