Changes in the Spectral Properties of a Plasma Membrane Lipid Analog During the First Seconds of Endocytosis in Living Cells

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ABSTRACT *N*-[5-(5, 7-dimethyl Bodipy)-1-pentanoyl]-D-*erythro*-sphingosylphosphorylcholine (C_5-DMB-SM), a fluorescent analog of sphingomyelin, has been used in a study of the formation of very early endosomes in human skin fibroblasts. This lipid exhibits a shift in its fluorescence emission maximum from green (~515 nm) to red (~620 nm) wavelengths with increasing concentrations in membranes. When cells were incubated with 5 μM C_5-DMB-SM at 4°C and washed, only plasma membrane fluorescence (yellow-green) was observed. When these cells were briefly (≤1 min) warmed to 37°C to allow internalization to occur, and then incubated with defatted bovine serum albumin (back-exchanged) at 11°C to remove fluorescent lipids from the plasma membrane, C_5-DMB-SM was distributed in a punctate pattern throughout the cytoplasm. Interestingly, within the same cell some endosomes exhibited green fluorescence, whereas others emitted red-orange fluorescence. Furthermore, the red-orange endosomes were usually seen at the periphery of the cell, while the green endosomes were more uniformly distributed throughout the cytoplasm. This mixed population of endosomes was seen after internalization times as short as 7 s and was also seen over a wide range of C_5-DMB-SM concentrations (1–25 μM). Control experiments established that the variously colored endosomes were not induced by changes in pH, membrane potential, vesicle size, or temperature. Quantitative fluorescence microscopy demonstrated that the apparent concentration of the lipid analog in the red-orange endosomes was severalfold higher than its initial concentration at the plasma membrane, suggesting selective internalization (sorting) of the lipid into a subset of early endosomes. Colocalization studies using C_5-DMB-SM and either anti-transferrin receptor antibodies or fluorescently labeled low-density lipoprotein further demonstrated that this subpopulation of endosomes resulted from receptor-mediated endocytosis. We conclude that the spectral properties of C_5-DMB-SM can be used to distinguish unique populations of early endosomes from one another and to record dynamic changes in their number and distribution within living cells.

INTRODUCTION

Eukaryotic cells utilize a variety of vesicular transport mechanisms to import molecules into the cell interior, including uptake by caveolae, nonclathrin-coated vesicles, clathrin-coated pits and vesicles, macropinosomes, and phagosomes (reviewed in Anderson, 1993; Gruenberg and Maxfield, 1995; Lamaze and Schmid, 1995). These different vesicle types are used for different functions and are thought to have unique protein compositions relevant to their specialized roles. Although it is generally accepted that the protein compositions of the different vesicle types are different from one another, the distribution and internalization of plasma membrane lipid components are less clear. Some studies have suggested that certain lipids may be organized in "microdomains" within the plane of the plasma membrane (reviewed in Thompson and Tillack, 1985; Edidin, 1992; Glaser, 1993; Jacobson et al., 1995; Wolf, 1995) or enriched in certain membrane specializations such as caveolae (Brown and Rose, 1992; Parton, 1994; Murata et al., 1995). However, to date there has been no study of the dynamic changes that may occur in the organization of lipids during the initial stages of endocytosis.

One approach for studying lipid transport along the endocytic pathway involves the use of fluorescent lipid analogs (reviewed in Pagano and Sleight, 1985; Koval and Pagano, 1991; Hoekstra and Kok, 1992; Koval, 1993; Rosenwald and Pagano, 1993). In this method one of the naturally occurring fatty acids of a phospholipid or sphingolipid is replaced with a short-chain fluorescent fatty acid. The resulting fluorescent lipid analogs can be readily integrated into the outer leaflet of the plasma membrane bilayer of intact cells by spontaneous monomeric lipid transfer at low temperature. The cells are then washed, and the lipids are observed to move into endocytic vesicles in a temperature- and energy-dependent manner. Temporal changes in the distribution of a given lipid can be correlated with changes in its metabolism, and the amount of lipid analog in the plasma membrane versus intracellular compartments

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Abbreviations used: Bodipy, brand name for boron dipyrromethene difluoride; C_5-DMB, *N*-[5-(5, 7-dimethyl Bodipy)-1-pentanoyl]; C_5-DMB-Cer, *N*-[5-(5, 7-dimethyl Bodipy)-1-pentanoyl]-D-*erythro*-sphingosine; C_5-DMB-SM, *N*-[5-(5, 7-dimethyl Bodipy)-1-pentanoyl]-sphingosylphosphorylcholine; C_6-NBD, *N*-[6-[(7-nitrobenz-2-oxa-1, 3-diazol-4-yl)amino]caproyl]; DF-BSA, defatted bovine serum albumin; DiI, 3,3′-dioctadecylinodocarbocyanine; DOPC, dioleoyl phosphatidylcholine; HCMF, 18 mM Hepes-buffered calcium- and magnesium-free Puck's saline, pH 7.4, without indicator; HMEMB, 10 m*M* 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid-buffered MEM, pH 7.4, without indicator, containing 0.5 m*M* each choline chloride, ethanolamine, serine, and (myo)inositol; LDL, low-density lipoprotein; LPDS, lipoprotein deficient serum; LUV, large unilamellar vesicles; R/G, ratio of red to green fluorescence; SUV, small unilamellar vesicles; Tf, transferrin.

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can be quantified by a variety of techniques (Struck and Pagano, 1980; van Meer et al., 1987; McIntyre and Sleight, 1991). With such an approach the internalization of various fluorescent lipid analogs labeled with *N*-[6-[(7-nitrobenz-2-oxa-1, 3-diazol-4-yl)amino]caproic acid (C_6-NBD-) has been studied in a number of different cell types. These include NBD analogs of phosphatidylcholine (Sleight and Pagano, 1984), sphingomyelin (SM; Koval and Pagano, 1989, 1990; Mayor et al., 1993), glucosylceramide (Kok et al., 1989, 1991, 1995), galactosylceramide (Mayor et al., 1993), thioglucosylceramide (Schwarzmann and Sandhoff, 1990), and ganglioside GM_1 (Sofer et al., 1996).

Although the studies cited above utilized C_6-NBD lipids, lipids labeled with other fluorophores have also been used. Lipids labeled with *N*-[5-(5, 7-dimethyl Bodipy)-1-pentanoic acid] (C_5-DMB) are a particularly attractive alternative to NBD-labeled lipids because the Bodipy fluorophore (a) has a higher fluorescence yield and is more photostable than NBD (Johnson et al., 1991) and (b) is less polar than NBD and therefore is probably better anchored in the membrane bilayer than NBD, which loops back to the membrane–water interface (Chattopadhyay and London, 1987; Wolf et al, 1992). Futhermore, lipids labeled with C_5-DMB fatty acids exhibit shifts in their fluorescence emission maxima from green to red wavelengths with increasing concentration in membranes. Thus, it is possible to distinguish membranes within the same cell that contain different concentrations of a fluorescent lipid and, using quantitative microscopy, to estimate the apparent concentration of the lipid in a particular organelle. This approach has been used to study the localization of *N*-[5-(5,7-dimethyl Bodipy)-1-pentanoyl]-D-*erythro*-sphingosine (C_5-DMB-Cer) at the Golgi apparatus of cultured cells (Pagano et al., 1991) and the internalization and sorting of C_5-DMB-glucosylceramide from the plasma membrane to the Golgi apparatus of human skin fibroblasts (Martin and Pagano, 1994).

In the present study we report on the use of *N*-[5-(5,7-dimethyl Bodipy)-1-pentanoyl]-sphingosylphosphorylcholine (C_5-DMB-SM) to study plasma membrane lipid internalization during the initial stages of endocytosis. Previous studies from our laboratory demonstrated that, when this analog was integrated into the plasma membrane bilayer of human skin fibroblasts and internalized *via* endocytosis, endosomes ranging in color from green to red-orange were formed (Martin and Pagano, 1994). In the present study we explore the nature of these variously colored endosomes and present evidence for sorting of this lipid analog into distinct populations of endocytic vesicles within the first few seconds of endocytosis.

MATERIALS AND METHODS

Cells and cell culture

Normal human skin fibroblasts (5659C) were obtained from the Coriell Institute, Human Genetic Mutant Cell Repository (Camden, NJ) and cultured as described (Martin and Pagano, 1994). In colocalization studies employing fluorescent low-density lipoprotein (LDL), cells were grown in a culture medium containing 10% lipoprotein-deficient serum (LPDS; Per-Immune, Inc, Rockville, MD) (Martin et al., 1993) for two to three days before use to "upregulate" the number of LDL receptors at the cell surface (Brown and Goldstein, 1986). All experiments were performed using monolayer cultures grown to ~30–~50% confluency on acid-etched glass cover slips (Martin and Pagano, 1994).

Fluorescent lipid analogs and bovine serum albumin complexes

C_5-DMB-SM was obtained from Molecular Probes (Eugene, OR) and found to be a mixture of the D-*erythro* and L-*threo* isomers. These isomers were separated and purified by thin-layer chromatography as described (Koval and Pagano, 1989) and stored in stock solutions of chloroform/methanol (19:1, v/v) at −20°C. The concentrations of the C_5-DMB-SM stock solutions were determined by phosphorous analysis (Rouser et al., 1966). Except as noted, the D-*erythro* isomer of C_5-DMB-SM was used.

Complexes of C_5-DMB-SM with defatted bovine serum albumin (DF-BSA) were prepared as described (Pagano and Martin, 1988; Martin and Pagano, 1994). These complexes contained 5 (or 25) μM each of the fluorescent lipid and DF-BSA in 10 mM 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid-buffered MEM, pH 7.4, without indicator, containing 0.5 mM each choline chloride, ethanolamine, serine, and (myo)-inositol (HMEMB), and were diluted to a final working concentration using HMEMB. For most experiments, 5 μM lipid–DF-BSA complex was used.

Cell labeling with the fluorescent sphingolipids

Cell cultures were washed with ice-cold HMEMB, transferred to an ice-water bath for 10 min, and then incubated with 5 μM C_5-DMB-sphingolipid–DF-BSA complex for 30 min to label the plasma membrane. The cell monolayers were then washed with cold HMEMB and warmed to 37°C for various times (7 s to 10 min) to induce endocytosis. These relatively short incubations at 37°C were performed by addition of 1.5 ml of prewarmed HMEMB to the cell monolayers, after which the culture dishes were "floated" upon a 37°C water bath. To stop endocytosis the warm medium was replaced with ice-cold HMEMB and the culture dishes were transfered to an ice-water bath. To view the labeled endosomes it was first necessary to remove the fluorescent lipid present at the plasma membrane by "back-exchange" (Koval and Pagano, 1989, 1990; Martin and Pagano, 1994), using DF-BSA. Except as noted (refer to Fig. 3), back-exchange was carried out as follows: Cells were treated with an ATP-depleting medium containing 5% DF-BSA for 30 min at 4°C and then incubated in the same medium at 11°C (six changes, 10 min each). ATP-depleting medium was prepared with glucose-free HMEMB and contained 10 μM carbonyl cyanide chlorophenylhydrazone (Sigma), 2 mM iodoacetate, 5 mM NaN_3, and 50 mM deoxyglucose. Cell monolayers were then washed with cold ATP-depleting medium and observed under the fluorescence microscope using a temperature-controlled stage maintained at 4°C.

Fluorescence microscopy and image processing

Fluorescence microscopy was performed with an inverted microscope (IM-35; Carl Zeiss, Inc.) equipped with a Planapo 100× (1.3 N.A.) objective, epifluorescence optics with filter combinations appropriate for the Bodipy fluorophore (Pagano et al., 1991), and an electronic shutter at the mercury lamp (100 W) housing. For routine observations samples were excited at 450–490 nm and observed in a spectral window corresponding to green + red wavelengths ($\lambda_{em} \geq 520$ nm; see Pagano et al., 1991). Color photomicrographs were obtained with Kodak Ektachrome 400 film (5–6-s exposure), which was pushed one stop during processing.

For quantitative microscopy, samples were excited with attenuated blue light (450–490 nm), and observations were made in the green (520–560 nm) and red (>620 nm) regions of the spectrum (Pagano et al., 1991). We used a KS-1380 image intensifier (Videoscope International, Ltd., Washington, D.C.) coupled to a CCD camera (KP-M1; Hitachi Denshi, Ltd.,

Japan) to obtain images through the microscope. The intensifier was operated at medium gain and in the linear portion of its sensitivity range. Samples were located under phase prior to image acquisition. Thirty-two fluorescence video images of C_5-DMB-SM-treated cells were digitized (16 bits) and averaged using the Metamorph Image Processing system (Universal Imaging Corp., Media, PA). Images of the same cell, first in the red and then in the green channel, were collected, followed by red and green images of blank areas of the culture dish, which were used for background subtraction.

Following background subtraction, a region within a single cell containing at least 100 endosomes was selected, and the red and green images were aligned. A threshold level was then determined such that all the endosomes were included, and a mask (Bright et al., 1987) defined by that threshold value isolated the endosomes within that region so the ratio of red to green fluorescence (R/G) of individual endosomes could be calculated. This procedure was repeated on images collected from five or six different cells in the same (or identically treated) culture dish(es). The resulting data were combined and plotted as histograms. Similar procedures were used to quantify R/G for the plasma membrane.

To estimate the concentration of C_5-DMB-SM in the plasma membrane and endosomes, giant vesicles (Luan et al., 1995), prepared from dioleoylphosphatidylcholine (DOPC; Avanti Biochemicals, Alabaster, AL), and various concentrations of C_5-DMB-SM were used as calibration standards (see Pagano et al., 1991). The R/G ratio of these vesicles was then determined under the fluorescence microscope as described above for fluorescently labeled cells.

Definition of green and red-orange endosomes

Throughout the text the terms "green" and "red-orange" are used to describe the color of the variously colored endosomes that formed upon internalization of C_5-DMB-SM from the plasma membrane and were observed by conventional fluorescence microscopy through the green + red microscope channel ($\lambda_{em} \geq 520$ nm; Pagano et al., 1991). In quantitative terms, we found that endosomes that appeared green always had an R/G ≤ 0.2, whereas endosomes that appeared red-orange had an R/G > 0.2.

Colocalization of C_5-DMB lipids with transferrin receptors

Monolayer cultures were washed with ice-cold HMEMB, and, after 10 min at 4°C, a monoclonal anti-human transferrin (Tf) receptor antibody (B3/25; Boehringer Mannheim Biochemicals, Indianapolis, IN) was added (final dilution, 1:30); the cells were further incubated for 30 min at 4°C. Excess antibody was removed by washing with 0.1% DF-BSA in HMEM, and the cells were then incubated with 5 μM C_5-DMB-SM–DF-BSA for 30 min at 4°C. The cells were then washed in HMEMB, incubated with a 1:30 dilution of TRITC-conjugated goat anti-mouse immunoglobulin (Cappel, West Chester, PA) for 30 min at 4°C, and washed. The monolayer cultures were then warmed to 37°C for various times (see above) and then chilled and back-exchanged as described above before observation and photography. The distribution of Tf receptors was visualized using a 546-nm excitation filter and a 620-nm long-pass emission filter. Fluorescence video images were acquired as described above. In control experiments using cells in which only the Tf receptors were labeled, no fluorescence was detected in the Bodipy red channel (data not shown).

Colocalization of C_5-DMB lipids with LDL receptors

Monolayer cultures that were grown in 10% LPDS were washed with ice-cold HMEMB and kept at 4°C for 10 min. The cells were then incubated with 5 μM C_5-DMB-SM–DF-BSA and 2 μg/ml DiI-LDL (Molecular Probes, Inc., Eugene, OR) for 30 min at 4°C. The cells were then washed in ice-cold HMEMB for 5 min, incubated for 7 s or 10 min at 37°C, chilled, and back-exchanged in ATP-depleting medium. These procedures were identical to those described above under in the subsection entitled "Cell labeling with the fluorescent sphingolipids." We then removed any DiI-LDL remaining at the cell surface by acid stripping (Hopkins and Trowbridge, 1983) by incubating the cells in 0.2 M acetic acid and 0.5 M NaCl (pH 2.4) for 3–4 min at 4°C. The cells were then washed in ATP-depleting medium, as described above, and observed under the fluorescence microscope. DiI fluorescence was observed by exciting the specimens with optics appropriate for Rhodamine fluorescence. In control experiments using cells in which only DiI-LDL was present, no fluorescence was detected in the green or the red Bodipy channels (data not shown).

Effect of membrane potential

The effect of membrane potential on the fluorescence emission properties of C_5-DMB-SM in artificial lipid vesicles was examined as follows. Small unilammellar vesicles (SUVs; Kremer et al., 1977) or large unilammelar vesicles (LUVs; Hope et al., 1985) were formed from DOPC in 18 mM Hepes-buffered calcium- and magnesium-free Puck's saline. SUV and LUV with an asymmetric distribution of C_5-DMB-SM were then formed (Wolf et al., 1992) by injection of a 100-μl aliquot of a 0.4–4 mM stock solution of C_5-DMB-SM in ethanol into 2 ml of SUV or LUV during vortex mixing. Ethanol was then removed by dialysis against 18 mM Hepes-buffered calcium- and magnesium-free Puck's saline. The final concentration of lipid in these solutions was ~1 mM, of which 2–20 mol % was C_5-DMB-SM. Valinomycin and K^+ were then used to induce an inside positive membrane potential across the bilayer (Venema et al., 1993), which was measured with a potential-sensitive anionic dye (Oxonol IV; Molecular Probes, Inc., Eugene, OR; Apell and Bersch, 1987). The fluorescence emission spectra of Oxonol IV and C_5-DMB-SM were recorded using an SLM 8100C spectrofluorometer (SLM/Aminco, Urbana, IL).

The effect of membrane potential on the fluorescence emission properties of C_5-DMB-SM in cells was examined as follows. Cultures were washed with ice-cold HMEMB and then incubated with 1 mM ouabain (Sigma) in HMEMB for 3 h at 37°C. The cells were then washed, incubated with 5 μM C_5-DMB-sphingolipid–DF-BSA for 30 min at 2°C to label the plasma membrane, and washed. The R/G of the plasma membrane was then determined as described above.

Effect of vesicle size

To study the effect of vesicle size on C_5-DMB-SM fluorescence we obtained the fluorescence emission spectra for SUVs (~30 nm diameter) and for various LUVs ranging from 70 to 90 nm in diameter, which were formed from DOPC and 20 mol % C_5-DMB-SM.

Other procedures

Cells were washed with HMEMB and fixed for 30 min at ~22°C with 0.5% glutaraldehyde and 3% formaldehyde.

RESULTS

C_5-DMB-SM labeling of the plasma membrane

We first studied the effect of the C_5-DMB-SM concentration on plasma membrane labeling in human skin fibroblasts. Cells were incubated with 1–25 μM C_5-DMB-SM–DF-BSA for 30 min at 4°C. The cells were then washed and observed under the fluorescence microscope at 4°C in the green + red channel. The plasma membrane appeared green to yellow-green for all the lipid concentrations that were

tested, except for 25 μM, which was red-orange in color (Fig. 1). This was confirmed by quantitative low-light-level measurements of the R/G ratio at the plasma membrane. As shown in Fig. 2, almost all of the plasma membrane had an R/G ≤ 0.1 when cells were incubated with 2–10 μM C_5-DMB-SM, whereas when 25 μM C_5-DMB-SM was used the percentage of plasma membrane area with R/G ≥ 0.1 increased to ~94%. These results indicate that a significant shift of plasma membrane fluorescence to red wavelengths occurred only in cells incubated with a relatively high concentration (25 μM) of C_5-DMB-SM.

Back-exchange of C_5-DMB-SM from the plasma membrane

Previous studies using C_6-NBD lipids to label the plasma membrane showed that only a small amount of fluorescence is internalized during endocytosis, whereas large amounts remain at the plasma membrane, interfering with the observation of intracellular structures that become labeled (Koval and Pagano, 1989, 1990). This was also found to be the case when C_5-DMB-SM was used. For C_6-NBD lipids one can overcome this problem by extracting the fluorescent lipid from the plasma membrane by back-exchange. During back-exchange the C_6-NBD lipids, which are more water soluble than their endogenous counterparts, are selectively extracted from the plasma membrane and partitioned into a suitable acceptor (e.g., lipid vesicles or DF-BSA) by monomeric transfer (Nichols and Pagano, 1982). However, application of this procedure to C_5-DMB-lipid-labeled cells is more difficult, presumably because the C_5-DMB lipids exchange much more slowly than C_6-NBD lipids.

To optimize the back-exchange procedure in the present study we incubated cells with 2–25 μM C_5-DMB-SM–DF-BSA for 30 min at 4°C to label the plasma membrane. The cells were then incubated with DF-BSA or lipid vesicles under various conditions. We found that ≥99% of the plasma membrane fluorescence could be removed during six sequential incubations (10 min each) with 5% DF-BSA at 11°C (data not shown).

Effect of back-exchange conditions on the distribution of C_5-DMB-SM in early endosomes

When cells were incubated with 5 μM C_5-DMB-SM at 4°C, washed, warmed to 37°C, and back-exchanged with 5% DF-BSA, the C_5-DMB-SM was distributed in a punctate pattern throughout the cytoplasm. This internalization was temperature and energy dependent (data not shown), and by analogy to previous studies using C_6-NBD-sphingomyelin (Koval and Pagano, 1989, 1990) it is assumed that the labeled structures corresponded to endocytic vesicles. Although fluorescently labeled endosomes could readily be observed with C_5-DMB-SM, we noted that the size and the color of the labeled endosomes varied dramatically, depending on the back-exchange conditions that were used. This phenomenon is shown in Fig. 3, in which the effects of fixation and energy depletion on the back-exchange procedure were examined.

To examine the effects of *fixation*, monolayer cultures were incubated with 5 μM C_5-DMB-SM at 4°C, warmed to 37°C for 30 s, chilled to stop endocytosis, and back-exchanged with 5% DF-BSA (6 × 10 min at 11°C) to remove plasma membrane fluorescence. The cells were then fixed and observed under the fluorescence microscope in the green + red channel. As shown in Fig. 3 *A*, only green endosomes were seen. However, if the cells were fixed immediately after the 37°C incubation but before back-exchange, a mixed population of smaller endosomes that were green and red-orange in color was observed within the same cell (Fig. 3 *B*). To examine the effects of *energy depletion*, cells were labeled with 5 μM C_5-DMB-SM/DF-BSA at 4°C, washed, and warmed to 37°C for 30 s. The plasma membrane staining was removed by back-exchange with 5% defatted BSA in the presence or absence of ATP-depleting medium (see Materials and Methods). As shown in Fig. 3 *C*, when back-exchange was carried out with an ATP-depletion medium, a mixed population of endosomes was seen that was very similar to that observed when cells were fixed before back-exchange (Fig. 3 *B*). On the other hand, only larger green endosomes were observed when back-exchange was performed in regular medium (Fig. 3 *D*). Results similar to those shown in Fig. 3 *B* and *C* were obtained when cells were treated with 0.1 mM NEM during back-exchange (data not shown). These results suggest that endosome fusion could occur during back-exchange at 11°C unless the process was arrested by fixation, ATP depletion, or NEM treatment (see Discussion).

Unless otherwise noted, all subsequent experiments involving back-exchange were performed in the presence of the ATP-depleting medium. A typical result obtained after 30 s of internalization is shown in Fig. 4, in which a conventional fluorescence photomicrograph and a low-light-level fluorescence ratio image are shown. Interestingly, the red-orange endosomes were often present at the cell periphery, whereas the green endosomes were distributed more evenly throughout the cytoplasm. Finally, it should be noted that, although a red shift in plasma membrane fluorescence was seen only when cells were incubated with 25 μM C_5-DMB-SM–DF-BSA (see Figs. 1 and 2), cells containing both green and red-orange endosomes were readily observed after initial incubations with C_5-DMB-SM at concentrations as low as 1 μM (data not shown). Most experiments in the present paper were carried out with cells treated with 5 μM C_5-DMB-SM–DF-BSA.

Time course of C_5-DMB-SM internalization

We next quantified the R/G ratio in endosomes that were formed during various times at 37°C (Fig. 5). First, cells were incubated with 5 μM C_5-DMB-SM–DF-BSA, and then endocytosis was induced for 0–2 min. All samples

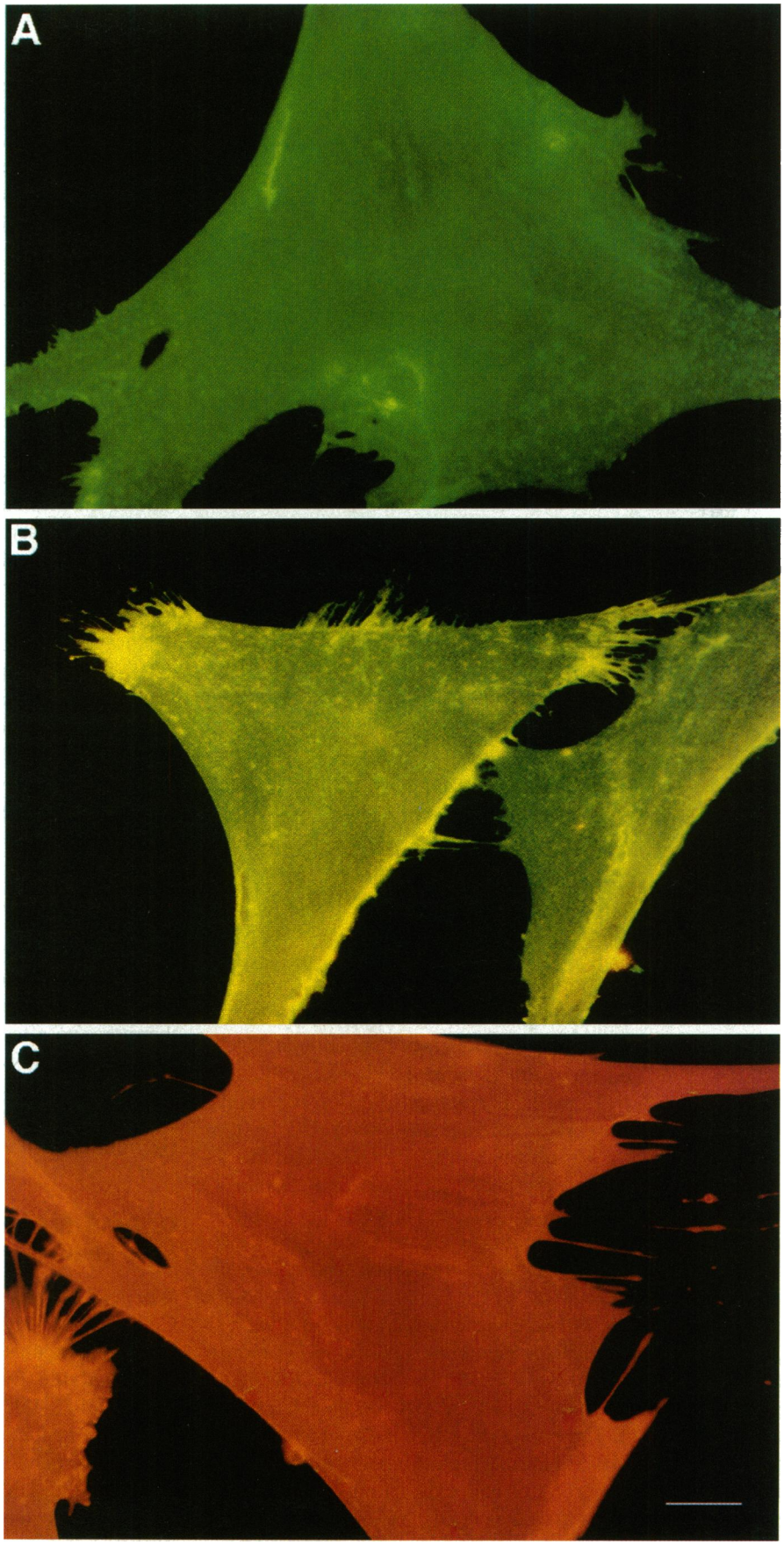

FIGURE 1 Spectral shift in plasma membrane fluorescence after labeling cells with various concentrations of C_5-DMB-SM. Cells were incubated with *A*, 2; *B*, 5; or *C*, 25 μM C_5-DMB-SM for 30 min at 4°C, washed, and photographed. Bar, 10 μm.

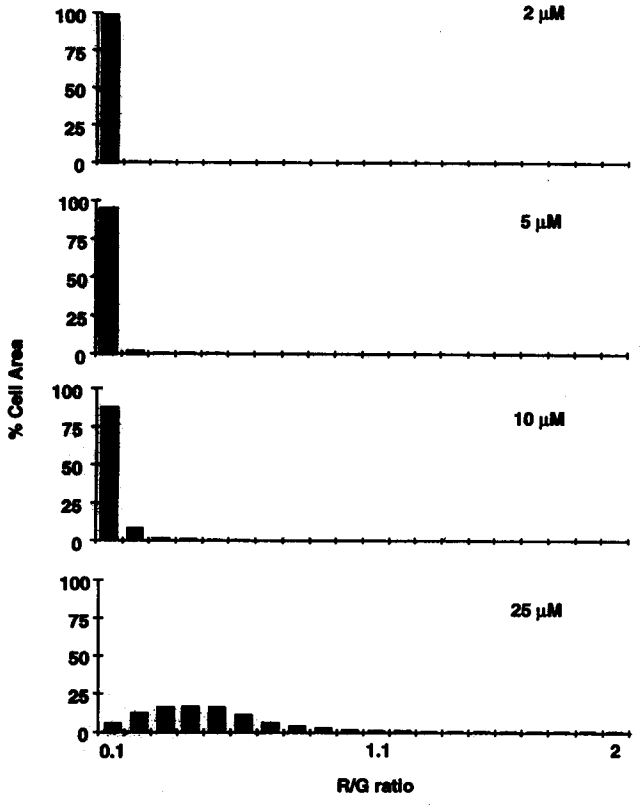

FIGURE 2 R/G of the plasma membrane after incubations with various concentrations of C_5-DMB-SM. Cells were incubated with the indicated concentration of C_5-DMB-SM for 30 min at 4°C, washed, and the R/G ratio of the plasma membrane was determined (see Materials and Methods).

were back-exchanged, except for control samples (0 min at 37°C) in which the R/G ratio for the plasma membrane was measured. As shown in Fig. 5 *A*, the R/G ratio of the plasma membrane before endocytosis was very low, with ~96% of the membrane area having an R/G ≤ 0.1 and only 3.9% having an R/G > 0.1. After a brief (7-s) incubation at 37°C the R/G ratio in endosomes increased dramatically, as shown in Fig. 5 *B*. Here, the percentage of endosomes with R/G ≤ 0.1 was only 18%, whereas those with R/G > 0.1 increased to 82%. Furthermore, with increasing times of endocytosis up to 30 s the peak values of the R/G ratios were found to shift toward higher values (Fig. 5 *B–D*, *arrows*). Interestingly, with longer times of endocytosis the peak values of the R/G ratios shifted back toward lower values (Fig. 5 *D–G*, *arrows*).

We were able to estimate the concentration (mol %) of C_5-DMB-SM in the plasma membrane and in the 7-s endosomes by using lipid vesicles formed from DOPC and containing various amounts of C_5-DMB-SM as calibration standards (Fig. 6). These data show that the apparent molar density of C_5-DMB-SM in the plasma membrane was ≤1 mol %, whereas, after 7 s of endocytosis, only 15% of the endosomes contained a similar concentration of C_5-DMB-SM. Instead, the apparent concentration of C_5-DMB-SM in ~84% of the endosomes had increased to >1 mol %.

Control experiments for the spectral shift of C_5-DMB fluorescence

We examined the fluorescence emission spectra of lipid vesicles formed from DOPC and various amounts (2–20 mol %) of C_5-DMB-SM. At low mole fractions a single fluorescence emission peak at ~515 nm was observed, while with increasing concentrations this fluorescence decreased in intensity and a second peak of fluorescence appeared at ~620 nm (data not shown). These results were virtually identical to those previously obtained with C_5-DMB-Cer (Pagano et al., 1991). We carried out a number of control experiments to determine whether the spectral shift in C_5-DMB-fluorescence could be induced by changes in temperature, membrane potential, vesicle curvature, or pH.

Cells were incubated with 5 μM C_5-DMB-SM/DF-BSA at 4°C to label the plasma membrane, washed, and warmed to 37°C for 0–30 s, but were not back-exchanged. The R/G ratio of the plasma membrane was then quantified. The R/G ratio of the plasma membrane remained <0.1, indicating that the 37°C incubation by itself could not induce a change in the spectral properties of C_5-DMB-SM at the plasma membrane.

No change in the fluorescence emission spectra of C_5-DMB-SM in artifical lipid vesicles was detected when the membrane potential was varied from 0 to 100 mV (data not shown). Furthermore, when cells were incubated with C_5-DMB-SM and the membrane potential was depolarized with oubain (Xu and Adams, 1992; Fuchs et al., 1989; see Materials and Methods), no change in R/G ratio at the plasma membrane was seen (data not shown).

No differences in fluorescence emission spectra were detected between SUVs (~30-nm diameter) and various LUVs ranging from 70 to 90 nm in diameter, which were formed from DOPC and 20 mol % C_5-DMB-SM, demonstrating that vesicle curvature did not affect the fluorescence emission properties of C_5-DMB-SM (data not shown). Furthermore, no effect of pH on C_5-DMB-SM fluorescence in lipid vesicles was detected between pH 5 and 7.4. The latter result is consistent with those of previous studies, demonstrating that the Bodipy fluorophore is not pH sensitive (Karolin et al., 1994; Kang and Haugland, 1989).

C_5-DMB-SM stereochemistry and the R/G ratio of early endosomes

We next examined the effect of the stereochemistry of C_5-DMB-SM on the R/G ratio in early endosomes when cells were incubated with the D-*erythro versus* the L-*threo* isomer of the fluorescent lipid. First, we quantified the amount of C_5-DMB-SM that became cell associated during a 30-min incubation at 4°C and found that the uptake was nearly identical when cells were incubated with 5 μM D-*erythro* C_5-DMB-SM (213.2 ± 11.3 nmol C_5-DMB-SM/mg cell protein) or with 6 μM L-*threo* C_5-DMB-SM (201.9 ± 4.6 nmol C_5-DMB-SM/mg cell protein). These concentrations were then used to label cells at 4°C. The

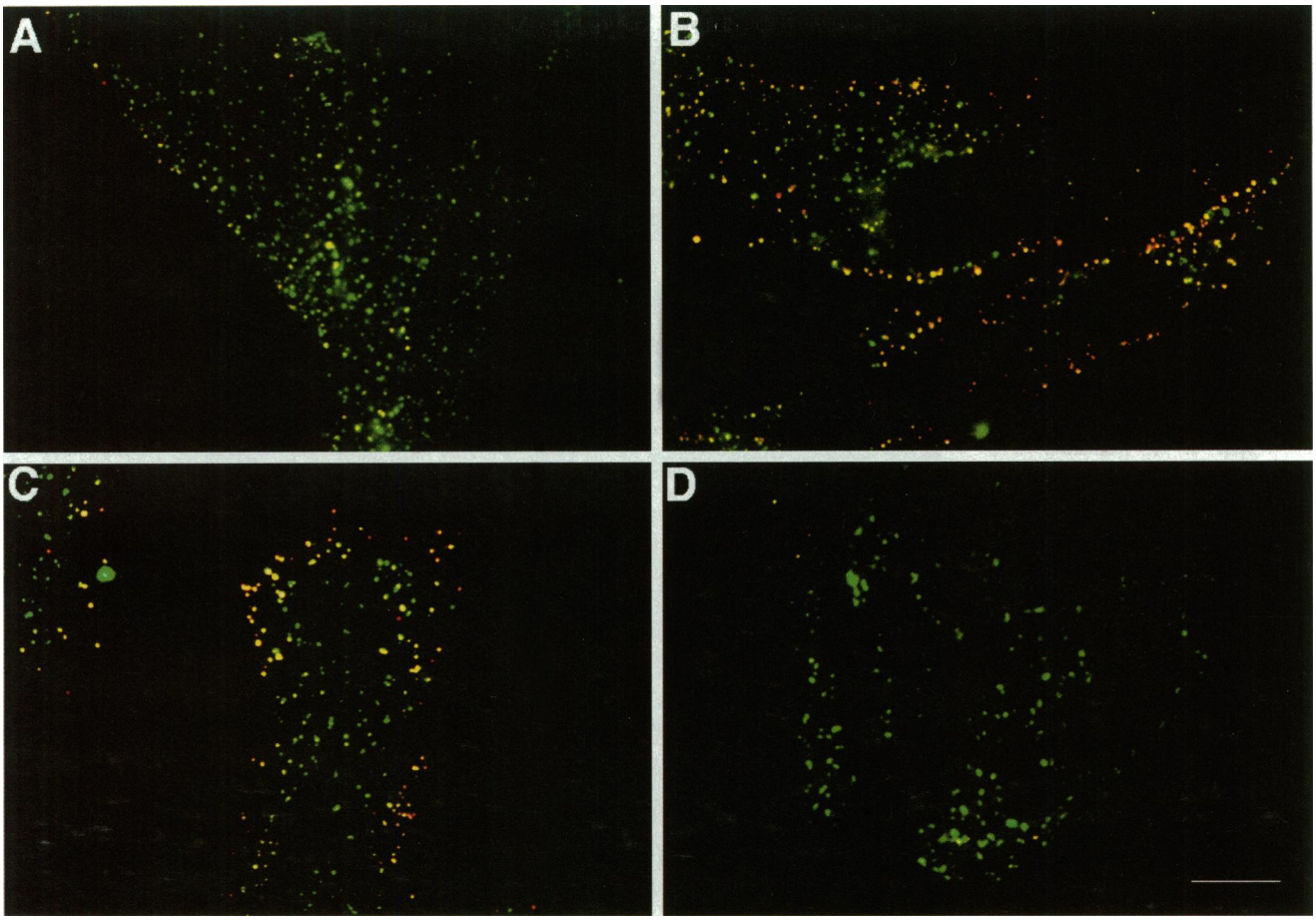

FIGURE 3 Inhibition of endosome–endosome fusion during back-exchange by ATP depletion or fixation. Cells were incubated with 5 μM C_5-DMB-SM for 30 min at 4°C, washed, and incubated in HMEM for 30 s at 37°C. The cells were then back-exchanged (6 × 10 min at 11°C) with 5% DF-BSA. In *A* cells were fixed *after* back-exchange; in *B* fixation was performed *before* back-exchange. In *C* back-exchange was performed in an ATP-depleting medium (see Materials and Methods), and in *D* no ATP depletion was used. Bar, 10 μm.

cells were then washed, warmed to 37°C for 15 s, and back-exchanged, and the R/G ratio of the resulting endosomes was quantified. As shown in Fig. 7, the D-*erythro* form of C_5-DMB-SM resulted in endosomes with a 2–3-fold higher R/G ratio than when the L-*threo* isomer was used.

Co-localization of C_5-DMB-SM labeled endosomes with markers for receptor-mediated endocytosis

To learn more about the nature of the variously colored endosomes formed during internalization of C_5-DMB-SM, we compared the distribution of the internalized C_5-DMB-SM with two different markers for receptor-mediated endocytosis. In one set of experiments, cells were double-labeled with 5 μM C_5-DMB-SM and a monoclonal antibody raised against the exoplamsic domain of the human Tf receptor (B3/25; Hopkins and Trowbridge, 1983). After incubation with a Rhodamine-conjugated secondary antibody, the cells were incubated at 37°C for either 7 s or 10 min and back-exchanged (Fig. 8). For the 7-s endosomes the Tf receptors were always observed to co-localize with the red endosomes but not with the green endosomes. With longer times of endocytosis the Tf receptors began to appear in green endosomes as shown in Fig. 8 for a 10-min chase period. In a second set of experiments co-localization studies were performed similar to those described above but with C_5-DMB-SM and fluorescently labeled LDL. In these experiments it was necessary to "upregulate" the number of LDL receptors expressed on the cell surface by growing the cells in a lipoprotein-deficient culture medium. In control studies using cells labeled with only C_5-DMB-SM, both green and red-orange endosomes were detected over various times of endocytosis with no apparent differences using cells grown in either normal serum or in LPDS (data not shown). Next, cells were double-labeled with 5 μM C_5-DMB-SM and 2 μg/ml DiI-LDL and warmed for either 7 s or 10 min at 37°C (Fig. 9). For the 7-s endosomes most of the DiI-LDL was observed to co-localize with the red endosomes but not with the green endosomes, while at later times the DiI-LDL was also present in green endosomes as shown in Fig. 9 for a 10-min chase period. These results indicate that both the Tf receptor and DiI-LDL were initially internalized into endosomes containing high concentrations

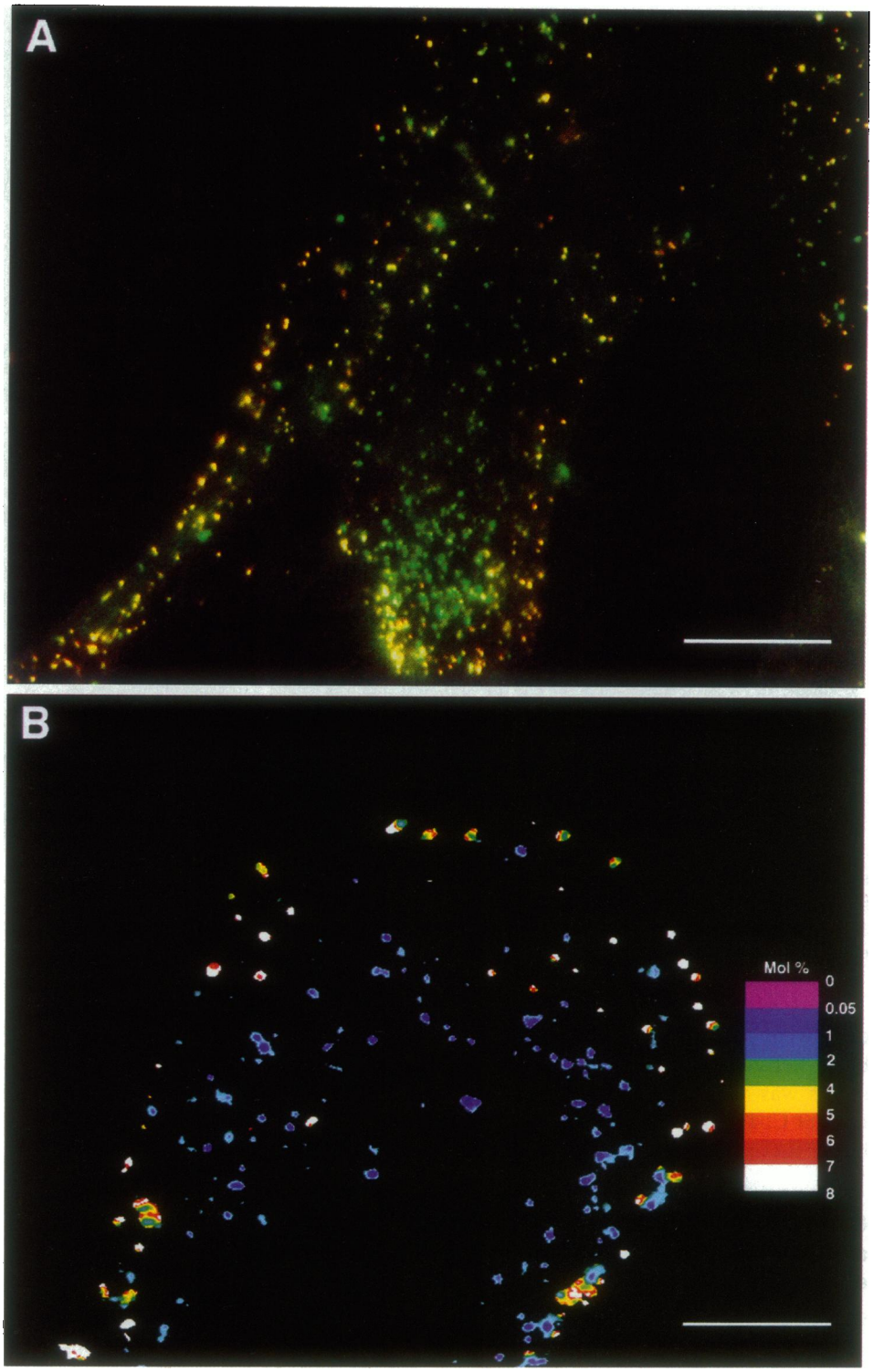

FIGURE 4 Internalization of C_5-DMB-SM from the plasma membrane results in the formation of green and red-orange endosomes within the same cell. Cells were incubated with C_5-DMB-SM, washed, warmed to 37°C for 30 s, and back-exchanged with DF-BSA in the presence of ATP-depleting medum (see Materials and Methods). *A*, Conventional photomicrograph showing fluorescence in the green + red regions (λ_{ex} = 450–490 nm, $\lambda_{em} \geq$ 520 nm) of the spectrum. Bar, 20 μm. *B*, Ratio image calculated from low-light-level fluorescence images and displayed in pseudocolor. From these data the concentrations of C_5-DMB-SM (and the corresponding percentages of endosomes) were calculated to be 0–1 mol % (10%), 1–2 mol % (40%), 2–5 mol % (35%), and >5 mol % (15%). Note the presence of red-orange endosomes (high R/G) at the cell periphery and the more uniform distribution of green endosomes throughout the cytoplasm. Bar, 10 μm.

of C_5-DMB-SM but subsequently could be chased into endosomes containing lower concentrations of the fluorescent lipid.

DISCUSSION

In this study we used a Bodipy-labeled sphingomyelin analog, C_5-DMB-SM, to study the transport of a plasma membrane lipid into early endosomes within living cells. Bodipy-labeled lipids are especially useful for such a study because their fluorescence emission maxima shift from green to red wavelengths with increasing concentration in membranes (Pagano et al., 1991), thus providing additional information about the nature of the labeled endosomes. This unique property of the Bodipy fluorophore allowed us to distinguish two populations of very early endosomes (green

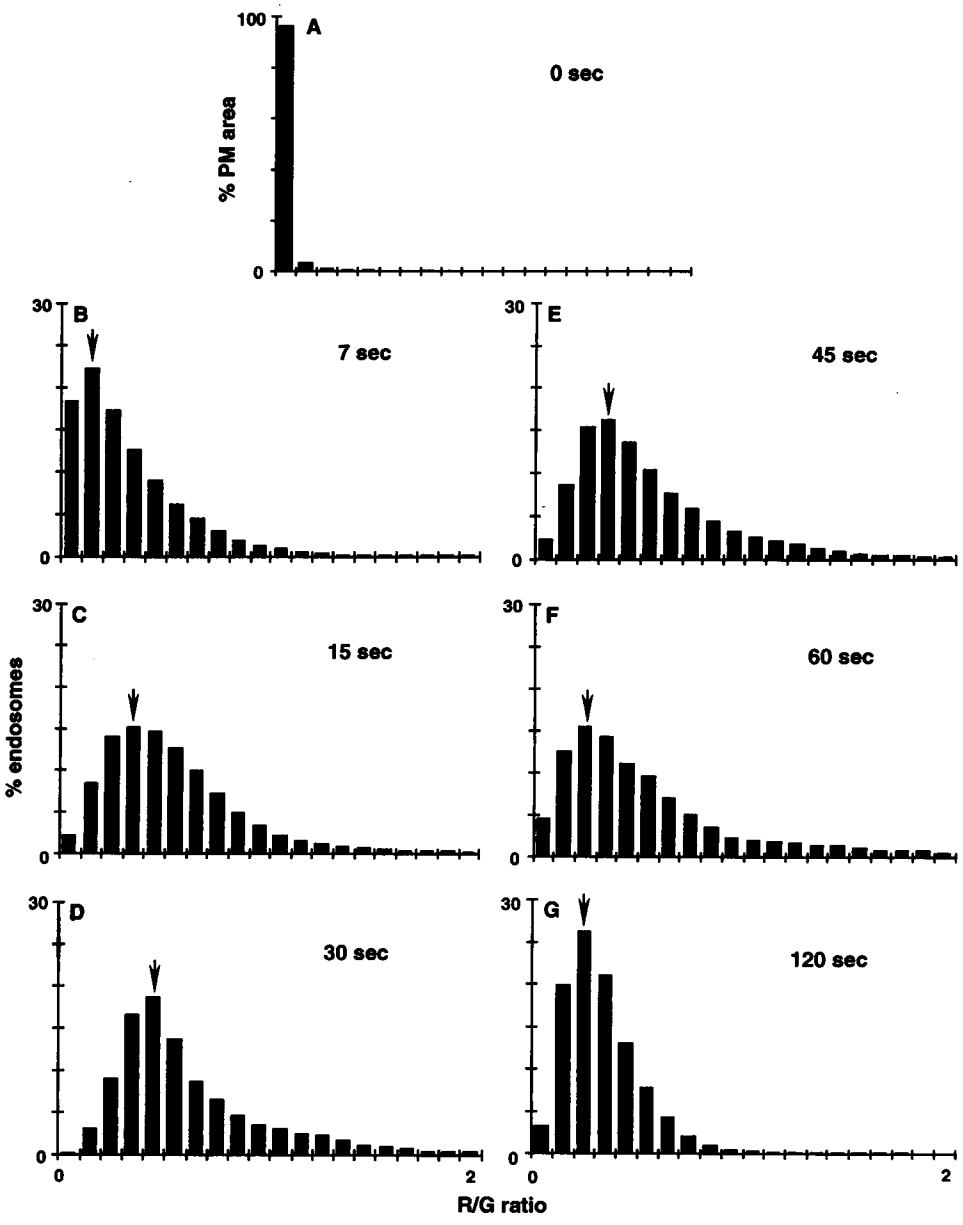

FIGURE 5 Dynamic change in the R/G ratio during endocytosis. Cells were labeled with C_5-DMB-SM, washed, and warmed to 37°C for *A*, 0; *B*, 7; *C*, 15; *D*, 30; *E*, 45; *F*, 60; or *G*, 120 s. The cells were then back-exchanged, and the R/G ratio was determined for ≥500 endosomes in five or six different cells. The R/G values were then plotted as a function of the percent of *A*, plasma membrane (*PM*) area or *B–G*, endosome number. The arrows indicate the peak value of R/G for each data set.

and red-orange) within the same cell on the basis of their fluorescence emission. Furthermore, we have provided evidence that the fluorescent lipid was selectively concentrated ("sorted") into one population of endosomes during the initial stages of endocytosis.

Inhibition of endosome–endosome fusion during back-exchange

When cells were incubated with C_5-DMB-SM at 4°C, washed, and warmed to 37°C for short periods of time (≤2 min), numerous endocytic vesicles were observed within the same cell. These endocytic vesicles could only be visualized after the relatively large amount of C_5-DMB-SM which remained at the plasma membrane following endocytosis was back-exchanged. Although previous studies using another sphingomyelin analog also required back-exchange to visualize endosomes (Koval and Pagano, 1989, 1990), we were concerned that endosome–endosome fusion might occur during the long incubations at 11°C which were required for back-exchange of C_5-DMB-SM in the present study. Fusion during back-exchange would alter the distribution of the fluorescent lipids in the labeled endosomes such that they no longer accurately reflected their distribution immediately after endocytosis (i.e., prior to back-exchange). Indeed, both green and red-orange small endosomes were readily seen if cells were fixed before back-exchange or if back-exchange was performed in an ATP-depleting medium, whereas only larger green endosomes were observed when fixation was performed after back-exchange or when back-exchange was carried out without ATP depletion (Fig. 3). Addition of NEM to the back-exchange medium at concentrations reported to inhibit vesicle fusion reactions

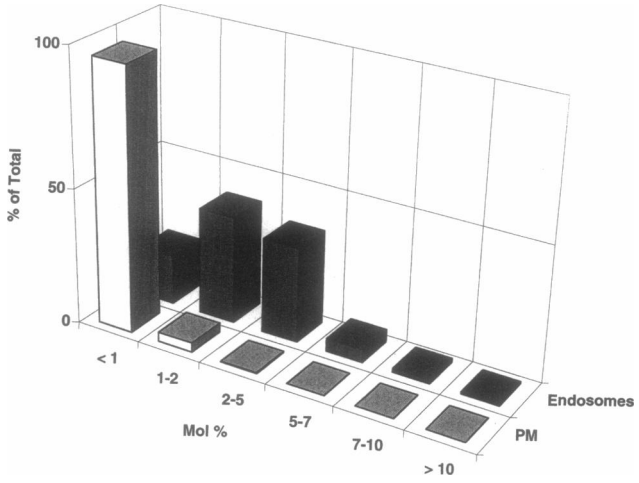

FIGURE 6 Enrichment of fluorescent SM in early endosomes. Cells were incubated with C_5-DMB-SM, washed, warmed to 37°C for 7 s, and back-exchanged. The apparent concentration of C_5-DMB-SM in the plasma membrane (*PM*) and endosomes was then determined from the R/G ratio using a calibration curve derived from the R/G ratio of a series of lipid vesicles containing different known amounts of C_5-DMB-SM (see Materials and Methods). % of Total represents the percent of PM area or percent of endosomes.

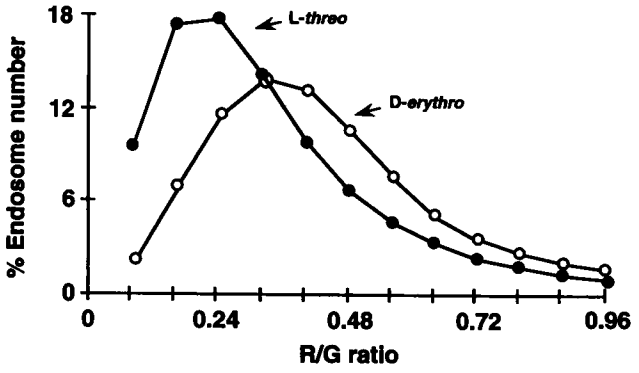

FIGURE 7 R/G fluorescence ratio of early endosomes formed upon internalization of D-*erythro* versus L-*threo* C_5-DMB-SM from the plasma membrane. Cells were incubated with D-*erythro* or L-*threo* C_5-DMB-SM at 4°C, washed, warmed to 37°C for 15 s, and back-exchanged. The R/G ratio of the endosomes was then determined as in Fig. 5.

(Rothman, 1994) gave virtually identical results to those shown in Fig. 3 *B* and *C*. Taken together, these data strongly suggest that endosome–endosome fusion can occur during back-exchange at 11°C unless this process is arrested by fixation, ATP depletion, or NEM treatment. As a result, all the experiments described in this paper used ATP depletion during the back-exchange procedure.

Internalization of C_5-DMB-SM from the plasma membrane reveals two populations of early endosomes

We found both green and red-orange endosomes in cells following periods of endocytosis as short as 7 s. The presence of red-orange endosomes was particularly surprising because the fluorescence at the plasma membrane was green. Control experiments demonstrated that the spectral shift from green to red wavelengths was not due to temperature, membrane potential, vesicle size, or pH. Furthermore, the presence of green and red-orange endosomes within the same cell, often right next to each other, rules out an artifact that is due to non-uniform illumination of the specimen or to photobleaching of selected regions of the cell. Although we are unable to eliminate the possibility that a protein component of the red-orange endosomes is responsible for the spectral shift in C_5-DMB-SM fluorescence, it is most likely that the variously colored endosomes reflect differences in concentration of the fluorescent lipid among the different endocytic vesicles because (a) increasing concentrations of C_5-DMB-Cer (Pagano et al, 1991) or C_5-DMB-SM (this study) in artificial lipid vesicles result in a green-to-red spectral shift in fluorescence and (b) plasma membrane fluorescence shifted from green to red wavelengths upon incubation of cells with increasing concentrations of C_5-DMB-SM (Figs. 1 and 2). Finally it should be noted that after short periods of endocytosis (e.g., 7 s), both internalized Tf receptors and fluorescent LDL, two independent markers for receptor-mediated endocytosis, were found exclusively in the red-orange endosomes, identifying them as constituents of the clathrin-mediated endocytic pathway.

Dynamic changes in the concentration of C_5-DMB-SM during endocytosis

We used low-light-level fluorescence microscopy and image processing to quantify changes in the R/G ratio of endosomes and found that with increasing times of endocytosis up to 30 s the fraction of endosomes with high R/G ratios increased, whereas with longer times of endocytosis (45–120 s) these ratios shifted back toward lower values, most likely because of endosome–endosome fusion. The fusion of red-orange endosomes either with pre-existing, non-fluorescent endosomes or with green endosomes would dilute the concentration of C_5-DMB-SM in the resulting membrane and lead to a spectral shift toward green wavelengths. Consistent with this interpretation, we also found that over longer periods of time (e.g., 2–10 min) both DiI-LDL and the labeled Tf receptors, which initially were present only in red-orange endosomes, were gradually chased into the green population of endosomes (Figs. 8 and 9). We conclude that endosome–endosome fusion was detected only after 30 s of internalization of the fluorescent lipid from the plasma membrane.

Our data also suggest that the different populations of endosomes seen after brief periods of endocytosis (e.g., 7 s) were derived independently from the plasma membrane: (a) Both green and red-orange endosomes were seen at the earliest time points (e.g., 7 s); however, only the red-orange population of endosomes contained Tf receptors and DiI-LDL. This means that the red-orange endosomes could not

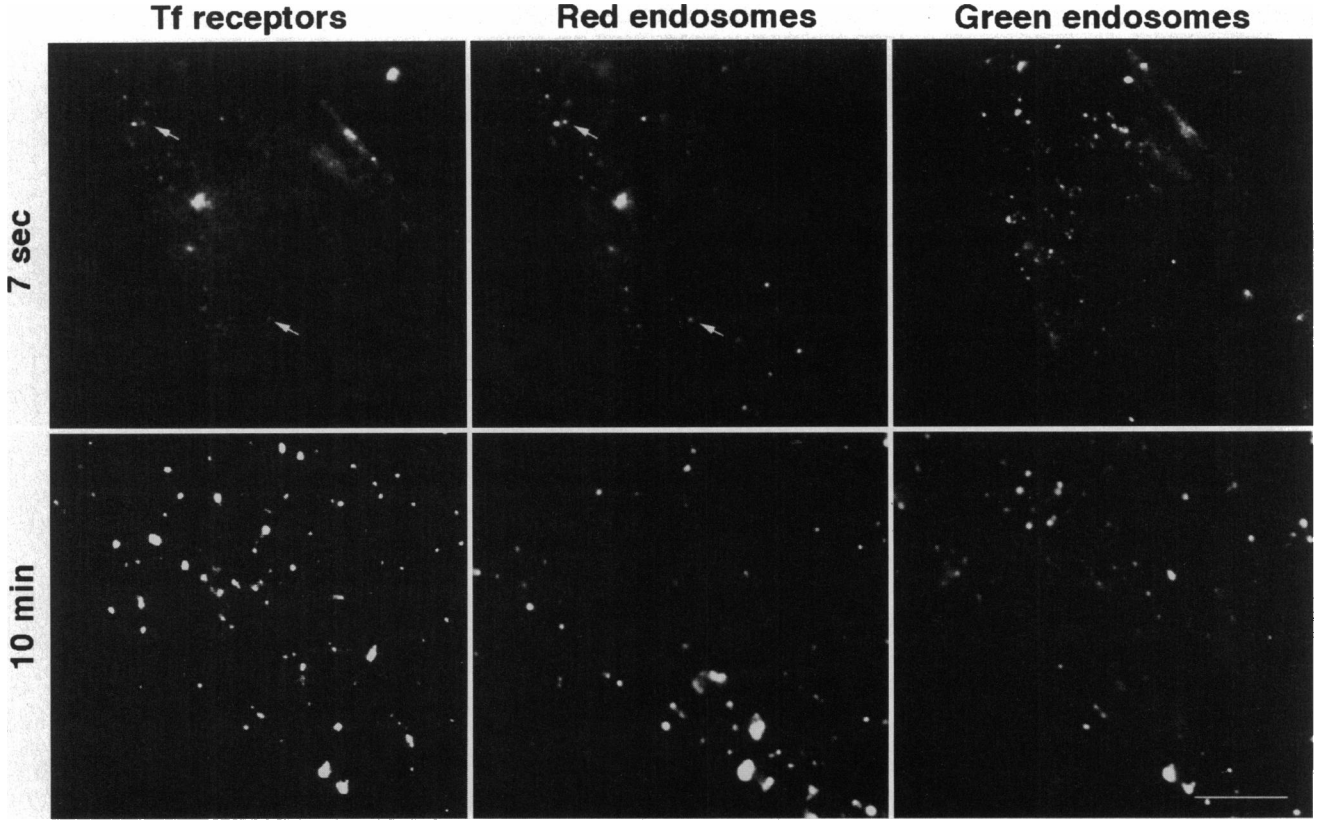

FIGURE 8 Tf receptors co-localize with red endosomes at early times of internalization, but are later transferred to green endosomes. Cells were doubly labeled with an anti-Tf receptor antibody and C_5-DMB-SM at low temperature, washed, warmed to 37°C for 7 s or 10 min, and back-exchanged. At each time point, photomicrographs corresponding to Tf receptors and red and green endosomes in the same cell were obtained. Note the excellent co-localization (e.g., at the arrows) of Tf receptors with red endosomes at 7 s and the redistribution of Tf receptors into green endosomes at 10 min. Bar, 10 μm.

have been derived from green endosomes since the latter lacked these markers. Rather, the red-orange endosomes must have been derived directly from the plasma membrane. (b) Green endosomes were seen in large numbers at early-times points (e.g., ~80% of total at 7 s) but lacked Tf receptors and DiI-LDL. This result could obtain if the green endosomes were derived from the red-orange endosomes and the Tf receptors and DiI-LDL were excluded from the green endosomes during vesicle formation. However, this possibility seems unlikely because at later times these markers appeared in the green endosomes rather than being *excluded* from them (Figs. 8 and 9). Furthermore, as discussed above, our data suggest that little endosome–endosome fusion occurred during the first 30 s of internalization of C_5-DMB-SM from the plasma membrane. Thus, it seems likely that the green endosomes were also derived directly from the plasma membrane.

Several experiments were performed to obtain further information about the green endosomes: (a) We attempted to label the endosomes that formed during a 30-s incubation at 37°C, using a fluorescent dextran (Koval and Pagano, 1990) as a marker for fluid phase uptake. However, even using very high concentrations of the dextran (10 mg/ml) no fluid phase uptake could be detected during this brief incubation. (b) We also attempted to inhibit uptake by receptor-mediated endocytosis by acidification of the cytosol (Sandvig et al., 1987), potassium depletion (Larkin et al., 1983), or hypertonic shock (Heuser and Anderson, 1989) to study the green endosomes in the absence of the red-orange endosomes, but in each case no internalization of C_5-DMB-SM was observed. To study this issue further it may be of interest to examine C_5-DMB-SM internalization in the recently described HeLa cell line, which expresses a temperature-sensitive mutant form of dynamin, resulting in inhibition of receptor-mediated endocytosis and elevation of fluid phase pinocytosis at the nonpermissive temperature (Damke et al., 1995). Thus, while we were able to identify the red-orange population of endosomes as constituents of the receptor-mediated endocytic pathway, the precise nature of the green endosomes is unknown.

Lipid sorting into red-orange endosomes

One of the most striking findings in the current study is the observation that C_5-DMB-SM was concentrated into one population of endosomes during the initial stages of membrane internalization. Based on quantitative measurements

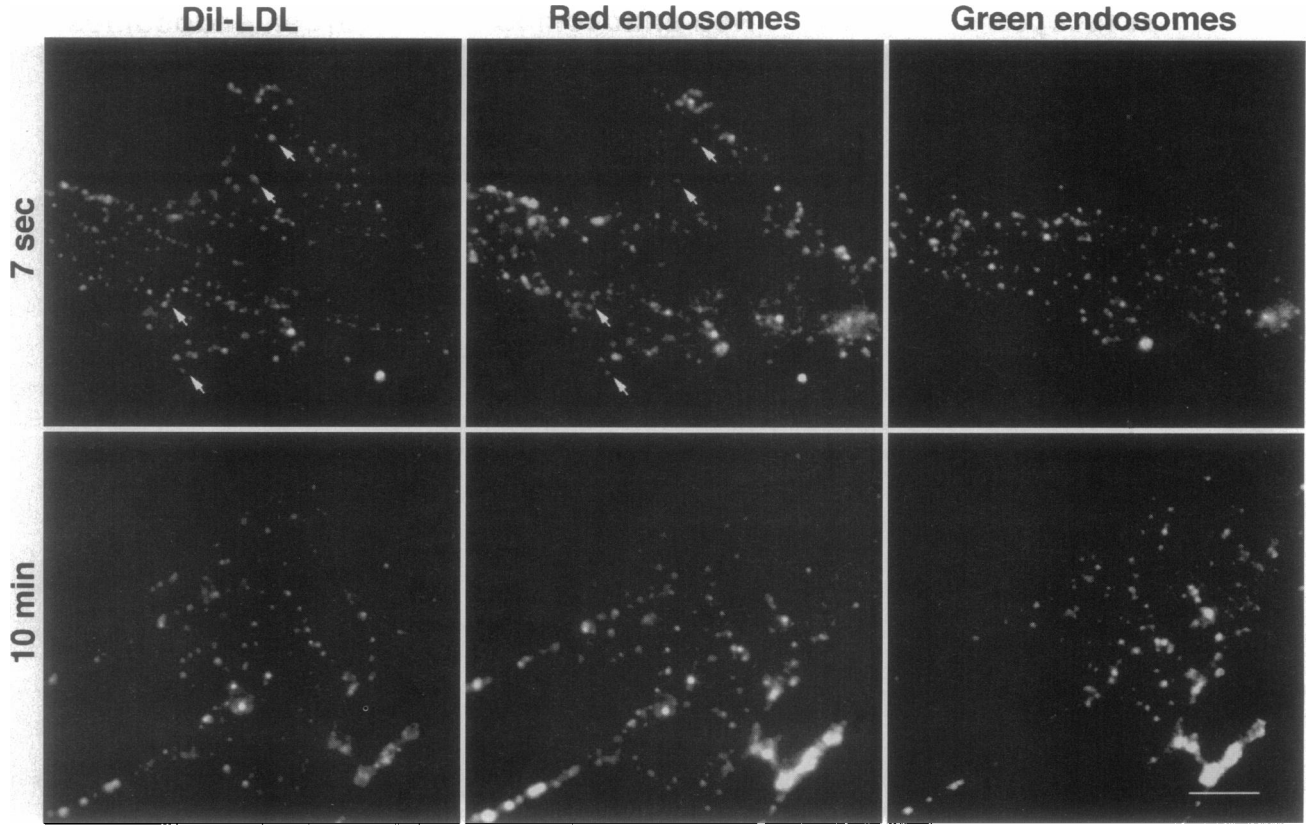

FIGURE 9 Fluorescent LDL was internalized into red endosomes at early times, but was later transferred to green endosomes. Cells were doubly labeled with DiI-LDL and C_5-DMB-SM at low temperature, washed, warmed to 37°C for 7 s or 10 min, and back-exchanged. The cells were then acid stripped to remove any DiI-LDL bound to the cell surface (see Materials and Methods) and observed under the fluorescence microscope. At each time point, photomicrographs corresponding to DiI-LDL and red and green endosomes in the same cell were obtained. Note the co-localization of DiI-LDL with the red (but not with the green) endosomes (e.g., at the arrows) at 7 s and the appearance of DiI-LDL in the green endosomes at 10 min. Bar, 10 μm.

of the R/G ratio we estimate that the apparent molar density of C_5-DMB-SM in the plasma membrane was <1 mol %, whereas after 7 s of endocytosis ~82% of the endosomes had a concentration of 1–10 mol % (Fig. 6). The precise mechanism by which C_5-DMB-SM becomes highly enriched in clathrin-coated vesicles is not known. However, when similar studies were carried out using the non-natural L-*threo* isomer of C_5-DMB-SM, a much smaller red shift was observed than when the natural D-*erythro* isomer was used (Fig. 7). This result suggests that the "enrichment" of C_5-DMB-SM in endosomes was not an artifact of the Bodipy fluorophore but rather was a property of the sphingolipid backbone and of interactions of the lipid with endogenous membrane components. One possible explanation for the formation of the variously colored early endosomes from C_5-DMB-SM is that it reflects the organization of this fluorescent lipid into "microdomains" (reviewed in Thompson and Tillack, 1985; Edidin, 1992; Glaser, 1993; Jacobson et al., 1995; Wolf, 1995) at the plasma membrane before internalization. However, we were unable to detect any red "patches" of fluorescence at the cell surface by viewing plasma membrane labeled cells through the red microscope channel. This suggests that C_5-DMB-SM may be concentrated or "sorted" into vesicles immediately prior to their fission from the cell surface. Regardless, the spectral properties of C_5-DMB-SM allow us to distinguish unique populations of early endosomes from one another and to record dynamic changes in their number and distribution within living cells. Such studies will be of particular interest in the future, using other cell types such as neurons and polarized epithelial cells whose plasma membranes are differentiated into distinct regions with specialized functions.

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