

Sorting of Sphingolipids in the Endocytic Pathway of HT29 Cells

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Abstract. The intracellular flow and fate of two fluorescently labeled sphingolipids, 6-[N-(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]hexanoyl glucosyl sphingosine (C₆-NBD-glucosylceramide) and C₆-NBD-sphingomyelin, was examined in the human colon adenocarcinoma cell line HT29. After their insertion into the plasma membrane at low temperature and subsequent warming of the cells to 37°C, both sphingolipid analogues were internalized by endocytosis, but their intracellular site of destination differed. After 30 min of internalization, C₆-NBD-glucosylceramide was localized in the Golgi apparatus, as demonstrated by colocalization with fluorescently labeled ceramide, a Golgi complex marker, and by showing that monensin-induced disruption of the Golgi structure was paralleled by a similar perturbation of the fluorescence distribution. By contrast, C₆-NBD-sphingomyelin does

not colocalize with the tagged ceramide. Rather, a colocalization with ricin, which is internalized by endocytosis and predominantly reaches the lysosomes, was observed, indicating that the site of delivery of this lipid is restricted to endosomal/lysosomal compartments. Also, in monensin-treated cells no change in the distribution of fluorescence was observed. Thus, these results demonstrate that (sphingo)lipid sorting can occur in the endocytic pathway. Interestingly, the observed sorting phenomenon was specific for glucosylceramide, when compared to other glycolipids, while only undifferentiated HT29 cells displayed the different routing of the two lipids. In differentiated HT29 cells the internalization pathway of sphingomyelin and glucosylceramide was indistinguishable from that of transferrin.

SORTING and recycling are well-known phenomena in intracellular protein trafficking, occurring during biosynthesis and endocytosis (1, 11, 13). The importance of these kind of processes in the concomitant intracellular flow of lipids is now also gradually emerging. Several sphingolipids have been shown to reappear at the plasma membrane by a recycling mechanism, after exogenous membrane insertion and subsequent internalization via the endocytic pathway (5, 6). In polarized cells, sorting of two newly synthesized sphingolipids, derived from a common precursor, has been shown to occur, as reflected by lipid specific, out-bound trafficking to apical, and basolateral membrane domains (14). However, it remains to be resolved whether lipids are also subject to sorting during inbound cellular trafficking, i.e., in endocytic events.

During endocytosis, an extensive flow of membranes takes place. To monitor the trafficking pathways of lipids during this process, advantage can be taken of the intracellular processing of certain ligand-receptor complexes. This processing involves delivery to endosomal compartments, sorting, and either recycling to the plasma membrane or further movement down the endocytic pathway towards the lysosomal system (1, 12).

In the present study, it is shown that two fluorescently la-

beled sphingolipids, C₆-NBD-glucosylceramide and C₆-NBD-sphingomyelin, are sorted during internalization along the endocytic pathway in HT29 cells. Using transferrin (Tf)¹ and ricin (in conjunction with their receptors) as intracellular traffic markers, evidence is presented indicating that C₆-NBD-glucosylceramide is transported to the Golgi apparatus, whereas C₆-NBD-sphingomyelin follows the endocytic pathway, down to the endosomal/lysosomal system. Furthermore, when the trafficking of these sphingolipids was compared in an undifferentiated and a differentiated HT29 cell type, the latter being derived by clonal selection, it could be shown that this sorting phenomenon only occurred in the undifferentiated cell type. This distinct difference between the two cell types may imply that lipid transport routes and sorting phenomena are related to differentiation of cells.

Materials and Methods

Materials

1-β-D-glucosylsphingosine, cerebroside type II, N-palmitoyl-DL-dihydro-lactocerebroside, sphingosylphosphorylcholine, D-sphingosine, iron-free Tf, and monensin were purchased from Sigma Chemical Co. (St. Louis, MO). 6-[N-(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]hexanoic acid and lis-

1. *Abbreviations used in this paper:* DECA, [[[7-(diethylamino)coumarin-3-yl]carbonyl]methylamino]; LR, lissamine rhodamine B sulfonylchloride; Tf, transferrin.

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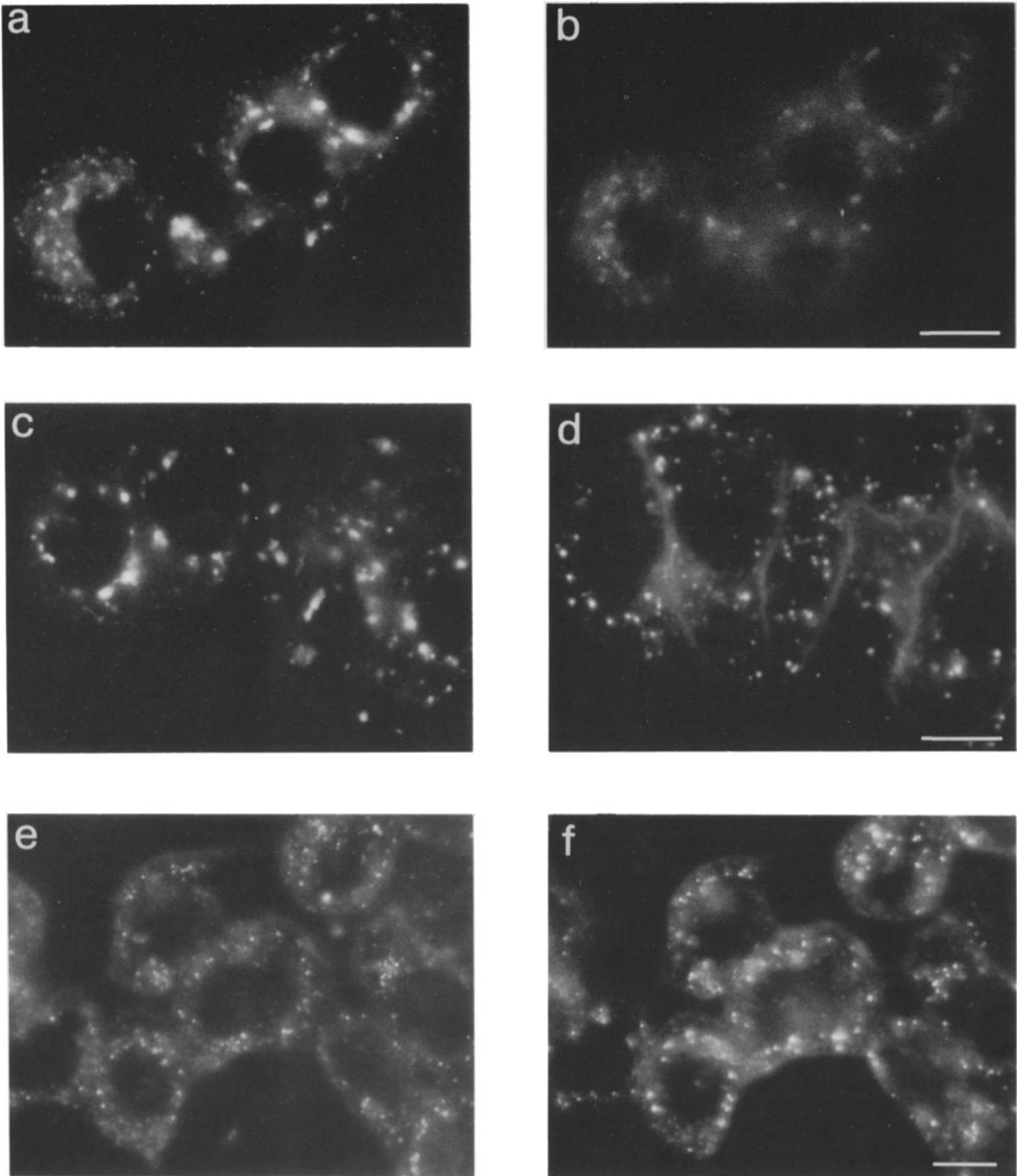


Figure 1. Intracellular localization of sphingolipids in undifferentiated HT29 cells. Cells were labeled with 4 μM C₆-NBD-glucosylceramide (*a* and *c*), C₆-NBD-galactosylceramide (*e*), or C₆-NBD-sphingomyelin (*g* and *i*), as described in Materials and Methods. Internalization was allowed to proceed for 30 min at 37°C, followed by a back-exchange procedure at 2°C. Colabeling studies were performed with either DECA-ceramide (*b* and *h*) or LR-ricin (*d*, *f*, and *j*) as described in Materials and Methods. Note that C₆-NBD-glucosylceramide colocalizes with DECA-ceramide (*a* and *b*), but not with LR-ricin (*c* and *d*). C₆-NBD-sphingomyelin colocalizes with LR-ricin (*i* and *j*), but not with DECA-ceramide (*g* and *h*). Bars, 10 μm .

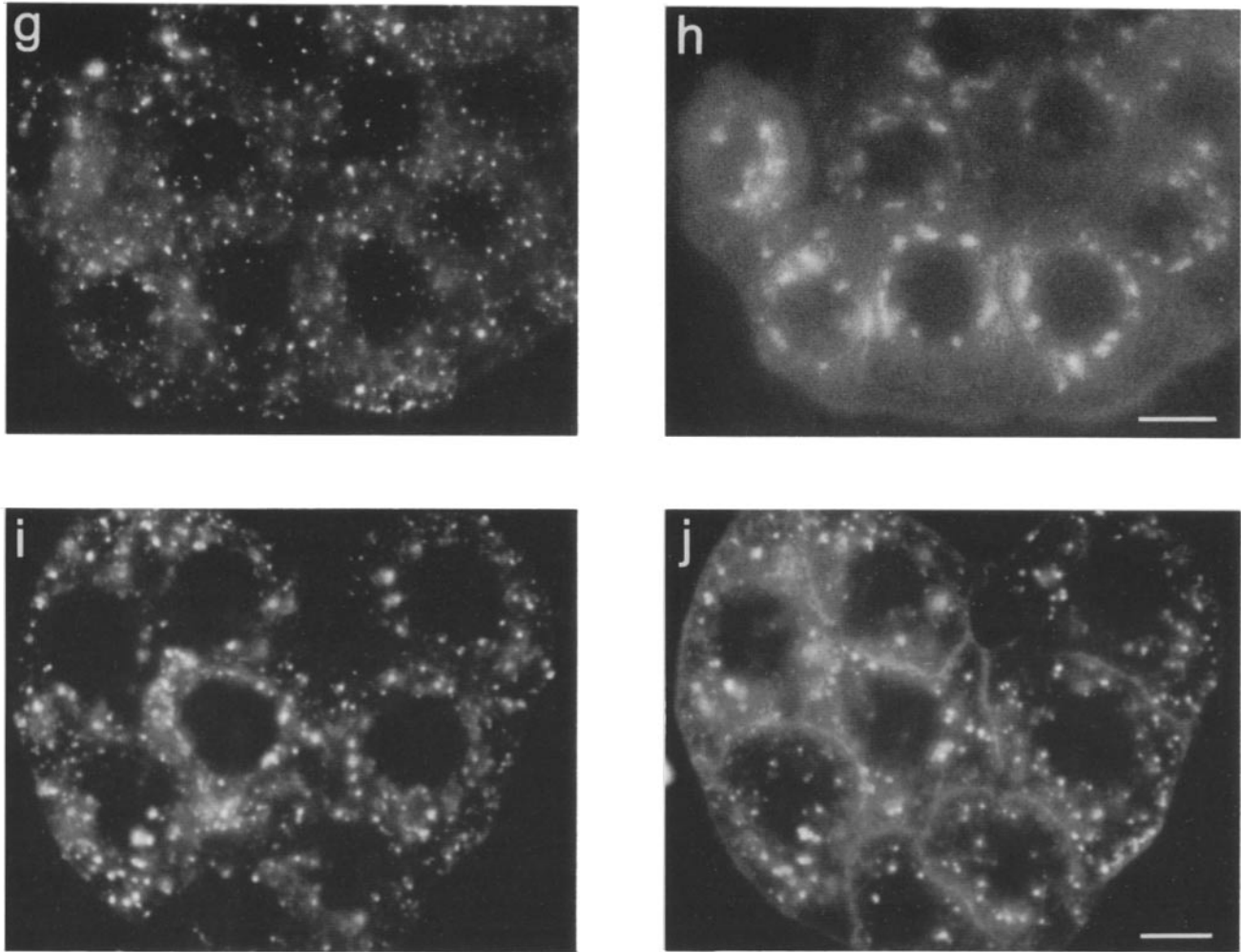


Figure 1.

samine rhodamine B sulfonylchloride (LR), 10% (wt/wt) on celite, were from Molecular Probes Inc. (Eugene, OR). [[[7-(diethylamino)coumarin-3-yl] carbonyl]methylamino]-ceramide was a gift from Dr. G. van Meer (University of Utrecht, The Netherlands) and ricin RCA60 was a gift from Dr. E. Nicolas (Université René Descartes, Paris, France).

Synthesis of C_6 -NBD-labeled Sphingolipids

C_6 -NBD-glucosylceramide, C_6 -NBD-galactosylceramide, C_6 -NBD-lactosylceramide, C_6 -NBD-sphingomyelin, and C_6 -NBD-ceramide were synthesized from C_6 -NBD and 1- β -D-glucosylsphingosine, 1- β -D-galactosylsphingosine, 1- β -DL-lactosyldihydrosphingosine, sphingosylphosphorylcholine, and D-sphingosine, respectively, as described (4). 1- β -D-galactosylsphingosine and 1- β -DL-lactosyldihydrosphingosine were prepared (3) from cerebroside (type II) and *N*-palmitoyl-DL-dihydroxylactocerebroside, respectively. The C_6 -NBD-lipids were quantitated spectrophotometrically in a fluorometer (model MPF-43; Perkin-Elmer Corp., Norwalk, CT) with an excitation wavelength of 465 nm and an emission wavelength of 530 nm, by reference to known amounts of C_6 -NBD-phosphatidylcholine.

Protein Labeling

Saturation of Tf with iron was carried out by the procedure of Van Renswoude et al. (15). Diferric Tf and ricin were conjugated with LR as described elsewhere (5).

Cell Culture and Membrane Insertion of Fluorescent Lipids

Monolayer cultures of HT29 cells were grown in DME, supplemented with 10% (vol/vol) FCS in a water-saturated atmosphere of 5% CO_2 /95% air.

Experiments were carried out 24–48 h after passage. C_6 -NBD-lipid insertion was carried out at 2°C. Before labeling, the cells were cooled to 2°C (30 min). Appropriate amounts of C_6 -NBD-lipid, stored in chloroform/methanol (2:1 [vol/vol]), were dried under nitrogen, and subsequently solubilized in absolute ethanol. An aliquot of the ethanolic solution (0.5% final concentration) was injected into Hank's solution (pH 7.4) under vigorous vortex mixing. This solution was then added to the cells. Alternatively, several microscopy experiments were carried out using C_6 -NBD-lipid containing liposomes for labeling, as described elsewhere (5). Both procedures used for labeling yielded identical results.

Back-Exchange for Membrane-inserted Lipid Analogues

Back-exchange was carried out by incubating the cells for 30 min at 2°C with 5% (wt/vol) BSA in Hank's solution, followed by extensive washing with Hank's solution. This procedure was repeated twice.

Metabolic Studies

Procedures used for carrying out metabolic studies were analogous to those described below for the microscopy experiments, allowing direct correlation of the results. After the 37°C incubation (and extensive washing), a back-exchange procedure was carried out at 2°C, in order to separate the plasma membrane NBD-lipid fraction from the intracellular pool. Both fractions were analyzed as described below.

Lipid Extraction, Analysis, and Quantification

Lipids were extracted by the procedure of Bligh and Dyer and analyzed by two-dimensional TLC on silica gel 60 HPTLC plates (Merck & Co., Rah-

way, NJ), using $\text{CHCl}_3/\text{CH}_3\text{OH}/20\%$ (wt/vol) NH_4OH [70:30:5] as the running solvent system in the first dimension and $\text{CHCl}_3/\text{CH}_3\text{OH}/86\%$ (vol/vol) HAc [90:40:14] in the second dimension, allowing separation of five different $\text{C}_6\text{-NBD-lipid}$ spots associated with HT29 cells. To positively identify $\text{C}_6\text{-NBD-galactosylceramide}$ as one of the $\text{C}_6\text{-NBD-lipids}$ synthesized from $\text{C}_6\text{-NBD-ceramide}$ in HT29 cells, the TLC-system as described by Van Meer et al. (14) was employed. For quantification, individual spots were scraped from the plates and after addition of 3 ml of a 1% (vol/vol) Triton X-100 solution, shaken vigorously for 1 h at 37°C. After spinning down the silica particles, the supernatant was measured by reference to a standard curve, as described above. Corrections were made for differences in efficiency of removal of different types of lipids from the silica. Data are expressed as the means (\pm SEM) of triplicate measurements.

Microscopy

Cells were grown on glass coverslips in 35-mm Petri dishes. Before experiments, the cells were cooled (30 min) and washed several times with ice-cold Hank's solution. For colabeling studies with fluorescently labeled ricin, the cells were labeled with LR-ricin (15 $\mu\text{g}/\text{ml}$) for 30 min at 2°C. Internalization of membrane-bound ricin (after washing away free ricin) was initiated by adding warm Hank's solution (37°C) and allowed to proceed for 60 min. Thereafter, the cells were again cooled to 2°C, followed by labeling with the $\text{C}_6\text{-NBD-lipid}$ for 30 min at 2°C. After washing, internalization was started again by adding warm Hank's solution and terminated after 30 min by replacing warm buffer with ice-cold one. Finally, a back-exchange procedure was done at 2°C, followed by a 0.1 M lactose wash (30 min at 2°C) to remove cell surface-bound ricin.

For colabeling studies with fluorescently labeled Tf, the cells were first labeled with the $\text{C}_6\text{-NBD-lipid}$ for 30 min at 2°C, followed by washing with Hank's solution and subsequent labeling with LR-Tf (0.2 mg/ml). Internalization was allowed to proceed for 30 min, after which a back-exchange procedure was performed at 2°C. Colabeling studies with DECA-ceramide were done as described above for LR-Tf, except that the cells were first labeled with DECA-ceramide (1 μM), as described for the $\text{C}_6\text{-NBD-lipids}$, followed by labeling with the $\text{C}_6\text{-NBD-lipid}$. The labeling concentration of the $\text{C}_6\text{-NBD-lipids}$ was 4 μM .

Monensin experiments were performed by preincubating the cells with the drug (10^{-5} M) for 30 min at 2°C. Thereafter, the cells were labeled with the $\text{C}_6\text{-NBD-lipid}$ followed by internalization and back-exchange. Throughout these experimental treatments the drug was present. Metabolic inhibition experiments were performed as described (10). Briefly, cells were preincubated at room temperature for 5 min with 5 mM azide/50 mM 2-deoxyglucose, followed by NBD-sphingolipid labeling at 2°C and subsequent warming of the cells to 37°C in the presence of the drugs. Acidification of the cytosol was achieved by incubating the cells for 30 min at 2°C with 5 μM nigericin in an isotonic KCl buffer solution (140 mM KCl, 2 mM CaCl_2 , 1 mM MgCl_2 , 0.5 mM KH_2PO_4 , 20 mM HEPES, pH 5.5). All subsequent washings and incubations were carried out with the same nigericin-containing buffer solution. After experimental manipulation, the glass coverslips were removed from the Petri dishes and mounted for microscopy. Fluorescence microscopy was performed with a Leitz Orthoplan microscope equipped with a Leitz Vario Orthomat 2 photography system, and the following filter sets: purple excitation, BP 340-410/LP 470 (DECA-channel); blue excitation, BP 450-490/LP 515 (NBD-channel); and green excitation, BP 530-560/LP 580 (Rh-channel). Photomicrographs were taken at 10–30-s exposure times using Kodak Tmax P3200 film that was processed at 12.800 ASA.

Results and Discussion

$\text{C}_6\text{-NBD-Sphingolipid Metabolism in HT29 Cells}$

When HT29 cells are incubated with $\text{C}_6\text{-NBD-ceramide}$, either at 2°C or 37°C, the lipid is taken up into the cells and at 37°C accumulates in the Golgi apparatus where several NBD-sphingolipids are synthesized. Similar observations have been described for other cell types (7, 14). The two main lipid products synthesized are $\text{C}_6\text{-NBD-glucosylceramide}$ and $\text{C}_6\text{-NBD-sphingomyelin}$. However, the cells also synthesize $\text{C}_6\text{-NBD-galactosylceramide}$, $\text{C}_6\text{-NBD-lactosylceramide}$, and a third, as yet unidentified $\text{C}_6\text{-NBD-sphingolipid}$ (Babia, T., J. W. Kok, and D. Hoekstra, manuscript in preparation).

$\text{C}_6\text{-NBD-Sphingolipid Internalization}$

$\text{C}_6\text{-NBD-glucosylceramide}$ and $\text{C}_6\text{-NBD-sphingomyelin}$ can be inserted into the plasma membrane of HT29 cells at 2°C with the ethanol-injection procedure. Under these conditions both lipids only label the plasma membrane and, in contrast to their precursor $\text{C}_6\text{-NBD-ceramide}$, do not gain access to intracellular membranes. However, when the cells are warmed to 37°C the lipids are internalized, but their intracellular sites of destination are different.

As shown in Fig. 1, $\text{C}_6\text{-NBD-glucosylceramide}$ is transported to the Golgi apparatus. It colocalizes with DECA-ceramide, a fluorescent derivative of ceramide, which, like the $\text{C}_6\text{-NBD-derivative}$, can be used as a vital stain for the Golgi apparatus (Fig. 2; Ref. 8). Because of its distinct fluorescent properties, DECA-ceramide is a convenient probe for double-labeling studies with NBD-labeled lipids.

Further support for the Golgi localization of $\text{C}_6\text{-NBD-glucosylceramide}$ comes from experiments in which monensin was used as a reagent that can disrupt the structural organization of the Golgi complex. As a result of such a treatment (Fig. 2), the Golgi complex in HT29 cells, normally visualized as large, irregularly shaped vesicles that are distinctly located around the nucleus, becomes vesiculated and more spread over the cell. As shown in Fig. 2, in comparison to its distribution in nontreated cells (Fig. 1), the distribution of $\text{C}_6\text{-NBD-glucosylceramide}$ is similarly disrupted in the presence of monensin.

In contrast to $\text{C}_6\text{-NBD-glucosylceramide}$, internalized $\text{C}_6\text{-NBD-sphingomyelin}$ did not colocalize with DECA-ceramide, implying that sphingomyelin is not transported to the Golgi apparatus. Instead, it was found in an intracellular compartment that can be labeled by LR-ricin which is taken up into the cells by endocytosis. This result thus indicates that this lipid is transported and confined to the endosomal/lysosomal system. Entirely consistent with this notion was the observation that the labeling pattern of NBD-sphingomyelin was not changed after a treatment with monensin that causes disruption of the Golgi (Fig. 2).

Back-exchange experiments revealed that after 30 min at 37°C ~10–15% of the initial membrane-inserted lipid fraction is located intracellularly. The internalization of both lipids is temperature dependent and can be inhibited in the presence of metabolic inhibitors (5 mM azide/50 mM 2-deoxyglucose) (Fig. 3) or when the intracellular pH is lowered to 5.5 with the aid of nigericin (not shown). Therefore, internalization is energy dependent and accomplished by endocytosis (2, 10). It is highly relevant to note here that on the time scale of the experiments no appreciable breakdown of the $\text{C}_6\text{-NBD-sphingolipids}$ had taken place, as revealed by lipid analyses. The relevance of this notion stems from the fact that the observed labeling of the Golgi complex by glucosylceramide might (in part) have been the result of initial lysosomal degradation of glucosylceramide to ceramide, followed by transfer and accumulation of this degradation product in the Golgi compartment (8, 9). The ceramide, in turn, could then have been reutilized (6, 9) for synthesis of glucosylceramide (and sphingomyelin, see below). However, this possibility can be ruled out. Analysis of the intracellular pool of NBD-lipid revealed that after an incubation period of 30 min at 37°C, 91.3% (\pm 1.4%) of the intracellular fraction was still present as $\text{C}_6\text{-NBD-glucosylceramide}$. Furthermore, 6.9% (\pm 0.9%) was identified as $\text{C}_6\text{-NBD-ceramide}$,

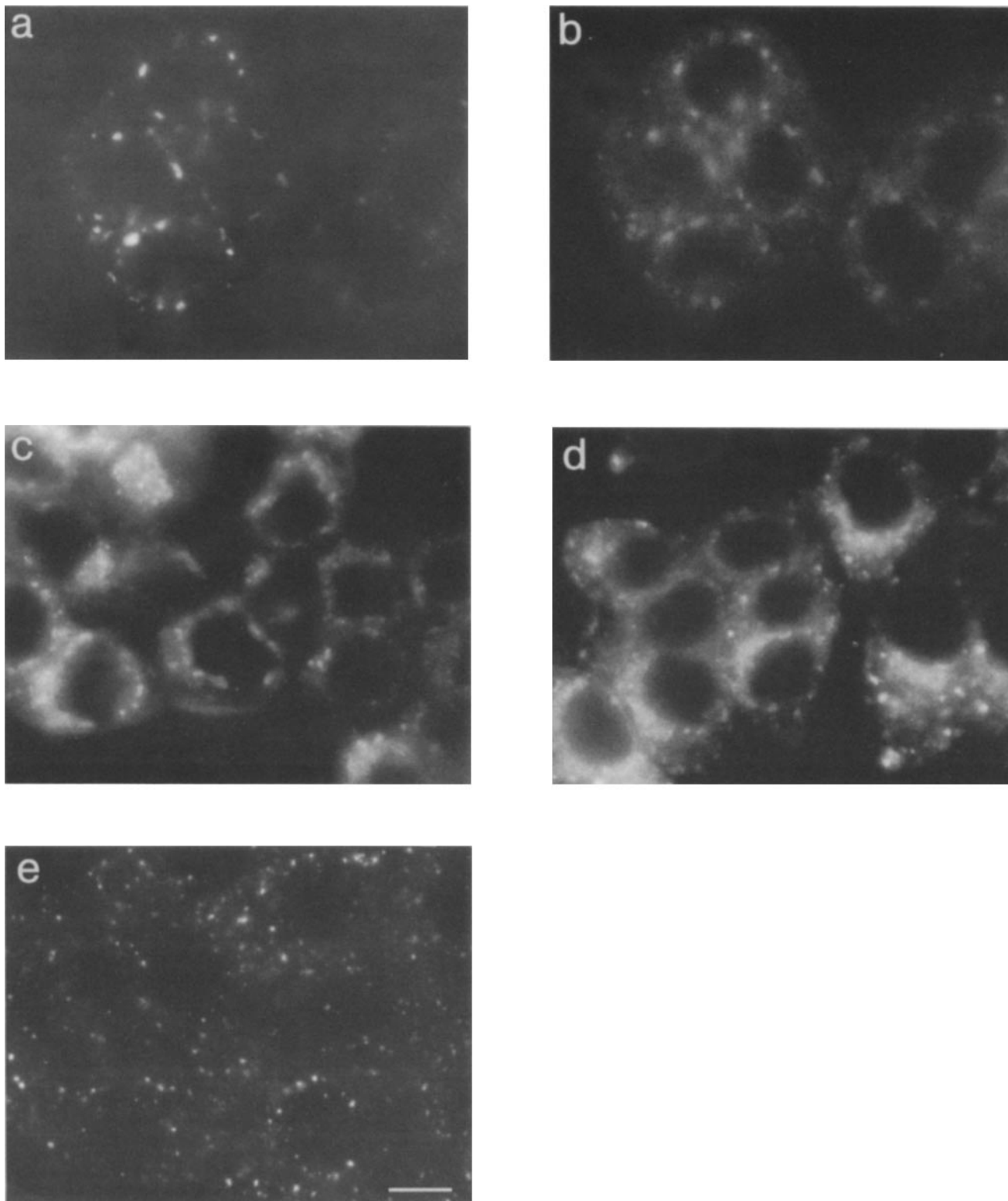


Figure 2. Effect of monensin on NBD-sphingolipid fluorescence distribution. (*a* and *b*) Cells were first labeled with DECA-ceramide (1 μM), followed by labeling with NBD-ceramide (4 μM). Thereafter, the cells were incubated at 37°C for 30 min. Both NBD-ceramide (*a*) and DECA-ceramide (*b*) accumulate in the Golgi-apparatus and thus show the same fluorescence distribution. (*c*–*e*) Cells were labeled with 4 μM C₆-NBD-ceramide (*c*), C₆-NBD glucosylceramide (*d*), or C₆-NBD-sphingomyelin (*e*) as described in Materials and Methods. Subsequently, the cells were preincubated for 30 min at 2°C with 10 μM monensin, followed by an incubation at 37°C for 30 min in the presence of the drug. Finally, a back-exchange procedure was carried out. Note the similarity in fluorescence distribution between NBD-ceramide (*c*) and NBD-glucosylceramide (*d*) in monensin-treated cells, and the difference with control cells (*a* and Fig. 1, *a* and *c*, respectively). There is no effect on NBD-sphingomyelin fluorescence distribution (*e* compared to Fig. 1, *g* and *i*). Bar, 10 μm .

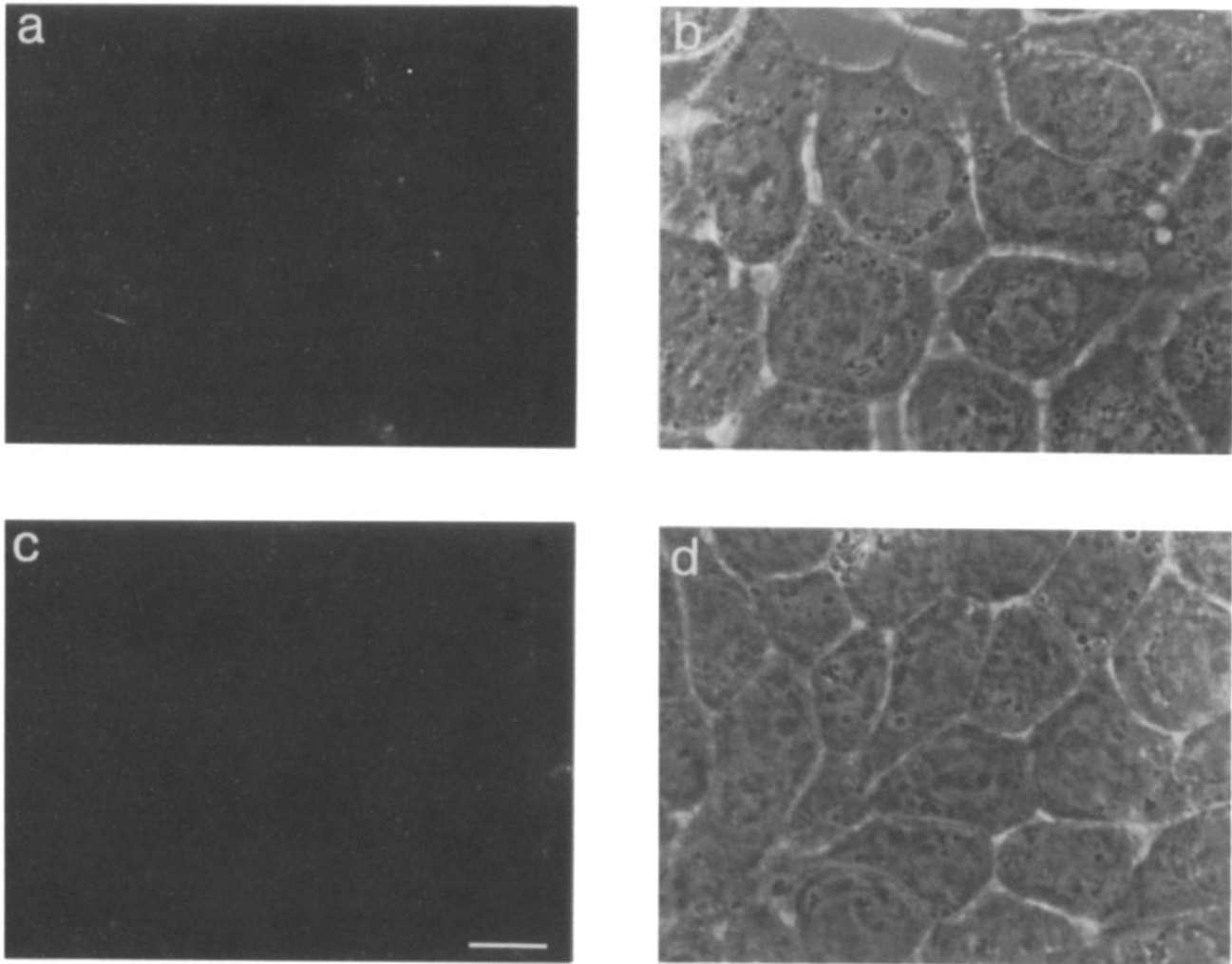


Figure 3. Inhibition of internalization by metabolic inhibitors. Cells were preincubated for 5 min at room temperature with metabolic inhibitors (5 mM azide/50 mM 2-deoxyglucose). Thereafter the cells were labeled with 4 μ M C₆-NBD-glucosylceramide (*a* and *b*) or C₆-NBD-sphingomyelin (*c* and *d*), followed by an incubation at 37°C for 30 min in the presence of the inhibitors. Finally, a back-exchange procedure was carried out. *b* and *d* show the corresponding phase-contrast images. Bar, 10 μ m.

whereas only 1.9% (\pm 1.1%) of the intracellular NBD-lipid fraction has been reused for synthesis to C₆-NBD-sphingomyelin. It should also be noted that the lipid present in the plasma membrane consisted exclusively ($99.6 \pm 0.3\%$) of C₆-NBD-glucosylceramide (as determined by analysis of the back-exchanged lipid fraction). When the sphingolipid precursor, C₆-NBD-ceramide, was supplied to the cells for a similar incubation period (30 min at 37°C), both glucosylceramide and sphingomyelin were synthesized (as noted above), while the ratio of glucosylceramide to sphingomyelin in which they were produced is 1.3 (\pm 0.1).

Taken together, it can thus be calculated that only 2.5% of the intracellular glucosylceramide fraction (which amounts to 91.3%) can at most have been derived from degradation and resynthesis. These considerations imply, therefore, that the fate of the original and intact C₆-NBD-glucosylceramide is monitored, as it is transported from the plasma membrane to the Golgi complex. It thus follows that C₆-NBD-glucosylceramide and C₆-NBD-sphingomyelin are actively sorted in HT29 cells during endocytic uptake. Whether early endosomes play a pivotal role in this lipid

sorting event—analogue to events in ligand/receptor trafficking—remains to be established. In this context it is interesting to note, however, that the sorting process as such seems to be rather specific. This became apparent when the trafficking of C₆-NBD-glucosylceramide was compared to that of the closely related sphingolipids C₆-NBD-galactosylceramide and C₆-NBD-lactosylceramide, both of which can be synthesized by the HT29 cells (see above). Of these three glycolipids, only C₆-NBD-glucosylceramide is transported to the Golgi apparatus. Both C₆-NBD-galactosylceramide and C₆-NBD-lactosylceramide, similarly as observed for C₆-NBD-sphingomyelin, were processed along the endosomal/lysosomal route (Fig. 1).

Interestingly, when the trafficking of C₆-NBD-glucosylceramide and C₆-NBD-sphingomyelin, occurring in undifferentiated HT29 cells, was compared to that in a differentiated type of HT29 cells (isolated by clonal selection; Babia, T., J. W. Kok, and D. Hoekstra, manuscript in preparation), sorting between the lipids no longer occurred. As shown in Fig. 4, in differentiated cells both C₆-NBD-sphingolipids followed the endocytic pathway during internalization and

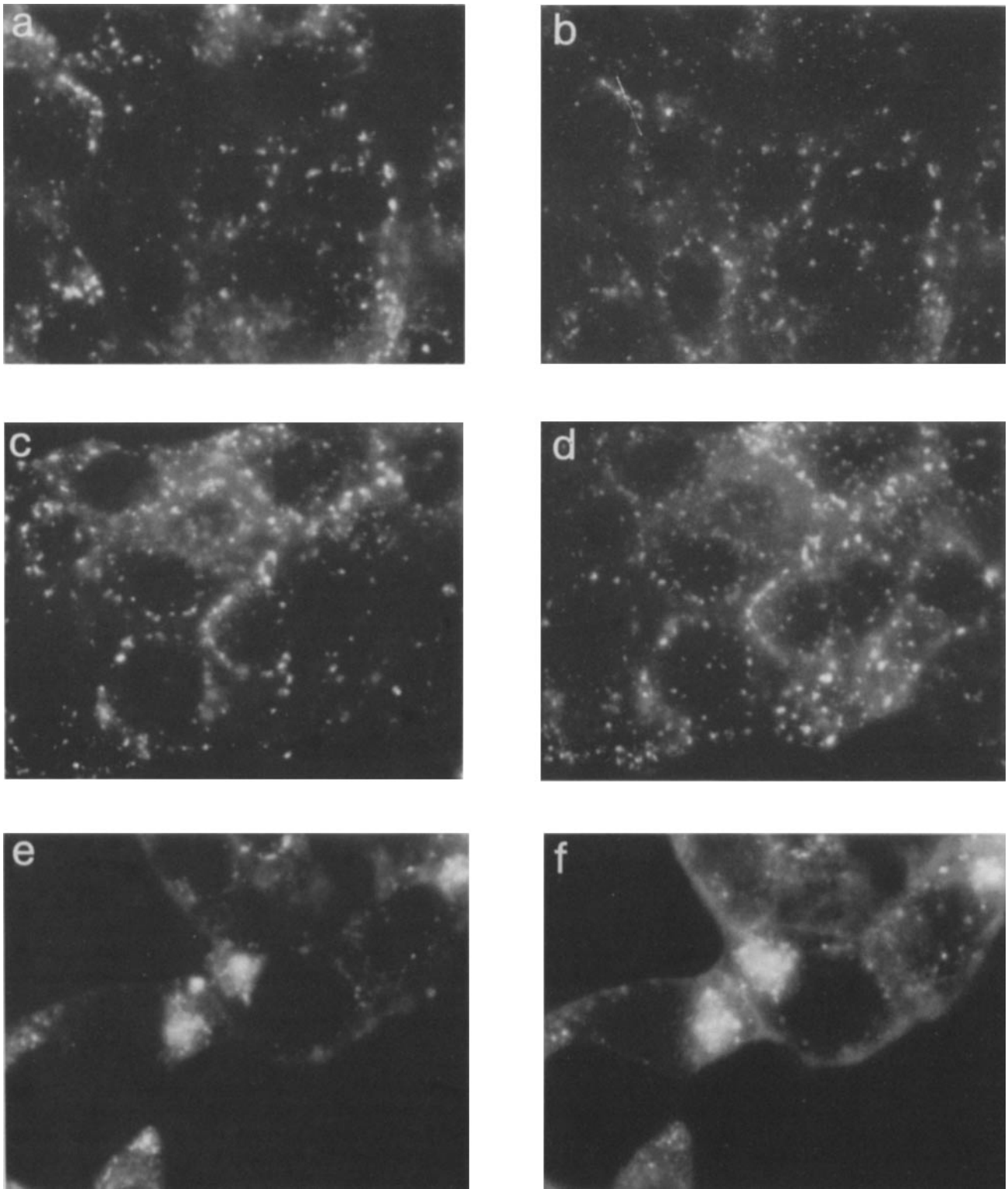


Figure 4. Intracellular localization of sphingolipids in differentiated HT29 cells. Cells were labeled with $4 \mu\text{M}$ C₆-NBD-glucosylceramide (*a*, *e*, and *i*) or C₆-NBD-sphingomyelin (*c* and *g*), as described in Materials and Methods. Internalization was allowed to proceed for 30 min at 37°C, followed by a back-exchange procedure at 2°C. Colabeling studies were performed with LR-Tf (*b* and *d*), LR-ricin (*f* and *h*), or DECA-ceramide (*j*), as described in Materials and Methods. Both C₆-NBD-glucosylceramide and C₆-NBD-sphingomyelin colocalize with both LR-Tf and LR-ricin. Note that C₆-NBD-glucosylceramide does not colocalize with DECA-ceramide (*i* and *j*), as opposed to their colocalization in undifferentiated HT29 cells (Fig. 1, *a* and *b*). Bar, 10 μm .

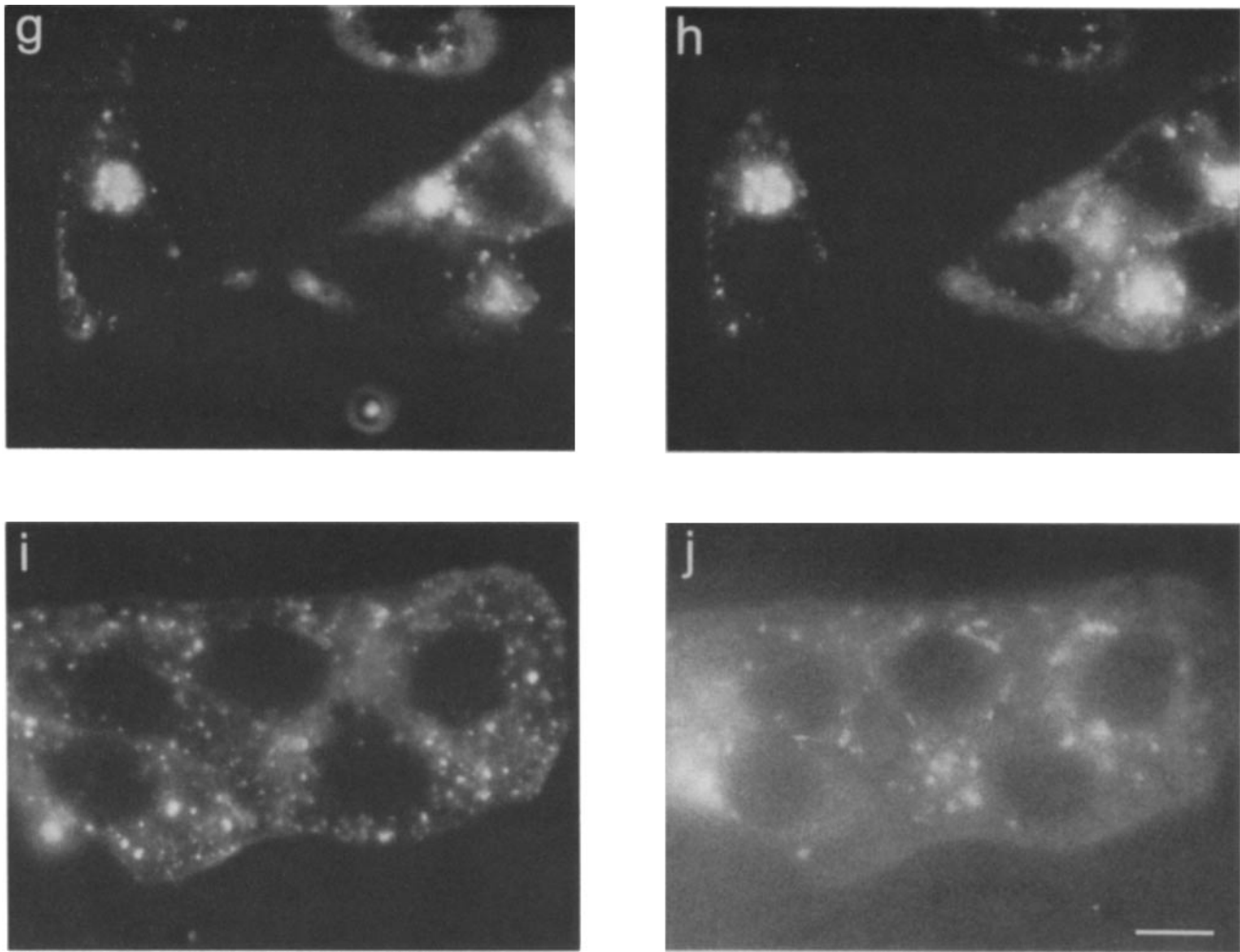


Figure 4.

perfectly colocalized with rhodamine-labeled Tf, a well-known marker for receptor-mediated endocytosis (15). Colocalization was also observed in double-labeling studies with LR-ricin as the endocytic marker. In this case, however, both sphingolipids and ricin were often found to colocalize in bright juxtannuclear patches of fluorescence (Fig. 4). This typical distribution pattern of endocytic vesicles may have been induced by the ricin, since no such patches were observed in double-labeling studies with Tf. In contrast to its localization in undifferentiated HT29 cells (Fig. 1; see above), C_6 -NBD-glucosylceramide does not colocalize with DECA-ceramide in this cell type, because the lipid is not transported to the Golgi apparatus (Fig. 4).

In summary, the results show that two C_6 -NBD-sphingolipids, glucosylceramide and sphingomyelin, are actively sorted during endocytosis in undifferentiated HT29 cells. This sorting process is lipid specific, since out of several glycolipids only C_6 -NBD-glucosylceramide is sorted from C_6 -NBD-sphingomyelin. Furthermore, this phenomenon appears to be related to the state of differentiation of the cells, being a property of the undifferentiated cells only.

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