Limited and Selective Transfer of Plasma Membrane Glycoproteins to Membrane of Secondary Lysosomes

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Abstract. Radioactive galactose, covalently bound to cell surface glycoconjugates on mouse macrophage cells, P388D$_1$, was used as a membrane marker to study the composition, and the kinetics of exchange, of plasma membrane-derived constituents in the membrane of secondary lysosomes. Secondary lysosomes were separated from endosomes and plasma membrane on self-forming Percoll density gradients. Horseradish peroxidase, taken up by fluid-phase pinocytosis, served as a vesicle contents marker to monitor transfer of endosomal contents into secondary lysosomes. Concurrently, the fraction of plasma membrane-derived label in secondary lysosomes increased by first order kinetics (k = [56 min]$^{-1}$) from <0.1% (background level) to a steady-state level of ~2.5% of the total label. As analyzed by NaDodSO$_4$ PAGE, labeled molecules of M_r 160–190 kD were depleted and of M_r 100–120 kD were enriched in lysosome membrane compared with the relative composition of label on the cell surface. No corresponding selectivity was observed for the degradation of label, with all M_r classes being affected to the same relative extent. The results indicate that endocytosis-derived transfer of plasma membrane constituents to secondary lysosomes is a limited and selective process, and that only ~1% of internalized membrane is recycled via a membrane pool of secondary lysosomes.

FLUID-PHASE pinocytosis leads to a high rate of internalization of plasma membrane, most of which is recycled to the cell surface within minutes (reviewed in references 7, 17, 29, and 33). The extent is not known to which internalized membrane contributes to the membrane of endosomes as a prelysosomal compartment (cf. 11, 23). Although endosomes are thought to be a major site from which internalized membrane is recycled, it remains to be determined whether the recycling pathway also leads through the membranes of secondary lysosomes, at least for a part of internalized membrane (cf. 6).

The soluble contents of endosomes are delivered mainly into secondary lysosomes. This transfer of vacuole contents implies that at some stage(s) fusion of endosomes, or of their derivatives, with secondary lysosomes must occur. Direct evidence for fusion between cell surface-derived membrane and secondary lysosomes, leading to a mutually accessible intravacuolar space, has been obtained by demonstrating that plasma membrane constituents can be labeled with a marker that has previously been delivered into secondary lysosomes (21, 27). Much of this labeled membrane is subsequently recycled to the cell surface within minutes. This shows that a substantial fraction of internalized membrane remains available for rapid recycling even after fusion with secondary lysosomes (cf. 36).

For membranes in fusion, membrane fluidity allows a rapid mixing of membrane constituents (15). Therefore, to the extent that rapid membrane recycling occurs after fusion with lysosomes, membrane constituents must be actively prevented from mixing or must be rapidly sorted out. Examples of nonmixing of membranes in fusion at the cell surface have been reported (4, 24, 25, 32).

The present study examines whether transfer of vacuole contents between endosomes and secondary lysosomes results in an admixing of cell surface-derived membrane constituents to the membrane of secondary lysosomes. If so, the membrane of secondary lysosomes should include a pool of plasma membrane constituents in exchange with the cell surface via fusion/fission with endosomes or their derivatives.

A previous study addressed this question explicitly and found ". . . little, if any, plasma membrane proteins in lysosomes, . . ." (2). We have used cell surface glycoconjugates, covalently labeled with radioactive galactose (31), as a membrane marker to measure the kinetics and the composition of plasma membrane constituents becoming associated with membrane of secondary lysosomes.

Materials and Methods

Cell Culture

Cells of the mouse macrophage cell line, P388D$_1$ (12), were grown at 37°C into suspension from a confluent monolayer culture in RPMI-1640 medium, containing 10% heat-inactivated fetal bovine serum, 100 IU/ml of penicillin,

0.1 mg/ml streptomycin, and 0.25 μg/ml of fungizone (Flow Laboratories, Irvine, Ayrshire, Scotland), buffered in 7% CO_2. After a growth period of not longer than 24 h in fresh medium, cells were collected from suspension by centrifugation for 10 min at 200 *g* in the cold and washed twice in Hepes saline (HS;[1] 10 mM Hepes [pH 7.4] and 140 mM NaCl). For experimental reincubation, ~80–120 × 10^6 cells were resuspended at ~4 × 10^6 cells/ml in RPMI medium without serum, buffered with Hepes (10 mM [pH 7.4]), and mildly agitated in a 100-ml Erlenmeyer flask in a shaking waterbath at 37°C.

Cell Surface Labeling

Labeling was done on ice, essentially as before (1, 31). Washed cells were resuspended to ~10^8 cells/ml in HS containing either UDP(6-^{3}H)galactose (ammonium salt; 3.2 μM, 15.6 Ci/mmol; Amersham International, Amersham, UK) or UDP(U-^{14}C)galactose (lithium salt; 8.1 μM, 309 mCi/mmol; Amersham International), and $MnCl_2$ (5 mM). The reaction was started by adding galactosyltransferase (0.5 U/ml, from bovine milk, EC 2.4.1.22; Sigma Chemical Co., St. Louis, MO). After ~15–30 min, the reaction was stopped by 10-fold dilution in HS, followed by washing three times with 10 ml HS.

Removal of Label from Cell Surface

Enzymatic hydrolysis of cell surface label was done essentially as before (1, 31). Membrane flow was arrested by cooling on ice. The cells were washed twice in 10 ml Imidazole saline (100 mM Imidazole [pH 6.8] and 50 mM NaCl), resuspended in the same buffer at ~10^8 cells/ml and the reaction was started by adding β-galactosidase (0.5 U/ml, from *Diplococcus pneumoniae*, EC3.2.1.23, also containing neuraminidase; a gift from Rudolf Weil, Sandoz Ltd., Vienna, Austria). After ~30 min on ice, the fraction of label released was determined by comparing the radioactivity in the total cell suspension to that in the supernatant after pelleting the cells by centrifugation. The cells were then washed twice in 10 ml HS.

Internalization of Label and Pinocytic Uptake

These assays were carried out as described, yielding quantitatively the same results as reported (not shown; see 1). In this study horseradish peroxidase (HRP; Sigma Chemical Co.) was used as a fluid-phase marker, as well as fluorescein-conjugated dextran (40,000-mol-wt; Pharmacia, Inc., Uppsala, Sweden). Both markers gave exactly the same result for pinocytic uptake (not shown).

Subcellular Fractionation

Fractionation was done on a self-forming Percoll gradient, in a similar way as described (8). All steps were done at 4°C. After washing in HS, cells were resuspended (10^8 cells in 2 ml) in homogenization buffer (0.25 M sucrose, 2 mM EDTA, and 10 mM Hepes [pH 7.4]) and homogenized to ~50% disruption with 50 strokes in a tight-fitting glass Dounce homogenizer. The homogenate was centrifuged (1000 *g*, 10 min) to remove unbroken cells. At this step ~50% of the total label was recovered in the supernatant. The supernatant (1 ml) was layered over 10 ml of a 27% Percoll (Pharmacia Inc.) solution in homogenization buffer, underlayered with 0.5 ml of a 2.5 M sucrose solution. Centrifugation was done in a rotor (model SW41Ti; Beckman Instruments Inc., Fullerton, CA) for 2 h at 17,000 rpm. Fractions of ~0.5 ml each were collected by piercing the bottom of the tube.

As it turned out, very small relative amounts of radioactivity were to be measured in the high density fractions. Therefore, extreme care was necessary to assure that the lower part of the centrifugation tubes had not experienced any prior exposure to the bulk of the label, i.e., neither during loading (overlayering of the organelle suspension), nor during centrifugation (swing-out instead of fixed-angle rotor), and also not during sampling of the fractions (sampling from bottom to top by piercing the bottom of the tube).

Enzyme Assays

HRP was measured in a similar way as described (26). An aliquot of 50 μl was taken from each gradient fraction and added to 0.5 ml of substrate solution (2,2-azino-di-3-ethylbenzthiazoline sulphonate [ABTS; Amersham International], 0.55 mg/ml, 0.003% H_2O_2, 20 mM phosphate-citrate [pH 4.3], 150 mM NaCl, and 0.1% Triton X-100). After 30 min at 20°C, the reaction was stopped by addition of 0.5 ml of citric acid (100 mM) containing NaN_3 (0.01%). Precipitated Percoll was removed by centrifugation. *N*-acetylglucosaminidase (GlcNAc-ase) was measured as described (9). An aliquot of 20 μl was taken from each gradient fraction and added to 200 μl substrate solution (2 mM *p*-nitrophenyl-*N*-acetyl-β-d-glucosaminide, Sigma Chemical Co., 25 mM phosphate-citrate buffer [pH 5.0], 150 mM NaCl, and 0.1% Triton X-100). After 1 h at 37°C, the reaction was stopped by addition of 0.8 ml of a NaOH solution (50 mM). For both assays the reaction product was measured within 4 h, at 420 and 400 nm, respectively, against blank samples prepared from the respective gradient fraction of a blank gradient obtained by overlayering with homogenization buffer only.

Membrane Preparations

Total membrane was prepared as described (1). Cells were resuspended in 10 mM Tris-HCl (pH 7.8), 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride, at a concentration not exceeding 10^7 cells/ml and disrupted by sonication. The total membrane fraction was collected by centrifugation at 10^5 *g* for 60 min in a Beckman SW41Ti rotor. For membrane of secondary lysosomes the high density gradient fractions (Nos. 2–6, cf. Fig. 1) were pooled (~2 ml) and diluted into 10 ml of Tris-HCl buffer (as above) and organelles disrupted by sonication. The suspension was centrifuged (as above), the membrane pellet collected from the top of a Percoll pellet, suspended in 10 ml of the same buffer, and recentrifuged to further reduce the Percoll in the membrane sample. After this procedure ~70% of the original amount of radioactivity was recovered with the membrane pellet (the lost radioactivity was not due to soluble material, but rather to incomplete pelleting of membrane in the presence of remaining Percoll).

Polyacrylamide Gel Electrophoresis

Membrane proteins were dissociated by heating at 90°C for 3 min in 1% SDS, 0.1 M 2-mercaptoethanol. Electrophoresis was carried out in gradient slab gels (250 × 180 × 1.5 mm/5–13% polyacrylamide/0.1% SDS) with a discontinuous buffer system at 5 mA for 48 h at room temperature. Molecular weight standards used were standard mixtures (SDS-6H and Dalton Mark VII-L, Sigma Chemical Co.). Bands were stained with Coomassie Brilliant Blue (Sigma Chemical Co.). Gels were dried and lanes cut into 2.5-mm slices, which were converted to, and separated as, ^3H_2O and $^{14}CO_2$ in a sample oxidizer (model 306; Packard Instrument Co. Inc., Downers Grove, IL) before determining radioactivity. Where radioactivity profiles were compared for different membrane samples, these were labeled with ^{3}H vs. ^{14}C and run in the same lane to eliminate all errors arising from possible inhomogeneities in the gel and from slice cutting. However, this turned out not to be absolutely necessary because, for the same membrane sample in different lanes, the relative amounts of radioactivity in equivalent slices varied <8%. Autoradiographs were prepared by soaking the gel in Amplify™ (Amersham International) for 30 min before drying. The dried gel was then exposed to preflashed FUJI RX x-ray film (FUJI Photo Film Co. Ltd, Tokyo, Japan) for 14 d at −70°C.

Results

Fluid-phase pinocytosis and concurrent membrane internalization was measured as before (1). Upon reincubation at 37°C, after labeling on ice, the cells resumed endocytosis at a constant rate of 2–3 μm³/(cell × min) as measured with HRP or fluorescein-conjugated dextran as fluid-phase marker. Concurrent membrane internalization and recycling led to a redistribution of label between the cell surface and intracellular membranes (steady-state distribution 83:17, respectively). Label on the cell surface was quantified by its susceptibility to removal using β-galactosidase (31). As described before (1), the kinetics of redistribution were biphasic indicating compartmentation of internalized label (not shown).

At various times, after labeling of the cell surface and resumption of membrane flow, we measured the relative amounts of both the pinocytic contents marker and the plasma membrane marker that could be cosedimented with a high density organelle fraction, containing secondary lysosomes. As Fig. 1 shows, this fraction could be separated

1. *Abbreviations used in this paper*: GlcNAc-ase, *N*-acetylglucosaminidase; HRP, horseradish peroxidase; HS, Hepes saline.

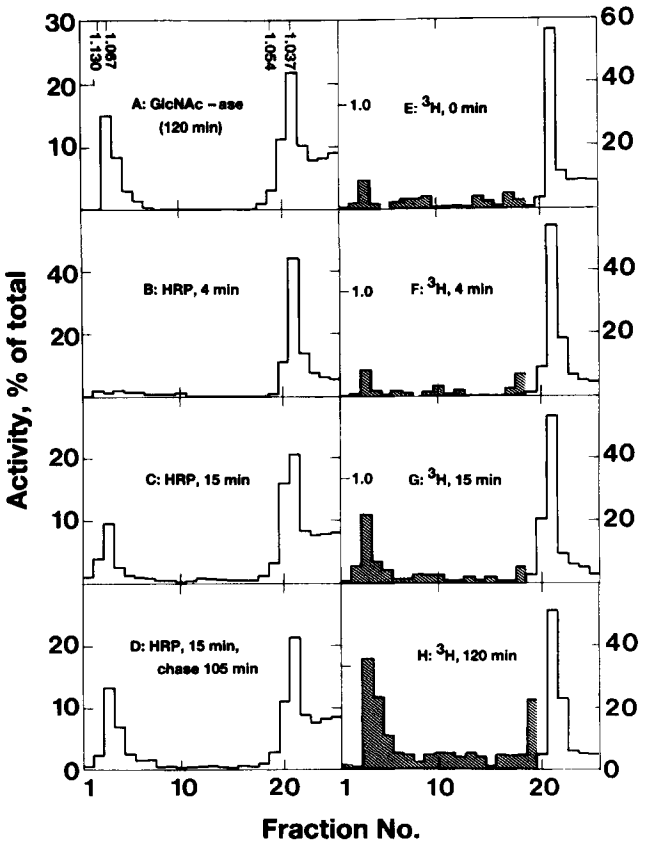

Figure 1. Transfer of vesicle contents and membrane constituents from endosomes to secondary lysosomes. Cells were labeled with [^{3}H]galactose on the cell surface and reincubated in the presence of fluid-phase pinocytosis marker, HRP. After various times, membrane flow was stopped, ~85% of the label on the cell surface was enzymatically removed, cells were fractionated, and secondary lysosomes were separated from lighter subcellular fractions on a self-forming Percoll gradient in a swing-out rotor (see Materials and Methods). (*A*) The activity of the lysosomal enzyme, GlcNAc-ase, served to identify secondary lysosomes in the high density fractions (3–6); the activity (72% of the total) in the low density fractions (18–22), and in the overlay volume (fractions 23–25) is ascribed to broken lysosomes; samples were from the identical gradient as in *D* and *H*; the densities as indicated were the positions of density-marker beads. (*B*) The activity of the contents marker, HRP, served to identify endosomes; after 4 min of HRP uptake, no HRP activity was recovered in secondary lysosomes, in agreement with a previous report (28); this profile shows that endosomes were not sedimenting into the high density fractions. (*C*) After 15 min of pinocytic uptake, 16% of the total HRP was recovered with secondary lysosomes. (*D*) After 15 min of HRP uptake, cells were resuspended in HRP-free medium for a chase period of 105 min; HRP (72% of the total) recovered in the low density fractions and in the overlay volume is ascribed to broken lysosomes, as in *A*. [^{3}H]Galactose, covalently bound to plasma membrane glycoconjugates at the cell surface, served as a marker to identify plasma membrane-derived membrane constituents in secondary lysosomes. (*E*) Cells were fractionated immediately after labeling on the cell surface, before internalization of label; the shaded profile, representing a 33-fold enlargement (cf. left ordinate), shows the low background of label measured in high-density fractions. (*F*) After 4 min of internalization, no increase of label was observed in secondary lysosomes. (*G* and *H*) After 15 and 120 min, respectively, small, but increasing, relative amounts of label were recovered with secondary lysosomes. Profiles from identical gradients are: *A*, *D*, and *H*; *B* and *F*; *C* and *G*. The shaded areas in panels *E–H* refer to the left ordinates, enlarged 33-fold. ^{3}H in the high density fractions from the bulk of the label, using self-forming Percoll density gradients. For human fibroblasts, it was shown (18) that plasma membrane, endoplasmic reticulum, and the Golgi marker, galactosyltransferase, all sedimented at a model density of 1.040 g/ml in Percoll density gradients, compared with typical secondary lysosomes sedimenting at 1.070 g/ml. The data in Fig. 1 agreed with these values.

The high density fractions (nos. 2–6, cf. Fig. 1) contained secondary lysosomes as characterized by the lysosomal enzyme, GlcNAc-ase (6, 35). Only 28% of the enzyme activity was found in the high density fractions (Fig. 1 *A*). The complementary 72% was found at the top of the gradient, partially sedimenting with the plasma membrane (^{3}H profile, Fig. 1 *E*) and endosomes (HRP profile, Fig. 1 *B*), and as soluble product in the upper three fractions making up the overlayered volume (see Materials and Methods). No hydrolase activity was observed at intermediate positions with no signal above background as measured using a gradient with a blank solution as overlayered volume. We concluded that a fraction of 28% of the secondary lysosomes was recovered intact after cell fractionation (cf. Fig. 1 *D* for HRP, and Fig. 1 *H* for ^{3}H, in the identical gradient).

Fig. 1, *B–D*, illustrates the transfer of HRP as fluid-phase marker from endosomes to secondary lysosomes. After 4 min of HRP uptake, practically no HRP was found in the high density fractions (Fig. 1 *B*). This is in agreement with a previous morphometric observation that HRP is not delivered to secondary lysosomes until about 5 min after uptake (28). After 15 min of uptake, 16% of the total HRP was recovered in the high density fractions (Fig. 1 *C*). After a further 105 min in the absence of external HRP, one would expect that the bulk of HRP had been chased from endosomes into secondary lysosomes (75% of pinocytosed HRP was transferred to secondary lysosomes after 30 min, 8). As shown in Fig. 1 *D*, 28% of the total HRP activity was recovered in the high density fractions. The complementary 72% of HRP activity, found in the low density fractions, could be ascribed to breakage of secondary lysosomes during cell fractionation. In the identical gradient the lysosomal marker enzyme, GlcNAc-ase, showed quantitatively the same distribution (28:72, see above and Fig. 1 *A*).

The identical set of gradients was analyzed for the distribution of the plasma-membrane marker [^{3}H]galactose-labeled glycoconjugates (Fig. 1, *E–H*). When cells were fractionated immediately after labeling on the cell surface, before any internalization of label, no significant amount of label was found in the high density fraction (Fig. 1 *E*, shaded area as enlarged 33-fold, cf. left ordinate). Neither was any significant amount of label observed in these fractions when cells were fractionated after 4 min of membrane flow (Fig. 1 *F*). Only when cells were allowed to internalize label for longer periods, could a small but increasing amount of label be recovered in the high density fractions (Fig. 1, *G* and *H*).

Based on the above information, in combination with three further similar experiments, we measured the kinetics describing the transfer of plasma membrane marker to secondary lysosomes. Fig. 2 shows the results. The values were corrected for breakage of secondary lysosomes using the

tions in *E–H* is shown in Fig. 2 (▽) as percentage of the total label, after having been corrected for lysosome breakage and for removal of cell surface label.

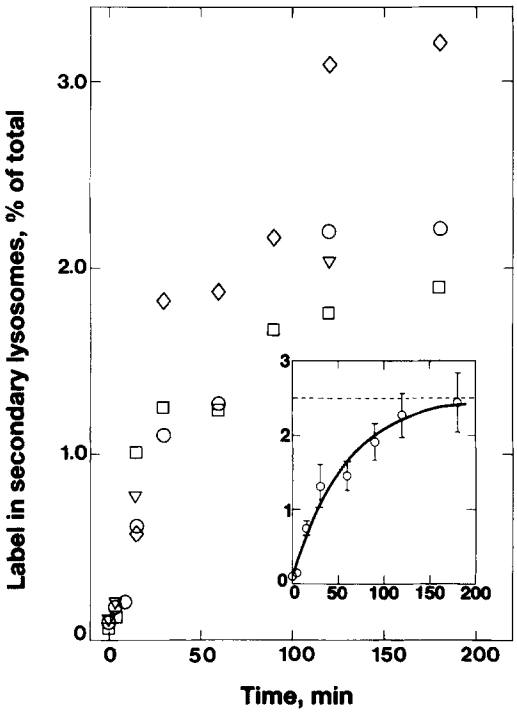

Figure 2. Plasma membrane-derived [^{3}H]galactose in secondary lysosomes after various times of membrane internalization during fluid-phase pinocytosis. Secondary lysosomes were separated from low density membrane fractions on self-forming Percoll gradients as illustrated in Fig. 1 and as described in Materials and Methods. To calculate the amount of label in secondary lysosomes as a percentage of the total cell-bound label, the relative amounts in the high density fractions were summed (e.g., Fig. 1 *H*: fractions 3–6 contained 1.8% of the total label recovered in the gradient), corrected for loss of label due to breakage of secondary lysosomes (using the relative recovery of GlcNac-ase in the high density fractions, as measured for each gradient, e.g., Fig. 1 *A* displays 28% of the total activity in fractions 3–6, therefore corrected ^{3}H value becomes 1.8%/0.28), and corrected for the amount of label that was enzymatically removed from the cell surface prior to cell fractionation (to reduce the background, e.g., after 120 min of membrane internalization, 68% of the total cell-bound label was removed from the cell surface during treatment with β-galactosidase at 0°C for 30 min, therefore corrected ^{3}H value becomes 1.8%/0.28 × (1–0.68) = 2%, cf. ▽ at t = 120 min). The different symbols refer to independent experiments (▽ refers to the data illustrated in Fig. 1). (*Inset*) Average values followed first-order kinetics as indicated; k = (56 min)$^{-1}$, 95% confidence interval k = (168 min)$^{-1}$ to k = (33 min)$^{-1}$. Label accumulated in secondary lysosomes to a steady-state level of 2.5% of the total cell-bound label, 95% confidence interval, 2–3%.

relative recovery of GlcNAc-ase activity as a measure for the degree of breakage. Membrane of broken lysosomes did not seem to band in the high density fractions. After additional homogenization to various degrees of samples from the same cell-free organelle suspension, extrapolation to 100% breakage (0% recovery of high density GlcNAc-ase) indicated only background levels (0.1–0.2%) of label in the high density fractions. Anyhow, small amounts of broken lysosomes banding at high density would render the results as upper values (overcompensated for loss). Although there was a considerable variation between the results of independent experiments, a plot of the averages suggested first-order steady-state kinetics. Plasma membrane marker entered the membrane of secondary lysosomes with a residence time of ~56 min, reaching a steady state with 2.5% of the total label being in secondary lysosomes (Fig. 2, inset).

We attempted to do similar measurements using the lactoperoxidase-mediated iodination technique for labeling cell surface proteins, as applied previously to the plasma membrane of macrophages (16). However, in view of the very low relative amounts of radioactive marker to be measured in secondary lysosomes, the background level in all membrane fractions was too high to allow reliable observations.

Realizing that a small but measurable fraction of plasma membrane marker entered the membrane of secondary lysosomes, we determined whether this process was selective for only certain species of labeled plasma membrane glycoconjugates. We compared the labeling profiles of SDS PAGE molecular weight distributions for membrane of secondary lysosomes and plasma membrane (Fig. 3, *A* and *D*). A very obvious difference was observed. In comparison with the labeling profile obtained for the plasma membrane, the profile for membrane of secondary lysosomes showed a depletion in the M_r-range of ~160–190 kD and an enrichment in the range of 100–120 kD. Some smaller differences in the two profiles might also be real.

In view of lysosomes being degradative organelles, this result could arise from differences in the susceptibility to degradation of the various labeled molecular species. Intralysosomal degradation could be partially inhibited by the addition of ammonia (reviewed in reference 3). Therefore, as a control, we measured the labeling profile of lysosome membrane when label had been internalized in the presence of ammonia (10 mM NH_4Cl, 2 h, 37°C). Within experimental accuracy, the same profile was observed as in the absence of ammonia (Fig. 3 *B*).

Another possibility was that those labeled M_r-species which were recovered with lysosomal membrane in an enriched (depleted) mode were subject to degradation to a larger (lesser) extent. As before (1), label was lost from the cells into the medium at a rate of 3.5%/h. We therefore compared the labeling profiles on SDS gels for total membrane preparations when prepared immmediately after labeling the plasma membrane and when prepared after 8 h of membrane flow (~28% loss of label). As Fig. 3 *C* shows, no obvious differences were found between the two profiles. This suggests that loss of label involved all labeled molecular species to a similar extent. A comparison of the profiles in Fig. 3 *C* with those obtained for plasma membrane in Fig. 3, *A* and *B* gave an indication of the degree of reproducibility between different experiments.

Discussion

The results show the composition and the kinetics of cell surface-derived membrane constituents becoming associated with membrane of secondary lysosomes. Quantitative conclusions can be made concerning the process of maintaining membrane specificity, in this particular case between the plasma membrane and secondary lysosomes. Two aspects can be distinguished. First, the degree of membrane specificity is illustrated in terms of the employed membrane marker. Second, quantification of membrane recycling via secondary lysosomes has mechanistic implications.

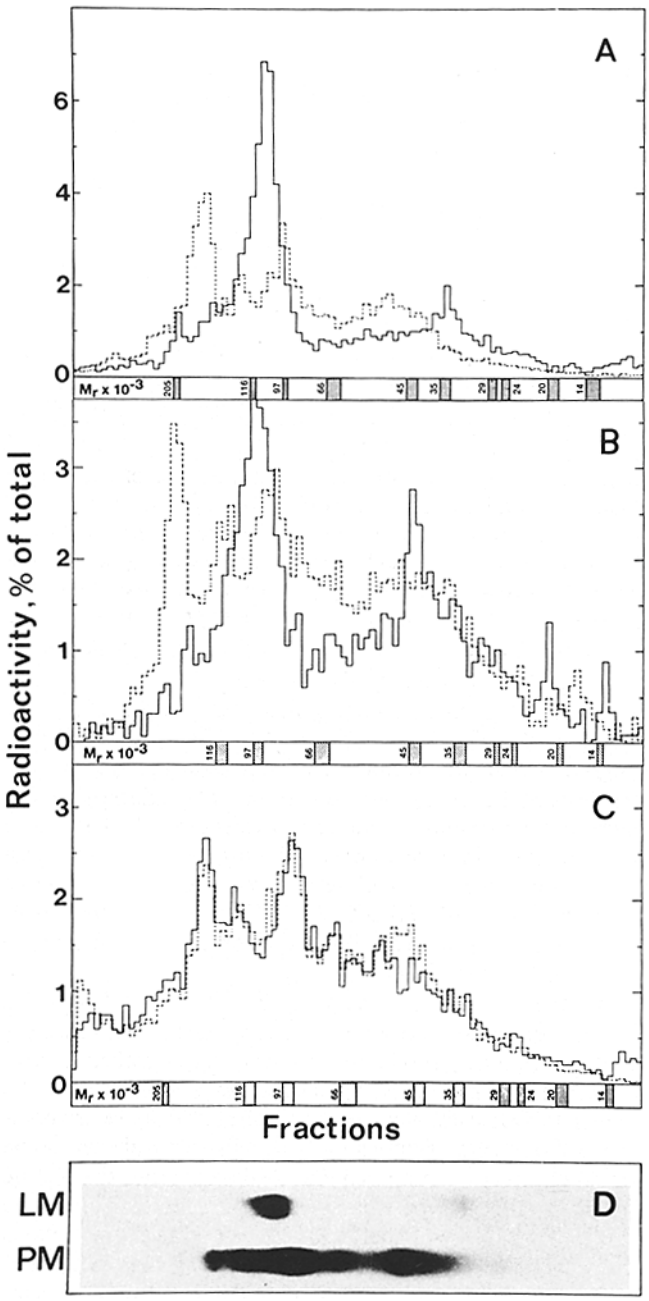

Figure 3. Composition of labeled plasma membrane glycoconjugates on SDS PAGE gels. (*A*) Plasma membrane-derived label on membranes of secondary lysosomes (solid line, [^{3}H]galactose-labeled, prepared after 120 min of postlabeling membrane flow), in comparison with label on the plasma membrane immediately after labeling at the cell surface (broken line, [^{14}C]-galactose-labeled). The two profiles were obtained from the same lane on the gel. (*B*) Same as in *A*, except that label on membranes of secondary lysosomes (solid line) was internalized in the presence of NH_4Cl (10 mM). (*C*) Label on total membrane fractions prepared either immediately after labeling at the cell surface (broken line, [^{3}H]galactose-labeled; cf., broken line profile in *A* and *B*) or after 8 h of membrane flow and ~28% loss of label (solid line, [^{14}C]galactose-labeled); the two samples were run on the same lane. The profiles from *A–C* were from independent membrane preparations and run on separate gels. (*D*) Autoradiographs represent lysosome membrane (*LM*) from the same batch as used for *A* (solid line), and plasma membrane (*PM*) from the same batch as used for *C* (broken line). These samples were run on the same gel as in *C*.

Composition and Relative Abundance of Plasma Membrane-derived Molecules in Lysosome Membrane

We have used plasma membrane glycoconjugates, covalently labeled with [^{3}H]galactose at the cell surface, as a membrane marker. The results concerning membrane specificity can therefore be considered only in terms of those membrane constituents that are susceptible to labeling by this method. More than 20 different M_r-species are labeled (cf. Fig. 6 in reference 1). This is also evident from the labeling profiles for plasma membrane in Fig. 3. The labeled molecules seem to be integral membrane proteins as judged by their resistance to solubilization (<5%) by either pH 11, or 3 M NaCl, or 1% mercaptoethanol (unpublished data). During fluid-phase pinocytosis, all labeled M_r-species seem to become internalized to the same relative extent (1; this is also the case for the internalization of iodinated cell surface molecules, 16). In contrast, the composition of label observed in membrane of secondary lysosomes differs significantly from that on the plasma membrane (Fig. 3, *A* and *D*). In lysosome membrane, labeled molecules of the M_r-range 100–120 kD are enriched whereas for the range 160–190 kD a depletion is observed in comparison with label on the plasma membrane.

Because lysosomes are degradative organelles, the possibility must be considered that the different labeling profile observed for secondary lysosomes is due to selective degradation. Label is lost into the medium with a $t_{1/2}$ ~20 h, affecting all labeled M_r-species to the same relative extent (Fig. 3 *C*; similar results have been reported for iodinated plasma membrane proteins, 21). The loss of label can be explained by the normal turnover rates of membrane proteins ($t_{1/2}$ = 10–100 h, cf. Table VI in reference 19). The loss can only partially be ascribed to β-galactosidase activity because only 30% of the released label is TCA-soluble (1). Endogenous β-galactosidase is secreted by P388D$_1$ cells (10) although we have not detected such activity, either cell bound or in the medium. Only ~25% of the loss seems to be due to lysosomal degradation, since it is susceptible to inhibition by the presence of ammonia. Although we cannot rule out entirely the possibility of selective degradation being partially the cause of the observed labeling profile of lysosomal membrane, it seems unlikely for the following reasons. First, quantitatively the same profile is observed when the experiment is repeated in the presence of ammonia (Fig. 3 *B*). Second, no evidence for selective degradation is observed (Fig. 3 *C*). Third, other authors have previously reported very similar differences between lysosomes and plasma membrane when isolated membranes are labeled with NaB^3H_4 (Fig. 2 in reference 2).

What are the implications of this observation for the membrane specificity of plasma membrane with respect to membrane of secondary lysosomes? Obviously, the M_r-range 160–190 kD represents labeled molecules specific for plasma membrane. Label in the M_r-range 100–120 kD represents molecular species that are present in both membranes. A 100-kD molecular mass lysosomal membrane antigen has recently been found to be present also in a prelysosomal compartment (endosomes) and on the plasma membrane in cul-

tured prolactin cells (34), or in fibroblasts (14) (cf. 13). Mechanistically, two different possibilities must be distinguished. First, the 100–120 kD molecules may be solely cell-surface derived, becoming "mixed" into the membrane of primary lysosomes, perhaps because they play a role during the interaction between endosomes and lysosomes. Second, the 100–120 kD molecules may be inherent also to the membrane of primary lysosomes and consequently, there is no need to discriminate against the admixing of these molecules when they are of cell surface origin. This latter argument cannot account for the different relative amounts (per membrane area, cf. below) of this protein in lysosomes compared to plasma membrane. A choice between the two possibilities depends on the biochemical characterization of purely primary lysosomes, which is not feasible at present.

An estimate can be made concerning relative membrane specificity. Cell surface–derived label accumulates in secondary lysosomes to a steady-state level of 2.5% of the total cell-associated label. Label can be expected to redistribute between the interacting membranes according to the relative abundances of the different, normally untagged, molecular species. For example, in the case of redistribution between membranes of the same composition, the steady state is reached when all membranes have the same surface density of label. This has been proven directly by morphometry (using the present label as autoradiographic marker) for the plasma membrane, pinosomes and phagosomes in Dictyostelium (5). In peritoneal macrophages, the relative membrane areas of the plasma membrane, pinosomes, and of secondary lysosomes, have been determined by morphometry as 100:12:18, respectively (28). Therefore, with respect to the relative membrane area of secondary lysosomes, the membrane proteins represented by the label are about six times more abundant on the plasma membrane and pinosome membrane (97.5% of label on 112/130 of the total area) than on membrane of secondary lysosomes (2.5% on 18/130 of total area). This factor is an average for all labeled M_r-species, not considering the observed selectivity.

Transfer Rate of Plasma Membrane-derived Molecules to Lysosome Membrane

Cell surface–derived label enters the membrane of secondary lysosomes by first order kinetics (Fig. 2). During endocytosis-derived membrane traffic, 2.5% (steady-state level) of the total label flows to and from lysosomes once every 56 min ($k = [56\ min]^{-1}$). This result can be related to the rate of membrane internalization as observed during a kinetic analysis for the same cells and labeling technique (1; see also reference 28, and Table I in reference 33): After labeling on the cell surface, subsequent membrane flow leads to a redistribution of label between the plasma membrane and intracellular membranes, until a steady-state distribution is reached when 83% of the label is on the cell surface; this indicates that the plasma membrane makes up 83% of the total amount of exchanging membrane constituents; from the rate at which the steady state is established it has been calculated that this cell surface pool is internalized about three times per hour, i.e., $3 \times 83\% = 250\%$ of the total label is internalized per hour (and recycled, as has been directly demonstrated, 1). Therefore, by way of comparison (2.5%/56 min vs. 250%/h), it can be concluded that only ~1% of the label internalized at any one moment (i.e., relative rate,

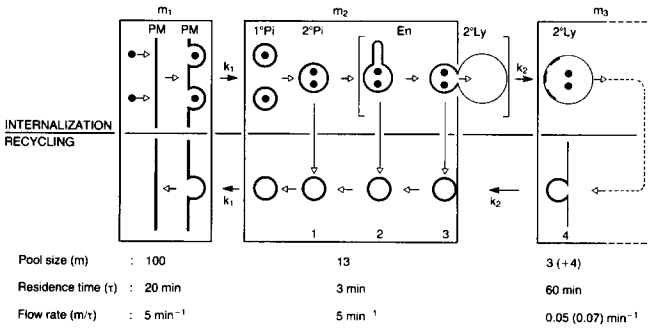

Figure 4. Schematic presentation of intracellular membrane flow and compartmentation. Cell surface–derived membrane is indicated by thick lines. Probable sites for membrane recycling are indicated by numbers 1–4. *1.*, during fusion among primary (1° P_i) and secondary (2° P_i) pinosomes; morphometric data on the reduction of surface to volume ratio (28) suggest that ~50% of pinosome membrane may be liberated at this site (cf. Fig. 2 in reference 33). *2.*, during processing of endocytic contents (concentrating) and transfer to endosomes (En); it is not known to what extent endosome membrane is derived from internalized membrane and to what extent they fuse directly with secondary lysosomes (2° Ly) (this uncertainty is symbolized by the square brackets). *3.*, during transfer of endocytic contents into 2° Ly. *4.*, during processing of 2° Ly; only this step involves "recycling via a membrane pool of 2° Ly", and implies mixing and sorting of membrane constituents; according to the present results only ~1% of internalized membrane is recycled via this route. Based on the kinetics of redistribution to intracellular membranes, of label applied at the cell surface, three membrane compartments have been resolved (1, cf. 28, 33). Membrane flows between the plasma membrane (PM, pool size $m_1 = 100$ relative area) and two consecutive intracellular compartments (pool sizes, $m_2 = 13$ and $m_3 = 7$, respectively) at rates k_1 and k_2 (dimension [membrane area/time]), being the same in both directions (homeostasis). PM is internalized once every 20 min ($k_1 = 100/20$ [relative area/min]), into a first intracellular pool (m_2) amounting to 13% of the cell surface pool size. The bulk of internalized membrane ($k_1/(k_1 + k_2)$ ~99%) is recycled directly from this compartment after a residence time of ~3 min (m_2/k_1). This rapid recycling involves steps 1–3, which have not been resolved kinetically, biochemically, or morphometrically. Only the small complementary fraction of internalized membrane ($k_2/(k_1 + k_2)$ ~1%) subsequently enters a second intracellular membrane pool (m_3), amounting to 3(+4)% of m_1 (the portion 3% has been allocated to membrane of 2°Ly in the present study), from where it is recycled after a residence time of ~60 min.

not steady state) subsequently enters the pool in membranes of secondary lysosomes. Three interpretations for this result can be considered (cf. Fig. 4).

First, only 1% of internalized membrane may suffice to transfer the bulk of pinosome contents to secondary lysosomes (regurgitation of fluid-phase marker by exocytosis was <0.01%, unpublished results). The following assessment makes this seem unlikely. Morphometrically, a 50% reduction in the surface to volume ratio of pinosomes due to pinosome–pinosome fusion, before fusion with lysosomes, has been observed (28, cf. Fig. 2 in reference 33). Thereby, 50% of pinosome membrane can be liberated for recycling, the remaining 50% comprising pinosomes with a 10-fold increased volume per pinosome. In fibroblasts, this volume increase can be calculated as ~2.6-fold (28, cf. 33). Also in fibroblasts, flow cytometry suggests an early increase in fluorescence marker per isolated pinosome of about six to eightfold, due to either an increase of vesicle volume or to

an increased concentration of the dye, or both (22). A comparison (2.6 vs. six to eight) suggests a threefold increase in concentration of pinosome contents in fibroblasts. In macrophages, morphometric analysis suggest a sevenfold increased concentration of fluid-phase marker after delivery into secondary lysosomes (28, cf. 33). On average, a corresponding decrease in pinosome volume, can make a further 40% of internalized membrane available for recycling before pinosome–lysosome fusion, leaving ~10% (minus 1%) unaccounted for.

Second, pinosome contents may be transferred to secondary lysosomes via an intermediate type of organelle. Possible intermediates have been discussed in terms of their density, intermediate between pinosomes and secondary lysosomes (18, 30, 37). Such a characterization does not exclude modification of pinosomes as discussed in the previous paragraph. The membrane composition of such an intermediate organelle can be either similar to and derived from pinosome membrane (cf. 11, 17, 23, 29), or similar to lysosome membrane, or intermediate to both. Concerning the problem of maintaining membrane specificity, this, respectively, either leaves the problem unchanged as considered in this article (cf. below), or places it one step earlier in the endocytic pathway, or splits it up between two intracellular sites, in a ratio depending on the relative differences of the membranes involved.

Third, internalized membrane is partially (>1%) involved in direct fusion with secondary lysosomes during transfer of endocytic contents. In view of the present results, such an explanation requires that during fusion, only a limited mixing of membrane constituents takes place, allowing for rapid and selective recycling of the bulk of internalized membrane involved (step 3 in Fig. 4). This is in full agreement with previous observations of direct fusion between cell surface–derived membrane structures (endosomes) and secondary lysosomes, with subsequent rapid and large scale recycling (21, 27). A discrepancy between the present results and an earlier study can also be explained by this model. In order to detect lysosome-specific membrane constituents of secondary lysosomes, the membrane composition of latex bead–containing phagolysosomes has been measured in comparison with the composition of plasma membrane (20). In terms of susceptibility to ^{125}I-labeling, no difference has been found in spite of the fact that in both membranes a set of >20 different protein species can be labeled (20). In terms of the present consideration it is possible that during fusion between latex-containing phagosomes and lysosomes, lysosomal enzymes are transferred instead of the bulky, indigestible latex beads, and yet, in the absence of membrane mixing, no detectable amounts of lysosome-derived membrane enter the phagosome membrane. In Dictyostelium, latex bead–containing phagosomes remain fully linked to the recycling pathway, and their membrane undiluted in terms of surface density of [^{3}H]galactose labeling; this occurs via rapid and extensive membrane exchange with newly formed pinosomes (5).

Fig. 4 shows the implications of the third interpretation. Accordingly, the term "recycling via secondary lysosomes" must be differentiated. At least four steps of membrane recycling can be distinguished. The first two steps indicate recycling before fusion with lysosomes and probably involve >50% of internalized membrane. This recycling is rapid, occurring within a few minutes after internalization (1, 28, 33). Third, the bulk of the remaining membrane is recycled after fusion with secondary lysosomes. This recycling is also rapid and has not been distinguished kinetically, nor biochemically, from the first two steps, suggesting that membrane constituents do not mix or are rapidly resorted. Fourth, only ~1% of internalized membrane is recycled strictly via the membrane pool of secondary lysosomes after a residence time of ~50–60 min. Sorting of this fraction may lead into the pathway of de novo synthesized membrane constituents.

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