Molecular Size-Fractionation during Endocytosis in Macrophages

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Abstract. The sorting of macromolecules within and between membranous organelles is often directed by information contained in protein primary or secondary structure. We show here that absent such structural information, macromolecules internalized by endocytosis in macrophages can be sorted by size. After endocytosis, small solute probes of fluid-phase pinocytosis were recycled to the extracellular medium more efficiently than large solutes. Using macropinosomes pulse labeled with fluorescent dextrans, we examined the ability of organelles to exchange solute contents. Dextran exchange was optimal between organelles of similar age, and small dextrans exchanged more efficiently than large dextrans. Efferent solute movement, from lysosomes or phagolysosomes toward the plasma membrane, occurred through the same endocytic vesicles as afferent movement, toward lysosomes and this movement was solute size dependent. Remarkably, uniform mixtures of different-sized dextrans delivered into lysosomes separated into distinct organelles containing only one dextran or the other. Thus, the dynamics of endosomes and lysosomes were sufficient to segregate macromolecules by size. This intracellular size fractionation could explain how, during antigen presentation, peptides generated by lysosomal proteases recycle selectively from lysosomes to endosomes for association with class II MHC molecules.

THE traffic of macromolecules within and through vesicular organelles is directed both by the dynamics of the organelles and by information contained in the structure of the macromolecules. Light and electron microscopic studies of endocytic organelle dynamics indicate a high degree of communication between endosomes and lysosomes, mediated by vesicle fusion and fission and by microtubule-mediated organelle movements. In most cells, solute internalized by fluid-phase endocytosis moves sequentially through early endosomes and late endosomes into lysosomes, with some percentage of that solute leaving the cell via vesicular recycling. Macromolecules that take other paths carry information in their structure that enhances either retention in endosomes or movement into other compartments. Such sorting information is often contained in protein primary or secondary structure, and works by selectively directing molecules into transport vesicles or by mediating associations with other molecules that are so directed (Sandoval and Bakke, 1994).

Some molecular sorting may occur without such signals, however. As antigen-presenting cells, macrophages process many different and essentially unrecognized proteins. Proteins delivered to lysosomes via endocytosis are degraded to peptides, which then recycle to endosomes or to other nonlysosomal compartments, where they associate with MHC class II molecules (Germain, 1994). Peptide movement from lysosomes to endosomes indicates a sorting process, in that these small molecules are recycled but the lysosomal enzymes are not. The great variety of peptides that travel this route makes it unlikely that sorting information in their primary structure directs them along that path.

This selective traffic from lysosomes to endosomes could be explained if movement through endocytic compartments were size-selective; that is, if small molecules recycled from lysosomes more easily than large molecules did. There is some evidence for this. Buckmaster et al. (1987) found that small fluid-phase probes recycled from CHO cell lysosomes better than large probes. Wang and Goren (1987) observed that small dyes preloaded into macrophage lysosomes by endocytosis moved more readily into phagosomes than did larger dyes. This indicates that the nature of organelle fusion and fission can permit some size selectivity in molecular movement between organelles, thereby suggesting a generic mechanism that could serve in antigen presentation.

Here we describe size-dependent solute sorting at several levels inside macrophages. Fluid-phase solute probes move through endocytic compartments differently depending on their size. Small solutes recycle from macrophages to the extracellular medium more efficiently, and move more easily between endocytic organelles than do large solutes. Efferent solute movement, from lysosomes toward the plasma membrane, occurs via the same organelles that mediate the afferent movement, from the plasma membrane toward lysosomes. Moreover, uniform mixtures of different-sized fluorescent dextrans can segregate by size into distinct lysosomes. These observations indicate a size-selective communication between

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endocytic organelles that can produce both size-selective recycling from lysosomes to endosomes and molecular size-fractionation within lysosomes.

Materials and Methods

Macrophages

Murine bone marrow-derived macrophages were obtained as described previously (Swanson, 1989). Femurs from female, C3H-HeJ mice were removed, their marrow extruded, and then the exudate was cultured in medium that promotes growth and differentiation of macrophages (Bone marrow culture medium: 30% L cell-conditioned medium, a source of macrophage colony-stimulating factor (M-CSF)[1], 20% heat-inactivated FBS, DMEM). After 6 or 7 d of culture, macrophages were harvested from dishes and plated onto 12-mm circular coverslips or 24-well culture dishes, at 5×10^4 and 3×10^5 cells per well, respectively. Cultures were then incubated overnight in medium lacking M-CSF (DME-10F: DMEM with 10% heat-inactivated FBS). Experiments were performed the next day (day 7 or 8).

Fluid-Phase Endocytosis (Pinocytosis)

Fluid-phase endocytosis by macrophages in tissue culture wells was measured as described by Swanson (1989). Cells were incubated in Ringer's saline (RB: 155 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 2 mM Na_2HPO_4, 10 mM glucose, 10 mM Hepes, pH 7.2, 0.5 mg/ml BSA) containing both lucifer yellow (LY) and fluorescein dextran, average mol wt 4,000 or 150,000 (FDx4 or FDx150, respectively), all at 0.5 mg/ml. After various incubation times to allow pinocytosis, dishes were drained and immersed into 2 × 1 l PBS (137 mM NaCl, 3 mM KCl, 6 mM Na_2HPO_4, 1 mM KH_2PO_4) plus 1 mg/ml BSA, and then 1 l PBS, all at 4°C for 5 min each. Dishes were then drained and the cells lysed in 0.5 ml lysis buffer containing 0.1% Triton X-100 and 50 mM Tris, pH 8.5. For pulse-chase experiments, the washed wells were incubated in warm Ringer's saline without BSA for variable times, and then wells were drained and the cells lysed with Triton X-100. The fluorescence of the lysates was measured using an SLM/Aminco 500C spectrofluorometer. LY was measured at excitation 430 nm and emission 580 nm, and fluorescein was measured at excitation 495 nm and emission 514 nm. These wavelengths allowed selective measurement of the two fluorophores; as evidenced by the fact that when their concentrations were similar, each fluorophore gave less than 5% signal at the other fluorophore's wavelengths. Protein was measured using the BCA assay (Pierce Chemical Co., Rockford IL).

Dextran Mixing

Delivery of fluorophores into macropinosomes was measured microscopically. Macrophages plated onto coverslips were incubated 1 min with RB containing 1 mg/ml Texas red dextran, average mol wt 10,000 (TRDx10) plus 1,000 U/ml recombinant human macrophage colony-stimulating factor (M-CSF). Cells were then washed and chased for a defined but variable interval (I_{TF}), pulsed 1 min with fluorescein dextran, average mol wt 10,000 (FDx10) plus M-CSF in RB, washed again, and incubated for another variable interval in RB (I_F). Cells were fixed as described previously (Swanson, et al., 1992), and then coverslips were washed and assembled for microscopy in glycerol plus phenylenediamine. For each coverslip, 50 TRDx-positive macropinosomes were scored for the presence of FDx, and 50 separate FDx-positive macropinosomes were scored for the presence of TRDx. On any given day, 8–12 such preparations would be obtained. The cumulative data from 200 separate experimental measurements were pooled.

To examine the size dependence of dextran mixing, we measured the ability of different sizes of FDx to reach TRDx-labeled macropinosomes. Afferent movement of FDx, from younger organelles into older macropinosomes, was measured by pulse-labeling cells 1 min with TRDx10 plus M-CSF, washing and chasing for 1–3 min in RB, and then pulse-labeling cells again with FDx10 or FDx150 (average mol wt 150,000), washing and chasing for 1–3 min. The total chase time after the TRDx10 pulse (I_T) was always 5 min, and the chase following the FDx pulse (I_F) varied. Cells were fixed and examined as described above, scoring 50 TRDx-positive macropinosomes for the presence of FDx. Efferent movement of FDx, from older organelles into younger macropinosomes, was measured by pulse-labeling cells 1.5 min with FDx10 or FDx150, washing and chasing for variable periods, then pulse-labeling cells 1 min with TRDx10 plus M-CSF and chasing for 5 min before fixing and scoring 50 TRDx-positive macropinosomes for the presence of FDx. Here, too, I_T was constant but the total chase interval following the FDx pulse (I_F) was variable. Figs. 3 *A* and 4, *A* and *C* show these experimental protocols schematically.

To measure efferent movement from phagolysosomes to late endosome-like macropinosomes, macrophages were allowed to phagocytose sheep erythrocytes that contained FDx. Sheep erythrocytes were loaded by osmotic rupture with FDx4, FDx10, or FDx150 using methods described previously (Knapp and Swanson, 1990). The resealed ghosts were then opsonized with rabbit anti-sheep erythrocyte IgG, resuspended in RB, and presented to macrophages at 4°C for 5 min to allow binding. Cells were warmed to 37°C to allow phagocytosis and intracellular delivery to lysosomes. At various times afterward these cells were pulse-labeled 1 min with TRDx10 and M-CSF, washed, and chased 5 min, and then fixed for microscopic determination of FDx in TRDx-positive macropinosomes.

Dextran Segregation

To measure dextran segregation after phagocytosis, sheep erythrocytes were loaded by osmotic rupture with 4 mg/ml TRDx10 and either FDx10 or FDx150, and then opsonized with rabbit anti-sheep erythrocyte IgG, resuspended in RB, and presented at 4°C to macrophages to allow binding. Cells were warmed to 37°C to allow phagocytosis and were then fixed at various intervals thereafter. They were examined by confocal fluorescence microscopy to determine the distributions of TRDx and FDx. Images were displayed as dual color fluorescence in order to examine coincident distributions of fluorophores.

To observe segregation of fluid-phase tracers, macrophages on coverslips were incubated with mixtures of TRDx10 or TRDx70 plus FDx10 or FDx70, each at 1 mg/ml in RB. After 30 min, cells were washed several times with RB, incubated another 60 min in RB, and then fixed and observed by confocal fluorescence microscopy as described above.

Materials

Female, C3H-HeJ mice were obtained from Jackson Laboratories (Bar Harbor, ME). Human recombinant macrophage colony-stimulating factor (M-CSF) was a generous gift of Genetics Institute (Cambridge, MA). TRDx10 was obtained from Molecular Probes (Eugene, OR). Fluorescein dextrans were purchased from Sigma Chem. Co. (St. Louis, MO), and were further size-fractionated by gel permeation chromatography (Sephadex G25 or Sephacryl S-300, Pharmacia-LKB Biotechnology, Piscataway, NJ), to remove low molecular weight contaminants. All other reagents were purchased from Sigma Chem. Co., unless otherwise indicated.

Results

Differential Movements of Fluorescein Dextran and Lucifer Yellow

If movement through endocytic compartments were size-selective, then different sizes of probes for fluid-phase pinocytosis should display different kinetics of accumulation in macrophages. To compare the kinetics of two different sizes of solute probe, macrophages were incubated with a mixture of fluorescein dextran, average mol wt 150,000 (FDx150) and lucifer yellow, mol wt 460 (LY), and after various intervals, cells were lysed to measure accumulation of FDx150 and LY.

The kinetics of pinocytosis were different for the two probes. The initial rate of accumulation, a measure of fluid influx, was greater for LY than for FDx150, indicating that it was entering cells more efficiently (Fig. 1 *A*). Accumulation of both probes decreased within an hour to lower rates

1. *Abbreviations used in this paper*: FDx, fluorescein-labeled dextran; LY, lucifer yellow; M-CSF, macrophage colony-stimulating factor; TRDx, Texas red-labeled dextran.

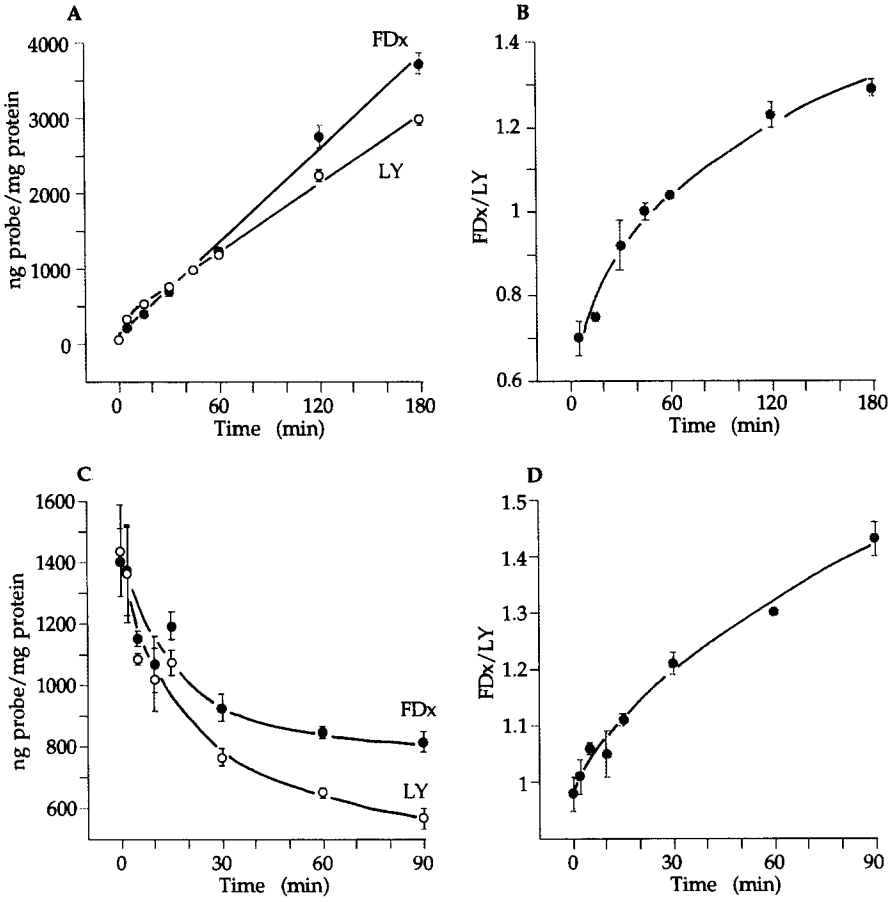

Figure 1. Fluid phase solute accumulation and efflux in macrophages. Accumulation (*A* and *B*): macrophages were incubated with a mixture of 0.5 mg/ml FDx150 and 0.5 mg/ml LY for the indicated times before washing and lysis to measure cell-associated fluorescence. (*A*) At early time points, LY accumulation (*open circles*) exceeded FDx150 accumulation (*closed circles*), but the long-term, linear rate of LY accumulation was less than that of FDx150. (*B*) Cellular content of FDx150 relative to LY increased with incubation time, indicating greater cellular retention of FDx150. Efflux (*C* and *D*): macrophages were incubated 30 min with a mixture of FDx150 and LY, and then were washed and incubated in buffer without fluorophores. At the indicated times, buffer was removed and cells were lysed for measurement of residual cell-associated fluorescence. The loss of LY was greater than that of FDx150 (*C*), and the ratio of FDx150 to LY increased with incubation time (*D*). Similar results were obtained on three separate occasions; bars indicate mean ± SD.

that were maintained for many hours thereafter. Earlier work showed that these linear rates represent net accumulation after solute influx and efflux (Besterman et al., 1981; Swanson et al., 1985). Despite the greater rate of LY influx, its linear, long-term rate of accumulation was less than that of FDx150 (Fig. 1 *A*). This indicated that, compared to LY, a greater percentage of the internalized FDx150 was remaining inside the cells.

The different rates of accumulation were more clearly evident when the data were replotted as the ratio FDx/LY (Fig. 1 *B*). At early time points, the ratio was less than one, reflecting the more efficient influx of LY. The continual increase in the ratio to values greater than one meant that FDx was retained more efficiently inside macrophages than LY was.

One way that FDx150 could enter macrophages less efficiently than LY, but accumulate at a greater rate, would be if its rate of efflux were substantially less than that of LY. We compared efflux of FDx150 and LY after preloading macrophage endocytic compartments with a mixture of the two fluorophores. LY efflux exceeded FDx150 efflux (Fig. 1 *C*). Here again, the ratio FDx/LY increased with incubation time, indicating that LY recycled to the medium more efficiently than did FDx150 (Fig. 1 *D*).

It was possible that the differential accumulation of LY and FDx150 reflected a selective adsorption of one or the other probe to intracellular membranes. To establish that both influx and efflux of these fluorophores were bona fide fluid phase solute movements, we compared the accumulation and efflux at different probe concentrations. As shown in Fig. 2, accumulation and efflux of both molecules were directly proportional to probe concentration, indicating that they behave as probes of fluid-phase solute movement. Consistent with the data of Fig. 1, less FDx150 than LY recycled during the chase period (16 vs 34%, respectively). Most importantly, the ratio FDx/LY increased uniformly, changing from 1.0 to 1.4 at all concentrations of probe added (Fig. 2 *C*). Thus, the differential retention of the two probes showed no adsorptive or saturable component.

As an additional measure of solute size effects on fluid-phase endocytosis kinetics, we compared LY with a smaller FDx, average mol wt 4,000 (FDx4), using the protocol of Fig. 2. A similar concentration dependence for accumulation and efflux were obtained, indicating that FDx4 was also behaving as a fluid-phase solute marker. Moreover, FDx4 recycled slightly more efficiently than FDx150 (Fig. 2 *D*), showing again that the efficiency of recycling depended on the size of the probe used to measure fluid-phase endocytosis. This result is similar to that of Buckmaster et al. (1987), who observed a molecular size-dependent efflux of solute probes from CHO cells.

Size-Selective Communication between Compartments

Macrophages internalize solutes very rapidly, taking in the equivalent of their cell volume every 2 h (Steinman et al., 1976; Swanson et al., 1985). This indicates correspondingly high rates of endosome fusion and fission reactions. A slight bias, related to molecular size, in the efficiency of content

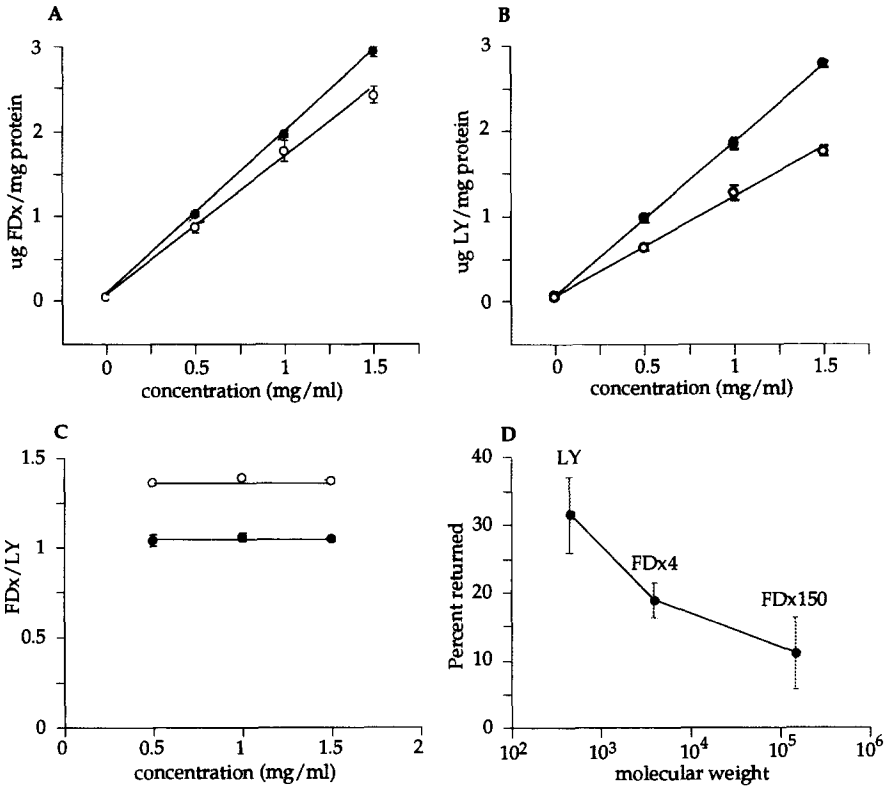

Figure 2. FDx and LY report fluid phase solute movements for both accumulation and efflux. (*A–C*) Macrophages were incubated with mixtures of FDx150 and LY at the indicated concentrations for 60 min (*closed circles*) or 60 min plus 60 min chase (*open circles*). For both FDx150 (*A*) and LY (*B*), the cell-associated fluorescence was proportional to the concentration of fluorophore provided to the cells. (*C*) Replotting the data of *A* and *B* to indicate the ratio of FDx to LY shows that the differential loss of the two probes was not due to adsorptive (i.e., concentration dependent) retention of either fluorophore. *A–C* show one representative experiment; data are mean and SD of triplicate measurements. (*D*) Cumulative data from six such experiments (LY, $n = 6$; FDx4, $n = 3$; FDx150, $n = 3$) indicate that the percent of fluorophore returned to the medium during the 60-min chase period is inversely related to probe molecular weight. Bars indicate mean ± SD.

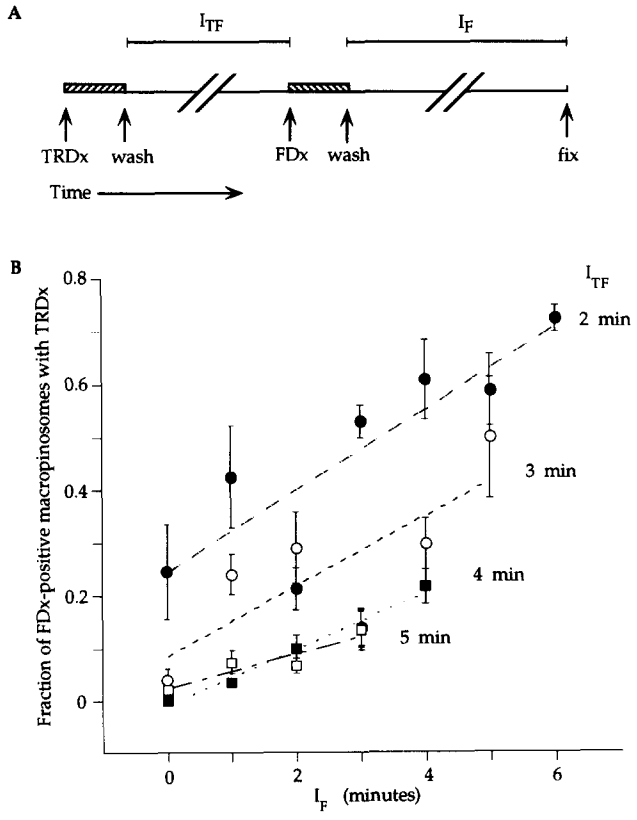

Figure 3. Organelles of similar age exchange contents continuously. Experimental design (A): macrophages on coverslips were incubated 1 min with TRDx10 plus M-CSF, washed and incubated for a variable period without fluorophore (I_{TF}), incubated 1 min with FDx10 plus M-CSF, washed, and incubated for another variable period (I_F) before fixation and scoring by fluorescence microscopy. For each coverslip, 50 FDx-positive macropinosomes were scored for the presence of TRDx. Cumulative data (*B*): As the interval between pulses (I_{TF}) decreased, the rate of content mixing during the second interval (I_F) increased. For $I_{TF} = 2$ min (*closed circles*), the slope was 0.08 per minute; for $I_{TF} = 3$ min (*open circles*), 0.06 per minute; for $I_{TF} = 4$ min (*closed squares*), 0.05 per minute; for $I_{TF} = 5$ min (*open squares*), 0.03 per minute. For each point shown, $n = 3$ or 4 coverslips measured; bars indicate mean ± SEM.

transfer during these reactions could, with repetition, lead to a mechanism for molecular fractionation. With this in mind we asked, first, how much content mixing occurs between endocytic organelles and, second, is there a size dependence to this process?

Content exchange between organelles was measured using macropinosomes as indicators. Macropinosomes are large endocytic vesicles formed as a consequence of cell surface ruffling. In macrophages, these can be induced by addition of M-CSF (Racoosin and Swanson, 1989). Briefly pulsing cells with fluorescent dextrans during macropinocytosis allows labeling of a cohort of vesicles that can be traced morphologically by fluorescence microscopy (Racoosin and Swanson, 1993). Using pulse-chase regimens with two distinguishable probes one can mark and identify specific age groups of organelles and monitor their interactions. For example, in Fig. 3 *A*, macrophages were pulsed with TRDx, chased, pulsed with FDx, chased again, fixed, and observed by fluorescence microscopy. If there were no mixing, or if mixing occurred only between organelles of exactly the same age, one would see two distinct fluorescently labeled com-

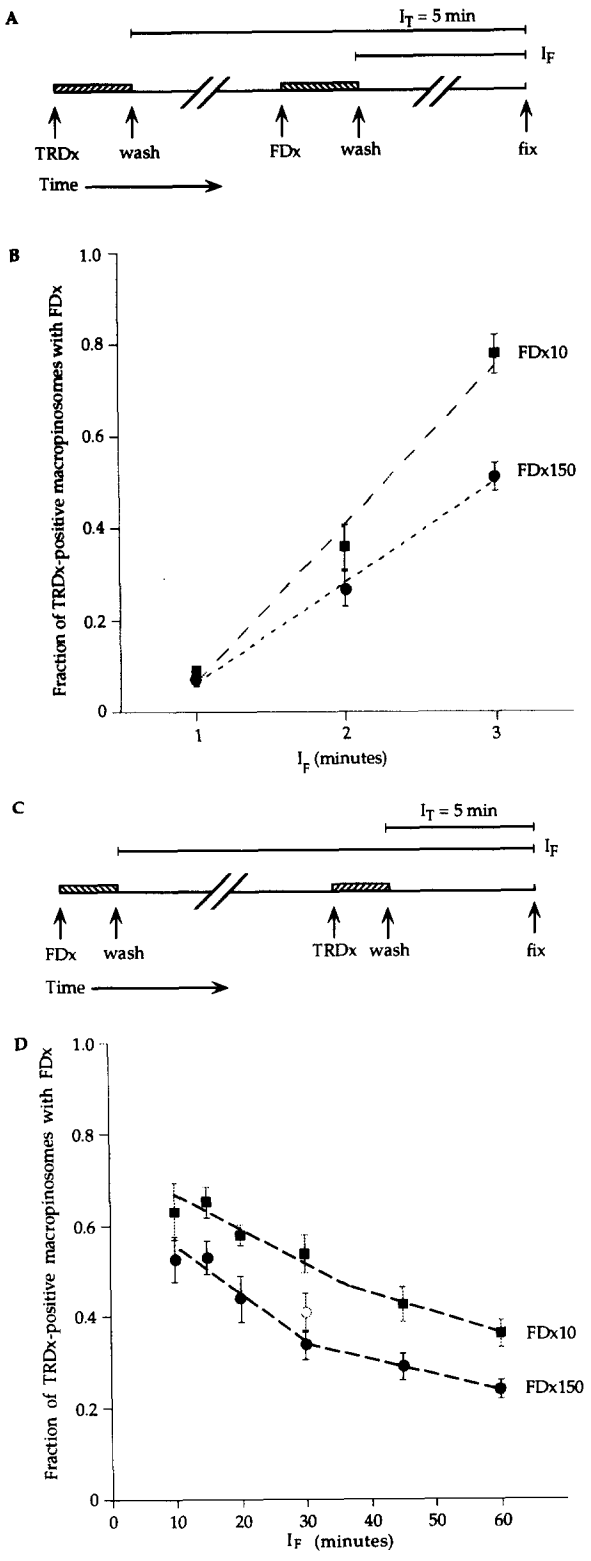

Figure 4. Size-dependent exchange of dextrans between compartments. Movement into older macropinosomes (*A* and *B*): macrophages were incubated 1 min with TRDx10 plus M-CSF, were washed and chased, incubated with FDx10 or FDx150 for 1 min, washed and chased again before fixation. In all conditions, the interval between the removal of TRDx10 and fixation (I_T) was 5 min; whereas the interval I_F was variable. For each coverslip, 50 TRDx-positive macropinosomes were scored for presence of FDx. FDx150 (*closed circles*) labeled these macropinosomes less readily than did FDx10 (*closed squares*). Movement into younger macropinosomes (*C* and *D*): macrophages were incubated 1.5 min with FDx10 or FDx150, were washed and chased for a variable period, incubated with TRDx10 plus M-CSF for 1 min, washed, and chased for 5 min, and then fixed and scored as in *A* and *B*. FDx150 moved less readily than FDx10 from older organelles into the 5-min macropinosomes. For data shown in solid symbols, cells were provided the same concentration of FDx (normalized to fluorescence); the open circle indicates the mixing when the concentration of added FDx150 was three times more fluorescent than the FDx10. Bars indicate mean ± SEM.

partments, with no macropinosomes containing both dyes. But if the contents of an older endosome or macropinosome could get its contents into a younger macropinosome (efferent movement), a percentage of FDx-positive macropinosomes would also contain TRDx. Conversely, if a newly formed endosome could deliver its contents to an older macropinosome (afferent movement), a percentage of TRDx-positive macropinosomes would also contain FDx. The dynamics of the organelle interactions would then be obtainable by varying the chase intervals.

The cumulative data from many measurements indicated extensive communication between macropinosomes and other endocytic compartments, including both older and younger organelles. To score efferent movement, for each experimental condition we located 50 phase-bright, FDx-positive macropinosomes and scored each for the presence of TRDx (Fig. 3 *B*). For afferent movement, we located 50 phase-bright, TRDx-positive macropinosomes and scored each for the presence of FDx (data not shown). The plots of these data were very similar, indicating that both methods of scoring indicated considerable mixing between organelles of similar age. The shorter the interval between pulses, I_{TF}, the higher the rate of content mixing during the subsequent chase period. This meant that macropinosomes exchanged contents best with other endocytic organelles of similar age, but that limited exchange also occurred with organelles of different ages. The small but measureable exchange of dextrans between organelles of different ages could provide routes for both afferent and efferent solute movement through the system. This high rate of mixing contrasts with the observations of Hewlett et al. (1994), who observed very little mixing between macropinosomes and other organelles inside fibroblasts, using FDx, average mol wt 70,000 (FDx70). It may be that they saw little mixing because they were using a larger probe (see below); or it may be that in fibroblasts macropinosomes communicate with other organelles less than they do in macrophages.

We next examined the size-selective movement of contents between organelles by measuring delivery of FDx from pulse-labeled compartments of different ages into "5 minute" macropinosomes containing TRDx10 (I_T = 5 min; Fig. 4, *A* and *C*). Earlier studies indicated that 5-min macropinosomes have many features of late endosomes: they contain rab7 and lysosomal membrane glycoprotein A (lgpA), but do not yet contain significant levels of the lysosomal hydrolase cathepsin L or of TRDx delivered from preloaded lysosomes (Racoosin and Swanson, 1993). Therefore, we could ask how efficiently different sizes of FDx moved from endosomes or lysosomes into these late-endosome–like macro-

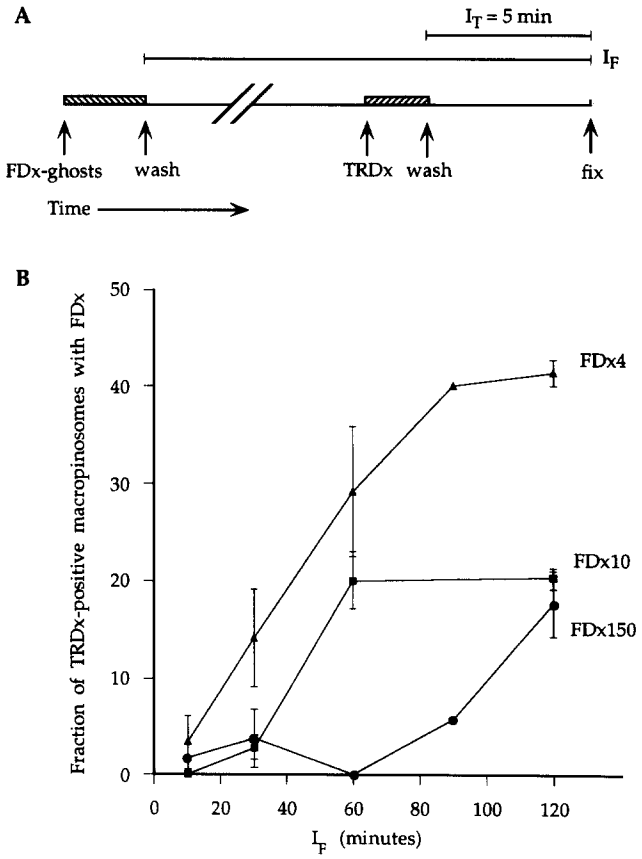

Figure 5. Efferent movement of FDx from phagolysosomes into macropinosomes. (*A*) Macrophages were allowed to bind opsonized sheep erythrocytes that had been loaded with FDx4, FDx10, or FDx150. At various intervals after warming, cells were pulse-labeled for 1 min with TRDx10 and M-CSF, washed and chased for 5 min, and then fixed and observed by fluorescence microscopy. The fraction of TRDx10-positive macropinosomes containing FDx indicated retrograde movement of FDx from phagolysosomes to earlier compartments. (*B*) Initially after phagocytosis few macropinosomes labeled with FDx of any size, as most of it was still contained within intact erythrocytes. As FDx was released, macropinosome labeling increased, indicating that efferent movement of FDx occurred via the organelles of afferent movement. This efferent movement was dependent on the size of the FDx.

pinosomes, both in the afferent direction, from younger organelles into older ones, and in the efferent direction, from older organelles into younger ones.

To compare the afferent movements of large and small FDx, cells were pulse-labeled with TRDx10 then either FDx150 or FDx10 (protocol in Fig. 4 *A*). All TRDx-positive macropinosomes were 5-min-old when fixed; and the FDx-positive organelles were younger. In all of the mixing experiments, labeling of macropinosomes was a cumulative process, in that a macropinosome could receive fluorescent dextran at any point during the second chase period. In this experiment, the variable lengths of the second chase, I_F, meant that different periods were available for fusion. Nonetheless, it was clear that for each pulse-chase regimen FDx10 moved more readily between compartments than did FDx150 (Fig. 4 *B*). It should be noted parenthetically that the low level of mixing observed at I_F = 1 min demonstrates that the washing procedures were sufficient to provide clean experimental separation of the pulses.

We next measured the size dependence of efferent movement, from variously preloaded compartments into the 5-min macropinosomes (protocol in Fig. 4 *C*). Here the second chase period, I_T, was always 5 min, so the periods for cumulative interaction were constant. With intermediate chase intervals of I_F (10–20 min), there was considerable delivery of FDx into TRDx-positive macropinosomes. As I_F increased, the first pulse moved further into the lysosomal compartment, and labeling of TRDx-positive macropinosomes with FDx from these compartments decreased. For all chase intervals, however, movement from older compartments into the younger macropinosomes was more efficient with FDx10 than with FDx150 (Fig. 4 *D*).

It was possible that the FDx10 and FDx150 had intrinsically different fluorescence quantum yields, and the differences observed were simply a result of FDx150 being less fluorescent than FDx10. To address this, the concentrations of FDx10 and FDx150 added to cells had been adjusted using a fluorometer, to obtain final solutions that were equally fluorescent. As an additional control for such fluorescence dominance effects, we added in one set of experiments a threefold higher concentration of FDx150 than FDx10 (i.e., 3× more fluorescent), yet still observed less efferent movement of the larger dextran (*open circle*, Fig. 4 *D*).

Recycling from Lysosomes

Although it was evident from the results of Fig. 4 *D* that efferent and afferent solute traffic could occur via the same organelles, this did not necessarily indicate a route of recycling from lysosomes. An alternative explanation of that figure would be that organelles fuse efficiently with others of similar age, and the mixing observed at longer I_F periods simply reflected a slow clearance of FDx from intermediate compartments into lysosomes. That is, the mechanism of solute delivery to lysosomes could be such that a pulse of FDx would spread into all endocytic compartments and clear slowly, forming a tail of FDx in the late endosomes. The mixing observed in Fig. 4 *D* would not then represent efferent movement from lysosomes, but rather continued fusion with similarly aged organelles.

As a more direct measure of efferent movement, we examined the redistribution of fluorophore released from inside phagolysosomes. Macrophages were allowed to phagocytose opsonized sheep erythrocytes that had been osmotically loaded with FDx of various sizes. At various times after phagocytosis, cells were pulse-labeled with TRDx10 and M-CSF, and then chased for 5 min and fixed (Fig. 5 *A*). During the chase intervals, FDx was released into the lumen of the phagolysosome after lysosomal hydrolases degraded the erythrocyte membranes. This FDx was still restricted to the lumen of the endocytic compartments, but was otherwise free to move into any communicating organelles, or to leave the cell via recycling vesicles. Thus, an increase in the fraction of TRDx-positive, 5-min macropinosomes that contained fluorescein indicated efferent movement, from phagolysosomes toward earlier compartments. At short intervals I_F, before significant release of FDx had occurred, few TRDx-positive macropinosomes contained FDx. As FDx was released, however, macropinosome labeling increased, and the rate and extent of this increase were size dependent

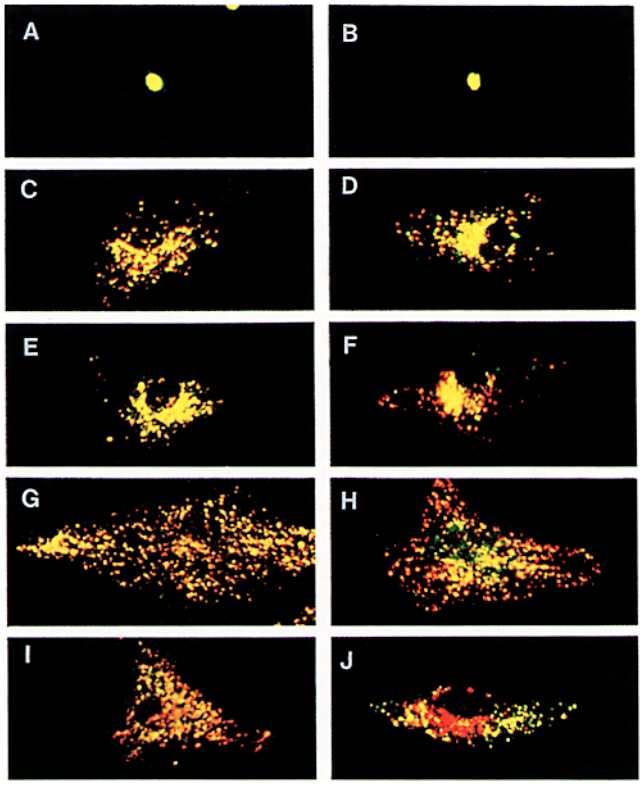

Figure 6. Size-dependent segregation of dextrans within phagolysosomes. (*A–F*) Macrophages were allowed to phagocytose opsonized sheep erythrocytes loaded with TRDx10 plus either FDx10 (*A*, *C*, and *E*) or FDx150 (*B*, *D*, and *F*). At various intervals after phagocytosis, cells were fixed and observed by fluorescence confocal microscopy. 5 min after phagocytosis, TRDx and FDx distributions coincided (yellow-orange phagosomes, *A* and *B*). At 60 and 120 min after phagocytosis, the FDx150 and TRDx10 appeared sometimes in separate red or green compartments (*D* and *F*), while the TRDx10 and FDx10 distributions remained coincident (*C* and *E*). (*G–J*) Size-dependent segregation of dextrans after pinocytosis. Macrophages were incubated with mixtures of dextrans for 30 min, and then were washed and incubated 60 min in unlabeled buffer before observation of dextran distributions by confocal fluorescence microscopy. (*G*) TRDx10 and FDx10, (*H*) TRDx10 and FDx70, (*I*) TRDx70 and FDx70, and (*J*) TRDx70 and FDx10. Mixtures of different-sized dextrans segregated into different organelles, whereas mixtures of similar-sized dextrans did not segregate.

(Fig. 5 *B*). Thus, the molecules released inside phagolysosomes moved out of the macrophages through the same organelles that were carrying solutes into the cell.

In summary, the mixing experiments of Figs. 4 and 5 indicate that the efferent flow of fluid-phase solutes occurs via the same organelles that carry solutes in the afferent direction, and that movement in both directions is size dependent.

Segregation of Dextrans after Delivery into Lysosomes

The processes of size-dependent solute movement could fractionate uniform mixtures of different-sized dextrans. This was best demonstrated by allowing macrophages to phagocytose sheep erythrocytes loaded with mixtures of TRDx10 plus either FDx10 or FDx150 (Fig. 6). Before and just after phagocytosis, these erythrocytes appeared by confocal fluorescence microscopy as large, yellow-orange particles, indicating the uniform mixture of TRDx and FDx inside these phagosomes (Fig. 6, *A* and *B*). Remarkably, one hour after phagocytosis, macrophages that had phagocytosed erythrocytes loaded with different-sized dextrans (TRDx10 and FDx150) contained some organelles with only TRDx10 (red), some with only FDx150 (green) and many that still contained both (Fig. 6 *D*). This segregation persisted 2 h after phagocytosis (Fig. 6 *F*). In contrast, the cells presented similar-sized probes contained primarily yellow-orange lysosomes (Fig. 6, *C* and *E*). Thus, the dextrans could be segregated by size after delivery to the lysosomes.

We also observed segregation after fluid-phase endocytosis. Macrophages were allowed to pinocytose mixtures of fluorescein- and Texas red–labeled dextrans, then were incubated for 1 h before observation by confocal fluorescence microscopy. When the dextrans were different sizes, FDx70 plus TRDx10, or FDx10 plus TRDx70, distinctly labeled organelles could be observed (Fig. 6, *H* and *J*). In contrast, no such segregation was observed when dextrans were the same size, FDx10 plus TRDx10, or FDx70 plus TRDx70 (Fig. 6, *G* and *I*). This indicates that the segregation was related to dextran size and not to some chemical feature of the fluorophores employed.

Discussion

We have identified a novel feature of molecular traffic through macrophage endocytic compartments. Unlike larger molecules that move primarily to lysosomes, small molecules contained in endocytic vesicles may diffuse both upstream and downstream between endosomes and lysosomes. Most remarkably, the differential movements of large and small solutes through endocytic compartments can fractionate them by size into separate organelles.

This molecular sorting occurs with no signals in the structure of the molecules other than their size. Size selectivity appears at several levels of endocytic traffic: during influx, en route to lysosomes, and within the lysosomal compartment. We suggest that the different paths taken by different sizes of macromolecules through those compartments results from an iterative fractionation process involving high rates of fusion and fission between vesicles. By such a mechanism, a small size bias in the efficiency of transport within and through endocytic organelles, repeated many times, could segregate solutes of different sizes. The phenomenon itself tells us something important about the normal mechanisms of endocytic vesicle traffic. It also has implications for related processes, such as Golgi vesicle traffic and antigen presentation.

Possible Mechanisms for Sorting by Size

Molecular size–fractionation could be achieved by amplifying a size-dependent bias in the ability of molecules to move within or between vesicular organelles. A small difference could become an efficient mechanism of segregation if the process occurred repeatedly and frequently inside the cell. This is essentially a chromatographic mechanism, most similar to gel-permeation chromatography. Similar iterative fractionation mechanisms have been suggested for receptor traffic within endosomes (Dunn et al., 1989).

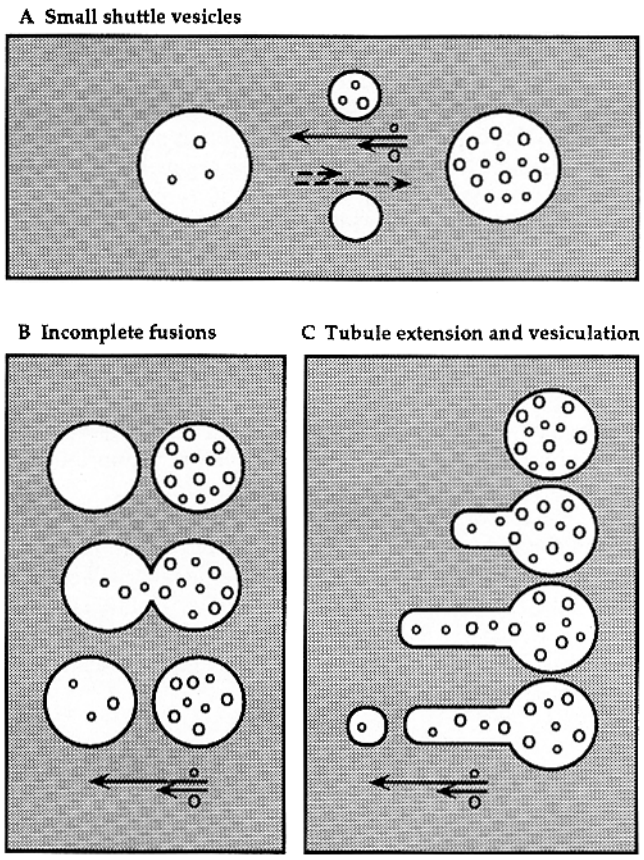

Figure 7. Possible mechanisms for size-selective molecule movement through endocytic compartments, based on the idea that organelle traffic or communication transfers small molecules from one compartment to another more readily than large molecules. Each panel shows how small molecules (o) could transfer better than large ones (O). (*A*) Vesicles mediating organelle traffic could have a larger capacity for small molecules, in that small molecules have access to more of the organelle lumen than large molecules do. Small vesicles would therefore carry more small molecules per transfer event. (*B*) Organelle communication could involve transient and incomplete fusions between organelles, with narrow pores for communication that allow differential transfer of macromolecules from one lumen to another. (*C*) Extension into tubules, perhaps on cytoplasmic microtubules, could create long, narrow lumens along which macromolecules would diffuse. This diffusion could create transient, molecular size–dependent concentration gradients that would deliver small molecules to the distal tip more quickly than large molecules. Vesicle formation at that distal tip would then lock in the concentration difference.

It is possible that there are size-selective molecular filters along the endocytic pathway that exclude molecules larger than a threshold size. However, for the size range of dextrans used here, there was no apparent size cutoff for movement through these compartments. Rather, the larger the molecule, the less it exchanged with other compartments. It seems more likely to us that the fractionation is a consequence of a molecular size–dependent bias in organelle communication.

There are several ways to create such a bias (Fig. 7). First, the relative carrying capacity of vesicles for macromolecules will decrease as vesicle diameter decreases, such that small vesicles provide less volume to large molecules than to small molecules. When the position of a molecule is indicated by its center of mass, that molecule can get no closer to the wall of an enclosing vesicle than its own molecular radius. As the molecular radius, r_s, approaches the radius of a spherical vesicle, r_v, the space that can be occupied by that molecule, $4\pi(r_v-r_s)^3/3$, becomes measurably less than the lumenal volume of the vesicle, $4\pi r_v^3/3$. By such calculations, molecules such as BSA or FDx70 (r_s = 34 Å) would have access to only 81% of the lumen of a 100-nm diameter spherical vesicle and 65% of a 50-nm vesicle. Smaller molecules would have access to a greater portion of vesicle volume than would large molecules, and this differential access would provide a differential carrying capacity for small vesicles. The carrying capacity of very large vesicles would not be much affected by molecular size, but most cellular organelles are small enough that the size of their molecular contents should affect their capacity. Hence, small vesicles should transport small molecules more efficiently than large molecules (Fig. 7 *A*). These effects would also be modulated to some extent by the geometries of the vesicles as they form.

For endocytosis, such a bias could operate during both influx and efflux. Coated vesicles are small enough that they could provide measurably less accessible volume to FDx150 than to LY. Consequently, 1,000 100-nm-coated vesicles formed per minute would carry 80% less FDx than LY from extracellular medium containing an equal mixture of the two molecules. If vesicles that return membrane and fluid to the cell surface were smaller than the vesicles carrying membrane into the cell, the larger probe would have a greater disadvantage on the efferent route than on the afferent route. Hence, FDx150 would accumulate more than LY.

This physical constraint should also affect transport through the Golgi network, where movement from one cisterna to another is thought to occur by 60-nm shuttle vesicles (Rothman and Orci, 1992). The rate of transport through the Golgi via these vesicles would necessarily be less for large soluble macromolecules than for small ones because there must be less room for them in each vesicle. The rate of bulk flowthrough the Golgi was measured using tripeptides (Wieland et al., 1987). It has been argued that because some macromolecules move more slowly through the Golgi, they must contain retention signals in their primary structure (Rothman and Orci, 1992). An alternative explanation would be that macromolecules move through more slowly simply because they are larger than tripeptides; their large size limits the volume available to them inside shuttle vesicles and consequently limits the number of molecules that can be carried per shuttle event.

A second mechanism for creating a size bias during endocytic traffic could occur if organelle fusions permitted only partial exchange of organelle contents (Fig. 7 *B*). Others have observed that organelle identities are not entirely lost when two vesicles fuse together. Willingham and Yamada (1978) described the interactions of lysosomes with macropinosomes as a kind of feeding frenzy, with the smaller lysosomes contacting and apparently tearing off portions of the shrinking macropinosome. They aptly named the process "pyrranhalysis". Their observations indicate that fusion between organelles can occur by docking and formation of a fusion pore, a joining of the two lipid bilayers with a narrow, aqueous connection between the two lumens, followed by disengagement of the two organelles into discrete compart-

ments. A transient, aqueous fusion pore could permit size-selective exchange of macromolecules, acting as a partial barrier to diffusion. Such a mechanism is consistent with our observations that movement between organelles is size-selective in both afferent and efferent directions.

Finally, a molecular size bias could be created in endocytic organelle traffic by including a size-restrictive diffusion along tubular organelles (Fig. 7 *C*). Endosomes and lysosomes are often narrow tubulovesicular organelles that move along cytoplasmic microtubules via microtubule-associated motors such as kinesin and cytoplasmic dynein (Matteoni and Kreis, 1987; Hollenbeck and Swanson, 1990). Tubules can form rapidly in these processes. Although it has not been measured, we expect that macromolecule diffusion through the lumens of these narrow compartments (50–75-nm diameter; Luo and Robinson, 1992; Swanson et al., 1987) is size dependent. Consequently, rapid tubule extension along microtubules might first set up molecular size–dependent diffusion gradients inside the tubule lumen, and then excision of the tubule or vesicle at the distal end would lock in the concentration differences at that end of the lumen.

Any combination of these biasing mechanisms could create small molecular concentration gradients within an organelle lumen just before fission divides it into two organelles. Frequent repetition of biased fission and fusion events, together with a process that gradually changes the specificity of the fusion partners, could produce the size fractionation observed here.

Implications for Constitutive Endocytic Traffic of Macromolecules

Small solutes can move relatively freely through endocytic compartments in both afferent and efferent directions. Movement in both directions through the same organelles shows that solutes can recycle from lysosomes to the cell surface without requiring transport vesicles dedicated to that route. As endocytic vesicles of slightly different ages continually exchange their contents, the overlapping specificity of their interactions will provide an indirect route out of the cell.

The size selectivity of this process is important. Influx, movement between compartments, and efflux were all less efficient for large molecules than for smaller ones. This shows that in constitutive endocytosis, large molecules move directly toward lysosomes, but hydrolytic fragments derived therefrom may move out of the lysosomes into endosomes; and then out of the cell or back again into lysosomes. Eventually, molecules that are not recycled or degraded completely will accumulate in lysosomes, perhaps in less active organelles such as residual bodies.

Regardless of their size, a portion of the solutes internalized by endocytosis accumulate in lysosomes, and if the solute is undegradable, this accumulation continues indefinitely (Swanson, 1989). Indefinite linear accumulation, without approaching a steady state of zero net flux, indicates that fluid movement through endocytic compartments is asymmetric; with more solute molecules entering cells than can leave. We have suggested elsewhere that this asymmetry could be sustained if the vesicles carrying fluid into the cell were larger, or had a lower surface-to-volume ratio, than the vesicles recycling membrane and fluid to the extracellular medium (Swanson, 1989). By this arrangement, membrane movement could be balanced in the afferent and efferent directions, but a portion of the fluid volume would remain inside the cell. The water of this excess volume would leave across membranes, but impermeant and undegradable solutes would accumulate in lysosomes.

The evidence presented here supports this model. The fact that larger solute probes of endocytosis accumulate in cells at a higher rate than small probes indicates that, overall, efferent vesicles are smaller than afferent vesicles. Accordingly, large probes enter influx vesicles less efficiently than small probes, but have an even greater disadvantage during efflux. Consequently, more of the large probe entering the cell remains inside (Fig. 1).

Segregation of macromolecules after delivery to lysosomes also indicates that fractionation can occur within that compartment alone. It has been shown that lysosomes inside cells fuse readily with each other (Ferris et al., 1987). Size-fractionation within lysosomes suggests that they must also divide at high rates. An active fragmentation process may be essential to maintaining organelle dimensions.

The Contribution of Solute Sorting to Antigen Presentation

Size-selective efferent movement may contribute to antigen processing and presentation, in that it provides a mechanism whereby endosomes could receive antigenic peptide fragments from lysosomes without significant recycling of larger lysosomal enzymes or proteins. Harding and colleagues have shown that class II MHC molecules associate with antigen in macrophage endocytic compartments, in phagosomes and in organelles termed sacs (probably macropinosomes) (Harding et al., 1990), in association with tubular lysosomes (Harding and Geuze, 1992). They have also shown that degradation of protein to antigenic peptides occurs in lysosomes (Harding et al., 1991). It has remained a puzzle how the peptides generated in lysosomes return to the sacs for association with MHC molecules, without also returning the contents of the lysosomes.

Other kinds of compartments for antigen-presentation have now been identified, principally in B-cells (Amigorena et al., 1994; Tulp et al., 1994). These are like endosomes, but are more removed from the bulk of fluid-phase traffic. Here, too, it is not known how peptides are delivered into these compartments from degradative organelles.

According to the mechanism identified here, a membrane protein residing in an endosome, such as MHC class II, would be continually exposed to small solutes, moving both toward the lysosomes and back toward the plasma membrane. Peptides generated in lysosomes would move readily into endosomes, where they could either associate with resident membrane proteins, recycle completely from the cell, or return again to the lysosomes and more complete degradation. Endosomal class II MHC would be continually exposed to the peptides moving through this system.

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