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)), 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 x 10⁶ and 3 x 10⁵ cells per well, respectively. Cultures were then incubated overnight in medium lacking M-CSF (DME-10%; 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 MgCl₂, 2 mM CaCl₂, 2 mM Na₃HPO₄, 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 x 11 PBS (137 mM NaCl, 3 mM KCl, 6 mM Na₂HPO₄, 1 mM KH₂PO₄) plus 1 mg/ml BSA, and then 11 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 spectrophotofluorometer. 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 on coverslips were incubated 1 min with RB containing 1 mg/ml Texas red dextran, average mol wt 10,000 (FDx10) 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 (Iₓ), 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 (Iₛ). 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. After movement of FDx, from younger organelles into older 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, Iₓ was constant but the total chase interval following the FDx pulse (Iₛ) was variable. Figs. 3 A and 4, B and C show these experimental protocols schematically.

To measure efficient 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 FDx10 or FDx150 using methods described previously (Knap and Swanson, 1990). The resolubilized 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 (Sephades 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.
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_r) 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_r) decreased, the rate of content mixing during the second interval (I_r) increased. For I_r = 2 min (closed circles), the slope was 0.08 per minute; for I_r = 3 min (open circles), 0.06 per minute; for I_r = 4 min (closed squares), 0.05 per minute; for I_r = 5 min (open squares), 0.03 per minute. For each point shown, n = 3 or 4 coverslips measured; bars indicate mean ± SEM.
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 ($I_f$) was 5 min; whereas the interval $I_r$ 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.
positive organelles were younger. In all of the mixing experiments, labeling of macropinosomes was cumulative rather than a result of fusion. An alternative explanation of that figure would be that organelles fuse efficiently with others of similar size, and the mixing observed at longer IF 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. 4D 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. 5A). 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 fluorescent 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 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 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, 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.
just after phagocytosis, these erythrocytes appeared by con-
phagocytose sheep erythrocytes loaded with mixtures of
ganelles that were carrying solutes into the cell. This was best demonstrated by allowing macrophages to
fractionate uniform mixtures of different-sized dextrans.

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Segregation of Dextrans after Delivery into Lysosomes
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phagocytose sheep erythrocytes loaded with mixtures of
TRDx10 plus either FDx10 or FDx150 (Fig. 6). Before and
just after phagocytosis, these erythrocytes appeared by con-
focal fluorescence microscopy as large, yellow-orange parti-
cles, indicating the uniform mixture of TRDx and FDx inside
these phagosomes (Fig. 6, A and B). Remarkably, one
hour after phagocytosis, macrophages that had phagocy-
tosed 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 per-
sisted 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 endocyto-
sis. Macrophages were allowed to pinocytose mixtures of
fluorescein- and Texas red-labeled dextrans, then were in-
cubated 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 J). 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 mole-
cules contained in endocytic vesicles may diffuse both up-
stream and downstream between endosomes and lysosomes.
Most remarkably, the differential movements of large and
small solutes through endocytic compartments can fraction-
ate them by size into separate organelles.

This molecular sorting occurs with no signals in the struc-
ture 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 compart-
ment. We suggest that the different pathways 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 mecha-
nism, 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 mecha-
nisms 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).
used here, there was no apparent size cutoff for movement than a threshold size. However, for the size range of dextrans more likely to us that the fractionation is a consequence of molecule, the less it exchanged with other compartments. It seems through these compartments. Rather, the larger the mole-
ment through endocytic compartments, based on the idea that or-
transfer event. (B) Organelle communication could involve tran-
sient 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 lu-
mens 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 mole-
cule, 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, \( r_m \), approaches the radius of a spherical vesicle, \( r_v \), the space that can be occupied by that molecule, \( 4\pi(r_v - r_m)^2/3 \), becomes measurably less than the lumenal vol-
figure 7. Possible mechanisms for size-selective molecule move-
ment through endocytic compartments, based on the idea that or-
gane 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 lu-
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ume of the vesicle, \( 4\pi r_v^2/3 \). By such calculations, mole-
cules such as BSA or FDx70 (\( r_v = 34 \text{ Å} \)) 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 mole-
cules (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 mem-
brane into the cell, the larger probe would have a greater dis-
advantage 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 be-
cause 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 explana-
ion 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 endo-
cytic 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 macro-
pinosomes as a kind of feeding frenzy, with the smaller lys-
somes contacting and apparently tearing off portions of the shrinking macropinosome. They aptly named the process "pyrranhalysis". Their observations indicate that fusion be-
 tween 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-

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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 macrophagosomes) (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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References

Amigorena, S., J. R. Drake, P. Webster, and I. Mellman. 1994. Transient accumulation of new class II MHC molecules in a novel endocytic compartment in B lymphocytes. Nature (Lond.). 369:113–120.

Besterman, J. M., J. A. Airhart, R. C. Woodworth, and R. B. Low. 1981. Exocytosis of pinocytosed fluid in cultured cells: kinetic evidence for rapid turn-over and compartmentation. J. Cell Biol. 91:716–727.

Buckmaster, M. J., D. Lo Briaco, A. L. Ferris, and B. Storrie. 1987. Retention of pinocytosed solute by CHO cell lysosomes correlates with molecular weight. Cell Biol. Intl. Rep. 11:501–507.

Dunn, K. W., T. E. McGraw, and F. R. Maxfield. 1989. Iterative fractionation of recycling receptors from lysosomally destined ligands in an early sorting endosome. J. Cell Biol. 109:3303–3314.

Ferris, A. L., J. C. Brown, R. D. Park, and B. Storrie. 1987. Chinese hamster ovary cell lysosomes rapidly exchange contents. J. Cell Biol. 105:2703–2712.

Germain, R. N. 1994. MHC-dependent antigen processing and peptide presentation: providing ligands for T lymphocyte activation. Cell. 76:287–299.

Harding, C. V., and H. J. Geuze. 1992. Class II MHC molecules are present in macrophage lysosomes and phagosomes that function in the phagocytic processing of Listeria monocytogenes for presentation to T cells. J. Cell Biol. 119:531–542.

Harding, C. V., D. S. Collins, J. W. Slot, H. J. Geuze, and E. R. Unanue. 1991. Liposome-encapsulated antigens are processed in lysosomes, recycled, and presented to T cells. Cell. 64:393–401.

Harding, C. V., E. R. Unanue, J. W. Slot, A. L. Schwartz, and H. J. Geuze. 1990. Functional and ultrastructural evidence for intracellular formation of major histocompatibility complex class II-peptide complexes during antigen processing. Proc. Natl. Acad. Sci. USA. 87:5553–5557.

Hewlett, L. J., A. R. Prescott, and C. Watts. 1994. The coated pit and macropinocytic pathways serve distinct endosome populations. J. Cell Biol. 124:689–703.

Hollenbeck, P. J., and J. A. Swanson. 1990. Radial extension of macrophage tubular lysosomes supported by kinesin. Nature (Lond.). 346:864–866.

Knapp, P. E., and J. A. Swanson. 1990. Plasticity of the tubular lysosomal compartment of macrophages. J. Cell Sci. 95:433–439.

Luo, Z., and J. M. Robinson. 1992. Co-localization of an endocytic marker and acid phosphatase in a tubular/reticular compartment in macrophages. J. Histochem. Cytochem. 40:93–103.

Matteoni, R., and T. E. Kreis. 1987. Translocation and clustering of endosomes and lysosomes depends on microtubules. J. Cell Biol. 105:1253–1265.

Racoczy, E. L., and J. A. Swanson. 1989. Macropinocytosis and the function of acceptor (rM-CSF) stimulates pinocytosis in bone marrow-derived macrophages. J. Exp. Med. 170:1635–1648.

Racoczy, E. L., and J. A. Swanson. 1993. Macropinosome maturation and fusion with tubular lysosomes in macrophages. J. Cell Biol. 121:1011–1020.

Rothman, J. E., and S. S. Yamada. 1978. A mechanism for the destruction of pinosomes in cultured fibroblasts: pinophagocytosis. J. Cell Biol. 78:480–487.