Lysosomes Can Fuse with a Late Endosomal Compartment in a Cell-free System from Rat Liver

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Abstract. The passage of pulse doses of asialoglycoproteins through the endosomal compartments of rat liver hepatocytes was studied by subcellular fractionation and EM. The kinetics of disappearance of radiolabeled asialofetuin from light endosomes prepared on Ficoll gradients were the same as the kinetics of disappearance of asialoorosomucoid–horse radish peroxidase reaction products from intracellular membrane-bound structures in the blood sinusoidal regions of hepatocytes. The light endosomes were therefore identifiable as being derived from the peripheral early endosome compartment. In contrast, the labeling of dense endosomes from the middle of the Ficoll gradient correlated with EM showing large numbers of reaction product-containing structures in the nonsinusoidal parts of the hepatocyte.

In cell-free, postmitochondrial supernatants, we have previously observed that dense endosomes, but not light endosomes, interact with lysosomes. Cell-free interaction between isolated dense endosomes and lysosomes has now been reconstituted and analyzed in three ways: by transfer of radiolabeled ligand from endosomal to lysosomal densities, by a fluorescence dequenching assay which can indicate membrane fusion, and by measurement of content mixing. Maximum transfer of radiolabel to lysosomal densities required ATP and GTP plus cytosolic components, including *N*-ethylmaleimide–sensitive factor(s). Dense endosomes incubated in the absence of added lysosomes did not mature into vesicles of lysosomal density. Content mixing, and hence fusion, between endosomes and lysosomes was maximal in the presence of cytosol and ATP and also showed inhibition by *N*-ethylmaleimide. Thus, we have demonstrated that a fusion step is involved in the transfer of radiolabeled ligand from an isolated endosome fraction derived from the nonsinusoidal regions of the hepatocyte to preexisting lysosomes in a cell-free system.

UNDERSTANDING of vesicular traffic pathways in the cell and their control has increased markedly in recent years, very largely as a result of the development of cell-free systems which permit identification of the proteins required. Components which operate at many steps on the secretory pathway have thus been characterized and shown to include GTP-binding proteins (Novick and Brennwald, 1993), *N*-ethylmaleimide (NEM)[1]-sensitive factor, its soluble attachment proteins and, most recently, receptor proteins which confer specificity between vesicle and target (Sollner et al., 1993; Takizawa and Malhotra, 1993). Cell-free systems have also permitted considerable understanding of the initial stages of the endocytic pathway (Gruenberg and Howell, 1989; Smythe and Warren, 1991; Schmid, 1993). In vivo it is known that hepatocytes internalize ligands such as asialoglycoproteins via coated pits to a peripheral endosomal compartment from which receptors recycle. The ligand then appears in endosomal compartments further away from the sinusoidal plasma membrane and is finally digested in lysosomes (for reviews see Geuze et al., 1986 and references in Mullock et al., 1989). Subcellular fractionation on Ficoll and Nycodenz gradients of livers from rats given ^{125}I-labeled asialofetuin (ASF) i.v. has shown that the ligand appears sequentially in light endosomes, dense endosomes, and very dense endosomes plus lysosomes (Branch et al., 1987; Ellis et al., 1992).

However, there remains a fundamental question as to whether maturation or vesicular traffic (or some combination of the two) is responsible for the sequential appearance of ligand in compartments observed in vivo. The maturation theory (Murphy, 1991) suggests that an early endosome gradually turns into a late endosome and then a lysosome by sequential addition of material to and subtraction of material from the organelle containing the ligand. The vesicular transfer theory (Griffiths and Gruenberg, 1991), proposes the permanent existence of early endosomes, late endo-

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1. *Abbreviations used in this paper*: AMP-PNP, 5'-adenylylimidodiphosphate; ASF, asialofetuin; ASOR-HRP, asialoorosomucoid-horse radish peroxidase conjugate; bpIgA, biotinylated polymeric IgA; NEM, *N*-ethylmaleimide; R18, octadecylrhodamine β-chloride; RIPA, 1% sodium, 1% Triton X-100, 0.2% NP-40, 0.15 M NaCl, 0.05 M Tris, pH 7.5; STM, 0.25 M sucrose, 10 mM *N*-tris (hydroxymethyl)-methyl-2-aminoethane sulphonic acid and 1 mM Mg^{2+} (pH 7.4).

somes, and lysosomes as discrete compartments, through which incoming ligand is sequentially transferred by vesicular traffic analogous to that which mediates transfer of newly synthesized proteins through the stacked Golgi cisternae. The relationships between early and late endosomes in various tissue culture cells (BHK, CHO, HepG2, and MDCK), have been examined in many studies (for example Gruenberg et al., 1989; Hopkins et al., 1990; Stoorvogel et al., 1991; Bomsel et al., 1990; Dunn and Maxfield, 1992; Aniento et al., 1993), but there has been little work on the relationship between late endosomes and lysosomes, probably because of the lack of suitable, simple assays. Roederer et al. (1990) suggested that endosomes matured into lysosomes, since 3T3 cell endosomes appeared at the density characteristic of lysosomes, when examined on Percoll gradients after incubation with ATP. On the other hand, incubation under suitable conditions of rat liver postmitochondrial supernatants containing late endosomes which had been loaded with ^{125}I-labeled ASF showed that radiolabel subsequently appeared at lysosomal densities on Nycodenz gradients if, and only if, lysosomes were present (Mullock et al., 1989), and that early endosomes did not interact with lysosomes.

The liver postmitochondrial supernatant system previously described (Mullock et al., 1989) contained many components irrelevant to the interaction between endosomes and lysosomes. Moreover, movement of ^{125}I to lysosomal densities on gradients might be due merely to specific adhesion between lysosomes and labeled endosomes. We have, therefore, isolated lysosomes and late endosomes and examined their interactions not only by centrifugation on density gradients but also by an assay which can indicate membrane fusion and by a content mixing assay, both to decide whether a fusion step occurs or whether endosomes simply mature into lysosomes and to examine the necessary conditions.

Materials and Methods

Materials

ASF and asialoorosomucoid-horse radish peroxidase (ASOR-HRP) were radiolabeled as described by Mullock and Luzio (1992). Polymeric IgA, affinity purified from a rat hybridoma cell line (Peppard et al., 1984), was biotinylated using biotinamidocaproate *N*-hydroxysuccinimide ester (Bayer and Wilchek, 1990) and radiolabeled in the same way as ASF. Avidin and ASF were conjugated using 3-(2-pyridyldithio)propionic acid *N*-hydroxysuccinimide ester (Carllson et al., 1978). Octadecylrhodamine B-chloride (R18) was from Molecular Probes, Inc. (Eugene, OR). Rabbit anti-avidin (chicken's egg) and a matched preparation of nonimmune rabbit IgG were obtained from Dako (Glostrup, Denmark) and magnetic beads coated with goat anti-rabbit IgG from Advanced Magnetics Inc. (Cambridge, MA). ASOR-HRP was a gift from Dr. A. Hubbard (Johns Hopkins University, Baltimore, MD). A rabbit antiserum to the chicken cation independent mannose 6-phosphate receptor was a gift from Dr. B. Hoflack (EMBL, Heidelberg, Germany); a rabbit antiserum to bovine cation independent mannose 6-phosphate receptor was the gift from Dr. S. Pfeffer (Stanford University, Stanford, CA) (Pfeffer, 1987). Specifically purified rabbit anti-rat Ig α-chain was a gift from Dr. S. Hobbs (Institute for Cancer Research, Sutton, UK).

Comparison of Positions of Endosomes on Ficoll Gradients with Positions of Endosomes in the Cell

Livers were established in a perfusion apparatus (Perez et al., 1988) modified as in Ellis et al. (1992). For subcellular fractionation experiments, the liver received a single pass dose of ~0.03 μg ^{125}I-ASF (10^7–10^8 dpm). After continuing perfusion for the appropriate time as a chase, the liver was rapidly flushed out with ice-cold 0.25 M sucrose containing 10 mM *N*-tris (hydroxymethyl)-methyl-2-aminoethane sulphonic acid and 1 mM Mg^{2+} (pH 7.4) (STM). The liver was then homogenized and fractionated (Branch et al., 1987; Ellis et al., 1992).

For EM experiments livers were similarly perfused, with the addition of 25 μg/ml mannan to the perfusate mix (this had no effect on distribution of ^{125}I-ASF on gradients). A single pass dose of 50 μg ASOR-HRP was administered and perfusion continued for the appropriate time. The liver was then immersed in and washed out with ice-cold 0.15 M NaCl and fixed by perfusion with ice-cold 2% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.4. Liver slices were post-fixed in the same mixture. Peroxidase activity was detected and ASOR-HRP-containing structures in the various regions of the cell counted as described by Hoppe et al. (1985). 3,090 ligand-containing structures were scored in the 53 low power fields counted. ^{125}I-labeled ASOR-HRP was shown to fractionate exactly as did ^{125}I-labeled ASF.

Centrifugation Assay

Dense endosomes and lysosomes were isolated and centrifugation analyses of their interaction performed as previously described (Mullock and Luzio, 1992). Briefly, for the isolation of dense endosomes, postmitochondrial supernatants were prepared by homogenizing liver in 3 ml/g of STM and centrifuging at 1500 *g* for 10 min. The postmitochondrial supernatant was fractionated on 1–22% Ficoll gradients and the dense endosome peak was further purified on Metrizamide step gradients in a vertical rotor. Lysosomes were rapidly prepared by differential centrifugation, followed by a wash with 0.25 M KCl to precipitate mitochondria and further purification on a Percoll gradient (Maguire and Luzio, 1985). Cytosol was prepared by centrifuging postmitochondrial supernatant at 288,000 *g* for 45 min and, unless otherwise stated, was gel filtered through BioGel P6 columns. The protein concentration was ~20 mg/ml.

Centrifugation assays were performed using dense endosomes prepared from rats injected i.v. with ^{125}I-ASF 10 min before killing, since in the rat, in contrast to the perfused liver system, dense endosome peak labeling remained high for at least 10 min (Branch et al., 1987). The complete system contained dense endosomes from ~0.3 g liver and freshly isolated lysosomes from 0.6–0.9 g unlabeled liver resuspended in 0.5 ml of cytosol and incubated with 10 mM phosphoenolpyruvate, 35 U pyruvate kinase, and 1.3 mM GTP. After incubation, the mixture was chilled and analyzed on small Nycodenz gradients in a vertical rotor. Radioactivity appearing in fractions with a refractive index of 1.3785 or more was scored as associated with lysosomes, on the basis of the distribution of *N*-acetyl-β-glucosaminidase on these gradients.

Fluorescence Dequenching Assay

The fluorescence dequenching assay was performed as previously described (Mullock and Luzio, 1992). Isolated dense endosomes suspended in STM were loaded with self-quenching concentrations of R18 by addition of an ethanolic solution of R18 at room temperature (Mullock and Luzio, 1992). Free R18 was removed on small columns of Sepharose CL-4B. The loaded endosomes were incubated under appropriate conditions and increases in fluorescent emission at 590 nm measured (excitation wavelength 560 nm).

Content Mixing Assay

For the content mixing assay, dense endosomes were prepared from a rat which had received an i.v. injection of ~1–3 nmol of avidin-ASF 6 min before killing. Labeling time was reduced from the 10 min used in the centrifugation assay to ensure lower possible contamination with vesicles labeling at later times especially those found at the bottom of the gradient (Fig. 1). Material from the dense endosome peak of Ficoll gradients was diluted with STM and sedimented, then resuspended in the appropriate medium, usually cytosol, and either used immediately or frozen in liquid N_2.

Lysosomes for the content mixing assay were loaded with ^{125}I-labeled biotinylated polymeric IgA (bpIgA) by administering ~50 pmol (3–4 × 10^8 dpm) i.v. 35–45 min before killing the rat. When leupeptin was preinjected, 0.3 mg in PBS were injected i.v. 15 min before the labeled bpIgA. 7 ml of the postmitochondrial supernatant was layered over a step gradient composed of 4 ml 45% Nycodenz, 14 ml 20% Nycodenz, and 14 ml 20% Ficoll and centrifuged in a vertical Beckman VTi rotor as described previously (Ellis et al., 1992). Material from the interface between the 45 and 20% Nycodenz layers was diluted with STM, sedimented, resuspended in the appropriate medium for assay, and used immediately.

The content-mixing assay contained 100 μl of a dense endosome suspension containing avidin-ASF, 100 μl of a lysosome suspension containing ^{125}I-labeled bpIgA and 25 μl of a 1 mg/ml solution of biotinylated insulin

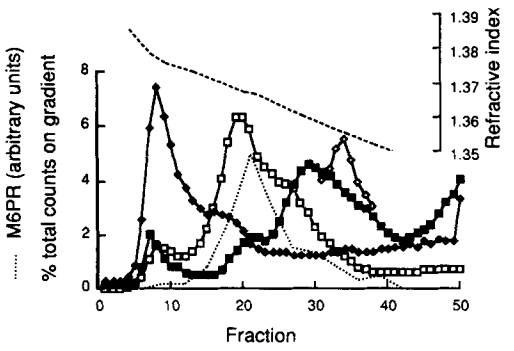


Figure 1. Movement with time of ^{125}I-ASF through subcellular fractions of perfused rat liver. After a single pass dose, livers were homogenized and postmitochondrial supernatants were fractionated on Ficoll gradients. (■) 1 min after dose; (□) 4 min after dose; (♦) 15 min after dose; (◇) position of sinusoidal plasma membrane peak (determined by adding radiolabeled ligand to a liver homogenate at 4°C); (----) refractive index; (·····) mannose 6-phosphate receptor (M6PR, arbitrary units, determined after SDS-PAGE and immunoblotting of every third fraction from fraction 6 to fraction 48.

or of 0.3 mg/ml biocytin. Both suspensions were prepared at concentrations roughly twice those of postmitochondrial supernatant assuming 100% recovery of organelles, so that on mixing the original concentration was approximately restored. For optimal activity the suspensions were in cytosol (gel filtered as for the centrifugation assay) and an ATP-regenerating system composed of 10 μl of an equal mix of 0.8 M phosphocreatine and 4 mg/ml creatine kinase was added. Incubations were routinely for 10 min at 37°C. Dilution with 400 μl 0.25 M sucrose containing 10 mM *N*-tris (hydroxymethyl)-methyl-2-aminoethane sulphonic acid, pH 7.4, and protease inhibitors (final concentrations benzamidine 1 mM, PMSF 1 mM, pepstatin 10 μM, *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)-butane 10 μM, EDTA 1 mM) was followed by addition of a 10 × RIPA detergent mix (giving final concentrations of 1% sodium deoxycholate, 1% Triton X-100, 0.2% NP40, 0.15 M NaCl, and 0.05 M Tris, pH 7.5). The lysed mixtures were then incubated with 0.3 μl rabbit anti-avidin at 37°C for 2 h before addition of washed magnetic beads coated with goat anti-(rabbit IgG) suspended in 1 × RIPA. Incubation at 4°C overnight was followed by five washes with 1 × RIPA before counting. Total possible capture of ^{125}I was measured by omitting the unlabeled biotinylated blocking agent, background trapping of ^{125}I was measured by substituting nonimmune rabbit Ig for anti-avidin.

NEM Treatment and Production of Protected and Depleted Cytosol

Treatment with NEM was carried out at 4°C for 15 min, after which sufficient DTT was added to neutralize the NEM. NEM-sensitive factor-protected cytosol was gel filtered in the presence of 0.5 mM ATP at 4°C; NEM-sensitive factor-depleted cytosol was filtered without ATP and incubated at 37°C for 15 min (Block et al., 1988).

Enzyme and Protein Assays

N-acetyl-β-glucosaminidase was assayed according to Maguire et al. (1983). Glucose 6-phosphatase was estimated by incubation with 5 mM glucose 6-phosphate in 120 mM dimethylglutarate, 10 mM EDTA, and 10 mM sodium (+) tartrate, pH 6.5. The reaction was stopped with TCA to 2.5% and phosphate determined according to Itaya and Ui (1966). SDS-PAGE, immunoblotting, and densitometry of blots were essentially as described in previous work from this laboratory (Abraha et al., 1988). Protein was measured according to Lowry et al. (1951) or using the BCA protein assay reagent (Pierce, Rockford IL). Where appropriate, data are presented as mean ± SEM with the number of experiments given in brackets.

Results

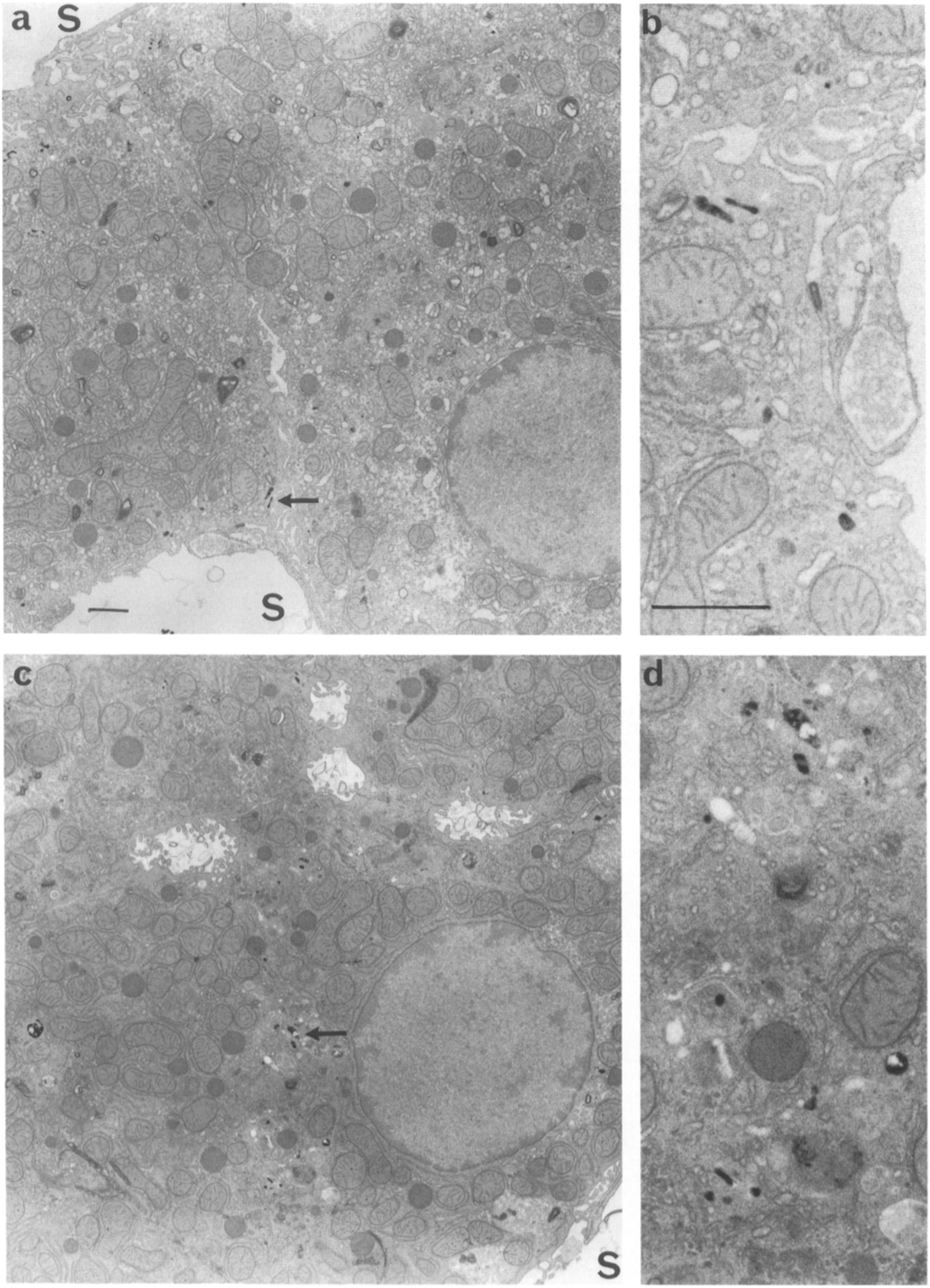
Previously we have shown that after injection of ^{125}I-ASF into a rat, radiolabel appeared first in endocytic vesicles which fractionated at the light end of Ficoll gradients and which we have named light endosomes. With increasing time after injection, more label appeared in the middle of the gradient in vesicles which we have named dense endosomes. We have further shown that dense endosomes interacted with lysosomes in a cell-free system, but that light endosomes did not take part in this interaction (Mullock et al., 1989). We have now correlated these gradient fractions with ligand-containing structures in the hepatocyte and demonstrated that the interaction between dense endosomes and lysosomes includes membrane fusion and content mixing. In order to correlate gradient fractions with endosomes seen by EM, tracer ligands must be administered as a pulse dose followed by an accurately timed chase. We therefore established livers in a perfusion system using a single pass format to give a pulse dose of ligand, followed by continuous perfusion with unlabeled medium to chase the ligand through successive endosomal compartments (Perez et al., 1988; Ellis et al., 1992).

The Dense Endosome Peak Contains Endosomes From the Nonsinusoidal Regions of the Cell

Fractionation of livers on 1–22% Ficoll gradients at accurately measured time intervals after a single pass dose of ^{125}I-ASF gave patterns like those shown in Fig. 1. Following a 1-min chase with unlabeled perfusion medium after the single pass dose, most radioactivity was found near the light end of the gradient between refractive indices 1.3600 and 1.3660, the light endosome region. We have previously shown that blood sinusoidal plasma membrane can be distinguished from light endosomes on these gradients, fractionating largely between refractive indices 1.3550 and 1.3599 (Branch et al., 1987). Four minutes after a single pass dose, the peak of ^{125}I-ASF was found in the dense endosome region in the middle of the gradient (refractive indices 1.3661–1.3729). Thereafter radiolabel increasingly appeared in the region at the bottom of the gradient, which contains lysosomes. ^{125}I-labeled ASOR-HRP fractionated in exactly the same way as ^{125}I-ASF. SDS-PAGE and immunoblotting across Ficoll gradients showed that the cation-independent mannose 6-phosphate receptor was present in endosomal fractions, especially in the dense endosome region. The mannose 6-phosphate receptor was not detectable (i.e., <10% of that found in endosomal fractions) in lysosomal fractions separated from endosomes on Nycodenz gradients.

Comparison of electron micrographs made from livers which had been chased with unlabeled perfusion medium for 1 and 4 min after a single pass dose of ASOR-HRP (Fig. 2) showed that while most membrane bound structures containing HRP reaction product were close to the sinusoidal surfaces of hepatocytes after 1 min (*a* and *b*), by 4 min much labeled material appeared in the bile canalicular and perinuclear regions (*c* and *d*).

Numbers of ASOR-HRP-containing structures in different regions of the hepatocyte were compared with proportions of total ^{125}I-ASF found in the fractions from Ficoll gradients after the same time interval. The loss of ASOR-HRP containing structures from the regions within 1.6 μm of a blood sinusoid followed the same time course as the loss of ^{125}I from the light endosome region (Fig. 3), indicating that dense endosomes were not peripheral early endosomes but were derived from regions of the cell further from the sinusoidal site of ligand entry.


a
S
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d

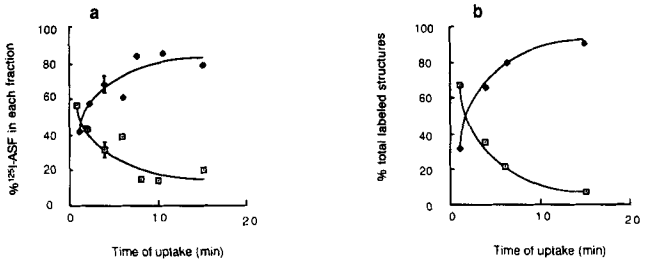

Figure 3. Comparison of (*a*) the proportion of ^{125}I–ASF in the light endosome region on Ficoll gradients with (*b*) the proportion of ASOR–HRP-containing structures found within 1.6 μm of a liver sinusoid at increasing times after a single pass dose of ligand in perfused livers. (*a*) (□) proportion of total postmitochondrial supernatant ^{125}I–ASF found in the light endosome region (refractive index 1.3600–1.3660). The error bars relate to four separate perfusions. Other points are means of duplicate perfusions except for 8 and 10 min which were single perfusions; (♦) proportion of ^{125}I–ASF found in denser endosome regions. (*b*) (□) proportion of total ASOR–HRP-containing structures found within 1.6 μm of the sinusoidal face; (♦) proportion of total ASOR–HRP-containing structures deeper in the hepatocyte.

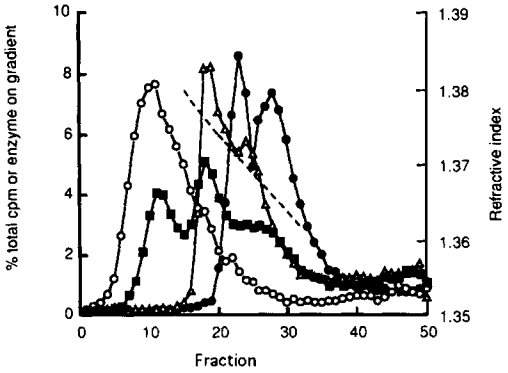

Figure 4. Interaction of purified ^{125}I–ASF loaded dense endosomes with lysosomes. Purified ^{125}I–ASF-labeled dense endosomes were incubated for 30 min at 37°C, with cell filtered cytosol, GTP, and an ATP-regenerating system in the absence (△) or presence (■) of lysosomes before centrifugation on Nycodenz gradients. (●) incubation at 0°C in the presence of lysosomes. (○) *N*-acetyl-β-glucosaminidase, the marker enzyme for lysosomes, measured on the samples which had been incubated at 37°C in the presence of lysosomes. (-----) refractive index.

Isolated Dense Endosomes Interact with Isolated Lysosomes In Vitro

Dense endosomes, which had been loaded with ^{125}I–ASF in vivo, were taken from a Ficoll gradient and further purified on a Metrizamide step gradient (Mullock et al., 1983) to remove much of the contaminating smooth ER (Table I). Such dense endosomes were resuspended in Bio-Gel treated cytosol and mixed with lysosomes isolated from another rat, in order to recreate the essential features of the postmitochondrial supernatant used in earlier work (Mullock et al., 1989). Samples applied to the Nycodenz gradients shown in Fig. 4 contained, in addition, an ATP-regenerating system and 1.3 mM GTP. As in the earlier work, interaction between endosomes and lysosomes was measured as a movement of radiolabel from the endosomal to the lysosomal position on the gradient. Radioactivity appearing at a refractive index of 1.3785 or above was scored as truly lysosomal (up to and including fraction 16 in the gradients illustrated in Fig. 4). If kept in ice, radiolabel remained in the endosomal position. Metrizamide purified dense endosomes always showed a biphasic distribution appearing as a double peak on Nycodenz gradients (in contrast to the single peak originally observed on Ficoll gradients, [Branch et al., 1987]), but both parts of the peak appeared to be capable of association with lysosomes. Incubation at 37°C in the presence of lysosomes markedly increased the proportion of total radiolabel found in the lysosomal region of the gradient (an increase from 1.0 to 28.6% of total label on the gradients in the example shown [Fig. 4]). However, after incubation at 37°C in the absence of lysosomes, the radiolabel appeared at a density intermediate between those typical of endosomes and lysosomes on Nycodenz gradients. Separation of the same mixture of labeled dense endosomes, cytosol, GTP, and an ATP-regenerating system on Ficoll gradients after incubation at 37°C showed clearly that radiolabel moved on incubation to a lighter density (data not shown). Lysosomes would go to the bottom of such a Ficoll gradient. Dense endosomes do not therefore mature to lysosomes on incubation with cytosol, GTP, and an ATP-regenerating system.

In addition to lysosomes, maximal movement of radiolabel

Table I. Purification of Dense Endosomes

Fraction	Protein	^{125}I*	Glucose 6-phosphatase	*N*-acetyl-β-glucosaminidase
		% homogenate		
Postmitochondrial supernatant	48.5 ± 1.90 (3)	74 ± 0.6‡ (3)	56 ± 6.4 (3)	77.9 ± 4.06 (3)
Pool loaded to Metrizamide gradient	2.9 ± 0.36 (3)	56 ± 5.5 (3)	18 ± 0.7 (3)	4.8 ± 0.40 (3)
Dense endosomes	0.5 ± 0.07 (3)	26 ± 3.7 (3)	4 ± 0.3 (3)	0.7 ± 0.17 (3)

* Rats received 125I-ASF 10 min before killing. At this time, the major part of the radiolabel is in dense endosomes (Branch et al., 1987).
‡ Assuming that all radioactivity in the nuclear pellet is trapped postmitochondrial supernatant.

Figure 2. Intracellular localization of ASOR–HRP in hepatocytes after a single pass dose to perfused livers. The liver was perfusion fixed after 1 min (*a* and *b*) or 4 min (*c* and *d*) and processed for HRP cytochemistry. (*a*) Reaction product is found mostly close to sinusoids (*S*) in small vesicles and tubules. (*b*) Enlarged view of arrowed vesicles in *a*. (*c*) Reaction product appears deeper in the cell in vesicles and multivesicular bodies. (*d*) Enlarged view of arrowed vesicles in *c*. Bar, 1 μm.

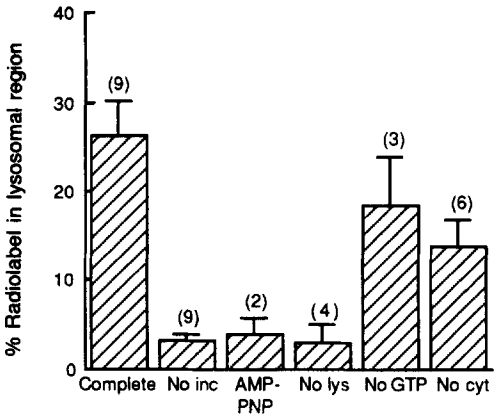

Figure 5. Requirements for interaction of ^{125}I-loaded dense endosomes with lysosomes. The complete system contained, in addition to endosomes and lysosomes, gel-filtered cytosol, an ATP-regenerating system, and 1.3 mM GTP. When AMP-PNP (2 mM) was added, the ATP-regenerating system was omitted.

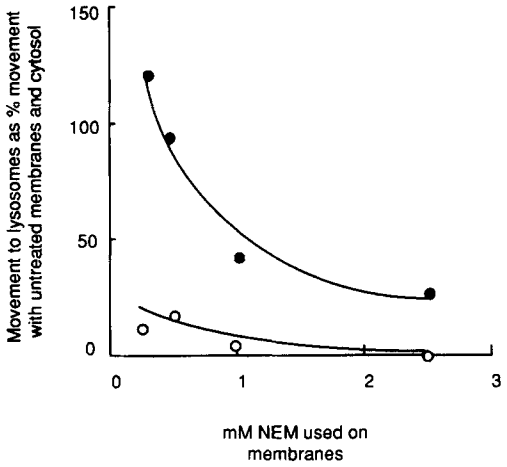

Figure 6. Involvement of NEM-sensitive factor(s) in ASF movement to the lysosomal position. Purified ^{125}I-ASF-loaded dense endosomes and unlabeled lysosomes were separately treated with NEM for 15 min at 0°C before suspension in either gel-filtered cytosol which had been depleted of NEM-sensitive factor(s) (○) or in cytosol, gel filtered in the presence of 0.5 mM ATP to protect NEM-sensitive factor(s) (●). Samples were incubated for 30 min at 37°C in the presence of GTP and an ATP-regenerating system before analysis by centrifugation on Nycodenz gradients.

from the endosomal to the lysosomal region required cytosol and GTP, although some movement occurred in their absence (Fig. 5). An ATP-regenerating system was routinely added, although variable amounts of movement occurred in its absence. Since all components of the system were prepared from fed rats and the lysosome preparation contained visible glycogen, this was hardly surprising. However, substituting 10 U of apyrase for the ATP-regenerating system, reduced the movement of radiolabel to the lysosomal position by ~70%. Alternatively, 2 mM 5'-adenylylimidodiphosphate (AMP-PNP) in place of the ATP-regenerating system prevented any increase in radiolabel in the lysosomal position after 30 min incubation at 37°C (Fig. 5).

Interaction between Dense Endosomes and Lysosomes Is Sensitive to NEM

Although NEM was without effect in the crude postmitochondrial supernatant system which we previously used (Mullock et al., 1989), the present system was NEM sensitive. Concentrations of NEM required to pretreat endosomes and lysosomes to produce inhibition of interaction varied with the pretreatment of the cytosol. Using cytosol depleted of NEM-sensitive factors as described by Block et al. (1988), interaction was inhibited by pretreatment with 0.25 mM NEM; with ATP-protected cytosol such NEM-pretreated organelles behaved normally (Fig. 6).

Data from Fluorescence Dequenching Assays Is Consistent with a Membrane Fusion Event Occurring when Isolated Dense Endosomes Interact with Lysosomes

Because the density shift assay could be measuring association rather than fusion, a fluorescence dequenching assay capable of measuring fusion between membranes was developed. This relied on the subcellular fractions used being pure and could not be used in the presence of cytosol (see below). This assay measured an increase in fluorescence due to dequenching which would occur after fusion between a membrane loaded to self-quenching concentrations with the fluorescent lipid R18 and unlabeled membrane. Dense endosomes, which had been thus labeled with R18, showed increasing dequenching with time of incubation with lysosomes at 37°C (Fig. 7). Mixtures kept in ice showed little or no increase in fluorescence. R18 was not liberated into solution by incubation of R18-labeled dense endosomes with lysosomes, since the mixture after incubation could be run through a column of Sepharose 4B, which binds free R18, with no loss of fluorescence. The R18 in the dense endosome–lysosome mixture was also acid precipitable and sedimented when the membranes were spun down.

The amount of dequenching was proportional to the quantity of lysosomes added (data not shown). Incubation with

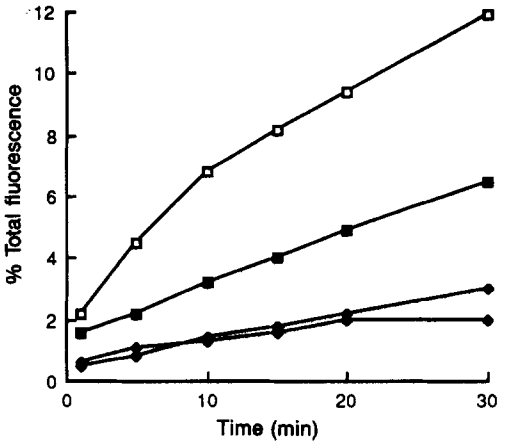

Figure 7. Dequenching of R18-loaded dense endosomes by lysosomes. R18-labeled dense endosomes (25 μg protein) were incubated at 37°C with lysosomes (1 mg protein) in the absence (□) or presence (■) of 2 mM AMP-PNP; R18-labeled dense endosomes incubated with mitochondria (1 mg protein) (◆); R18-labeled dense endosomes incubated with unlabeled dense endosomes (1 mg protein) (◇).

similar protein concentrations of crude mitochondria or of the same preparation of dense endosomes without R18 label produced little increase in fluorescence (Fig. 7). Light endosomes were equally ineffective. Addition of an ATP-regenerating system to these assays produced little change in the fluorescence dequenching. However, addition of apyrase or the ATP analogue AMP-PNP (Fig. 7) reduced the observed dequenching. Incubation of R18-labeled dense endosomes with cytosol in the absence of lysosomes caused a large increase in fluorescence. This increase in fluorescence was probably due to the presence of exchange proteins (see Discussion); cytosol was therefore excluded from experiments with R18-loaded membranes.

Interaction of Isolated Dense Endosomes with Isolated Lysosomes In Vitro Includes Content Mixing

In order to confirm that a fusion step was involved in the transfer of ligand from isolated dense endosomes to lysosomes, a content mixing assay was devised using dense endosomes and lysosomes loaded with ligands taken up by receptor mediated endocytosis. A ligand resistant to digestion was needed for lysosomal loading; rat biotinylated pIgA (bpIgA) was chosen.

Polymeric IgA is transcytosed across rat hepatocytes from blood to bile, but in experiments using ^{125}I-polymeric IgA, it was observed that 30–50% of the ligand was delivered to lysosomes, where it was relatively resistant to lysosomal hydrolysis (Schneider, 1982; Perez et al., 1988). We found that radiolabeled Ig heavy and light chains could be immunoprecipitated using a specific anti rat Ig α-chain antibody from lysosomes prepared 50 min after i.v. injection of ^{125}I-labeled rat polymeric IgA. ^{125}I-labeled bpIgA was therefore used to load liver lysosomes. Lysosomes were usually prepared using a step gradient for convenience, since contamination with mitochondria and rough ER was unimportant in this assay. Immunoblotting experiments using two different antibodies for the detection of the cation-independent mannose 6-phosphate receptor showed that it was just detectable in the lysosomes prepared on the step gradient, but was at the limit of detection and did not exceed 10% of the total receptor in the postmitochondrial supernatant. The yield of lysosomes from the step gradient was ~50% as compared to the 20% of the Maguire and Luzio (1985) method. However, lysosomes prepared by the latter method, as for centrifugation and fluorescent dequenching experiments, were also used to show content mixing.

When isolated lysosomes were incubated with avidin–ASF-loaded dense endosomes under appropriate conditions, ^{125}I radiolabel could be immunoprecipitated using an anti-avidin antibody. ASF-avidin-^{125}I-bpIgA complexes could only have formed within a membrane-bound compartment(s), since the incubations were carried out in the presence of a large excess of biotinylated insulin or biocytin to mop up any leaked avidin. Initially incubations were carried out for 30 min. However, measurement of total possible ^{125}I immunoprecipitation, by lysing the components in the absence of a biotinylated blocking agent (Fig. 8) showed that digestion of the loaded ligands was occurring during the in vitro incubation. This digestion was not all occurring in lysosomes. Incubation of avidin–ASF-loaded dense endosomes alone, followed by addition of ^{125}I-bpIgA and immunoprecipitation, showed that dense endosomes contained enough

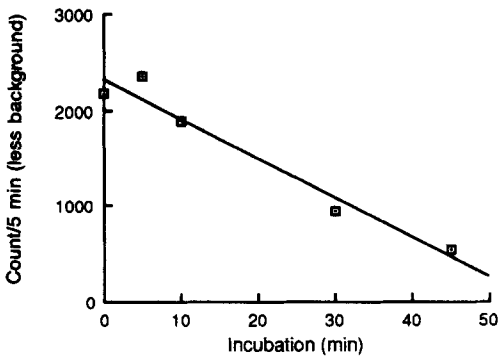

Figure 8. Digestion of loaded ligands during in vitro incubation. ^{125}I-biotinylated polymeric IgA loaded lysosomes prepared on a step gradient were incubated at 37°C with avidin–ASF-loaded dense endosomes in gel-filtered cytosol with an ATP-regenerating system, before solubilization with RIPA and immunoprecipitation with anti-avidin.

proteases to reduce the amount of immunoprecipitable radiolabeled avidin–ASF. Attempts to prevent this digestion and the similar digestion of bpIgA in lysosomes by addition of protease inhibitors or agents which increase lysosomal pH (5 μM nigericin with 50 mM KCl, 20 μM monensin, or 20 mM ammonium chloride) to the incubation mixtures were ineffective. Preinjection of leupeptin into the rat used to make loaded lysosomes also failed to prevent the progressive loss of total immunoprecipitable radiolabel during the assay. Content mixing was therefore expressed as a percentage of the total possible immunoprecipitation after the same length of incubation and the standard incubation period was restricted to 10 min. Under these conditions, content mixing between endosomes and lysosomes was clearly demonstrable (Fig. 9), in spite of variation between animals (lysosomes from 26 rats and dense endosomes from 15) and the fact that two competing processes were at work. As shown in Fig. 9, substitution of STM for cytosol largely prevented content mixing. Pretreatment with 2 mM NEM inhibited content

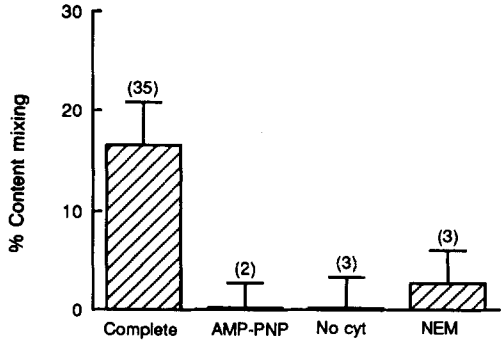

Figure 9. Content mixing between dense endosomes and lysosomes. Avidin–ASF-loaded dense endosomes were incubated at 37°C with ^{125}I-bpIgA loaded lysosomes in gel-filtered cytosol with an ATP-regenerating system and excess biotinylated blocking agent. Solubilization with RIPA was followed by immunoprecipitation with anti-avidin. Results are expressed as a percentage of total precipitable counts after the same incubation period in the absence of biotinylated blocking agent. AMP-PNP was 2 mM in the absence of the ATP-regenerating system. NEM was 2 mM.

mixing markedly. The substitution of 2 mM AMP-PNP for the ATP-regenerating system prevented content mixing.

Discussion

Cell-free interaction between dense endosomes and lysosomes isolated from rat livers has been shown to occur under conditions which do not permit maturation of endosomes to lysosomes. The interaction results in content mixing between endosomes and lysosomes, indicating that lysosomes are not simply formed by maturation of endosomes but continue to receive incoming ligand.

The endosomes used in these experiments were the dense endosomes from the central region of 1–22% Ficoll gradients, which we previously demonstrated (Mullock et al., 1989) to be those involved in interaction with lysosomes in postmitochondrial supernatants. These dense endosomes have now been shown to come from the nonsinusoidal regions of the hepatocytes. The early labeled endosomes near to the sinusoidal plasma membrane have been clearly identified with the light endosomal fraction from near the top of 1–22% Ficoll gradients, which did not interact with lysosomes (Mullock et al., 1989). Pulse labeling in perfused livers showed that dense endosomes became loaded with ^{125}I-labeled ASF after the light endosomes but retained their ASF relatively briefly, the radioactivity then appearing at the bottom of the gradient or as acid-soluble breakdown products. The dense endosomes prepared in the present study were, therefore, derived from a transiently labeled compartment found in the perinuclear and bile canalicular regions of the cell and contained ligand destined for lysosomes. They contained mannose 6-phosphate receptors but little of the lysosomal enzyme *N*-acetyl-β-glucosaminidase. The data from both the present and previous experiments (Branch et al., 1987) suggests that asialoglycoproteins travel through the early endocytic compartments to late endosomes faster in hepatocytes in situ than is the case for fluid phase markers in tissue culture cells (Bomsel et al., 1989; Gruenberg et al., 1989; Parton et al., 1989). We believe that the isolated dense endosomes used in the present cell-free experiments are unlikely to be endosomal carrier vesicles (Gruenberg et al., 1989; Bomsel et al., 1990) despite the transient labeling, since they are derived from a nonsinusoidal area within the hepatocyte and our cell-free interaction/fusion assays are not affected by the presence of taxol (data not shown) in contrast to cell-free fusion of endocytic carrier vesicles with late endosomes.

When ^{125}I–ASF-loaded dense endosomes were incubated in vitro with lysosomes and an ATP–regenerating system, the ^{125}I became specifically associated with lysosomes as shown by analysis on density gradients. Addition of purified lysosomes was essential for this changed position of the ^{125}I label on the gradient. Incubation of dense endosomes with cytosol and an ATP-regenerating system, but no lysosomes, resulted in a small density increase on Nycodenz gradients, but not to the density of the lysosomes. It should be noted that the Nycodenz gradients used were dense enough to keep lysosomes in the linear region and hence as a defined density band. Our conclusion that incubated endosomes did not attain lysosomal densities on Nycodenz gradients was borne out by examination on Ficoll gradients, when the incubated endosomes moved to a lighter density, i.e., further away from the lysosomal position. (The observed densities of subcellular organelles depend, of course, on the nature of the density gradient medium used for their separation). Rat liver dense endosomes did not mature into lysosomes under any cell-free incubation conditions used.

Optimal interaction between dense endosomes and lysosomes required the presence of cytosol and of GTP in addition to ATP, although some interaction occurred in their absence. Experiments with NEM indicated that NEM-sensitive factor(s) was required. Such requirements are similar to those shown for vesicle fusions in the Golgi complex (Melancon et al., 1987; Block et al., 1988; Balch, 1989; Schatz, 1989) or in the early part of the endocytic pathway (Diaz et al., 1989; Mayorga et al., 1989*a*,*b*; Wessling-Resnick and Braell, 1990; Woodman and Warren, 1991). However, although these requirements suggest that fusion may be involved in the interaction of dense endosomes with lysosomes, the centrifugation assay can only demonstrate interaction between the two particulate fractions.

Fluorescence dequenching has been used to show membrane fusion in a number of other systems (Hoekstra et al., 1984; Nadin et al., 1989; Hoekstra, 1990). In the present experiments cytosol alone caused dequenching of R18-loaded dense endosomal membranes, presumably due to the presence of phospholipid transfer proteins (reviewed by Rothman, 1990), and/or fatty acid-binding proteins (Storch and Bass, 1990). However, it was possible to carry out fluorescence dequenching experiments in the absence of cytosol, since in the absence of cytosol there was almost half the optimal interaction between dense endosomes and lysosomes measured by centrifugation. Under such conditions, R18-loaded dense endosomes were dequenched by lysosomes but not by mitochondria, unloaded dense endosomes, or light endosomes. The lysosomes used in these experiments had been washed with 0.25 M KCl and sedimented through Percoll during their purification; their ability to dequench seemed therefore most unlikely to be the result of a loosely adherent cytosolic component(s). The time course of the dequenching was similar to that shown for interaction between dense endosomes and lysosomes measured on density gradients (Mullock et al., 1989). Although addition of an ATP-regenerating system did not reliably increase dequenching, addition of AMP-PNP inhibited dequenching. The fluorescence dequenching data was consistent with membrane fusion occurring between dense endosomes and lysosomes, but the impossibility of examining the effects of cytosolic components and the need for pure subcellular fractions limited the usefulness of the assay. Fluorescence dequenching has also been criticized since it is difficult to be certain that it is the result of membrane fusion; under some circumstances R18 may not be randomly distributed in loaded membranes and in addition, spontaneous exchange of the probe between membranes has sometimes been reported (Stegmann et al., 1993).

In order to prove that a fusion event occurred, we developed an assay to demonstrate content mixing between dense endosomes and lysosomes. Altstiel and Branton (1983) reported fusion of alkali-stripped clathrin-coated vesicles and lysosomes, Mayorga et al. (1991) reported phagosome–endosome fusion and Racoosin and Swanson (1993) have recently reported macropinosome fusion with tubular lysosomes. However, this is the first report demonstrating

cell-free content mixing between endosomes and lysosomes. The main difficulty in developing a cell-free system was loading lysosomes with a test material that survived digestion for long enough to demonstrate content mixing. Polymeric IgA is a relatively resistant ligand; we therefore rapidly prepared lysosomes from rats loaded with biotinylated ^{125}I-labeled polymeric IgA. Receptor mediated endocytosis was also used to load avidin into dense endosomes as an ASF–avidin conjugate. Content mixing between these two loaded compartments after incubation in the presence of cytosol and an ATP-regenerating system was clearly demonstrated by the immunoprecipitation of radiolabel using an anti-avidin antibody, in spite of the presence of excess unlabeled biotinylated compounds in the incubation medium. Digestion of both loaded ligands during the course of incubation limited the feasible length of incubation and increased variability. The existence of digestion in the dense endosomes was consistent with reports of proteases in hepatocyte endosomes (Casciola-Rosen et al., 1992) but precluded the use of digestion as a simple measurement of fusion with lysosomes. However, unlike the fluorescence dequenching assay, this content mixing assay did not depend crucially on the purity of the preparations of the two fractions being reacted, since lysosomes and dense endosomes were specifically loaded by timed periods of receptor mediated endocytosis as well as being partially fractionated. The results show clearly that a fusion between two preexisting entities is occurring rather than a simple maturation.

Content mixing assays showed a much greater dependence on the presence of cytosol than did either the density shift or the fluorescence dequenching assays; this may well reflect the occurrence of dense endosome lysosome association and even membrane fusion before and under less stringent conditions than the final mixing of contents (Racoosin and Swanson, 1993). Using brief incubation periods (10 min), it was difficult to demonstrate reproducible dependence on the addition of an ATP-regenerating system, though, as with the density gradient system, this may not be surprising given that the subcellular fractions were prepared from fed rats and were contaminated with mitochondria, glycogen particles, and associated enzymes. As with both the other assay systems, AMP-PNP in the absence of the ATP-regenerating system inhibited activity.

In the cell-free assays described in this paper we are confident that lysosomes and not a subpopulation of late endosomes are the acceptors for interaction and content mixing with dense endosomes. Unlabeled lysosomes used in the centrifugation studies were prepared by a rapid method (Maguire and Luzio, 1985), which we have previously shown (Mullock et al., 1989), to produce a 20% yield of lysosomes containing less than 2% of the endosomes present in the original homogenate, measured by the content of endocytosed ligand. Content mixing was demonstrable with these lysosomes or with lysosomes prepared on a step gradient, which provided an ~50% yield and contained less than 10% of the total cation-independent mannose 6-phosphate receptor. Other studies using fractions from tissue culture cells have demonstrated homotypic fusion between late endosomes (Aniento et al., 1993). The efficiency of homotypic late endosome fusions in cell-free systems ranges from 7 (Bomsel et al., 1990) to 25% (Aniento et al., 1993) after 45 min incubation at 37°C. The maximum content of mannose 6-phosphate receptor in our lysosomal fractions was certainly not more than 10% of the total present in the whole postmitochondrial supernatant. Even if this represents the presence of a small number of late endosomes, it seems extremely unlikely that homotypic fusion of these alone could account for the observed content mixing (16.5 ± 4.3% after 10 min incubation).

Our results are consistent with either vesicular transfer of ligand between dense endosomes and lysosomes or direct fusion between these membrane-bound compartments. However, they clearly show that preexisting lysosomes do receive endocytosed ligand by means of a fusion event, rather than being necessarily derived de novo by maturation of a ligand-containing endocytic compartment.

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