Reconstitution of an Endosome–Lysosome Interaction in a Cell-free System

Barbara M. Mullock, William J. Branch, Margriet van Schaik, Lynne K. Gilbert, and J. Paul Luzio

Department of Clinical Biochemistry, University of Cambridge, Addenbrooke's Hospital, Cambridge CB2 2QR, United Kingdom

Abstract. A cell-free model for the transfer of endocytosed material to lysosomes is described. Rat liver late endosomes, loaded in vivo with radiolabeled ligand by intravenous injection shortly before killing the animal, showed a specific interaction with lysosomes when incubated at 37°C in the presence of cytosol and an ATP regenerating system. The location of the ligand, generally asialofetuin, was analyzed by isopycnic centrifugation on Nycodenz gradients. Appearance of radiolabel in the lysosomal position on such gradients was maximal after ~30 min at 37°C and required the provision of undamaged cytosol, lysosomes, and an ATP regenerating system. It could not be accounted for by nonspecific bulk aggregation of membranes. Transfer occurred only from late endosomes; radiolabel in early endosomes was unaffected. Digestion of the asialofetuin, as shown by the appearance of TCA-soluble radioactivity, occurred on incubation at 37°C and was increased by the provision of an ATP regenerating system.

THE processes of endocytosis whereby a variety of surface-bound ligands, membrane proteins, and fluid-phase molecules are internalized by eucaryotic cells have been well described morphologically and biochemically (Pastan and Willingham, 1985; Breitfeld et al., 1985; Geuze et al., 1986). Although it is clear that internalization occurs largely via coated pits and vesicles, the mechanisms by which endocytosed material is transferred to endosomes and then to lysosomes and other destinations within the cell are less well understood. Recently, cell-free systems have been used in a number of laboratories to investigate early endocytic pathway events (Davey et al., 1985; Gruenberg and Howell, 1986, 1987; Braell, 1987; Pearse, 1987; Woodman and Warren, 1988; Diaz et al., 1988). Several of these have used enzyme reactions to show vesicle fusion after mixing homogenates from different cell populations which had, respectively, endocytosed enzyme and substrate. Whereas previous cell-free experiments have demonstrated requirements for endosome fusion, the present work shows the subsequent interaction of endosomes (loaded with ¹²⁵I-asialofetuin [ASF][1]) and lysosomes in a cell-free system prepared from rat liver.

The passage of ASF through the endocytic pathway in hepatocytes is well described (Wall et al., 1980; Geuze et al., 1983; Berg et al., 1985; Van Berkel et al., 1986). It requires binding to asialoglycoprotein receptors (Schwartz et al., 1981; Drickamer et al., 1984; Steer and Ashwell, 1986), internalization via coated pits (Wall et al., 1980; Geuze et al., 1984), appearance in an early, peripheral endosomal compartment(s) (Wall et al., 1980; Geuze et al., 1983; Berg et

al., 1985; Branch et al., 1987a) where the ligand becomes dissociated from receptor (Geuze et al., 1983; Mueller and Hubbard, 1986), appearance in a late, deep-lying endosomal compartment(s) (Berg et al., 1985; Mueller and Hubbard, 1986; Branch et al., 1987a), and finally digestion in lysosomes (Berg et al., 1985). ASF movement through the various endosomal compartments in rat hepatocytes may be demonstrated by isopycnic centrifugation on vertical density gradients of Ficoll and Nycodenz, which respectively separate early from late endosomal compartments and endosomes from lysosomes (Branch et al., 1987a) and have been used in analysis of the present cell-free experiments.

Materials and Methods

Preparation of Loaded Postmitochondrial Supernatant

Rats were injected intravenously with ¹²⁵I-ASF (or when specified with a mixture of ¹³¹I-ASF and ¹²⁵I-polymeric IgA [pIgA]) as described by Branch et al. (1987a) 10 min before killing, unless otherwise stated. Livers were perfused in situ with cold 0.25 M sucrose, 10 mM N-tris(hydroxymethyl)-methyl-2-aminoethane-sulphonic acid (TES), pH 7.4, homogenized in 3 vol 0.25 M sucrose, 10 mM TES, 1 mM MgCl$_2$, pH 7.4, and centrifuged at 1,500 g for 10 min.

Transfer Reactions and Analysis by Centrifugation

0.5-ml aliquots of postmitochondrial supernatant were incubated with 1.3 mM vanadium-free ATP, 10 mM phosphoenolpyruvate, and 35 U of pyruvate kinase. After incubation the mixtures were chilled, layered over 4.5-ml linear gradients prepared from 45% wt/vol Nycodenz and 0.25 M sucrose both containing 10 mM TES, pH 7.4, and 1 mM EDTA, and centrifuged for 1 h in a vertical rotor (model TV-865; Sorvall Instruments Div., Newton, CT) at 219,000 g. 0.1-ml fractions were collected from the bottom of the tube, and refractive index measurements were used to determine the position of lysosomes on the gradients by comparison with the values for the peak of N-acetyl-β-glucosaminidase activity.

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1. *Abbreviations used in this paper:* ASF, asialofetuin; pIgA, polymeric IgA; TES, N-tris (hydroxymethyl)methyl-2-aminoethane-sulphonic acid.
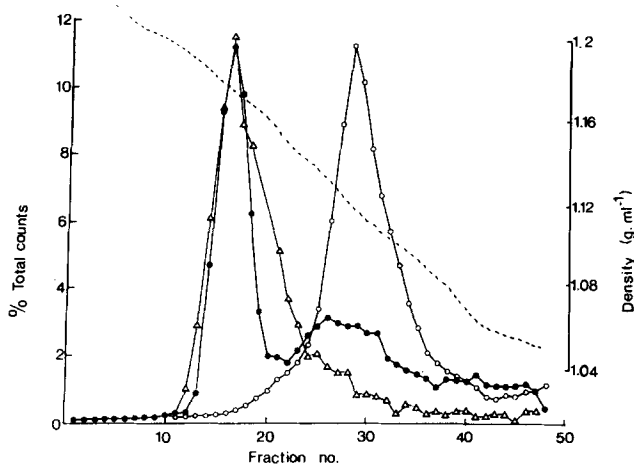
Figure 1. Transfer of ^{125}I-ASF from the endosomal to the lysosomal position on Nycodenz gradients. In this representative experiment, postmitochondrial supernatant from a rat which had received ^{125}I-ASF 10 min before killing was incubated with ATP and a regenerating system for 30 min at either 0°C (o) or 37°C (●) before fractionation on 0.25 M sucrose–45% wt/vol Nycodenz gradients. (– – – –) Density. Total radioactivities loaded on to the gradients were 1,104,000 and 1,125,000 cpm, respectively. (△) *N*-Acetyl-β-glucosaminidase activity (arbitrary units).

Time (min)

Figure 2. Time course of transfer of ^{125}I-ASF from the endosomal to the lysosomal position on 0.25 M sucrose–45% wt/vol Nycodenz gradients. Postmitochondrial fractions were incubated at 37°C with ATP and a regenerating system. Results are expressed as percentages of the total label on the gradient in the lysosomal (density >1.144; ●) and endosomal (density 1.090–1.130; o) positions and are means ± SEM with numbers of experiments in parentheses. The values for 0-min incubation are from 30 experiments.

For analysis using Ficoll gradients, 1-ml samples of postmitochondrial supernatant were similarly incubated. They were then diluted to 5 ml with 0.25 M sucrose, 10 mM TES, 1 mM $MgCl_2$, pH 7.4, and loaded over 30-ml linear gradients of 1–22% wt/vol Ficoll containing 0.25 M sucrose, 10 mM TES, and 1 mM EDTA, pH 7.4, over a 4-ml cushion of 45% wt/vol Nycodenz containing 10 mM TES, 1 mM EDTA, pH 7.4. The tubes were centrifuged for 1 h at 206,000 g in a rotor (model VTi 50; Beckman Instruments, Inc., Fullerton, CA) to separate dense and light endosomes (Branch et al., 1987a).

Preparation of Lysosome-depleted Postmitochondrial Supernatant and Lysosomes

10 ml of postmitochondrial supernatant prepared from the livers of rats injected with ^{125}I-ASF 10 min before killing was layered over 22% Ficoll, 0.25 M sucrose (19 ml), with a cushion (10 ml) of 45% Nycodenz (both containing 10 mM TES, 1 mM EDTA, pH 7.4) and centrifuged for 1 h at 206,000 g in a rotor (model VTi 50; Beckman Instruments, Inc.). Fractions were collected starting from the bottom of the gradient and counted to ascertain the exact position of the endosomes (light and dense) which, unlike the lysosomes, did not pass through the Ficoll. Fractions containing endosomes were then pooled with all the material of lower density from that gradient. Lysosomes were prepared according to Maguire and Luzio (1985).

Liver Perfusion

Livers were perfused according to Perez et al. (1988) in a controlled temperature cabinet. The liver was established in the apparatus at 37°C and a single pass dose of ^{125}I-ASF was administered. 5 min later, perfusion medium at the chosen temperature was substituted for medium at 37°C, and the cabinet heating was switched off. Over the next 5–10 min, the temperature of the medium leaving the liver dropped to the chosen temperature and perfusion was continued at this temperature for a total of 30 min after ASF addition. The liver was then removed, homogenized, and fractionated (Perez et al., 1988).

Assay Methods

N-Acetyl-β-glucosaminidase was assayed according to Maguire et al. (1983), and 5'-nucleotidase was assayed in the presence of 1% Triton X-100 according to Luzio and Stanley (1983). Protein was measured using a protein assay reagent (Bio-Rad Laboratories, D-8000 Munich, West Germany) after
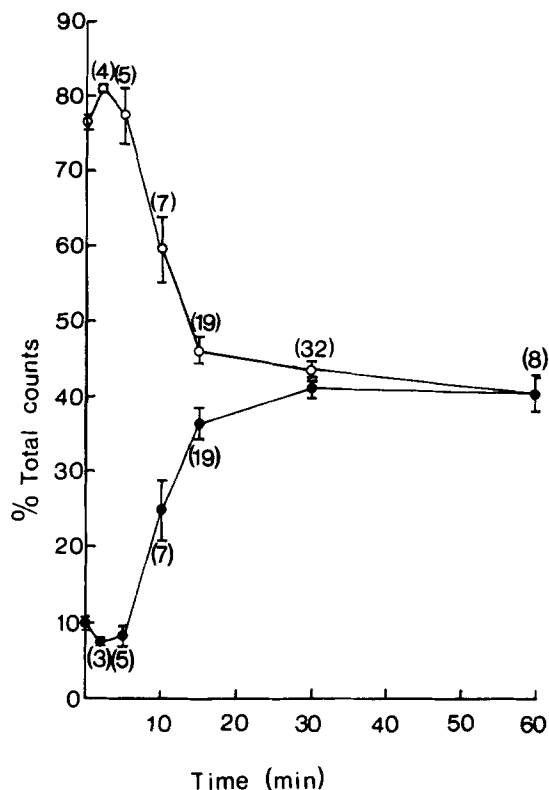
digestion in 0.4 M NaOH for 30 min at 37°C. ATP concentrations were measured by the luciferase method of Malaisse et al. (1978).

Results

Rat livers were loaded with ^{125}I-ASF by intravenous injection such that 10 min after injection the ligand was mostly (>60%) located in late endosomes, <8% associated with lysosomes, and ~20% with plasma membranes and early endosomes (Branch et al., 1987a). Immediate analysis of postmitochondrial supernatants prepared from such livers on Nycodenz gradients showed that the radioactivity appeared in a single broad peak of density ~1.09–1.13 clearly distinguished from the peak of activity of the lysosomal enzyme marker *N*-acetyl-β-glucosaminidase in the presence or absence of ATP and a regenerating system (Fig. 1). However, density gradient analysis of the postmitochondrial supernatant after a 30-min incubation at 37°C in the presence of ATP and a regenerating system showed that much (41 ± 1%; $n = 32$) of the radioactivity had moved to a peak density >1.14 coinciding with the peak of *N*-acetyl-β-glucosaminidase activity (Fig. 1) (Branch et al., 1987b). The distribution of radioactivity was almost unchanged by the addition of ATP and a regenerating system to the postmitochondrial supernatant if the temperature was kept at 0°C for up to 1 h.
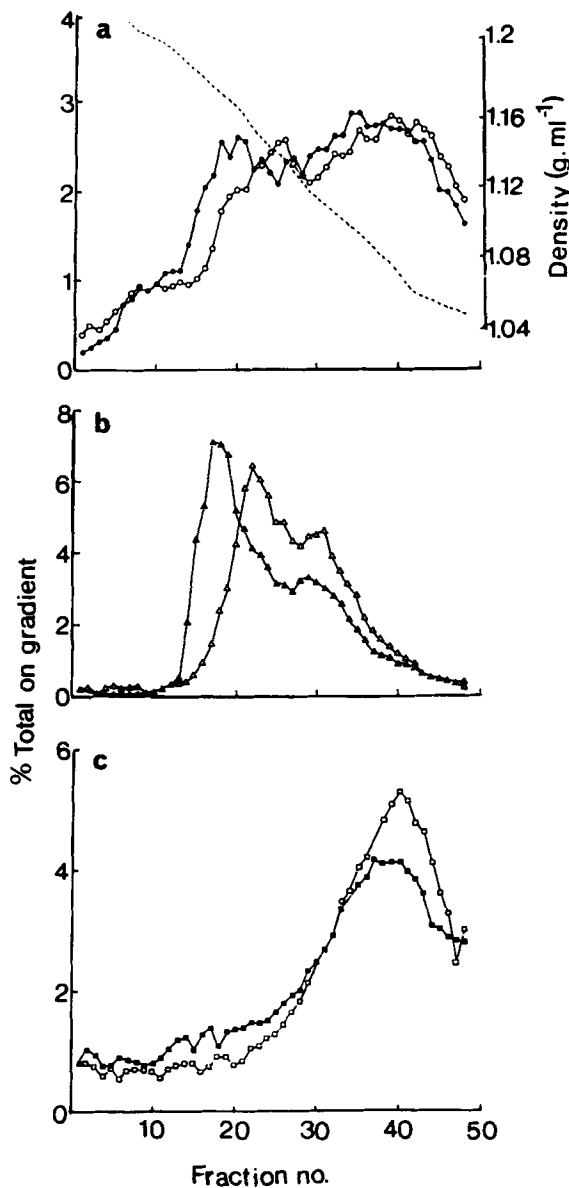
Figure 3. Specificity of transfer to the lysosomal position on Nyco-denz gradients. (a) Protein (O; ●) and (b) 5'-nucleotidase (△; ▲) distributions on 0.25 M sucrose-45% wt/vol Nycodenz gradients after 0-min (open symbols) or 30-min (closed symbols) incubation of postmitochondrial supernatant with ATP and a regenerating system at 37°C. (c) Distribution of ^{125}I-ASF added to the postmito-chondrial supernatant and incubated for 0 min (□) or 30 min (■) at 37°C. Representative experiments are shown. (- - - -) Density.

Incubation at 37°C for 30 min without ATP and a regenerating system transferred <15% of the radioactivity to the lysosomal position. ATP levels in the postmitochondrial supernatant were <15 μM; this was raised to 2.5 mM within 1 min of adding ATP and a regenerating system.

Examination of the time course of the transfer from the endosomal to the lysosomal position on Nycodenz gradients showed a lag during the first 5 min of incubation at 37°C followed by a rapid rise to a plateau within 15–30 min (Fig. 2). The redistribution of radioactivity was not due to a nonspecific bulk aggregation of membranes since the distribution of
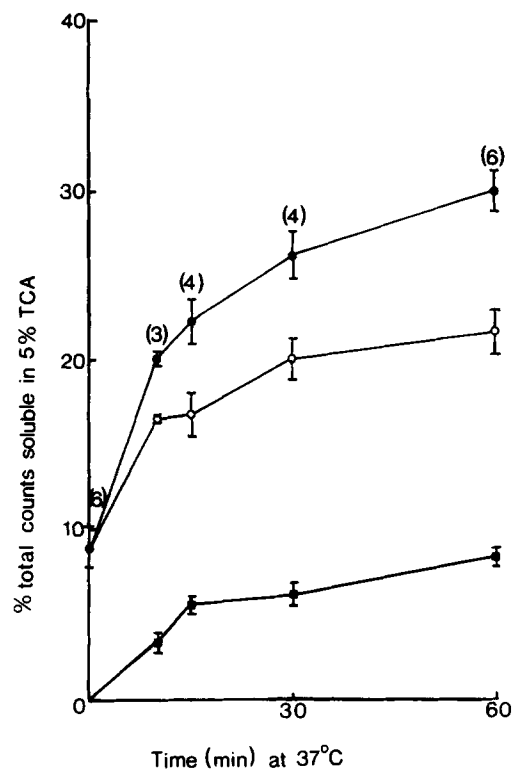

Figure 4. Time course of production of TCA-soluble radiolabel. Postmitochondrial fractions from rats which had received ^{125}I-ASF 10 min before killing were diluted with an equal volume of 0.25 M sucrose, 10 mM TES, pH 7.4, and incubated at 37°C with (●) or without (O) ATP and a regenerating system. Results are expressed as percentages of the total label in the postmitochondrial fractions and are means ± SEM with numbers of experiments in parentheses. (■) Difference between acid-soluble radiolabel production with and without ATP and a regenerating system.

protein across the gradient was little changed after a 30-min incubation at 37°C with ATP and a regenerating system (Fig. 3 a), and the distribution of 5'-nucleotidase, principally a plasma membrane marker but also found in endosomes (Branch et al., 1987a), was only slightly altered (Fig. 3 b). The transfer was not due to the preferential binding by lyso-somes of free ^{125}I-ASF released from the endosomes, since addition of free ^{125}I-ASF to an otherwise unlabeled post-mitochondrial supernatant resulted in a simple broad peak of radioactivity in the upper part of the gradient both before and after incubation with ATP and a regenerating system (Fig. 3 c).

If ASF were being transferred to lysosomes, its digestion would be expected to result in the production of TCA-soluble radioactivity. Initially ~9% of the total radiolabel in the postmitochondrial supernatant was acid soluble. Incubation at 0°C for 2 h with or without ATP and a regenerating system caused no significant change in this figure. However, incubation at 37°C produced an increase in acid-soluble radioactiv-ity, which was more rapid and sustained after initial addition of ATP and a regenerating system (Fig. 4).

Transfer Occurs from Late Endosomes

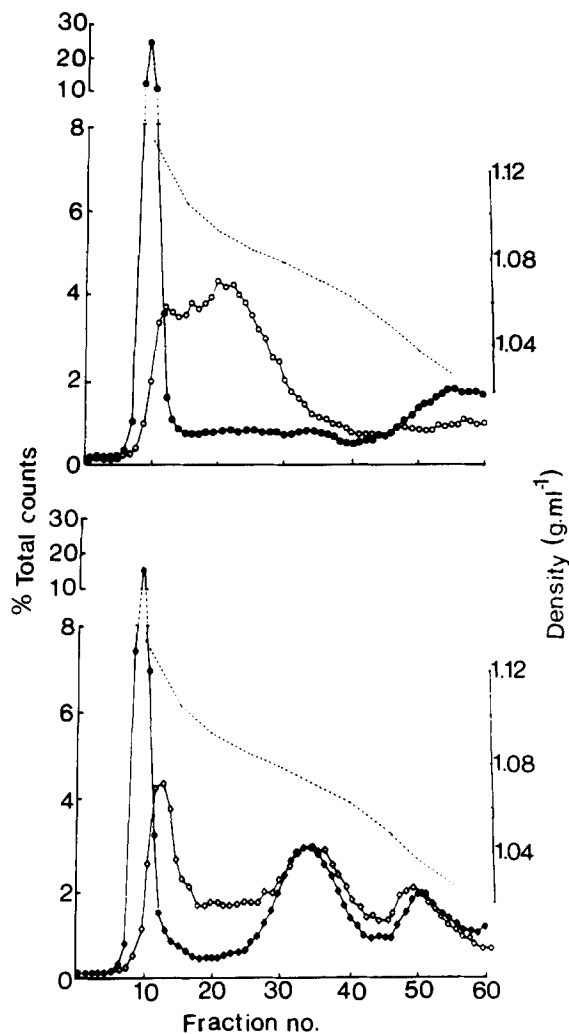To demonstrate that late endosomes were the species in-

Figure 5. Transfer of pIgA and ASF from late (dense) but not early (light) endosomes. Samples of postmitochondrial supernatant from a rat, which had received ^{125}I-pIgA ($\Diamond$; $\blacklozenge$) and ^{131}I-ASF (o; $\bullet$) intravenously 10 min before killing, were incubated with ATP and a regenerating system for 30 min at 0°C (*open symbols*) or 37°C (*solid symbols*). The samples were then loaded over linear gradients of 1–22% wt/vol Ficoll and centrifuged to separate dense and light endosomes. In this representative experiment, dense endosomes were present between tubes 17 and 26 and light endosomes between tubes 27 and 42. (- - - -) Density.

Figure 6. Effect of lysosome depletion and replacement on transfer of ^{125}I-ASF to the lysosomal position. Samples of lysosome-depleted postmitochondrial fraction were mixed with less than half their volume of buffered sucrose containing varying amounts of independently prepared lysosomes and incubated with ATP and a regenerating system at 37°C for 30 min before examination on 0.25 M sucrose–45% wt/vol Nycodenz gradients. The N-acetyl-β-glucosaminidase activities of the postmitochondrial supernatants and of the various mixtures of lysosome-depleted postmitochondrial fractions with buffered sucrose and lysosomes were also measured. The percentage of the total counts on each Nycodenz gradient found in the lysosomal position (determined by refractive index measurements on the fractions) is plotted against the percentage of the total postmitochondrial supernatant N-acetyl-β-glucosaminidase activity found in the mixture used for that gradient. (*) Gradients whose N-acetyl-β-glucosaminidase peak was less dense than the normal lysosomal region (i.e., intermediate between normal endosome and lysosome densities). Results shown are for lysosome-depleted postmitochondrial fractions from two different rats combined with lysosomes made from two additional rats.

volved in interaction with lysosomes, endosomes were loaded with a mixture of ^{125}I-pIgA and ^{131}I-ASF by intravenous injection of this mixture into the rat 10 min before killing. pIgA is initially taken up into the same light endocytic compartment as ASF, but unlike ASF, much pIgA is transcytosed to bile rather than passing on through the later endosomal compartments to the lysosomes (Geuze et al., 1986; Perez et al., 1988). The remainder of the pIgA follows the degradative pathway to lysosomes in the same way as ASF (Schneider, 1982; Perez et al., 1988). The doubly labeled postmitochondrial supernatants were analyzed on 1–22% wt/vol Ficoll gradients which separate plasma membrane, early (light) endosomes, and late (dense) endosomes from each other and from a peak at the bottom of the gradient containing lyso-
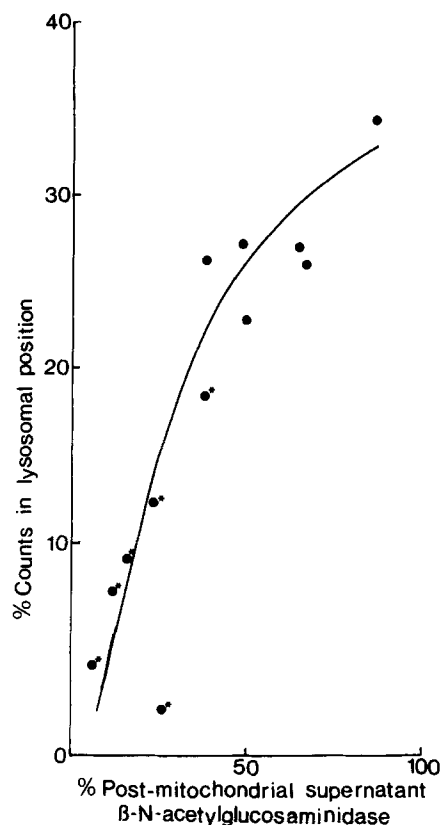
somes and very dense endosomes (Branch et al., 1987a; Perez et al., 1988). Comparison of such analyses of postmitochondrial supernatants that had been incubated with an energy source for 30 min at 0 or 37°C showed that only ASF and pIgA from the late (dense) endosome compartment moved to the end peak; the pIgA in the early (light) endosomes remained as in the sample kept at 0°C (Fig. 5). It is also clear on such Ficoll gradients that after incubation at 37°C the amount of ASF label at the top of the gradient was increased as would be expected if some ^{125}I-ASF was being degraded enzymically. This cannot be seen on the steeper Nycodenz gradients where the soluble region is not well enough separated from the endosomal region.

Experiments were performed with two ligands as described because movement of ASF into the dense endosome peak is so rapid that it is difficult to kill a rat quickly enough after injection to trap most of the ASF in the light endosome peak. However, since pIgA injected 10 min before killing may arguably not be in the same light endosomal compartment as that initially entered by ASF, even though they appear in the same position on Ficoll gradients, experiments were also performed with rats injected with ^{125}I-ASF 3 min before killing. At this time most of the label appears in the light endosomal region of the gradient, although some has already reached the denser compartments (Branch et al., 1987a). As expected, when such postmitochondrial supernatants were incubated with ATP and a regenerating system for 15 min, the proportion of the label found in the lysosomal region of Nycodenz gradients was markedly reduced (14.3 ± 3.09% as compared with 36.3 ± 1.98% [from 3 and 36 experiments, respectively] for the usual 10-min labeling time). Examination on Ficoll gradients showed that label moved only from the dense endosome region.

Transfer Requires Lysosomes as Acceptors

Postmitochondrial supernatants may be very effectively depleted of their lysosomes by centrifugation over a step of 22% Ficoll. Transfer of label to the normal lysosomal density position in such lysosome-depleted preparations was reduced to ~5% of the normal level. Addition of increasing amounts of intact lysosomes, prepared from another rat by isopycnic centrifugation in Percoll (Maguire and Luzio, 1985), before incubation at 37°C with a regenerating system produced proportionate increases in the amounts of ^{125}I-ASF appearing in the lysosomal position (Fig. 6). Lysosomes prepared by this method contain at most 2% of the liver endosomes, as shown by their preparation from a liver whose endosomes had been loaded with ASF. When few lysosomes were present, the peak of N-acetyl-β-glucosaminidase activity moved to a position between the normal lysosomal and endosomal positions after incubation at 37°C with an ATP-regenerating system. Some ^{125}I-ASF also moved into this intermediate position. The N-acetyl-β-glucosaminidase activity of samples kept at 0°C was in the usual lysosomal position. Preliminary experiments (data not shown) using electron microscope autoradiography on fractions from the lysosomal position of gradients after ^{125}I-ASF transfer (conditions as in Fig. 1) have shown a preponderance of silver grains associated with elongated electron-dense structures whose morphology resembles that of similar structures in purified rat liver lysosome preparations (Wattiaux et al., 1978).

Transfer Requires Cytosol

Loaded postmitochondrial supernatants could be centrifuged for 1 h at 190,000 g to pellet all particulate material and still transfer their label to the lysosomal position quite normally if incubated appropriately after remixing. The supernatant used for the resuspension of the pellet could come from a different rat and could have been stored overnight at 4°C or frozen. However, resuspension of the pelleted material in buffered sucrose with or without addition of BSA prevented normal transfer as did pretreatment of the supernatant with heat or protease (Table I). Treatment of cytosol or of whole postmitochondrial supernatant with 1.5 mM N-ethylmaleimide for 20 min at 4°C did not prevent subsequent transfer.

Table I. Transfer of ^{125}I-ASF to the Lysosomal Region on 0.25 M Sucrose–45% wt/vol Nycodenz Gradients after Resuspension of the Organelles from Postmitochondrial Supernatant

Resuspension medium	^{125}I-ASF in lysosomal region after incubation with ATP and a regenerating system for 30 min at 37°C
	%
Fresh cytosol	47.6 ± 2.9 (6)
Cytosol stored at −20°C (2 wk)	42.3 ± 4.8 (4)
0.25 M sucrose, 10 mM TES, 1 mM Mg^{2+}, pH 7.4	9.8 ± 1.4 (3)
0.25 M sucrose, 10 mM TES, 1 mM Mg^{2+}, pH 7.4, plus 40 mg/ml BSA	10.6 (2)
Cytosol trypsin treated (1 mg/ml; 60 min, 4°C)	11.5 (2)
Cytosol boiled 10 min	9.2 (2)

Numbers in parenthesis denote number of experiments.

Low Temperature Inhibition of Transfer

Transfer of ^{125}I-ASF to the lysosomal position after a 15-min incubation at different temperatures followed the curve shown in Fig. 7 a. For comparison we examined transfer of ^{125}I-ASF to lysosomes in whole livers given a single pass dose of ^{125}I-ASF at 37°C and then cooled to different temperatures in a perfusion apparatus. In the whole liver, however, ASF radiolabel left the lysosomes for the soluble region at the top of the gradient much more rapidly and completely than was the case in the in vitro system (see Discussion). The
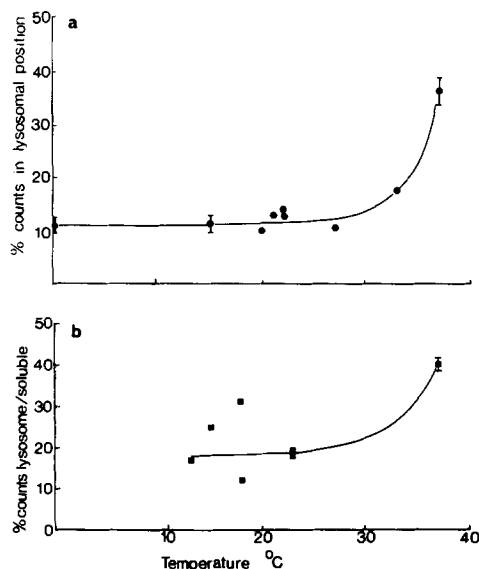

Figure 7. (a) Effect of lowered temperatures on in vitro transfer of ^{125}I-ASF from the endosomal to the lysosomal position on 0.25 M–45% wt/vol Nycodenz gradients (●) compared with (b) effect of lowered temperatures in a perfused liver preparation on movement of ^{125}I-ASF from the endosomal to the lysosomal plus soluble positions on Nycodenz gradients (■). Data points in a for 0, 15, and 37°C are means ± SEM from 18, 3, and 10 experiments, respectively; the remaining points are from individual experiments. The 37°C data point in b is from three perfused rat livers; the remaining points are from individual perfused livers.

data in Fig. 7 b therefore include radioactivity from both these regions. The overall temperature response appears very similar to that in the in vitro transfer system.

Discussion

The cell-free transfer of in vivo-endocytosed ^{125}I-ASF from the endosomal to the lysosomal position on density gradients, after incubation of postmitochondrial supernatants under suitable conditions, has been shown to specifically require lysosomes and undamaged cytosol and to involve dense (late) rather than light (early) endosomes. The time course of the process is consistent with the time taken for ^{125}I-ASF to appear in lysosomes in whole liver (Breitfeld et al., 1985). Large-scale nonspecific aggregation of membranes during incubation of postmitochondrial supernatants is ruled out by the minimal nature of the change in protein distribution which accompanies the movement of ^{125}I-ASF. Although 5'-nucleotidase is primarily a plasma membrane enzyme, ∼20–25% of the total cellular activity is found in endosomes (Branch et al., 1987a); the small change seen in the distribution of this enzyme was therefore expected. The concentration of radiolabel in the lysosomal region was not reproduced by incubation of free ^{125}I-ASF in unlabeled postmitochondrial supernatants; hence, the dense endosomes must interact directly with the lysosomes rather than simply releasing ^{125}I-ASF, which is then picked up from solution by the lysosomes.

Movement to lysosomes was accompanied by increased acid-soluble radioactivity as the ASF was digested. Some increase occurred on incubation at 37°C; this would be expected since some of the ASF label will already have passed the stage at which ATP is required on the path to lysosomes during the in vivo loading period. Fig. 2 shows that ∼10% of the label appeared in the lysosomal position in these experiments in the absence of any incubation at 37°C. This label would be expected to become acid soluble on incubation at 37°C even in the absence of ATP and a regenerating system in addition to the 9% of label which has already become acid soluble in vivo (Fig. 4). The rate and extent of ASF degradation was markedly greater in the presence of ATP and a regenerating system (Fig. 4). However, the initially rapid appearance of acid-soluble radioactivity slowed with time as also shown by Dunn et al. (1979) for intact lysosomes preloaded with ASF and incubated in vitro. Cysteine cathepsins seem to play a major part in ASF digestion since leupeptin markedly inhibits ASF breakdown (Kirschke and Barrett, 1987; Dunn et al., 1979; Branch et al., 1987a). These enzymes are irreversibly denatured at pH >7 and require the essential sulphydryl group in the active site to be fully reduced (Kirschke and Barrett, 1987). Intralysosomal pH is known to rise during incubation in vitro (Ohkuma et al., 1982) and, since the transfer system is insensitive to N-ethylmaleimide, thiol reagents are not added to our normal reaction mixture. Incubation conditions optimized for the transport reaction are therefore far from those best for lysosomal proteolysis in vitro.

We have shown the temperature inhibition effect displayed by the in vitro system to be very similar to the effects of temperature reduction on movement of ^{125}I-ASF radiolabel to lysosomes and the soluble region in perfused liver. Dunn et

al. (1980) and Marsh et al. (1983) similarly showed that incubation at temperatures <20°C prevented transfer of ligand from late endosomes to lysosomes in perfused liver and cultured cells, respectively. Our dense endosomes can probably be equated with the receptor-negative endosomes of Mueller and Hubbard (1986), which neither took up nor passed on ^{125}I-asialoorosomucoid at 16°C, and with the late endosomes of Schmid et al. (1988), which were involved in delivery of material to lysosomes and differed in polypeptide composition from early endosomes. The energy requirements of the present system are consistent with the findings of Tolleshaug et al. (1985) that lowering cell concentrations of ATP prevented transfer of asialoglycoproteins to lysosomes. In its resistance to N-ethylmaleimide, the present system differs from the cell-free transport of protein between successive compartments of the Golgi apparatus (Balch and Rothman, 1985; Rothman, 1987) and cell-free transport between early endosomes (Braell, 1987), although undamaged cytosol is required in all cases.

It seems likely that the movement of ^{125}I-ASF observed using the gradients is due to a specific association of dense endosomes with lysosomes which forms part of the normal process of lysosomal degradation in vivo. Since lysosomes are necessary in the system, both a simple increase in endosomal density and/or a maturation of endosomes into lysosomes as suggested in 3T3 cells (Roederer et al., 1987) would appear to be excluded. ASF is being exposed to lysosome enzymes, since breakdown to acid-soluble products is occurring under the conditions required for the transfer of endosomal label to the lysosomal position on gradients. Berg et al. (1985) and Kindberg et al. (1987) detected two types of lysosomes in cultured hepatocytes that differed in density on Nycodenz gradients. The gradient region, in which we observed transferred endosomal radioactivity, probably corresponds to their dense lysosomes which contain the bulk of the lysosomal enzymes and in which most digestion normally occurs. The light lysosomes, which they describe as particularly evident in conditions of high autophagic activity, may well be related to the N-acetyl-β-glucosaminidase activity and radiolabel we observed in the gradient region between the normal lysosomal and endosomal positions after incubating severely lysosome-depleted preparations with an ATP regenerating system at 37°C. In both this case and during high autophagic activity, the proportion of lysosomes to vesicles with material to be digested is low. Within the normal cell, substrate within the dense lysosomes is usually digested as rapidly as it is delivered, but in our preparation the rate of digestion falls off over 15–30 min as discussed above and a build-up of radioactivity in the lysosomal region results.

It is clear that the present system shows an energy-, cytosol-, and temperature-dependent, specific interaction between dense (late) endosomes and lysosomes in vitro. Although fusion of membrane vesicles (stripped, clathrin-coated vesicles) with lysosomes has been observed previously in vitro (Altstiel and Branton, 1983), it is not clear that this data is relevant to the present experiments. Further examination of our cell-free system, including a more detailed knowledge of the energy requirements and the determination of cytosolic components required for lysosome-endosome interaction should lead to a better understanding of the late stages in the transfer of endocytosed material to lysosomes.

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