Intracellular localization of β -glucuronidase in fibroblasts after direct transfer from macrophages

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The subcellular distribution of β -glucuronidase acquired by deficient human fibroblasts during co-culture with peritoneal macrophages was compared with that taken up by receptor-mediated endocytosis. Labelled enzyme taken up via receptors was located initially in a low-density endosomal fraction and was transferred to lysosomes within a few minutes. The β -glucuronidase acquired during 24 h of co-culture was present almost entirely within lysosomes and had a distribution profile identical with that of endogenous β -hexosaminidase. Monensin prevented transfer of radiolabelled enzyme from endosomes to lysosomes and had a similar effect on the distribution of enzyme acquired by direct transfer, causing β -glucuronidase to accumulate within endosomes. When the temperature was lowered from 37 °C to 19 °C, the rate of transfer of enzyme from endosomes to lysosomes was decreased during both direct transfer and indirect receptor-mediated endocytosis. These results show that a lysosomal enzyme acquired by direct transfer during cell-to-cell contact follows a similar intracellular route and has a similar distribution to that of enzymes taken up via cell-surface receptors.

INTRODUCTION

Transfer of molecules such as growth factors, hormones, neurotransmitters, cytokines and lysosomal enzymes from the cells in which they are produced to others in which they exert a biological effect is a widespread phenomenon (Neufeld *et al.*, 1975; Kahn, 1976; Kaplan *et al.*, 1977; Pastan & Willingham, 1981; Willingham & Pastan, 1984; Goldstein *et al.*, 1985). Lysosomal enzymes, for example, may be transferred from donor to recipient cells by two different processes (Dean & Diment, 1984). In the more clearly understood of these, enzymes secreted by donors can bind to receptors on recipient cells, are rapidly internalized and eventually become localized within lysosomes (Shepherd *et al.*, 1983). In an alternative process, donor cells such as lymphocytes or macrophages that are able to adhere to recipient cells can transfer enzymes directly to them by a mechanism for which cell-to-cell contact is obligatory (Olsen *et al.*, 1981, 1982; McNamara *et al.*, 1985). Cultured lymphocytes secrete very low amounts of lysosomal enzymes, and the β -glucuronidase present in or secreted by them is not taken up into deficient fibroblasts via receptors, but can be transferred to them only during direct contact (Olsen *et al.*, 1982; Dean & Diment, 1984). Peritoneal macrophages, however, are able to transfer enzyme both by secretion, which can be blocked by competitive inhibition, and by direct transfer, which does not take place when cells are separated by Millipore membranes (McNamara *et al.*, 1985).

Most lysosomal enzymes have oligosaccharide side chains that can bind to one or more of the several carbohydrate receptors present on recipient cells. Fibroblasts, for example, are able to recognize lysosomal enzymes containing mannose 6-phosphate groups (Kaplan *et al.*, 1977; Ullrich *et al.*, 1978), whereas macrophages have receptors of broader specificity that can bind

glycoproteins with mannose, *N*-acetylglucosamine or fucose residues (Stahl *et al.*, 1978, 1980; Shepherd *et al.*, 1981). Subsequent cell-disruption experiments followed by separation of intracellular organelles on density gradients of Percoll have shown that enzymes such as β -glucuronidase, which enter cells via these receptors, become associated initially with a light membrane fraction and are then transferred to lysosomes some 15–20 min after uptake (Merion & Sly, 1983; Wileman *et al.*, 1984). Entry into this intermediate compartment, whose vesicles are most commonly termed endosomes, is often extremely rapid, occurring within 1–2 min after commencement of endocytosis. Endosomes appear to play an important role in intracellular sorting, since ligands such as internalized lysosomal enzymes are separated from their receptors within the endosomal compartment before being transferred to lysosomes (Wileman *et al.*, 1985).

Little is known, however, about the intracellular localization of lysosomal enzymes acquired by the alternative process of direct cell-to-cell transfer. Lymphocytes and macrophages are both able to transfer enzymes directly to a variety of recipient cells with which they come into contact, including fibroblasts, neuronal and glial cells (Olsen *et al.*, 1981, 1982; McNamara *et al.*, 1985; Dean *et al.*, 1985b). The β -glucuronidase acquired by direct transfer from macrophages has been identified within recipient cells by specific cytochemistry (McNamara *et al.*, 1985) or indirect immunofluorescence (Dean *et al.*, 1985b), and appears to be concentrated in discrete granules with a punctate distribution, characteristic of that of lysosomes.

Enzymes transferred directly to recipient fibroblasts during cell-to-cell contact may therefore follow an intracellular pathway common to that of enzymes taken up by indirect receptor-mediated endocytosis. If enzymes acquired by direct transfer follow a different route, how-

ever, then this may well be reflected in a different distribution of newly transferred enzyme within recipient cells. The experiments described here were undertaken in order to determine whether the β -glucuronidase present in deficient fibroblasts after direct transfer is indeed contained within lysosomes, and if endosomes are involved as an intermediate during the process of direct transfer.

MATERIALS AND METHODS

Materials

Tissue-culture media, supplements and Dulbecco's phosphate-buffered saline (PBS) were supplied by Flow Laboratories, Rickmansworth, Herts., U.K., and sera were from Imperial Laboratories, Salisbury, Wilts., U.K. Percoll and density marker beads were obtained from Pharmacia (U.K.). Methylumbelliferyl β -D-glucuronide and 2-acetamido-2-deoxy- β -D-glucopyranoside enzyme substrates were purchased from Koch-Light Laboratories, Colnbrook, Bucks., U.K., and protein reagent and standards were from Bio-Rad Laboratories, Watford, Herts., U.K. Na¹²⁵I, UDP-[¹⁴C]galactose and [2-³H]AMP were supplied by Amersham International. Monensin was obtained from Calbiochem, Cambridge, U.K.

Cell culture

β -Glucuronidase-deficient fibroblasts, from a patient with mucopolysaccharidosis type VII (D.F.) were generously given by Dr. Adrian Sewell (Mainz, Germany). They were cultured in 175 cm² Falcon flasks in Dulbecco's minimal essential medium (DMEM) supplemented with 10% (v/v) foetal-calf serum, 0.37% NaHCO₃, 2 mM-glutamine, 50 units of penicillin/ml and 50 μ g of streptomycin/ml. Macrophages were elicited from CBA mice by injecting 2 ml of 1% (w/v) starch in PBS intraperitoneally, and were harvested 3 days later (Diment & Dean, 1983). They were resuspended in the above DMEM, supplemented by addition of 10% (v/v) conditioned medium collected from L929 cells and plated out in 60 mm-diam. dishes (3 $\times$ 10⁶ cells in 5 ml of medium). After 60 min at 37 °C, non-adherent cells were washed away with warm PBS and the remaining cells were cultured for a minimum of 4 days before use.

Purification of β -glucuronidase

The β -glucuronidase secreted by 3T3 fibroblasts was purified from culture medium by affinity chromatography on concanavalin A-Sepharose, followed by antibody affinity chromatography (Dean *et al.*, 1985a). Batches containing 100 μ g of protein were iodinated by using Iodobeads and 1 mCi of Na¹²⁵I (Dean *et al.*, 1985a).

Cell fractionation

β -Glucuronidase deficient fibroblasts (3 $\times$ 10⁶, in a 175 cm² flask) were collected by incubating them for 5 min at 37 °C with 3 ml of 0.25% (w/v) trypsin. The trypsin was neutralized with 10 ml of DMEM + 10% foetal-calf serum pre-cooled to 4 °C; the cells were collected by centrifugation, suspended in 2 ml of 0.25 M-sucrose/3 mM-imidazole, pH 7.5 at 4 °C, and disrupted by nitrogen cavitation after pressurization for 15 min at 65 lb/in² (450 kPa) in a nitrogen bomb. The homogenate was triturated through a Pasteur pipette and centrifuged at 800 g (r_{av} . 8 cm) for 5 min at 4 °C to remove nuclei and

remaining whole cells. Then 10 mg of bovine serum albumin was added to the postnuclear-supernatant solution as a carrier and the suspension layered over 39 ml of Percoll in 0.25 M-sucrose (density 1.06 g/ml) and centrifuged at 45000 g (r_{av} . 10.5 cm) for 60 min. Fractions (1 ml) were collected from the bottom of the resulting gradient at room temperature and assayed for enzyme activity or radioactivity. In some experiments those fractions containing endosomes or plasma membranes were pooled, layered over Percoll at a density of 1.04 g/ml and re-centrifuged for a further 60 min at 45000 g. Mouse peritoneal macrophages, were collected by incubating them at 4 °C for 5 min with 3 ml of PBS/0.02% EDTA and then gently scraping the culture dishes with a rubber policeman. The cells were suspended in 2 ml of the sucrose/imidazole homogenization buffer containing albumin and disrupted and fractionated as above.

Uptake of iodinated β -glucuronidase

β -Glucuronidase from 3T3 cells was used as a ligand for uptake into both fibroblasts and macrophages. Cells were incubated in monolayers at 37 °C or 19 °C with 10⁸ c.p.m. of β -glucuronidase added to 10⁷ cells grown either in 15 ml of medium in 175 ml flasks (fibroblasts) or in 3 ml of medium in 60 mm-diam. culture dishes (3 $\times$ 10⁶ macrophages/dish). After uptake, cells were washed three times with PBS at 4 °C and either fractionated immediately or chased for various periods of time by incubating them with unlabelled DMEM + 10% heat-inactivated foetal-calf serum. For binding experiments, fibroblasts were washed with serum-free DMEM cooled to 4 °C and left at this temperature for 15 min. Fresh cold medium (7.5 ml, containing 10⁸ c.p.m. of labelled β -glucuronidase) was added to each flask for 2 h at 4 °C, and the cells were washed three times in cold PBS and incubated with 3 ml of 0.25% trypsin for 10 min at 4 °C. Trypsin was neutralized with 10 ml of cold DMEM containing 10% foetal-calf serum, and the flasks were shaken vigorously and cells collected by centrifugation (1000 g for 10 min) at 4 °C. In those experiments in which 10 μ M-monensin was added, Ham's F12 medium buffered with 20 mM-Hepes was used in place of DMEM. Monensin was added to all media and buffers used for this series of experiments until cell disruption had been completed.

Co-culture experiments

Confluent cultures of β -glucuronidase-deficient fibroblasts were collected by trypsin treatment, resuspended in macrophage growth medium containing 5 mM-mannose 6-phosphate to inhibit receptor-mediated endocytosis (McNamara *et al.*, 1985) and plated out at a ratio of 1:10 on to macrophages in 60 mm-diam. Petri dishes containing 3 $\times$ 10⁶ cells in 5 ml of medium plus 5 mM-mannose 6-phosphate. After 24 h of co-culture, cells were washed in 5 ml of PBS and incubated with 3 ml of 0.25% trypsin for 5–10 min at 37 °C, to detach the fibroblasts. The trypsin was neutralized with 3 ml of complete medium, and the cells were then kept at 4 °C until fractionation had been completed. Almost 99% of the macrophages remained attached to the culture dishes after this procedure. The suspension of cells was centrifuged at 1000 g for 10 min in an MSE Centaur bench centrifuge, and then they were resuspended in 2 ml of 0.25 M-sucrose/3 mM-imidazole buffer, pH 7.4, layered

over 39 ml of a gradient of Percoll in 0.25 M-sucrose [prepared by centrifuging a solution of starting density 1.06 g/ml at 21000 g (r_{av} , 10.5 cm) in a Sorvall RC5C centrifuge for 40 min] and centrifuged at 2850 g (r_{av} , 8 cm) on an MSE Chillspin for 25 min. The top 5 ml of the gradient, which contained the fibroblasts and less than 20 % of the few remaining macrophages, most of which sedimented at a higher density, was collected, added to 45 ml of DMEM, centrifuged at 1000 g for 10 min, and the pellet resuspended in 2 ml of sucrose/imidazole buffer for subcellular fractionation as described above. The macrophages remaining on the plates after co-culture were washed once with PBS at 4 °C, removed by scraping in PBS/0.02 % EDTA at 4 °C with a rubber policeman, collected by centrifugation at 1000 g for 10 min, and resuspended in 2 ml sucrose/imidazole buffer. Both types of cell were disrupted by nitrogen cavitation at 65 lb/in² (450 kPa) for 15 min in a nitrogen bomb (Scientific and Medical Products, Didsbury, Manchester, U.K.).

Assays

The activities of β -glucuronidase and β -hexosaminidase were determined fluorimetrically with 4-methylumbelliferyl β -D-glucuronide and 4-methylumbelliferyl 2-acetamido-2-deoxy- β -D-glucopyranoside as substrates (Dean *et al.*, 1982). For both enzymes 1 unit of activity hydrolysed 1 nmol of substrate/h at 37 °C. Protein concentration was determined with the Bio-Rad reagent (Bradford, 1976) and bovine γ -globulin as a standard. Galactosyl transferase activity was used as a marker for Golgi membranes and assayed by measuring the transfer of labelled [¹⁴C]galactose from UDP-galactose to ovalbumin, by the method of Beaufay *et al.* (1974), in which 50 mM-Tris/HCl buffer, pH 7.4, was substituted for cacodylate/HCl buffer. Plasma membranes were detected by their 5'-nucleotidase activity (Avruch & Wallach, 1976) with [2-³H]AMP as a substrate.

RESULTS

Localization of lysosomal enzymes

The distribution of β -hexosaminidase and β -glucuronidase was used to determine the position of lysosomes within the gradient. Both enzymes were found to have an identical sedimentation profile after fractionation of donor macrophages, with more than 95 % of their activity being located in those fractions with maxima at a density between 1.10 and 1.13 g/ml. A much smaller proportion, comprising only 5 % of the total, was present in more buoyant fractions sedimenting at a density of 1.04 g/ml near the top of the gradient (results not shown). The hexosaminidase activity profile of β -glucuronidase-deficient fibroblasts was similar to that of the macrophages, except that only 70–80 % of their total activity sedimented as a lysosomal fraction, which had a slightly lighter buoyant density (1.08–1.10 g/ml) than that of macrophages. A higher percentage of the total activity (20–30 %) was present in the endosomal fraction (Fig. 1a). Fibroblasts 5'-nucleotidase activity, indicative of the presence of plasma membrane, was concentrated in those fractions of lowest density at the top of the gradient, but did overlap the endosomal peak, with a small additional peak of activity present in the lysosomal fraction (Fig. 1b). Galactosyltransferase activity, indicative of Golgi membranes, was present primarily in a buoyant fraction

Fig. 1. Fractionation of fibroblasts after uptake of ¹²⁵I-labelled β -glucuronidase

A typical sedimentation profile is shown of cells that had been (a) pulse-labelled for 10 min or (b) labelled for 10 min, washed, incubated at 37 °C for a further 20 min and then fractionated, as described in the Materials and methods section. Fractions were collected from the bottom of the gradient and analysed for radioactivity (■), β -hexosaminidase (●) and 5'-nucleotidase (◆). Density (---) was determined with Pharmacia marker beads.

at the top of the gradient, which overlapped with, but was distinct from, endosomes. A small secondary peak of activity sedimented with lysosomes (Fig. 2).

When fibroblasts were pulse-labelled for 10 min at 37 °C with ¹²⁵I-labelled β -glucuronidase before fractionation, more than 90 % of the cell-associated radioactivity was present in two fractions near the top of the gradient (Fig. 1a). The most buoyant of these corresponded to the peak of 5'-nucleotidase activity and was therefore presumably present at the cell surface; the other component sedimented at a density of 1.04 g/ml, indicating an endosomal distribution (Fig. 1a). In those cells that had been washed free of ligand after a 10 min pulse and then incubated for a further 20 min at 37 °C, most of the radioactivity (80 %) had moved to a fraction with a density equivalent to that of lysosomes (Fig. 1b), the peak of radioactivity being coincidental with that of endogenous hexosaminidase. Cell surface radioactivity was no longer present.

To confirm that endosomes were separated from plasma membranes during centrifugation, labelled enzyme was bound to fibroblasts at 4 °C. In some experiments this was followed by a subsequent incubation at 37 °C. When cells were fractionated immediately after

Fig. 2. Separation of endosomes from Golgi membranes

Confluent monolayer cultures of fibroblasts were collected by trypsin treatment, and pellets containing 10^7 cells were disrupted by nitrogen cavitation. A typical sedimentation profile is shown of cells that had been fractionated and assayed for β -hexosaminidase (●) and galactosyltransferase (▲) as described in the Materials and methods section.

Fig. 4. Distribution of β -hexosaminidase and β -glucuronidase after direct transfer

A typical sedimentation profile is shown after macrophages and fibroblasts had been co-cultured for 24 h in the presence of excess mannose 6-phosphate, separated, and the fibroblasts fractionated as described in the Materials and methods section. Each fraction was assayed for endogenous β -hexosaminidase (●) and acquired β -glucuronidase (▼).

Fig. 3. Separation of endosomes from plasma membranes

(a) Typical sedimentation profiles are shown of fibroblasts that had been labelled with β -glucuronidase and then fractionated on a gradient of starting density 1.06 g/ml. (b) Profiles obtained when the radiolabelled fractions from (a) were pooled and re-fractionated on a gradient of starting density 1.04 g/ml. ■—■, Radioactivity profile from cells to which labelled β -glucuronidase had been bound at 4 °C; ■·····■, radioactivity profile from cells that had been warmed to 37 °C after binding; ◆—◆, 5'-nucleotidase activity.

binding, two labelled components were separated near the top of the gradient, the most buoyant of which corresponded to the peak of 5'-nucleotidase activity (Fig. 3a). A different pattern of labelling was seen after binding followed by incubation for 10 min at 37 °C, with one major peak of radioactivity which sedimented at a density of 1.04 g/ml and corresponded to the secondary peak of hexosaminidase activity. When the labelled fractions from each gradient were pooled and re-fractionated on a second gradient, starting density 1.04 g/ml, differences were more clearly apparent. Most of the radioactivity from the binding experiments remained at the top of the gradient, closely associated with the peak of 5'-nucleotidase activity (Fig. 3b). In contrast, most of the radioactivity from the binding and warm-up experiments sedimented to the bottom of the gradient at a density of 1.03–1.05 g/ml, indicative of its location within endosomal vesicles.

Location of β -glucuronidase after direct transfer

Representative samples taken from cells separated after 24 h of co-culture were over 90% homogeneous, with few contaminating macrophages. Since the initial co-cultures had been set up at a ratio of 10:1 macrophages to fibroblasts, the remaining macrophages accounted for less than 1% of the original number present before trypsin treatment. Subsequent separation of fibroblasts from the few remaining macrophages on pre-formed gradients of Percoll as described in the Materials and methods section decreased contamination to less than 0.2%, so that the β -glucuronidase activity measured after co-culture was present almost entirely within recipient fibroblasts. When these cells were fractionated, more than 80% of the β -glucuronidase acquired by direct transfer to them was detected in a dense lysosomal fraction, in a pattern of distribution identical with that of endogenous β -hexosaminidase (Fig. 4). The remaining enzyme activity was located in an endosomal fraction, with none detectable either in the cytosol or at the cell surface.

Fig. 5. Effect of lower temperature on distribution of ^{125}I -labelled β -glucuronidase

Typical sedimentation profiles are shown of fibroblasts that had been incubated with ^{125}I -labelled β -glucuronidase for 24 h at either 19 °C (■····■) or at 37 °C (■——■) before homogenization and fractionation.

Fig. 6. Effect of monensin and lower temperature on distribution of β -glucuronidase acquired by direct transfer

Typical sedimentation profiles are shown of fibroblasts that had been co-cultured either for 24 h at 37 °C in the presence of monensin (▼——▼) or for 24 h at 19 °C (▼····▼). Fibroblasts were separated from macrophages, fractionated, and the β -glucuronidase activity of each fraction was determined as described in the Materials and methods section.

Effect of monensin and lower temperature on distribution of β -glucuronidase

Brief co-cultures to determine if the β -glucuronidase acquired by direct transfer did pass through an intermediate endosomal compartment before reaching lysosomes were not possible, since a minimum of 4 h co-culture was required to allow a measurable quantity of enzyme to be transferred (McNamara *et al.*, 1985). A series of experiments was therefore carried out in which cells were co-cultured for 24 h either in the presence of monensin, to entrap any β -glucuronidase which might enter endosomes, or at 19 °C to delay transfer of enzyme

from endosomes to lysosomes. In parallel experiments, fibroblasts were allowed to take up ^{125}I -labelled β -glucuronidase for 24 h at either 37 °C or 19 °C, and the distribution of label acquired at both temperatures was compared.

In cells incubated at 37 °C, almost 80 % of the radioactivity taken up was present in the lysosomal fraction after 24 h, with the remaining 20 % in endosomes (Fig. 5). Cells that had been incubated at 19 °C, however, showed a bimodal distribution of radioactivity, with 48 % of the total still retained in the endosomal fraction and the remainder in lysosomes. In those co-culture experiments carried out at 19 °C, there was a similar decreased degree of transfer to lysosomes, which accounted for less than 20 % of the total, the remainder being present either at the cell surface or in the endosomal fraction (Fig. 6). In cells that had been incubated with labelled β -glucuronidase and monensin at 37 °C, all of the radioactivity was retained in the endosomal fraction (results not shown), and no label was transferred to lysosomes. Similarly, after 24 h of co-culture with monensin, all of the β -glucuronidase activity acquired by fibroblasts was present in two buoyant fractions that corresponded to the cell-surface and endosomal compartments labelled in Fig. 1 in their position of elution (Fig. 6). No enzyme activity was detected in lysosomes.

DISCUSSION

Investigation of the processes involved in receptor-mediated endocytosis of a wide variety of ligands, including insulin, epidermal growth factors (Schlessinger *et al.*, 1978), low-density lipoprotein, β -hexosaminidase (Merion & Sly, 1983), β -glucuronidase and mannosylated bovine serum albumin (Wileman *et al.*, 1984), has shown that they all pass through intermediate vesicles before reaching lysosomes. These intermediate vesicles have been termed 'receptosomes' (Pastan & Willingham, 1981) or 'CURL' (Geuze *et al.*, 1983), but are more often known as 'endosomes' (Helenius *et al.*, 1983). Since endosomes have no well-defined enzyme markers of their own (Dickson *et al.*, 1983), they can be distinguished only by their buoyant density, by the absence of enzyme activities characteristic of other organelles, and by the presence in them of externally added ligands after short periods of labelling. Endosomal vesicles have an acid pH maintained by a proton pump (Tycko & Maxfield, 1982), which can be disrupted by the addition of proton ionophores such as monensin. This in turn has the effect of trapping ligands within endosomes and inhibiting their transfer to lysosomes (Merion & Sly, 1983; Wileman *et al.*, 1984). Transport from endosomes to lysosomes is also decreased greatly at temperatures below 20 °C (Wall & Hubbard, 1981; Merion & Sly, 1983; Beardmore *et al.*, 1987).

The endosomal fraction separated from β -glucuronidase-deficient human fibroblasts in this series of experiments had a buoyant density (1.04 g/ml) identical with that described previously by Merion & Sly (1983) for normal human lung fibroblasts fractionated on gradients of Percoll. Similarly, in both types of fibroblast a small percentage of the total β -glucuronidase present was located in the endosomal fractions. Endosomes were more dense than either Golgi or plasma membranes, although galactosyltransferase and 5'-nucleotidase activi-

ties did overlap them to some degree. Binding and subsequent warm-up experiments did show, however, that endosomes were a separate organelle distinct from plasma membranes. When both fractions were subjected to second sedimentations on gradients of lower starting density, the denser endosomal vesicles containing radio-labelled enzyme sedimented to the bottom of the gradient, whereas the lighter membranes remained near the top.

In fibroblasts that had been incubated for 10 min with ^{125}I - β -glucuronidase, no ligand was found in lysosomes, but was all present either at the cell surface or within the endosomal fraction. After a 20 min chase, however, some 80% of the radioactivity taken up had reached the lysosomes, with the remaining 20% still present in the endosomal compartment. These findings are analogous to those of Merion & Sly (1983), who showed substantial transfer of labelled β -hexosaminidase from endosomes to lysosomes within 30 min, but not within 15 min. Similarly, rabbit alveolar macrophages incubated for 5 min with ^{125}I - β -glucuronidase contained label only within their endosomes, but a substantial proportion of this was shown to have reached lysosomes after a 20 min chase (Wileman *et al.*, 1984).

In the direct transfer experiments described here we found that after 24 h of co-culture, during which receptor-mediated endocytosis was blocked with mannose 6-phosphate, nearly all the β -glucuronidase acquired by recipient cells was present within lysosomes. A small amount of activity was, however, also detected in a fraction corresponding to that of endosomes. Since the experiments with radiolabelled β -glucuronidase had shown that transfer of externally added ligand between endosomes and lysosomes took less than 30 min, attempts were made to delay this process in order to determine whether endosomes were indeed an intermediate compartment in the direct transfer process. When fibroblasts were continuously labelled for 24 h with ^{125}I - β -glucuronidase at 19°C or alternatively at 37°C in the presence of monensin, very little of the enzyme was transferred to lysosomes, but instead accumulated within endosomes, in agreement with the findings of Beardmore *et al.* (1987) or Merion & Sly (1983) and Wileman *et al.* (1984). Similarly, in our direct transfer experiments at 19°C or with added monensin, almost all of the β -glucuronidase activity acquired by the recipient fibroblasts was retained within endosomes or at the cell surface.

These results show that β -glucuronidase, a typical lysosomal enzyme, is transferred to lysosomes after passing through an endosomal compartment during direct transfer, but accumulates within endosomes when transfer is delayed by lower temperature or blocked with monensin in an analogous manner to receptor-mediated uptake of externally added ligands. It is therefore unlikely that direct transfer of enzymes takes place by means of gap junctions, cytoplasmic bridges, or by the direct transfer of whole lysosomes, since one might expect to see at least some of this transferred enzyme within the cell cytosol, and none of these mechanisms is likely to involve endosomes as an intermediate compartment. Enzyme present at the surface of donor cells may therefore become transferred directly from the surface of donor cells to the surface of recipients, and from there follow a similar route to that of enzyme taken up by receptor-mediated endocytosis. Alternatively, lysosomal

contents may be discharged into a protected cleft formed between both types of cell and taken up from there by pinocytosis in a similar way to that suggested previously (Dean *et al.*, 1988). The hypotheses require further investigation.

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REFERENCES

- Avruch, J. & Wallach, D. F. H. (1976) *Biochim. Biophys. Acta* **233**, 334–347
- Beardmore, J., Howell, K., Miller, K. & Hopkins, C. R. (1987) *J. Cell. Sci.* **87**, 495–506
- Beaufay, H., Amar-Costesec, A., Feytmans, E., Thines-Sempoux, D., Wibo, M., Robbi, M. & Berthet, J. (1974) *J. Cell Biol.* **61**, 188–200
- Bradford, M. (1976) *Anal. Biochem.* **72**, 248–254
- Dean, M. F. & Diment, S. (1984) *Biochem. Soc. Trans.* **12**, 524–526
- Dean, M. F., Olsen, I. & Muir, H. (1982) *Biochim. Biophys. Acta* **721**, 441–448
- Dean, M. F., Diment, S., Ostlund, C., Jenne, B. M. & Contractor, S. (1985a) *Biochem. J.* **229**, 213–219
- Dean, M. F., McNamara, A. & Jenne, B. M. (1985b) *J. Cell Sci.* **79**, 137–149
- Dean, M. F., Cooper, J. A. & Stahl, P. (1988) *J. Leucocyte Biol.* **43**, 539–546
- Dickson, R. B., Beguinot, L., Hanover, J. A., Richert, N. D., Willingham, M. C. & Pastan, I. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 5335–5339
- Diment, S. & Dean, M. F. (1983) *Biochim. Biophys. Acta* **782**, 165–174
- Geuze, J. J., Slot, J. W., Strous, G. J., Lodish, H. & Schwartz, A. L. (1983) *Cell* **32**, 38–44
- Goldstein, J. L., Brown, M. S., Anderson, R. G. W., Russell, D. W. & Schneider, W. J. (1985) *Annu. Rev. Cell Biol.* **1**, 1–39
- Helenius, A., Mellman, I., Wall, D. & Hubbard, A. (1983) *Trends Biochem. Sci.* **8**, 245–250
- Kahn, C. R. (1976) *J. Cell Biol.* **70**, 261–286
- Kaplan, A., Achord, D. T. & Sly, W. S. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 2026–2030
- McNamara, A., Jenne, B. M. & Dean, M. F. (1985) *Exp. Cell Res.* **160**, 150–157
- Merion, M. & Sly, W. S. (1983) *J. Cell Biol.* **96**, 644–650
- Neufeld, E. F., Lim, T. W. & Shapiro, L. J. (1975) *Annu. Rev. Biochem.* **44**, 357–376
- Olsen, I., Dean, M. F., Harris, G. & Muir, H. (1981) *Nature (London)* **291**, 244–247
- Olsen, I., Dean, M. F., Muir, H. & Harris, G. (1982) *J. Cell Sci.* **55**, 211–231
- Pastan, I. H. & Willingham, M. C. (1981) *Annu. Rev. Physiol.* **43**, 239–250
- Pastan, I. H. & Willingham, M. C. (1987) *Science* **214**, 504–509
- Schlessinger, J. Y., Schechter, M. C., Willingham, M. C. & Pastan, I. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 2659–2663
- Shepherd, V. L., Lee, Y. C., Schlesinger, P. H. & Stahl, P. D. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 1019–1022
- Shepherd, V. L., Stahl, P. D., Berud, P. & Rabinovitch, M. (1983) *J. Exp. Med.* **157**, 1471–1482
- Stahl, P. D., Rodman, J. S., Miller, M. J. & Schlesinger, P. H. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 1399–1403

- Stahl, P. D., Schlesinger, P., Sigardson, J., Rodman, J. & Lee, Y. C. (1980) *Cell* **19**, 207–215
- Tycko, B. & Maxfield, F. R. (1982) *Cell* **28**, 643–661
- Ullrich, R., Mersmann, G., Weber, E. & Von Figura, K. (1978) *Biochem. J.* **170**, 643–650
- Wall, D. A. & Hubbard, A. L. (1981) *J. Cell Biol.* **90**, 687–695
- Wileman, T., Boshans, R. L., Schlesinger, P. & Stahl, P. (1984) *Biochem. J.* **220**, 665–675
- Wileman, T., Harding, C. & Stahl, P. (1985) *Biochem. J.* **232**, 1–14
- Willingham, M. C. & Pastan, I. (1984) *Recent Prog. Horm. Res.* **40**, 569–587

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