Iodinated fibroblast β -glucuronidase as a ligand for receptor-mediated endocytosis

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1. Antibodies raised to human placental β -glucuronidase were shown to cross-react with the β -glucuronidase secreted by mouse 3T3 fibroblasts, but did not react with other lysosomal enzymes. 2. The β -glucuronidase secreted by 3T3 cells was purified 15000-fold by chromatography on an affinity column made from this antibody and resolved into a single component, of M_r 68000, by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. 3. Iodinated samples of purified enzyme were taken up into mouse peritoneal macrophages by receptor-mediated endocytosis at a rate similar to that calculated previously for unlabelled enzyme, and uptake was competitively inhibited by yeast mannan. 4. Binding of β -glucuronidase to macrophages was saturable, with a K_d of 7×10^{-9} l/mol, an affinity comparable with that calculated for the binding of mannosylated bovine serum albumin (K_d 1.3×10^{-9} l/mol), a ligand specific for mannose receptors. Four times as many molecules of mannosylated albumin (12000) as of β -glucuronidase (3000), however, bound to each cell. 5. This purification and iodination procedure did not therefore have any adverse effect on the uptake properties of secreted β -glucuronidase, and provides a ligand with which to investigate binding and specific endocytosis into a range of different types of cell.

Mouse 3T3 fibroblasts spontaneously secrete large amounts of many lysosomal enzymes when grown in medium supplemented with serum (Diment & Dean, 1983). Uptake experiments, using partially purified preparations of one of these enzymes, β -glucuronidase (EC 3.2.1.31), showed that this enzyme entered both β -glucuronidase-deficient human fibroblasts and partially deficient mouse macrophages by receptor-mediated endocytosis (Diment & Dean, 1983). Competitive-inhibition studies with sugars and sugar phosphates suggested that this secreted β -glucuronidase was taken up into fibroblasts via the mannose 6-phosphate receptors described by Kaplan *et al.* (1977) and Ullrich *et al.* (1977) and into macrophages via their mannose/*N*-acetylglucosamine receptors (Stahl *et al.*, 1978; Achord *et al.*, 1977). There was no species barrier to uptake (Dean & Diment, 1984), although enzyme was taken up into

macrophages at a slightly higher rate than into other fibroblasts.

The usefulness of such enzyme preparations as ligands to study uptake into other types of cell is, however, severely limited by the availability of suitable enzyme-deficient cells. In order to overcome this problem and to extend these uptake studies, the β -glucuronidase secreted by 3T3 fibroblasts was purified to homogeneity and iodinated. The uptake properties of this iodinated enzyme were then compared with those measured previously, by using non-iodinated β -glucuronidase in order to assess its usefulness as a more general ligand with which to study receptor-mediated endocytosis.

Materials and methods

Materials

Concanavalin A-Sepharose, CNBr-activated Sepharose 4B, Sephadex G-50 and Protein A-Sepharose were from Pharmacia Fine Chemicals, Uppsala, Sweden. Methylumbelliferyl β -D-glucur-

Abbreviations used: PBS, Dulbecco's modification of phosphate-buffered saline.

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onide and 4-methylumbelliferone were from Koch-Light Laboratories, Colnbrook, Bucks., U.K., and M_r markers from Sigma Chemical Co., Dorset, U.K. Amersham International, Amersham, Bucks., U.K., supplied ^{125}I -polyvinylpyrrolidone ($125\mu\text{Ci/ml}$) and Na^{125}I ($14.0\text{mCi}/\mu\text{g}$ of iodine), and Iodobeads were from Pierce and Warriner, Chester, Cheshire, U.K., and protein reagent and γ -globulin standard were from Bio-Rad Laboratories, Watford, Herts., U.K. Antibodies, Freund's adjuvant and other immunological reagents were obtained from Sera-Lab, Crawley Down, Sussex, U.K. Tissue-culture media, antibiotics, glutamine, sera and PBS were purchased from Flow Laboratories, Irvine, Ayrshire, Scotland, U.K.

Culture of cells

Mouse AKR macrophages and 3T3 fibroblasts were cultured as described by Diment & Dean (1983) in medium 199 supplemented with 5% (v/v) foetal-calf serum, 2mM-glutamine and 50 units of penicillin and 50 μg of streptomycin/ml.

Preparation of antibody affinity column

β -Glucuronidase was purified from human placentas by ion-exchange chromatography on DEAE-cellulose in borate buffer as described by Contractor & Oakey (1977). A rabbit was immunized with 2mg of this enzyme in Freund's complete adjuvant, followed by a second injection of a further 1mg in incomplete adjuvant 28 days later. The rabbit was bled after a further 4 weeks, the serum collected by centrifugation at 4°C and IgG isolated from it by affinity chromatography on a column of Protein A-Sepharose 4B.

The IgG was eluted from the column with 0.5M-NaCl/0.1M- NaHCO_3 , pH 8.3, and coupled to CNBr-activated Sepharose 4B (5mg/ml of gel) by rotating it for 2h at room temperature in the NaHCO_3 buffer. Groups that had not reacted were blocked with 0.2M-glycine, pH 8.0 at 4°C, and excess protein was washed away with bicarbonate buffer. The gel was finally packed into columns and conditioned by washing them with 3 bed volumes of 1% (w/v) bovine serum albumin in PBS.

Immunological methods

The homogeneity of the IgG fraction eluted from Protein A was determined by immunoelectrophoresis, by using 1% (w/v) agarose in 0.01M-barbitone buffer, pH 8.6, as described by Goudsward *et al.* (1978). Goat anti-rabbit whole serum and goat anti-rabbit IgG were used undiluted in the troughs. The immunoreactivity of the IgG with β -glucuronidase and other lysosomal enzymes was determined by the degree to which they were immunoprecipitated in the presence of *Staphylo-*

coccus aureus as described previously (Dean *et al.*, 1982), by using concentrated 3T3-cell-conditioned medium containing 300 units of β -glucuronidase, 400 units of β -hexosaminidase and 40 units of β -galactosidase/100 μl as a source of enzyme (one unit of each enzyme hydrolysed 1nmol of substrate/h at 37°C). The species specificity of the anti- β -glucuronidase antiserum was ascertained by double immunodiffusion in 1% (w/v) agarose (Ouchterlony, 1961) against partially purified preparations of β -glucuronidase from human placenta and from 3T3 fibroblasts. The precipitin lines were stained for β -glucuronidase activity with 6-bromo-2-hydroxy-3-naphthoic acid 2-methoxyanilide β -D-glucuronide ('ASBI-glucuronide') substrate and *p*-rosaniline as a simultaneous coupling reagent (Dean *et al.*, 1982).

Purification of β -glucuronidase

The β -glucuronidase secreted by unstimulated 3T3 fibroblasts was concentrated by ultrafiltration and heated at 65°C for 1h to inactivate bovine β -glucuronidase (Diment & Dean, 1983). Enzyme was then bound to the antibody affinity gel by rolling it for 16h at 4°C (2000 units of enzyme/ml of gel); the gel was collected by centrifugation at 1000g for 10min and packed in a 10ml column. Unbound proteins were removed by washing the column with 5 bed volumes of PBS, followed by 5 bed volumes each of 0.1M-glycine, pH 10.0, of 0.1M-sodium acetate, pH 4.5, and finally of 2M-NaCl in PBS, pH 7.4. The β -glucuronidase was eluted from the column with 5 bed volumes of 8M-urea in PBS, dialysed against three changes of 1 litre of 1M-NaCl and concentrated by ultrafiltration through a Diaflo PM100 filter to 4000 units/ml. Purified samples were stored at 4°C and used within a few days of preparation.

Iodination of β -glucuronidase and bovine serum albumin

For this, 1mCi of ^{125}I and six Iodo-Beads were added to 50–100 μg of β -glucuronidase in 1ml of 0.1M-phosphate buffer, pH 7.0. After 2h at room temperature, the reaction was stopped by removing the solution from the beads, which were then washed with a further 0.5ml of the phosphate buffer containing 0.2M-NaI. Free ^{125}I was separated from radiolabelled β -glucuronidase by chromatography on a 10ml column of Sephadex G-50, packed in 0.5M-phosphate buffer, pH 7.0. Fractions (1ml) were eluted in 0.5M-phosphate/0.2M-NaI, their radioactivity was determined in a Compugamma counter (LKB), and those containing β -glucuronidase were pooled. Bovine serum albumin was added to give a final concentration of 1mg/ml, and the labelled enzyme was stored at 4°C and used within a few days of

preparation. Batches of 100 μ g of mannosylated albumin (a gift from Dr. P. D. Stahl, Department of Physiology and Biophysics, Washington University, St. Louis, MO, U.S.A.) were iodinated with 1 mCi of 125 I by the chloramine-T method (Stahl *et al.*, 1978), and then processed in an identical manner.

Uptake and binding of β -glucuronidase

Cells (10^6 in 1 ml of medium per 35 mm Petri dish) were washed with 2×1 ml of warm PBS, and 1 ml of fresh medium 199 containing 125 I-labelled β -glucuronidase and 10% (v/v) foetal-calf serum was added to them. After incubation at 37°C for 2 h, the cells were washed with 2×1 ml of PBS and lysed by adding 2×0.5 ml of 0.1% (v/v) Triton X-100 in water, and scraping the dishes with a rubber policeman. The samples were clarified by centrifugation at 2000g for 10 min and counted for radioactivity in a Compugamma counter. Control dishes without cells were treated in an identical manner, and the radioactivity adsorbed non-specifically to the plastic (less than 0.03% of the amount added) was subtracted from all experimental values. To determine the rate of non-specific pinocytosis, uptake of 125 I-polyvinylpyrrolidone (10 μ g/ml) was measured by an identical procedure.

Binding experiments were carried out on confluent monolayers of cells grown in 1.5 cm-diameter Linbro multi-well culture dishes. These were washed with 2×1 ml of PBS, which had been pre-cooled to 4°C, then kept for 2 h in 1 ml of pre-cooled medium 199 containing 5% (v/v) heat-inactivated foetal-calf serum. Medium was then replaced with 0.4 ml of fresh pre-cooled medium containing either 125 I-labelled β -glucuronidase or mannosylated albumin; the cells were kept for a further 2 h until equilibrium binding had been reached, and then solubilized, and their radioactivity was counted as above. Non-specific ligand binding was determined by incubating cells with either 300 units of unlabelled β -glucuronidase from mouse liver or 1 mg of yeast mannan/ml, and was always less than 1% of the total radioactivity added.

Other methods

The activity of β -glucuronidase was assayed as described by Dean *et al.* (1982), when 1 unit of activity hydrolysed 1 nmol of substrate/h at 37°C. Protein was determined with the Bio-Rad reagent, as described by Bradford (1976), with bovine γ -globulin as a standard. Polyacrylamide-gel electrophoresis was carried out on slab gels by the method of Laemmli (1970) with a 5% (w/v) stacking gel and a 10% (w/v) running gel. The protein bands, separated, were stained with Coomassie Brilliant Blue.

Results

Specificity of antiserum

The antiserum raised against human placental β -glucuronidase gave a single precipitin line on double diffusion against the partially purified glucuronidase secreted by mouse 3T3 cells. This precipitin line was positive for β -glucuronidase activity when incubated with ASBI-glucuronide substrate and was present at dilutions of antiserum greater than 1:360. More than 95% of the activity present in concentrates of 3T3-cell-conditioned medium was immunoprecipitated by this antiserum in the presence of *S. aureus*, whereas, in contrast, all of the β -galactosidase or β -hexosaminidase activity present in the same extract remained in the supernatant solution. When the same test was used to screen other, less specific, monoclonal antibodies, however, which had been raised against mouse liver β -glucuronidase (B. K. Sahota & M. F. Dean, unpublished work), all three enzymes were precipitated with equal efficacy.

Purification and iodination of β -glucuronidase

After isolation of the IgG fraction by chromatography on Protein A-Sepharose, only one precipitin arc was visible after immunoelectrophoresis and diffusion against goat anti-rabbit whole serum. This arc was identical in mobility with that seen after immunoelectrophoresis against goat anti-rabbit IgG. More than 95% of the specific anti-(β -glucuronidase) IgG was coupled to CNBr-activated Sepharose under the conditions described, to produce an affinity ligand with the capacity to bind 2000–3000 units of 3T3-cell β -glucuronidase/ml of gel. Mouse 3T3-cell β -glucuronidase did not, however, bind to control columns which had been blocked with glycine buffer but to which no IgG had been coupled. Other lysosomal enzymes, such as β -galactosidase or β -hexosaminidase, which were present in the initial concentrates made from 3T3-cell-conditioned medium, did not bind to the affinity ligand and were washed straight through the column.

To determine if the β -glucuronidase present in foetal-calf serum could bind to the antibody and be eluted together with the 3T3-fibroblast enzyme, samples of serum were subjected to affinity chromatography before and after heat-inactivation, and any β -glucuronidase present in the fractions eluted was analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Although bovine β -glucuronidase from fresh serum bound to the antibody and was eluted with 8 M-urea, the heat-inactivated serum enzyme did not and was washed from the column in PBS (results not shown). In contrast, heating at 65°C for 1 h did not inactivate the β -glucuronidase secreted by 3T3

fibroblasts and had no effect on its binding to or elution from the column.

The results of a typical purification are shown in Table 1, with a specific activity of some 40000 units/mg of protein and a purification factor of more than 15000 being regularly obtained. Recovery of enzyme activity averaged 70% of that present in the original culture medium. Purified enzyme was resolved into a single protein band of M_r 68000 after sodium dodecyl sulphate/polyacrylamide-gel electrophoresis in the presence of mercaptoethanol. No other contaminating proteins were detected by staining with Coomassie Blue (Fig. 1).

Iodination of purified samples of β -glucuronidase under the conditions chosen produced preparations with an average specific radioactivity of $3.4\mu\text{Ci}/\mu\text{g}$ of protein, an activity slightly in excess of that required for a substitution of 1 atom of ^{125}I per molecule of β -glucuronidase ($3.2\mu\text{Ci}/\mu\text{g}$ of protein). After iodination, enzyme would no longer bind to the antibody affinity column, but still retained 50% of its enzymic activity. Iodination did not, however, degrade the molecule, since the

mobility of iodinated β -glucuronidase was identical with that of non-iodinated enzyme during sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, with a major band (R_F 0.45) which contained more than 80% of the total radioactivity recovered being detected by autoradiography (Fig. 2).

Uptake and binding studies

Uptake of ^{125}I -labelled β -glucuronidase into peritoneal macrophages was rapid, saturable and could be competitively inhibited (Fig. 3). The average degree of inhibition with yeast mannan (1 mg/ml) was 78%, resulting in a decrease in the rate of uptake from 10 to 0.65%/h per mg of cell protein, whereas the comparable rate of non-specific uptake calculated for ^{125}I -labelled poly-

Fig. 1. Electrophoresis of β -glucuronidase purified from 3T3 fibroblasts

Track 1 was taken from a slab gel stained with Coomassie Blue after electrophoresis of $5\mu\text{g}$ of 3T3-cell β -glucuronidase as described in the Materials and methods section. The gel was then scanned on an LKB 2202 laser densitometer. Track 2 containing polypeptide M_r markers is shown alongside for comparison.

Fig. 2. Autoradiograph of iodinated β -glucuronidase. Tracks 1 and 2 from the same gel contained 100000 d.p.m. of ^{14}C -labelled protein M_r markers and 15000 c.p.m. of ^{125}I -labelled β -glucuronidase respectively. The gel was dried and exposed to Kodak XAR-5 film for 18 days at -70°C without enhancing screens before development.

Table 1. Purification of 3T3 fibroblast β -glucuronidase

Medium from a number of cultures of 3T3 fibroblasts was pooled and purified by chromatography on a 2 ml column of antibody gel as described in the text.

Stage of purification	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Recovery (%)	Purification factor
Pooled medium	4400	1692	2.6	100	1
Medium concentrate	3784	774	4.9	86	1.9
Heat-treated medium	3432	561	6.1	78	2.3
Elate from antibody column	3085	0.078	39551	70	15212

Fig. 3. Uptake of 3T3-fibroblast β -glucuronidase into mouse peritoneal macrophages

The rates of uptake of increasing concentrations of enzyme were determined over a period of 2 h in the presence (●) or absence (■) of 1 mg of yeast mannan/ml as described in the Materials and methods section. Data shown represent the mean values from duplicate cultures in three separate experiments.

vinylpyrrolidone (batch no. C282299) was 0.9%/h per mg of cell protein. Specific binding at 4°C to β -glucuronidase-deficient human fibroblasts (GM151) was inhibited 100% by mannose 6-phosphate at a concentration of 10 mM, but completely unaffected by an equivalent concentration of fructose 1-phosphate and α -methyl mannoside or yeast mannan (1 mg/ml). Specific binding to macrophages at 4°C was saturable and, when plotted by the method of Scatchard (1949), there appeared to be a single class of binding sites with an apparent dissociation constant of 7.1×10^{-9} l/mol. These sites were capable of binding $3100 (\pm 400; \text{s.e.m.})$ molecules of β -glucuronidase/cell (Fig. 4). The affinity of the neo-glycoprotein ligand mannosylated bovine serum albumin for macrophages was of the same order of

Fig. 4. Binding of β -glucuronidase to mouse peritoneal macrophages

Cell surface binding was determined by incubating cells for 2 h at 4°C with iodinated ligand as described in the text. Non-specific binding, measured in the presence of 300 units of unlabelled β -glucuronidase/ml ($< 20\%$ of total binding), was subtracted from each data point. The insert shows the data replotted by the method of Scatchard (1949) and represents the mean values from duplicate cultures in three separate experiments.

magnitude at 1.3×10^{-9} l/mol, with approximately four times the number of molecules (12000 ± 850 s.e.m.) able to bind to each cell (Fig. 5).

Discussion

Experiments using unlabelled enzyme (Diment & Dean, 1983; Dean & Diment, 1984) had shown that fibroblast β -glucuronidase might have the capacity to be taken up by receptor-mediated endocytosis into a range of other types of cell. In these further experiments ^{125}I -labelled β -glucuronidase was chosen as a ligand to distinguish donor enzyme from endogenous activity, since similarly labelled preparations have been used to demonstrate both specific binding and receptor-mediated endocytosis. Rat preputial-gland β -glucuronidase, for example (Stahl *et al.*, 1978; Stahl & Gordon, 1982), and β -glucuronidase from *Dictyostelium discoideum* (Freeze *et al.*, 1983) have been iodinated and used to measure uptake into rabbit alveolar macrophages and human fibroblasts.

The partially purified β -glucuronidase used in previous experiments (Diment & Dean, 1983) was

Fig. 5. Binding of mannosylated bovine serum albumin to mouse peritoneal macrophages

Cell-surface binding was determined after 2 h at 4°C; non-specific binding was measured in the presence of 1 mg of yeast mannan/ml and subtracted from total binding. The insert shows the data replotted by the method of Scatchard (1949) and represents the mean values from duplicate cultures in three separate experiments.

of low specific activity (1000 units/mg) and was therefore unsuitable for iodination. β -Glucuronidases from rat liver lysosomes or preputial gland and from mouse and human fibroblasts have been purified, however, to a high degree of homogeneity by using antibody affinity chromatography (Owens *et al.*, 1975; Chern, 1977). So this method was chosen as the most likely to yield homogeneous preparations of secreted β -glucuronidase. Double immunodiffusion showed that the antiserum raised against human placental β -glucuronidase did cross-react with mouse enzyme and that the IgG isolated from this antiserum might therefore be used as an affinity ligand. When 8M-urea was used as an eluent, more than 90% of the bound enzyme was recovered from the column after removal of the urea by dialysis.

Iodination of antibody-purified enzyme with Iodo-beads was chosen as a mild procedure after which preparations still retained 50% of their enzymic activity. The partial decrease in activity probably resulted from modification of catalytically essential tyrosine residues, since a similar decrease in the activity of β -glucuronidase isolated

from mouse urine was observed by Homandberg (1982) after modification of tyrosine residues with reagents such as diazobenzenesulphonate. β -Glucuronidase was not, however, degraded to any significant extent by this procedure, since more than 80% of the radioactivity in iodinated samples ran as a single band with a mobility identical with that of non-iodinated enzyme during gel electrophoresis.

Iodination did not have any measurable adverse effect on the uptake properties of β -glucuronidase, since labelled enzyme was taken up into macrophages in a rapid and saturable manner, little different from that observed previously for unlabelled enzyme (Diment & Dean, 1983). Uptake could be competitively inhibited by yeast mannan, and was presumably, therefore, mediated by mannose receptors. Binding to GM151 fibroblasts was saturable and competitively inhibited by mannose 6-phosphate, but in contrast was unaffected by yeast mannan, α -methyl mannoside or fructose 1-phosphate, indicating that it was mediated by mannose 6-phosphate receptors. A similar pattern of inhibition had been demonstrated previously for uptake of unlabelled enzyme into β -glucuronidase-deficient human fibroblasts (Diment & Dean, 1983).

The dissociation constant calculated for binding of β -glucuronidase to macrophages (7.1×10^{-9} l/mol) was in agreement with the range of values calculated previously for binding of other lysosomal enzymes. Rome *et al.* (1979), for example, showed that binding of high-uptake α -L-iduronidase from human urine to fibroblasts had a K_d of 1.1×10^{-9} l/mol, β -glucuronidase from rat preputial gland bound to mouse peritoneal macrophages with a K_d of 5.4×10^{-9} l/mol (Stahl & Gordon, 1982), whereas the K_d for binding of β -glucuronidase from *D. discoideum* to human fibroblasts was 1.0×10^{-9} l/mol (Freeze *et al.*, 1983).

Only some 3100 molecules of secreted β -glucuronidase, however, bound to each macrophage, a lower value than those calculated for binding of other lysosomal enzymes. This was presumably because the relatively large size of β -glucuronidase (M_r 280000) may have allowed more than one receptor to be occupied or blocked by each molecule. In similar binding studies from other laboratories, an inverse relationship between M_r and number of molecules bound per cell appears to exist. For example, 11000 molecules of β -D-hexosaminidase from *D. discoideum* (M_r 140000) can bind to each fibroblast (Freeze *et al.*, 1980), whereas 15900 molecules of β -D-glucosidase (M_r 95000) or 14000 molecules of α -L-iduronidase (M_r 90000) can bind to the same cells (Freeze *et al.*, 1983; Rome *et al.*, 1979).

The binding affinity of the mannosylated albumin for macrophages was of a similar order of magnitude to that of β -glucuronidase, and was in agreement with previously calculated values for this ligand of 5.4×10^{-9} l/mol for binding to mouse peritoneal macrophages (Stahl & Gordon, 1982) and 9.8×10^{-9} l/mol for rat alveolar macrophages (Stahl *et al.*, 1980), using the same ligand. Almost four times as many molecules of mannosylated albumin (12000) as of β -glucuronidase were, however, able to bind to each cell. This in part may have been because of the much lower M_r of the mannosylated albumin (74000–75000). Alternatively, mouse peritoneal macrophages may express two different types of surface binding site, the more abundant of which is able to recognize mannosylated ligands and a smaller number of receptors able to bind ligands bearing mannose 6-phosphate. This latter possibility requires further investigation.

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