The egasyn gene affects the processing of oligosaccharides of lysosomal β -glucuronidase in liver

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The accumulation of the relatively large amounts of β -glucuronidase in microsomal fractions of normal mice depends on formation of complexes with the protein egasyn. Unexpectedly, it was found that the egasyn gene also affects the processing of β -glucuronidase, which is segregated to lysosomes. In egasyn-positive mice lysosomal β -glucuronidase from liver has a mean pI of 5.9 with a minor proportion at pI 5.4, whereas in egasyn-negative mice the proportion of the two lysosomal forms is reversed. Combined experiments measuring susceptibility to neuraminidase and to endoglycosidase H and specific binding to *Ricinus communis* lectin-agarose columns showed that the alterations in isoelectric point were associated with a decrease in complex oligosaccharides of lysosomal β -glucuronidase in egasyn-positive mice. Since this alteration occurs not only in a congenic strain carrying the *Eg*⁰ gene but also in several other inbred strains that are homozygous for this gene, it is considered to be a genuine effect of the *Eg* gene rather than other genes that might regulate oligosaccharide processing. Also, the alteration is likely to be a result of direct physical interaction of the egasyn protein and lysosomal β -glucuronidase, since a second lysosomal enzyme, β -galactosidase, which does not form complexes with egasyn, is unaffected. The results suggest a model in which egasyn not only causes accumulation of β -glucuronidase in the microsomal compartment but also acts upon the precursor to lysosomal β -glucuronidase to alter its interaction with trans-Golgi-apparatus processing enzymes.

INTRODUCTION

An interesting area of study in cell biology concerns mechanisms responsible for the sorting of proteins among subcellular organelles and within specific membranes or compartments of these organelles (Palade, 1983). The microsomal accumulation of β -glucuronidase in the mouse is an example of a system in which both biochemical and genetic evidence points to the importance of binding of a lysosomal enzyme with an accessory peptide in specific subcellular localization (Lusis & Paigen, 1977). A significant fraction (20–40%, depending on the strain examined) of total liver β -glucuronidase is stable in the microsomal compartment, where it is present as the high-molecular-mass precursor form (Brown *et al.*, 1981). This stable component is present together with a very small amount of precursor β -glucuronidase that is transiently in the microsomal compartment while en route to lysosomes. A variety of biochemical, genetic and immunological evidence has demonstrated the same β -glucuronidase polypeptide, with certain modifications, is present in both subcellular compartments (Lusis & Paigen, 1977). In mouse liver it has been shown that stable microsomal β -glucuronidase, designated X, is non-covalently associated with one to four molecules of a 64 kDa polypeptide called egasyn (Swank & Paigen, 1973). Egasyn serves to stabilize microsomal β -glucuronidase precursor as a microsomal component. Normal mice with egasyn have stable microsomal β -glucuronidase with a half-life of 4.5–5 days (Smith & Ganschow, 1978; Brown *et al.*, 1981). Mice

homozygous for the *Eg*⁰ mutation lack egasyn and also lack stable microsomal β -glucuronidase, though they have normal amounts of transient precursor β -glucuronidase (Brown *et al.*, 1981).

Belinsky *et al.* (1984) have suggested that liver microsomal β -glucuronidase is physiologically important in regulating the degree of glucuronidation of drugs and endogenous compounds.

The subcellular routing and post-translational processing of lysosomal enzymes is an area of active investigation (Hasilik & von Figura, 1984; Skudlarek *et al.*, 1984; Goldberg *et al.*, 1984; Creek & Sly, 1984; Shepherd & Stahl, 1984). Lysosomal enzymes are synthesized as high-molecular-mass precursor forms, which are inserted into the endoplasmic reticulum in a manner similar to secretory proteins. After removal of the signal sequence a pro-form of the enzyme traverses the endoplasmic reticulum and Golgi apparatus and is modified to a mature form either just before reaching the lysosome or within the lysosome itself. This intracellular transport, with the above-mentioned exception of microsomal β -glucuronidase, is complete in a few hours. There is evidence that high-mannose oligosaccharides are added to newly synthesized lysosomal enzymes in the endoplasmic reticulum and that the lysosomal recognition marker mannose 6-phosphate and terminal sugars are added in the Golgi apparatus to form complex oligosaccharides. The modifications with terminal sugars are, however, variable (Strawser & Touster, 1980), and the factors regulating the degree of modification are uncertain.

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In this paper we present evidence that β -glucuronidase traverses the trans-Golgi apparatus during transport to lysosomes and that the egasyn gene modifies this process. Thus this system represents an unusual method of modification of the oligosaccharide composition of a glycoprotein by a gene that specifies an accessory protein. These experiments were made possible because of the availability of a congeneric mutant strain that carries the *Eg*⁰ mutation.

EXPERIMENTAL

Animals

Mouse strain C57BL/6 was purchased from the Jackson Laboratory (Bar Harbor, ME, U.S.A.) or from Kantonales Tierspital (Zurich, Switzerland). The congeneric strain C57BL/6J YBR *Es-1*^b *Eg*⁰ (N15,F16) was constructed from F₁ heterozygotes between C57BL/6J and YBR *Eg*⁰ by 14 generations of back-crossing to C57BL/6J with selection for *Es-1*^{a/b} heterozygotes in each back-cross generation, followed by 15 generations of inbreeding to produce homozygotes. The *ES-1* gene is 1 cMorgan from *Eg* (Karl & Chapman, 1974), so that the probability of a recombination between *Eg* and *Es-1* was 1% per generation. Alleles of *Es-1* are co-dominantly expressed as an electrophoretic variation detectable in plasma. Thus we were able to score phenotypically *Es-1*^{a/b} heterozygotes in back-crosses without additional breeding tests. Two parallel back-crosses were used to lower the probability of losing the linked *Eg*⁰ allele during the back-cross breeding. After 14 generations of back-cross, the heterozygous progeny of each line were intercrossed to produce mice that were *Eg*^{0/0}-*Es-1*^{b/b} homozygotes, and these were subsequently inbred brother $\times$ sister. They are currently in the F16 generation. The probability of genes unlinked to *Eg* and derived from YBR being present in the congeneric is less than 0.01%. The average amount of genetic material linked to *Eg* and derived from the YBR donor in the congeneric strain is 15 cMorgans (Flaherty, 1981).

Inbred strains LTS/A and STS/A, also lacking detectable egasyn (Chapman *et al.*, 1979), were obtained from Dr. R. Nikkels and Dr. J. Hilgers of The Netherlands Cancer Institute, Amsterdam, The Netherlands. Inbred strains P/2 and CYPYM (Wassmer *et al.*, 1985), which have less than 10% of the normal amounts of egasyn (Medda *et al.*, 1986), were obtained from Dr. O. von Deimling of Universität Freiburg, Freiburg, Germany.

Enzyme assays

Tissues were homogenized in 10 vol. of 0.15 M-NaCl/0.02 M-Tris/HCl buffer, pH 7.4, in a Polytron homogenizer (Kinematica, Luzern, Switzerland) or a Teflon/glass homogenizer (Brandt *et al.*, 1975).

β -Glucuronidase and β -galactosidase activities were assayed by a fluorimetric procedure with 4-methylumbelliferyl β -D-glucuronide or β -D-galactoside as substrate (Brandt *et al.*, 1975). Neuraminidase was assayed with 4-methylumbelliferyl α -D-N-acetylneuraminic acid as substrate (Potier *et al.*, 1979). Mice of the SM/J inbred strain, known to have significantly lower liver neuraminidase specific activity (Potier *et al.*, 1979), were used as internal controls. One enzyme unit is the amount of enzyme forming 1 μ mol of product/h at 37 °C.

Staining for β -glucuronidase activity in gels was performed by the dye-coupling method of Hayashi *et al.* (1964), with Naphthol AS-BI β -D-glucuronide as substrate and Fast Garnet GBC salt (Sigma Chemical Co.) as coupling dye.

Isoelectric focusing

Isoelectric focusing was performed according to a procedure described by Owens *et al.* (1975), modified by Lusis & Paigen (1978), with a vertical slab-gel system. The gels contained 6% *NN'*-methylenebisacrylamide, 6 M-urea and 2% Ampholines. Before being stained, gels were incubated for at least 1 h in 0.2 M-sodium acetate buffer, pH 5.2, to wash out urea. β -Glucuronidase activity was detected after electrophoresis by the activity staining method described above.

Cell fractionation

Microsomal, lysosomal and soluble fractions were prepared by the osmotic-shock method (Brandt *et al.*, 1975). Contamination of the microsomal fraction with lysosomal β -glucuronidase and vice versa was tested by electrophoretically separating the microsomal and lysosomal β -glucuronidase enzyme forms (Beltramini-Guarini *et al.*, 1984) and was estimated to be less than 10%.

Purification of β -glucuronidase and determination of molecular mass

The β -glucuronidase forms were separated according to their isoelectric points by the Fast Protein Liquid Chromatography System of Pharmacia with a Mono P column HR 5/20. The applied samples were prepared from tissue homogenates supplemented by 0.5% Triton X-100, incubated at 55 °C for 20 min and centrifuged at 100000 g for 60 min. The supernatant was filtered through a 0.22 μ m filter before being loaded on the column. The column was equilibrated with starting buffer (0.025 M-Tris/HCl buffer, pH 6.7), the enzyme solution was loaded in starting buffer and elution was performed by switching to Polybuffer 74 adjusted to pH 5.0 by HCl and diluted 20-fold with water. Fractions were collected and assayed for β -glucuronidase activity.

Fractions of the various enzyme forms from the Mono P column were further purified on a concanavalin A-Sepharose column (0.5 ml bed volume, maximally loaded with 3 units of β -glucuronidase activity). The column was equilibrated with 20 mM-Tris/HCl buffer, pH 7.5. After the sample had been loaded, unbound protein was washed out with 2 ml of 20 mM-Tris/HCl buffer, pH 7.5, and bound protein was eluted with 3 $\times$ 0.5 ml of 0.5 M-methyl α -mannoside/0.02 M-Tris/HCl buffer, pH 8.5, at 45 °C. The recovered enzyme (yield more than 95%) was supplemented with Triton X-100 to give a final concentration of 1% (v/v), and approx. 2 activity units were immunoprecipitated with anti- β -glucuronidase antibody by overnight incubation at 4 °C. The immunoprecipitate was collected by centrifugation at 10000 g for 20 min and washed twice with the homogenization buffer containing 4% Triton X-100 and once with the homogenization buffer containing 0.1% SDS. The immunoprecipitate was then redissolved in the sample buffer described by Laemmli (1970) supplemented with 4.5 M-urea, and was subjected to SDS/polyacrylamide-gel electrophoresis. Protein was stained by Coomassie Blue R-250.

Enzyme treatments

β -Glucuronidase was first purified from whole liver or from microsomal and lysosomal fractions (Brandt *et al.*, 1975) by immunoaffinity columns of monospecific goat anti-(mouse β -glucuronidase) antibody (Watson *et al.*, 1981) covalently attached to Sepharose 4B. This method yielded approx. 60% recovery of applied β -glucuronidase activity and did not alter the relative proportions of the various β -glucuronidase isoelectric forms. For endoglycosidase H treatment, before isoelectric focusing, approx. 0.3 unit of β -glucuronidase activity was adjusted to pH 5.5 in 60 mM-sodium citrate buffer and treated for 20 h at 37 °C with 3 or 6 munits of endoglycosidase H in 0.1 ml final volume. For endoglycosidase H treatment before SDS/polyacrylamide-gel electrophoresis, immunoprecipitation, immunoprecipitates of the various β -glucuronidase forms were dissolved by boiling for 2 min in 10 μ l of 1% SDS in water. To this was added 40 μ l of 30 mM-sodium citrate buffer, pH 5.5, containing 1.5 munit of endoglycosidase H. The total mixture was incubated at 37 °C for 24 h, dissolved in urea/SDS, boiled and electrophoresed on an SDS/polyacrylamide gel (see above under 'Purification of β -glucuronidase and determination of molecular mass'). Addition of the proteinase inhibitor phenylmethanesulphonyl fluoride (1 mM) did not affect the results. For neuraminidase treatment, β -glucuronidase (0.2 unit) in 0.05 M-sodium citrate buffer, pH 5.2, was treated for 1.5 or 3 h at 37 °C with 10 munits of neuraminidase in 0.1 ml final volume. For alkaline phosphatase treatment, β -glucuronidase (0.2 unit) in 4 mM-MgCl₂/0.1 M-Tris/HCl buffer, pH 8.0, was treated for 20 h at 37 °C with 1 unit of alkaline phosphatase in 0.1 ml final volume. The alkaline phosphatase preparation had full activity with the artificial substrate *p*-nitrophenyl phosphate. Recoveries of enzyme activity were greater than 92% after treatment with endoglycosidase H, neuraminidase or alkaline phosphatase.

Ricinus communis lectin-agarose column chromatography

The percentage of liver β -glucuronidase of Eg^+/Eg^+ and Eg^0/Eg^0 mice that adheres specifically to *Ricinus communis* lectin-agarose and that is specifically eluted with 0.1 M-D-galactose was determined as described by Miller *et al.* (1981).

Samples (0.5 unit) of immunopurified β -glucuronidase were dialysed overnight against 0.1 M-sodium phosphate buffer, pH 6.0, containing 1 mg of bovine serum albumin/ml, 0.15 M-NaCl and 0.02% NaN₃ before application to the *Ricinus communis* lectin-agarose columns that had been equilibrated in the same buffer. Some samples were treated with neuraminidase type X as described above before application. Recovery of enzyme activity was 82–111%.

The properties of liver β -galactosidase that specifically adhered to *Ricinus communis* lectin-agarose columns were similarly determined after partial purification of β -galactosidase activity from a 10% (w/v) homogenate of liver by a heat and acid treatment (Brandt & Swank, 1976). Some samples were treated with 0.39 unit of neuraminidase type VI/ml of liver extract for 3 h before application to the lectin-agarose column. Recovery of enzyme activity was 77–120%.

Materials

Endoglycosidase H, purified from *Streptomyces plicatus*, was obtained from Health Research, Albany, NY, U.S.A. Neuraminidase (affinity-purified type X and type VI from *Clostridium perfringens*) and 4-methylumbelliferyl α -D-N-acetylneuraminic acid were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Alkaline phosphatase was *Escherichia coli* BAPF form from Worthington Corp., Freehold, NJ, U.S.A. *Ricinus communis* agglutinin I-agarose was from Vector Laboratories, Burlingame, CA, U.S.A. Sepharose-bound concanavalin A was from Pharmacia, Uppsala, Sweden.

RESULTS

Differences in properties of β -glucuronidase in Eg^+/Eg^+ and Eg^0/Eg^0 mice

Charge differences of liver β -glucuronidase. Triton X-100 extracts of total liver homogenates of microsomal and of lysosomal fractions (osmotic-shock fractionation; see the Experimental section) were subjected to isoelectric focusing, and β -glucuronidase was detected by activity staining. On focusing β -glucuronidase formed a multiple banding pattern that was subdivided into distinct pH ranges (Fig. 1). In liver extracts of Eg^+/Eg^+ mice (containing egasyn) two groups of bands with mean pI values of 6.4 and 5.9 were visible. In extracts of Eg^0/Eg^0 mice (deficient in egasyn) one major group of bands was at a mean pI of 5.4 and a minor group at a pI of 5.9. Focusing of the microsomal and lysosomal extracts separately revealed that the group of basic bands at pI 6.4 represented microsomal β -glucuronidase; the other two relatively acidic groups were differently charged forms of the lysosomal enzyme. It has likewise been found that the basic components are microsomal and the acidic components lysosomal in subcellular-fractionation studies of kidney. The most striking observation was that if microsomal β -glucuronidase was present (Eg^+/Eg^+ mice) most of the lysosomal enzyme was expressed as the less-acidic pI 5.9 form, and if the microsomal enzyme was absent (Eg^0/Eg^0 mice) most of the lysosomal enzyme was in the more-acidic pI 5.4 form.

By densitometric scans of gels stained for β -glucuronidase activity 75% of lysosomal β -glucuronidase of Eg^+/Eg^+ mice was in the pI 5.9 region and 25% at pI 5.4. In contrast, 23% of β -glucuronidase activity of Eg^0/Eg^0 mice was at pI 5.9 and 77% in the pI 5.4 region.

The shift to the more-acidic pI 5.4 form of lysosomal β -glucuronidase is highly likely to be under the control of the *Eg*-gene locus (rather than other genetic loci, including those that control expression of enzymes such as the various sugar transferases and glycohydrolases that modify glycoproteins). This is because of the nature of the genetic construction of the Eg^0/Eg^0 congenic mouse (see the Experimental section). Additional evidence that the *Eg* gene is responsible for the alteration in isoelectric point was derived (not shown) by examining other inbred strains that have alterations at the *Eg* gene that cause either a deficiency or absence of egasyn. Inbred mouse strains P/2 and CYPYM (Wassmer *et al.*, 1985) have less than 10% the normal liver egasyn contents (Medda *et al.*, 1986). They have a decrease in the amount of the pI 6.4 microsomal β -glucuronidase forms and, as in the Eg^0/Eg^0 congenic strain, they have a pronounced shift of lysosomal

Fig. 1. Isoelectric forms of β -glucuronidase of total liver extracts and of microsomal and lysosomal fractions of C57BL/6J Eg^+/Eg^+ and C57BL/6J Eg^0/Eg^0 mice

Subcellular fractions were adjusted to be equal in volume to the whole liver homogenates (10%, w/v) from which they were obtained, and equal volumes (60 μ l) of all samples were electrophoresed. β -Glucuronidase was detected by activity staining. Liver β -glucuronidase specific activity in Eg^0/Eg^0 mice is 60% of that of Eg^+/Eg^+ mice.

β -glucuronidase to the pI 5.4 form. Strains STS and LTS lack detectable egasyn (Chapman *et al.*, 1979), as evidenced by the lack of the microsomal pI 6.4 form, and show the same shift of lysosomal β -glucuronidase to the acidic pI 5.4 form as observed in the Eg^0/Eg^0 congenic strain. In all five strains with alterations at the *Eg* gene,

therefore, the shift to the more-acidic lysosomal β -glucuronidase occurs. Conversely, in at least 11 other strains (not shown) that have the Eg^+/Eg^+ genotype, we have observed no alteration in isoelectric point of lysosomal β -glucuronidase.

The correlation between the presence or absence of the basic microsomal enzyme and distinct net charges of the respective lysosomal enzyme was confirmed by comparing the focusing pattern of the enzyme in additional organs of both strains containing or lacking egasyn, i.e. containing or lacking microsomal β -glucuronidase (Fig. 2). Lung contains only trace amounts of microsomal enzyme in Eg^+/Eg^+ mice, and there is likewise little, if any, change in proportions of the pI 5.9 and pI 5.4 bands in normal and mutant mice. Spleen and brain of both normal and mutant mice lack pI 6.4 microsomal enzyme forms, and they have mainly the more-acidic pI 5.4 lysosomal enzyme. There was one exception. Despite the presence of some basic microsomal enzyme in normal mice, most of the kidney lysosomal enzyme of the Eg^0/Eg^0 mutant was in the pI 5.9 form and not in the acid pI 5.4 form.

Comparisons of molecular masses of the various enzyme forms. In addition to their net charge the various enzyme forms differ in their molecular masses (Fig. 3). The microsomal pI 6.4 and the lysosomal pI 5.9 enzyme forms were purified from livers of egasyn-containing normal mice (Eg^+/Eg^+). The lysosomal pI 5.4 form was purified from livers of normal and mutant mice (Eg^0/Eg^0). The microsomal enzyme (mean pI 6.4) (lane A) has a molecular mass of approx. 75 kDa, and the pI 5.9 (lane B) and pI 5.4 (lane D) lysosomal enzymes have molecular masses of approx. 71.5 and 73 kDa respectively. The pI 5.4 form from Eg^+/Eg^+ mice (lane C) contains some 71.5 kDa form in addition to the 73 kDa form as a result of incomplete separation of the large amount of pI 5.9 from the pI 5.4 form on the Mono P column. Other investigators (Tomino & Paigen, 1975;

Fig. 2. Isoelectric focusing of total extracts of organs of C57BL/6J Eg^+/Eg^+ and C67BL/6J Eg^0/Eg^0 mice

A 60 μ l sample of 10% (w/v) organ homogenate was electrophoresed in each case. β -Glucuronidase was detected by activity staining.

Fig. 3. SDS/polyacrylamide-gel electrophoresis of immunopurified liver β -glucuronidase

Lane A, microsomal pI 6.4 form, lane B, lysosomal pI 5.9 form, and lane C, lysosomal pI 5.4 form of C57BL/6 Eg^+/Eg^+ mice; lane D, lysosomal pI 5.4 form of C57BL/6 Eg^0/Eg^0 mice. Gels were stained with Coomassie Brilliant Blue. The components at 71–75 kDa are the β -glucuronidase subunits. The intensely stained band at 50 kDa is the heavy-chain subunit of anti- β -glucuronidase antibody. Molecular-mass standards (phosphorylase b, bovine serum albumin and ovalbumin) are at the left.

Tulsiani *et al.*, 1978; Brown *et al.*, 1981), have likewise found that microsomal β -glucuronidase is slightly larger than lysosomal β -glucuronidase, though a size difference between lysosomal forms of the enzyme in egagyn-positive and egagyn-negative mice has not been reported. Although other explanations are possible, it is likely that the 1.5 kDa lower molecular mass of the lysosomal enzyme in egagyn-positive mice is caused by lowered amounts of sialic acid and other terminal sugars of complex oligosaccharides (see below). Alterations in molecular mass related to the degree of sialic acid content have been observed with other glycoproteins (Baumann & Doyle, 1979).

Oligosaccharide properties of microsomal and lysosomal β -glucuronidase

Sialylation of oligosaccharides. In order to test for possible modification of neuraminic acid, immunopurified β -glucuronidases of Eg^+/Eg^+ and Eg^0/Eg^0 mice were treated with neuraminidase and the effect was monitored by isoelectric focusing. Neither the microsomal form nor the lysosomal pI 5.9 form in Eg^+/Eg^+ livers were changed by the neuraminidase treatment (Fig. 4). The lysosomal pI 5.4 glucuronidase of Eg^0/Eg^0 mice, however, was changed to a more basic net charge at pI 5.7, which was close to but not identical with that of the Eg^+/Eg^+ lysosomal β -glucuronidase. This indicated that the pI 5.4 lysosomal β -glucuronidase in Eg^0/Eg^0 mice is sialylated and therefore processed to a higher degree with terminal sugars than is lysosomal β -glucuronidase of Eg^+/Eg^+ mice. Doubling the neuraminidase concentrations caused no further changes in Eg^0/Eg^0 β -glucuronidase, indicating either the presence

Fig. 4. Isoelectric focusing of liver β -glucuronidase with and without neuraminidase treatment

of inaccessible sialic acid residues or that other differences in charged residues are present between lysosomal β -glucuronidase of Eg^+/Eg^+ and Eg^0/Eg^0 mice.

The increased amounts of sialic acid on lysosomal β -glucuronidase of Eg^0/Eg^0 mice is not due to lowered activities of lysosomal neuraminidase. When this enzyme was assayed in whole liver extracts, values of 0.633 ± 0.007 and 0.620 ± 0.032 unit/g of liver (means $\pm$ S.E.M.) were obtained from three individual Eg^+/Eg^+ and three Eg^0/Eg^0 mice respectively.

Relative galactose content of oligosaccharides. The preferential affinity of *Ricinus* lectin to galactose-containing oligosaccharides was used to determine whether one or the other of the β -glucuronidase forms contain exposed galactose residues. Total liver β -glucuronidases of Eg^+/Eg^+ and Eg^0/Eg^0 mice were chromatographed on *Ricinus* lectin-agarose columns and the proportions of bound and unbound enzymes were determined by activity measurement in the eluate before and after elution with buffer containing galactose. When β -glucuronidase was applied untreated on to the column, most of the enzyme of both strains did not bind to the column (Fig. 5a). After switching to the galactose-containing buffer only a small proportion of specifically bound enzyme could be detected. In contrast, after treatment with neuraminidase $37 \pm 7.1\%$ (mean $\pm$ S.D. for four experiments) of totally applied enzyme activity of Eg^0/Eg^0 enzyme was retained on the column and was removed by the specific ligand galactose (Fig. 5b). A much smaller proportion ($9.0 \pm 3.2\%$) of Eg^+/Eg^+ enzyme was retained and specifically eluted. Neither treatment with double the normal neuraminidase concentrations nor use of widely varied ratios of applied enzyme to total column-bound lectin altered these

Fig. 5. Chromatography on *Ricinus communis* lectin-agarose columns of untreated (a) and neuraminidase-treated (b) immunopurified liver β -glucuronidase

Galactose at 0.1 M and NaCl at 0.6 M were added to elute specifically and non-specifically adsorbed enzyme respectively. $\bigcirc$ — $\bigcirc$, C57BL/6J Eg^+/Eg^+ mouse enzyme; $\bullet$ — $\bullet$, C57BL/6J Eg^0/Eg^0 mouse enzyme.

proportions. In these experiments, total β -glucuronidase activity, including microsomal activity, was applied to the columns. Microsomal enzyme of Eg^+/Eg^+ is totally unadsorbed (see below). If the contribution of microsomal pI 6.4 enzyme (22% of total in strain C57BL/6J) is mathematically eliminated from the Eg^+/Eg^+ total enzyme applied to the column, the percentage of lysosomal enzyme adsorbed is 13%, which is still considerably less than the 37% value for Eg^0/Eg^0 mice.

In order to attribute lectin-binding and non-binding enzyme forms to the various forms identified by isoelectric focusing, the unadsorbed fraction and the adsorbed (specifically eluted with galactose) fraction of chromatographed glucuronidases of Eg^+/Eg^+ and Eg^0/Eg^0 mice were compared by isoelectric focusing (Fig. 6). The microsomal pI 6.4 form and the lysosomal pI 5.9 form were almost exclusively present in the unadsorbed fraction (Eg^+/Eg^+). The small amount of material in the adsorbed fraction of Eg^+/Eg^+ mice was slightly more acidic with a pI of approx. 5.7. In the Eg^0/Eg^0 β -glucuronidase the pI 5.7 form (generated from the pI 5.4 form by neuraminidase treatment) does not appear to be separated into different forms by lectin treatment.

In contrast with the β -glucuronidase results, no increase in the quantity of galactose residues of β -galactosidase of Eg^0/Eg^0 mice was detected by chromatography on *Ricinus* lectin-agarose columns (results not shown). A very high percentage of β -galactosidase activity was adsorbed on *Ricinus* lectin-agarose columns after neuraminidase treatment, in agreement with previous reports (Breen *et al.*, 1977) of large changes in electrophoretic mobility after neuraminidase treatment, but the proportion was equal in three normal ($83.7 \pm 3.5\%$; mean $\pm$ S.E.M.) and three mutant ($76.7 \pm 0.33\%$; mean $\pm$ S.E.M.) mice. The equal amounts of complex oligosaccharides in β -galactosidase in the two types of mice provide biochemical evidence

that enzymes that normally process oligosaccharides of lysosomal enzymes are identical in egasyn-positive and

Fig. 6. Isoelectric focusing of β -glucuronidase fractions unadsorbed and specifically adsorbed on *Ricinus communis* lectin-agarose columns

β -Glucuronidase was immunopurified from whole liver extracts and treated with neuraminidase. Total samples are those that were applied to the *Ricinus communis* lectin-agarose columns of Fig. 5. Unadsorbed samples are derived from fractions 4 of the elutions of Eg^+/Eg^+ and Eg^0/Eg^0 enzymes of Fig. 5(b). Adsorbed samples are fractions 17 (Eg^+/Eg^+) or 19 (Eg^0/Eg^0) of Fig. 5(b). β -Glucuronidase was detected by activity staining.

egasyn-negative mice. Thus the differences in β -glucuronidase processing can be ascribed to a specific effect of the egasyn gene, a conclusion in agreement with the genetic data.

Susceptibility to endoglycosidase H. The effects of endoglycosidase H treatment on the various β -glucuronidase forms was monitored by both isoelectric focusing of intact enzyme and by analysis of subunit molecular masses. Endoglycosidase H treatment of native immunopurified Eg^+/Eg^+ and Eg^0/Eg^0 β -glucuronidases followed by isoelectric focusing revealed that the microsomal enzyme form of Eg^+/Eg^+ β -glucuronidase was highly susceptible to this enzyme, which hydrolyses preferentially high-mannose oligosaccharides (Fig. 7). It focused with fewer bands in a higher pH range with one major band at approx. pH 6.8. In contrast, lysosomal forms from normal or mutant mice did not change in isoelectric point. That endoglycosidase H specifically affected the net charge of microsomal components was confirmed when individual microsomal and lysosomal components were isolated by the osmotic-shock procedure before endoglycosidase H treatment. It is unlikely that differential accessibility of oligosaccharides to endoglycosidase H explains these results, since basically the same β -glucuronidase tetramer is present at each sub-cellular site. Alkaline phosphatase treatment did not affect the isoelectric point of microsomal or lysosomal β -glucuronidase.

Analysis of molecular masses of β -glucuronidase subunits after endoglycosidase H treatment of SDS-denatured enzyme confirmed that endoglycosidase H affected the oligosaccharide structure of microsomal β -glucuronidase (Fig. 8) and also revealed differing effects on the two types of lysosomal β -glucuronidases. The original untreated bands of the microsomal and lysosomal enzyme forms from egasyn-positive mice at 75 kDa (lane B) and 71.5 kDa (lane D) respectively nearly completely disappeared, and in each case (lanes A and C) two new bands at approx. 1.5 and 3 kDa smaller appeared. In contrast, in agreement with other experiments, which indicated a more complex oligosaccharide structure, only a portion of the acidic 73 kDa lysosomal enzyme (lane F) from egasyn-negative mice was converted into just one band of 1.5 kDa lower molecular mass (lane E).

Fig. 7. Isoelectric focusing of liver β -glucuronidase with and without endoglycosidase H treatment

β -Glucuronidase was detected by activity staining.

Fig. 8. Relative effects of endoglycosidase H treatment on subunit size of immunopurified microsomal and lysosomal β -glucuronidases

The microsomal pI 6.4 form (lanes A and B) and the lysosomal pI 5.9 form (lanes C and D) were from C57BL/6 Eg^+/Eg^+ mouse liver, and the lysosomal pI 5.4 form (lanes E and F) from C57BL/6 Eg^0/Eg^0 mouse liver. Lanes A, C and E, with endoglycosidase H; lanes B, D and F, without endoglycosidase H. Gels were stained with Coomassie Brilliant Blue. The components of 68–75 kDa are the β -glucuronidase subunits. Doubling the 1.5 units of endoglycosidase H used in these experiments gave identical results. The intensely stained band at 50 kDa is the heavy-chain subunit of anti- β -glucuronidase antibody. Molecular-mass standards (phosphorylase b, bovine serum albumin and ovalbumin) are at the left.

Taken together, therefore, the endoglycosidase H experiments suggest that stable microsomal β -glucuronidase contains a high proportion of electronegatively charged high-mannose oligosaccharides. Lysosomal β -glucuronidase of egasyn-positive mice likewise has a high proportion of high-mannose residues, though, at least those susceptible to endoglycosidase H in the native enzyme, they are not charged, whereas lysosomal β -glucuronidase of egasyn-negative mice has comparatively few high-mannose residues, and these are not charged, as revealed by treatment of the native enzyme with endoglycosidase H.

DISCUSSION

These results suggest that the egasyn gene regulates the processing, not only of microsomal, but also of lysosomal β -glucuronidase. Furthermore, this altered processing is effected by an alteration in oligosaccharide composition with a shift to a lower content of complex oligosaccharides in lysosomal β -glucuronidase in egasyn-positive mice. The evidence for a decreased content of complex oligosaccharides includes a lowered specific binding to *Ricinus* lectin-agarose columns, insensitivity of isoelectric point to neuraminidase treatment and an increased loss of oligosaccharide from β -glucuronidase subunits after endoglycosidase H treatment. The 1.5 kDa lower molecular mass of the lysosomal β -glucuronidase subunit in egasyn-positive mice as compared with egasyn-negative mice is further evidence for an effect of egasyn on processing of β -glucuronidase. We have reported (Beltramini-Guarini *et al.*, 1984) that microsomal β -glucuronidase gradually disappears over several days

essentially genetically identical (less than 0.01% difference in genotype at genes unlinked to the egasyn gene). Any other putative gene that alters oligosaccharide processing as described would both have to be present in the YBR donor strain and would have to be located in either the very small amount (0.01%) of unlinked genes still present in the congenic or in the relatively small (15 cMorgans) amount of genes linked to the egasyn gene on chromosome 8. Secondly, five other inbred lines with mutant egasyn genes displayed the acidic lysosomal phenotype whereas 11 inbred lines with the normal egasyn genotype did not. Thirdly, it has recently been found (S. Medda, R. T. Swank & O. von Deimling, unpublished work) that even in a separate species, the rat, the same correlation holds: that is, microsomal-glucuronidase-negative rat strains have a more acidic form of lysosomal β -glucuronidase than have microsomal-glucuronidase-positive rat strains. It is extremely unlikely that other gene(s) that so alters oligosaccharide processing could by chance be distributed identically with the egasyn gene among these 16 separate inbred mouse lines and two species. Fourthly, except for kidney (see below), the negative expression of the *Eg* gene among organs is correlated with an increased content of complex oligosaccharides in lysosomal β -glucuronidase.

It is possible that the *Eg* gene could exert its effect on oligosaccharide processing of lysosomal glucuronidase indirectly rather than through direct interaction of egasyn and β -glucuronidase. For example, the egasyn protein could affect the activity of a glycosyltransferase or glycohydrolase. We believe these types of indirect effects are unlikely, for two reasons. First, assay of lysosomal neuraminidase, which removes terminal sialic acid residues, revealed no difference between normal and mutant mice that differ in degree of sialylation of β -glucuronidase. Secondly, the proportion of complex sugars in another lysosomal enzyme, β -galactosidase, which does not interact with egasyn (Lusis & Paigen, 1977; Medda & Swank, 1985), but which would be expected to be affected by the same oligosaccharide-modifying enzymes as lysosomal β -glucuronidase, are identical in egasyn-positive and egasyn-negative mice. A more likely explanation is that direct physical interaction of β -glucuronidase precursor and the egasyn protein in the microsomal complex shields β -glucuronidase from modifying enzymes or serves to redirect the precursor along an alternative subcellular route.

The organ survey showed that in almost all cases the presence of the basic pI 6.4 microsomal enzyme was correlated with the presence of the more-basic pI 5.9 lysosomal enzyme form, which has been shown to be less highly processed in the case of liver. Kidney homogenates of *Eg*⁰/*Eg*⁰ mice are an exceptional case that have only traces of microsomal enzyme together with predominantly the pI 5.9 rather than the pI 5.4 lysosomal enzyme form. A likely explanation is that lysosomal enzymes in general are less highly processed to forms with complex oligosaccharides in kidney. For example, Breen *et al.* (1977) and Lusis & Paigen (1976) have noted that there is extensive sialylation of β -galactosidase and α -galactosidase in mouse liver, but little or no sialylation in mouse kidney. Dizik & Elliott (1978) have likewise determined that α -mannosidase is sialylated in liver, but not in kidney. Lowered processing in mouse kidney could be due either to decreased incorporation of terminal sugar residues or to their more rapid removal. Potier

et al. (1979) have reported that acidic sialidase content is 2-fold higher in mouse kidney than in liver.

There is likewise a clear distinction between the oligosaccharide structure of β -glucuronidase of the microsomal and lysosomal fractions of egasyn-positive mice. The shift of isoelectric point of microsomal, but not lysosomal, β -glucuronidase to a more-basic position combined with the expected decrease in subunit molecular mass after endoglycosidase H treatment suggests the presence of specific electronegative oligosaccharides of the high-mannose or hybrid type (Varki & Kornfeld, 1983). The insensitivity of the oligosaccharides to neuraminidase treatment, however, indicates that they do not contain hybrid oligosaccharides with sialic acid. Also, the lack of absorption of microsomal enzyme on *Ricinus* lectin-agarose columns is evidence that the microsomal enzyme does not contain complex processed oligosaccharides. The chemical nature of the negative groups is uncertain. However, their non-susceptibility to alkaline phosphatase or neuraminidase suggests that stable microsomal glucuronidase does not have exposed mannose 6-phosphate or sialic acid groups. We cannot rule out the presence of groups sterically masked from alkaline phosphatase action, though this seems less likely, as another enzyme, neuraminidase, did cleave sialic acid residues from lysosomal β -glucuronidase. Also, recent experiments have indicated that the mannose 6-phosphate-inhibitable uptake of microsomal β -glucuronidase into fibroblasts is relatively inefficient (K. Pfister, unpublished work). It seems likely, therefore, that these egasyn-associated microsomal components contain 'covered' mannose 6-phosphate residues (Goldberg *et al.*, 1984). In fact, Mizuochi *et al.* (1981) have provided evidence that about 12% of the oligosaccharides of rat liver microsomal β -glucuronidase occur as a phosphodiester.

Tulsiani *et al.* (1978) and Himeno *et al.* (1978) have reported differences in the carbohydrate composition of microsomal and lysosomal β -glucuronidases of the rat, though the two reports differ somewhat in the quality and quantity of sugar differences.

There is discussion as to whether lysosomal enzyme precursors traverse the entire Golgi apparatus or only cis-Golgi elements while en route to lysosomes (Brown & Farquhar, 1984; Geuze *et al.*, 1984). It is of interest in this regard that a genetic mutation that alters the ultimate subcellular location of a lysosomal enzyme also alters its Golgi-apparatus-associated processing. Our data indicate that in egasyn-minus mice a large percentage of liver lysosomal β -glucuronidase has acquired sialic acid and/or galactose residues. There is little doubt, therefore, that a significant fraction of this enzyme has been modified by terminal transferases, which are thought to be localized to trans-Golgi elements (Dunphy *et al.*, 1981; Goldberg & Kornfeld, 1983; Deutscher *et al.*, 1983). In fact, these data are minimal estimates of the degree of terminal sugar modification, since there is substantial trimming of terminal sugars of lysosomal enzymes within the lysosome (Womack *et al.*, 1981; Swallow *et al.*, 1984). Fedde & Sly (1985) have likewise provided evidence that acid hydrolases, which are segregated to lysosomes, are exposed to trans-Golgi-apparatus processing enzymes.

These data suggest an unusual regulation by a microsomal accessory protein of the degree of oligosaccharide modification of a lysosomal enzyme distinct

from terminal sugar transferases or from receptors that translocate nucleotide sugars across Golgi vesicle membranes (Deutscher *et al.*, 1984) or from other conformation-dependent, species-specific and cell-specific methods (Hsieh *et al.*, 1983a; Kornfeld & Kornfeld, 1985).

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