Phenobarbital Induction of Egasyn: Availability of Egasyn In Vivo Determines Glucuronidase Binding to Membrane.

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SUMMARY: Murine egasyn, a protein which stabilizes the binding of β-glucuronidase to microsomal membranes, was induced 1.9 fold in liver by phenobarbital treatment. Accompanying this increase was an alteration of the subcellular distribution of liver β-glucuronidase, although total glucuronidase activity remained constant. In control mice 32.6 ± 4.6% of the activity was microsomal, while after four days of phenobarbital treatment 50.5 ± 3.1% was microsomal. Thus, the availability of egasyn appears to be an important factor in determining the proportion of glucuronidase distributed to either microsomes or lysosomes.

INTRODUCTION: Murine β-glucuronidase (EC 3.2.1.31) has become a model system for the study of mammalian enzyme processing (1-4). The enzyme is present in both the lysosomes and microsomes of several tissues, including liver and kidney (5). The enzyme at both sites is derived from a single structural gene, Gus, on chromosome 5 (6,7). The lysosomal form, called L, is a tetramer with a molecular weight of 280,000 (4). The microsomal form, when solubilized using Triton X-100, consists of a series of higher molecular weight complexes, known as M1, M2, M3 and M4 (2). These are composed of a glucuronidase tetramer core, termed X, complexed with from one to four chains of egasyn, a glycoprotein of molecular weight 64,000 (2, 8).

Biochemical and genetic evidence indicates that egasyn functions in stabilizing the binding of glucuronidase to the membranes of the endoplasmic reticulum (2, 9). There is a correlation in various tissues between the levels of microsomal glucuronidase and egasyn (8). Also, mouse strains containing the Eg^{o} mutation, resulting in the absence of microsomal glucuronidase, are deficient in egasyn, (8, 9). The presence of egasyn is determined by a gene, Eg, on chromosome 8 (10).

The L form of glucuronidase appears to arise from the X form by covalent modification. These two forms differ in charge and show a small difference in molecular weight (3).

Phenobarbital has been shown to induce a number of microsomal proteins in liver (11-13). We now show that it induces egasyn about two-fold. By perturbing egasyn levels using phenobarbital, we have been able to examine the relationship between egasyn levels and the amount of glucuronidase that is incorporated into endoplasmic reticulum. We find that after phenobarbital induction a significantly greater proportion of the glucuronidase activity is in the microsomal fraction, suggesting that the availability of egasyn is an important factor in the integration of glucuronidase into microsomal membranes. Our results are consistent with the model of glucuronidase processing proposed by Paigen, et al. (14).

METHODS

Animals and Homogenates. Inbred mice were from Jackson Laboratories (Bar Harbor, Maine). Tissues were homogenized in 10 volumes of 0.02M imidazole, 0.25M sucrose, pH 7.4, using a Polytron homogenizer. Homogenates were stored at -20° until use. Unless otherwise stated, all mice used were 6 week old females of inbred strain A/J.

β-Glucuronidase Assay. A fluorimetric assay, using 4-methylumbelliferyl-β-glucuronide (Sigma) was used. A properly diluted sample was incubated at 37° with 0.4 mM substrate and 0.1M sodium acetate, pH 4.6, in a volume of 0.1 ml. The reaction was stopped after 30 min by adding 1.0 ml of 0.1M sodium carbonate. The fluorescence was determined against a substrate blank using an Aminco fluorocolorimeter (catalog No. 4-7439) equipped with a Corning 7-60 excitation filter and a Kodak 2A emission filter. One unit of activity is that amount which will hydrolyze 1 μmole of substrate per hour.

Electrophoresis. For electrophoresis the detergent Triton X-100 was added to homogenates to a final concentration of 2% and the mixture centrifuged at 100,000xg for 30 min. Disc gel electrophoresis was carried out by the modified method of Clarke (15). Supernatant solutions (10 μl) were applied to 7% polyacrylamide gels, and 300 volts (constant voltage) was applied for 60 min. Glucuronidase activity was visualized in polyacrylamide gels by the method of Hayashi, et al. (16) using napthol-AS-BI-β-D-glucuronide as substrate. Gels were incubated at 37°C for 1 hour and staining was terminated by immersing the gels in a solution of 5% ethanol and 7% acetic acid. Gels were then scanned using a Gilford linear transport densitometer at 550 nm. Integration of the resulting curves gave values used to calculate the percentage of lysosomal versus microsomal forms of enzyme. Activity of individual M forms was estimated by measuring the height of appropriate peaks on the gel scan.

Protein Concentration. Protein concentration was determined by the method of Lowry et al. (17) using crystalline BSA as standard.

Phenobarbital Treatment. Sodium phenobarbital (0.5 ml of a 25 mg/ml solution containing 0.85% NaCl, adjusted to neutral pH) was injected intraperitoneally once daily for 4 consecutive days. Control mice received 0.5 ml injections of 0.85% NaCl. Twenty-four hours after the final injection the animals were sacrificed and tissue homogenates prepared. During preliminary experiments it was found that testosterone treatment enhanced slightly the effect of phenobarbital, although it did not affect either liver β-glucuronidase activity, glucuronidase subcellular distribution, or egasyn concentration within the interval of treatment. Similar observations on the effect of testosterone on phenobarbital sensitivity have been reported in other systems (18). In order to observe maximal phenobarbital effects, all mice, including control animals, were pelleted with dihydrotestosterone immediately prior to phenobarbital injection.

Radioimmunoassay of Egasyn. The method for the radioimmunoassay of egasyn was as previously described (8). The assay employs purified egasyn, labeled with iodine-125, and antiegasyn antibody, prepared in rabbits. During the assay the presence of the ionic detergent deoxycholate is necessary to unmask antigenic sites of egasyn. Assays were performed in 0.6 x 5 cm culture tubes (Kimble). Homogenates were made 2% in Triton X-100 and centrifuged at 100,000 xg for 30 min. The supernatant solutions, containing the solubilized egasyn, were used in the assay.

RESULTS

Egasyn levels in livers from control and phenobarbital treated mice were determined by radioimmunoassay (Fig. 1). The control mice contained 41.3 ± 8.5 µg egasyn per gram liver while the phenobarbital treated mice contained 79.4 ± 11.8 µg egasyn per gram liver, representing an induction of about 1.9 fold (Table I). An induction by phenobarbital of this magnitude is typical of several liver microsomal proteins (11, 12, 13). It has previously been demonstrated that egasyn is localized entirely in the microsomal fraction in mouse liver (8).

Phenobarbital treatment also resulted in an altered distribution of liver β-glucuronidase activity (Table I). Although the total activity remained constant, the proportion of the activity in the microsomal fraction increased significantly (from 32.6 ± 4.6% to 50.5 ± 3.1%) after four days of treatment (Table I). This increase was accompanied by a shift in the relative amounts of the M forms of the enzyme, with the greatest increased in the higher molecular weight forms (Fig. 2; Table 1). In contrast to liver, the proportion of microsomal β-glucuronidase in kidney remained constant upon phenobarbital treatment.

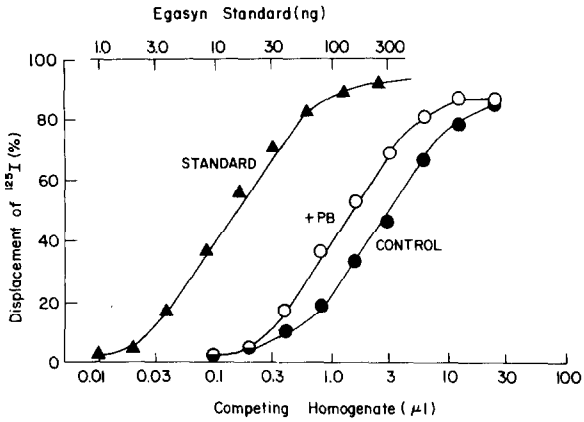

Fig. 1. Radioimmunoassay of Egasyn. Duplicate tubes contained 50 μl of antiegasyn IgG (diluted in 0.02M Tris, 0.15M NaCl, 2% sodium deoxycholate, pH 7.4) and 50 μl of sample (diluted in 0.02M tris, 0.15M NaCl, 0.1% Triton X-100 and 0.1% BSA, pH 7.4). The antiegasyn IgG added was that amount required to precipitate 50% of the labeled egasyn, representing about a 500-fold dilution of antiserum. The mixture was incubated 1 hr. at 37°, then overnight at 4°. To this was added 50 μl of [^{125}I]egasyn (4000 cpm) diluted in sample buffer. This was incubated for 1 hr at 37° and 72 hr at 4°. Egasyn bound to antibody was then separated from free egasyn by the addition of 40 μl control rabbit serum and 30 of goat antibody to rabbit IgG. The mixture was incubated for 1 hr at 37°, then for 4 hrs at 4°. The solution was centrifuged at 10,000 x g for 20 min. The supernatant solution was decanted and the pellet was washed with sample buffer. The radioactivity in the pellet was measured in a gamma counter. Binding displacement curves shown are for purified egasyn standard (△) and typical liver homogenates from a control mouse (●) and a mouse treated with phenobarbital (○).

DISCUSSION

Using phenobarbitol treatment, egasyn, a membrane associated glycoprotein which stabilizes the membrane binding of glucuronidase, has been induced 1.9 fold in mouse liver. The induction was accompanied by a significant shift (from lysosomes to microsomes) in the subcellular distribution of β-glucuronidase, although the total β-glucuronidase activity remained constant. In addition to the increase in microsomal activity, there was also change in the relative amounts of each of the microsomal forms. While M1 remained about constant, M4 increased 5 fold. This suggests that an important factor in determining the subcellular distribution of β-glucuronidase is the availability of egasyn.

Table I. Effect of Phenobarbital on Mouse Liver β-glucuronidase and Egasyn.

	Control (mean ± σ)	Phenobarbital Treated (mean ± σ)
β-glucuronidase activity (units/g liver)	9.12 ± 0.66	8.46 ± 0.69
% lysosomal	67.4 ± 4.6	49.5 ± 3.1
% microsomal	32.6 ± 4.6	50.5 ± 3.1
% M1	7.3 ± 1.5	8.6 ± 1.7
% M2	11.8 ± 1.7	16.2 ± 0.7
% M3	11.6 ± 2.1	17.0 ± 2.1
% M4	1.7 ± 2.4	8.7 ± 1.7
egasyn (μg/g liver)	41.3 ± 8.5	79.4 ± 11.8
protein (mg/g liver)	127 ± 9	163 ± 7

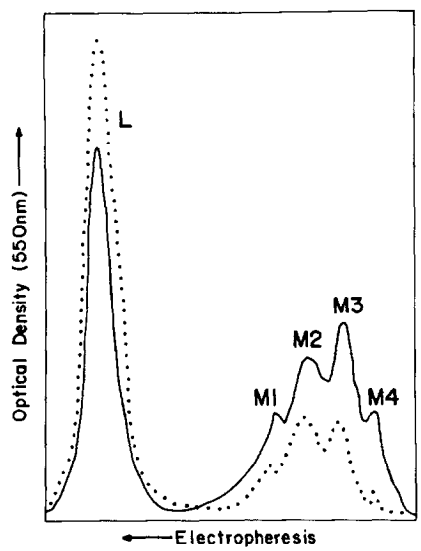

Fig. 2. Polyacrylamide gel electrophoresis of liver β-glucuronidase. Samples were high speed supernatants of Triton X-100 treated liver homogenates and contained 0.68 units of activity. All gels were run simultaneously and were stained with napthol-AS-BI-glucuronide. The fraction of activity present in lysosomal (L) and microsomal (M) forms was calculated as described in Methods. Phenobarbital treated (——); control (. . . .).

Increasing the level of egasyn appears to facilitate the integration of glucuronidase into microsomal membranes, leaving less of the enzyme available for packaging into lysosomes. We have attempted to estimate the magnitude of this effect. From the observations of Smith and Ganschow (19) indicating that the half-life of both microsomal and lysosomal glucuronidase in mouse liver is about six days, we calculate that about 37% of the enzyme in each fraction is replaced during the 4 days of phenobarbital treatment. In order to account for the change in distribution of activity (from 33% microsomal to 50% microsomal) over this time period at least 80% of the newly synthesized glucuronidase would have had to enter the microsomal fraction after phenobarbital induction. This can be compared to the value of about 30% seen before induction.

Some other aspects of the mechanism determining the distribution of glucuronidase are not yet well understood. Competition between glucuronidase and other proteins for egasyn is probably another important factor in specifying the distribution of glucuronidase. Only about 10% of the egasyn in mouse liver is associated with glucuronidase; the remainder appears to be complexed with other proteins in microsomal membranes (8). Also, in $\underline{Eg}^{o}$ mice, which lack both egasyn and microsomal glucuronidase, the amount of glucuronidase activity in lysosomes is not increased over that in normal strains (2, 8, 9). Thus, the glucuronidase which is integrated into microsomal membrane in normal strains is presumably degraded in $\underline{Eg}^{o}$ mice. These facts suggest that the availability of egasyn is not the only factor regulating the distribution of glucuronidase between microsomes and lysosomes.

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