

Luminal Location of the Microsomal β -Glucuronidase–Egasyn Complex

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Abstract. Mouse liver β -glucuronidase is stabilized within microsomal vesicles by complexation with the accessory protein egasyn. The location of the β -glucuronidase–egasyn complex and free egasyn within microsomal vesicles was investigated. Surprisingly, it was found that neither the complex nor free egasyn are intrinsic membrane components. Rather, both are either free within the vesicle lumen or only weakly bound to the inside of the vesicle membrane. This conclusion was derived from release studies using low concentrations of Triton X-100 or controlled sonication. Both the intact complex and free egasyn were

released in parallel with luminal proteins, not with intrinsic membrane components. Also, β -glucuronidase was protected from digestion by proteinase K by the membrane of microsomal vesicles. The hydrophilic nature of both the complex and free egasyn was confirmed by phase separation experiments with the detergent Triton X-114. Egasyn is one of an unusual group of esterases that, despite being located within the lumen or only weakly bound to the luminal surface of the endoplasmic reticulum, do not enter the secretory pathway.

β -Glucuronidase is an unusual acid hydrolase in that large amounts (20–40%) of stable enzyme exist in the microsomal compartment of liver in addition to the usual lysosomal location. A wide variety of biochemical and genetic information indicates that the same polypeptide is found at both subcellular sites and that complex formation with the accessory protein, egasyn, is required for stabilization of β -glucuronidase in microsomes (reviewed in 24). The system thus serves as a model for the specific subcellular localization of enzymes. Mutant mice that lack egasyn have no stable microsomal β -glucuronidase (14). More recent studies have shown that egasyn is associated exclusively with the precursor form of β -glucuronidase in microsomes (9) and that egasyn is identical with mouse esterase-22 (26, 27).

It has been postulated that egasyn stabilizes the β -glucuronidase complex in microsomes by serving as an integral membrane anchor peptide (24). Others (12) have suggested that microsomal β -glucuronidase serves in a membrane structural role. In this paper we present evidence that the microsomal β -glucuronidase–egasyn complex, rather than being an integral membrane component, surprisingly is either free within the lumen of microsomal vesicles or very weakly attached to the inside of these vesicles. These results suggest, in turn, that the binding protein, egasyn, stabilizes β -glucuronidase within the microsomal lumen.

Materials and Methods

Animals

C57BL/6J, *Mus musculus molossinus* and *Mus spretus* mice were raised in the animal facilities of Roswell Park Memorial Institute. Mice 2–4 mo old and of both sexes were used. Unless otherwise indicated, C57BL/6J mice were used.

Radiolabeling Experiments

Radiolabeling of membrane and secretory components of microsomes was done according to the method of Kreibich and Sabatini (20). Membrane phospholipids were labeled by injecting mice intraperitoneally with 100 μ Ci [3 H]choline 4 h before killing. Membrane proteins were labeled by intraperitoneal injection of 100 μ Ci [3 H]leucine at 72, 48, and 24 h before killing. Secretory proteins were labeled by intraperitoneal injection of 100 μ Ci [35 S]methionine 20 min before killing.

Isolations of Microsomes

Liver and kidney homogenates were made 10% in 0.3 M sucrose/20 mM imidazole pH 7.4 containing 1 mM EDTA. Homogenates were prepared in a 7-ml homogenizer (Dounce; Wheaton Scientific, Millville, NJ) by three passes of a 7-ml loose-fitting pestle followed by 10 passes with a 7-ml tight-fitting pestle. The homogenate was spun at 50 g for 10 min to remove unbroken cells. Microsomes were prepared from kidney and liver homogenates as previously described (9). The final microsomal pellet was washed with 20 mM imidazole pH 7.4 containing 1 mM EDTA, 5 mM phosphate, and 0.5 M KCl to remove any lysosomal enzymes nonspecifically sticking

to the outside of the microsomes. Identical results were obtained with or without EDTA and phosphate in these experiments.

Detergent Solubilization of Microsomes

Microsomes were solubilized with increasing detergent concentrations essentially as described by Kreibich and Sabatini (20). Microsomes were suspended in 20 mM imidazole pH 7.6 to give a final protein concentration of 3–6 mg/ml. Increasing concentrations of Triton X-100 (0–0.2%) were added to separate microsomal samples of 1 ml vol. After 5 min, at 0°C, samples were centrifuged at 100,000 g for 30 min. The supernatants containing released proteins were made 0.3 M in sucrose and 0.2% in Triton X-100 to stabilize lysosomal enzyme activity and either stored overnight at 0°C or used immediately. The pellets containing unsolubilized membranes were resuspended in 1 ml 0.3 M sucrose, 0.02 M imidazole containing 0.2% Triton X-100. 20- μ l samples of supernatant and resuspended pellet fractions were taken for radioactivity assays, enzymatic assays, or analysis of esterase or β -glucuronidase components on nondenaturing polyacrylamide gels.

Solubilization of Microsomes by Sonication

Microsomes were suspended at 3–6 mg protein/ml in 20 mM imidazole pH 7.6, and sonicated in one test tube for varying times at low power and 0°C in a water bath sonicator (model 7; Heat Systems-Ultrasonics Inc., Farmingdale, NJ). Aliquots were removed at each time point and centrifuged 30 min at 100,000 g. The pellets and supernatants were separated and treated the same as described for detergent solubilization.

Nondenaturing Polyacrylamide Gels

Nondenaturing 7% acrylamide gels were subjected to electrophoresis at pH 8.1 in a slab gel apparatus (model SE500; Hoefer Scientific Instruments, San Francisco, CA) at 300 V for 2 h according to Swank and Paigen (44). Gels were stained for β -glucuronidase activity using naphthol AS-BI β -D-glucuronide as substrate according to Hayashi (16) as modified by Medda and Swank (26). Other gels were stained for esterase activity using α -naphthyl acetate as described by Medda and Swank (26).

Immunoblotting

SDS-PAGE with 50 μ l (egasyn analysis) or 100 μ l (β -glucuronidase analysis) of supernatant and pellet fractions of detergent-treated microsomes (see above) was performed with Laemmli's (21) system. Western blotting was performed according to Towbin et al. (47) in transfer buffer containing 20% MeOH, 25 mM Tris, and 192 mM glycine (pH 8.3) by applying constant current (0.5 A) at 2°C. Proteins were electrophoretically transferred onto nitrocellulose membrane. Nonspecific background was removed by incubating the membrane with 20 mM Tris-HCl, pH 7.4, plus 0.5 M NaCl and 5% nonfat dried milk blocking solution for 1 h at room temperature (17). Goat anti-serum to mouse egasyn (29) or mouse β -glucuronidase (41) was diluted 1:10 in blocking solution and incubated with the transfer membrane overnight at room temperature. Peroxidase-conjugated rabbit anti-goat IgG was diluted 1:1,000 in blocking solution and membranes were incubated for 5 h at room temperature. After several washes of the membranes with 20 mM Tris-HCl, pH 7.5, peroxidase activity was visualized with H₂O₂ and 0.01% 4-chloro-1-naphthol (47).

Phase Separation of Microsomal β -Glucuronidase and Egasyn

Phase separation of liver microsomal proteins was performed in solutions of Triton X-114 according to Bordier (6). The final fractions of aqueous-phase proteins and detergent-phase proteins were concentrated 5–10-fold with Centricon-30 (Amicon Corp., Danvers, MA). Bacteriorhodopsin (from *Halobacterium halobium*; Sigma Chemical Co., St. Louis, MO) and BSA were used as hydrophobic and hydrophilic protein markers, respectively. Egasyn was purified as previously described (25) as modified by Medda and Swank (26).

Proteolytic Digestions of Microsomal Vesicles

Mouse liver microsomes from female C57BL/6J were suspended at 13 mg protein/ml in 20 mM imidazole-HCl, pH 7.4, containing 0.25 M sucrose, 50 mM KCl, and incubated with or without Triton X-100 (final concentration of 0.2% Triton X-100) at 0°C in the presence of 250 μ g/ml proteinase

K (Boehringer Mannheim GmbH, Federal Republic of Germany). After 30-min incubation at 0°C, proteinase K was inhibited with 1 mM phenylmethylsulfonyl fluoride. All samples were then adjusted to 0.2% Triton X-100 to solubilize microsomal vesicles and centrifuged at 100,000 g for 30 min at 4°C to remove insoluble material which interferes with subsequent electrophoreses. The supernatants were analyzed by nondenaturing PAGE (44) and stained for β -glucuronidase activity (26).

Assays

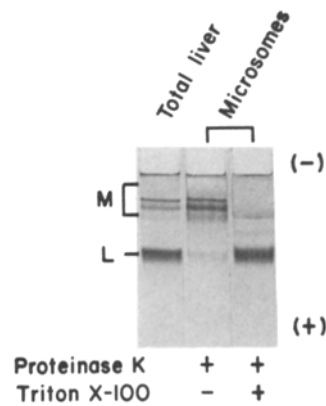
β -Glucuronidase and β -galactosidase were assayed fluorimetrically using, respectively, 4-methylumbelliferyl- β -D-glucuronide and 4-methylumbelliferyl- β -D-galactoside as substrates (8). 1 U of enzyme activity was defined as the amount of enzyme hydrolyzing 1 μ mol of substrate per hour at 37°C. NADH-cytochrome C reductase activity was determined by the method of Omura et al. (33) by measuring the initial rate of reduction of cytochrome C in a mixture containing 0.1 M Tris-HCl, pH 7.5, 30 μ M oxidized cytochrome C, and 44 μ M NADH. The change in optical density at 550 nm was measured at 30-s intervals at room temperature. Protein was determined by the Lowry method (22) or by the Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, CA). Radioactivity was measured by scintillation counting as described by Skudlarek and Swank (42).

Reagents

[³⁵S]Methionine, [³H]leucine, and [³H]choline were purchased from Amersham Corp., Arlington Heights, IL. Cytochrome C was type III (Sigma Chemical Co.). Reagents for gel electrophoresis were from Bio-Rad Laboratories. Nitrocellulose membranes (pore size, 0.1 μ m) were from Schleicher & Schuell, Inc., Keene, NH. Peroxidase-conjugated rabbit anti-goat IgG was from Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD. The proteinase inhibitors leupeptin, aprotinin, chymostatin, and pepstatin were from Sigma Chemical Co. All other reagents were from Sigma Chemical Co., Fisher Scientific Co. (Pittsburgh, PA), or J. T. Baker Chemical Corp. (Phillipsburg, NJ) and were the highest quality available. Specific anti- β -glucuronidase serum was previously described (42).

Results

The various intracellular forms of β -glucuronidase (24, 44) and egasyn-esterase (26, 27) can be resolved by nondenaturing electrophoresis at pH 8.1 and then staining the gels with appropriate substrates for β -glucuronidase or esterase activity. Mouse liver β -glucuronidase components are typically resolved into two main groups: a more anodal L or lysosomal component which is predominantly lysosomal in location and a cathodal M or microsomal group of three to four bands which are exclusively located in microsomes. The L component is the free glucuronidase tetramer of \sim 280 kD while the M forms, termed M₁–M₄, represent complexes between the slightly larger precursor form of the glucuronidase tetramer and from one to four molecules, respectively, of the accessory protein egasyn. It is possible to dissociate the microsomal M components to precursor form X by treatment with a variety of agents such as heat, urea, and organophosphorous compounds (28). When the nondenaturing acrylamide gels are stained with an esterase substrate, many components appear because of the many genetically distinct liver esterases (26, 27; Fig. 7 A). With appropriate controls, however, it is possible to identify egasyn complexed with β -glucuronidase in M forms as an esterase. Also, free egasyn or E form egasyn, can be identified as a series of more anodally migrating esterase bands (26, 27; Figs. 7 A and 8 B). E form egasyn is, like most other esterases, a microsomal component and is not complexed with β -glucuronidase or with any other protein (26). The submicrosomal location of the M form β -glucuronidase–egasyn complexes and E form egasyn–esterases were examined in these experiments.



nondenaturing polyacrylamide gels and stained for β -glucuronidase activity. A control untreated whole liver homogenate was included to determine the mobility of microsomal and lysosomal components.

Proteolytic Digestion of Microsomal Vesicles

Proteolytic digestion of sealed and detergent-disrupted microsomes with proteinase K was used to determine if microsomal β -glucuronidase is exposed on the surface of microsomal vesicles (Fig. 1). Without detergent treatment, the microsomal M forms (44) (complexed with egasyn) were not affected in their relative mobility on nondenaturing gels. However, upon disruption of microsomal vesicles with Triton X-100 before proteinase K treatment, microsomal M forms became susceptible to proteolytic modification. Interestingly, all M forms shifted in mobility to that of lysosomal form L (44). Thus, this experiment indicates that microsomal β -glucuronidase is protected from proteolytic attack by the microsomal membrane.

The change in properties of the microsomal M forms to that of the lysosomal L form by proteinase K digestion is similar to the results obtained by Lusic (23) and Owens et al. (35) after digestion of microsomal β -glucuronidase with carboxypeptidase, trypsin, or chymotrypsin. Thus it is possible that in vivo an exposed portion of β -glucuronidase precursor is readily susceptible to proteolytic attack, and removal of this exposed portion is the mechanism for generation of the mature lysosomal form of β -glucuronidase (11). In our experiments, it was found that conversion of M forms of β -glucuronidase to L form occurred in <1 min after exposure to proteinase K and that no further modification occurred during the 30-min incubation. Also, by activity assay 96% of β -glucuronidase was recovered after proteinase K digestion. This is in agreement with the known resistance (37) of lysosomal β -glucuronidase to proteolytic attack.

Microsomal β -Glucuronidase Is a Luminal Component

The proteolytic digestion experiment suggested that the β -glucuronidase-egasyn complex is within the microsomal membrane, is attached to the interior of the membrane, or is a luminal component.

To partially distinguish these possibilities, liver microsomal vesicles were treated with gradually increasing concentrations of the detergent Triton X-100. The solubilization of β -glucuronidase activity closely paralleled that of liver proteins rapidly labeled with [35 S]methionine (Fig. 2). The

majority of rapidly labeled liver proteins have been shown to be newly synthesized secretory proteins found within the lumen of the endoplasmic reticulum (20). Intrinsic membrane components, including NADH-cytochrome C reductase, an intrinsic protein of the endoplasmic reticulum membrane (20) and membrane phospholipids labeled with [14 C]choline, require higher Triton X-100 concentrations for solubilization (20). β -Glucuronidase was >80% solubilized before there was significant release of NADH-cytochrome C reductase or membrane phospholipids. In separate experiments (not shown), it was found that intrinsic membrane proteins, labeled by injection of [3 H]leucine 24–72 h before killing, were released in parallel with membrane phospholipids. The small increase in release of rapidly labeled liver proteins at higher Triton X-100 concentrations likely represents the expected small quantity of newly synthesized intrinsic membrane proteins.

The solubilization of β -glucuronidase activity from kidney microsomes with increasing concentrations of Triton X-100 (Fig. 3) was very similar to the release from liver microsomes with 50% release occurring at 0.03–0.04% Triton X-100 in each case. The release of total pulse-labeled proteins from kidney microsomes differs from the situation in liver since kidney synthesizes comparatively low levels of rapidly turning over secretory proteins found within the lumen of microsomal vesicles.

Since the detergent Triton X-100 might remove loosely bound hydrophobic proteins from membranes, the experiments were repeated with liver microsomes using an independent method of membrane disruption, low intensity sonication. The results (Fig. 4) mimicked the Triton X-100 results in that β -glucuronidase and pulse-labeled luminal secretory proteins were released in parallel and rapidly in comparison with intrinsic membrane components. There was no signifi-

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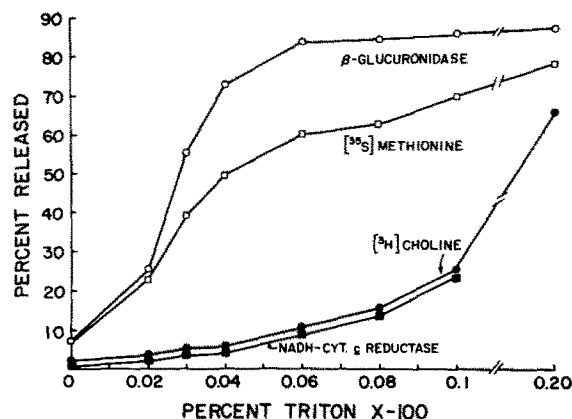


Figure 2. Release of β -glucuronidase from liver microsomes with Triton X-100. C57BL/6J mice were injected 4 h before killing with 100 μ Ci [3 H]choline to label intrinsic membrane phospholipids and 20 min before killing with 100 μ Ci [35 S]methionine to preferentially label luminal secretory proteins. Microsomal vesicles were isolated from liver by an osmotic shock procedure, which served to remove lysosomal β -glucuronidase activity (9), and were treated with increasing detergent concentrations according to the method of Kreibich and Sabatini (20). Unsolubilized membrane components were separated from solubilized components by centrifuging at 100,000 g for 30 min. β -Glucuronidase and NADH-cytochrome C reductase were assayed as described in Materials and Methods.

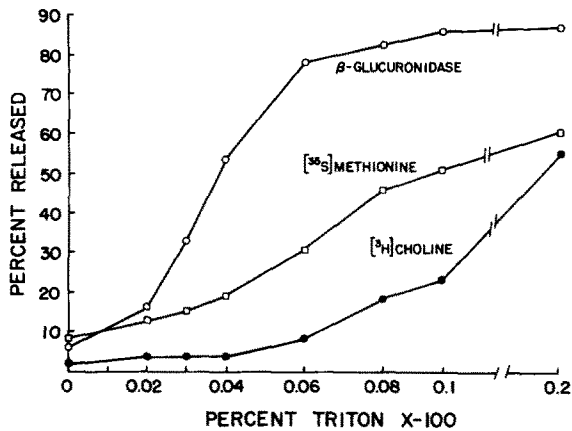


Figure 3. Release of kidney microsomal β -glucuronidase with Triton X-100. Microsomes were isolated from kidneys of mice treated with [3 H]choline and [35 S]methionine and treated with Triton X-100 as described in legend to Fig. 2.

cant loss of β -glucuronidase activity over the 1-h time course of sonication.

To test whether inactive forms of β -glucuronidase might separate as intrinsic membrane components, supernatant and pellet fractions of the Triton X-100 release experiments were analyzed with an antibody to β -glucuronidase after electrophoretic transfer to nitrocellulose membranes (Fig. 5). Microsomes were prepared from kidneys of female mice (DBA/2Ha) that were treated with testosterone for 21 d to fully induce kidney β -glucuronidase concentrations (45). Induced kidney was used as the source of microsomes since the β -glucuronidase concentration of liver microsomes was too low to analyze by this method. The β -glucuronidase antigen, which comigrates with purified microsomal β -glucuronidase, was released in parallel to enzymatically active β -glucuronidase (Fig. 6). All β -glucuronidase was released from the membrane at 0.08% Triton X-100, a concentration that caused only minimal release of intrinsic membrane components (Figs. 2 and 3).

The pattern of release of β -glucuronidase activity and there-

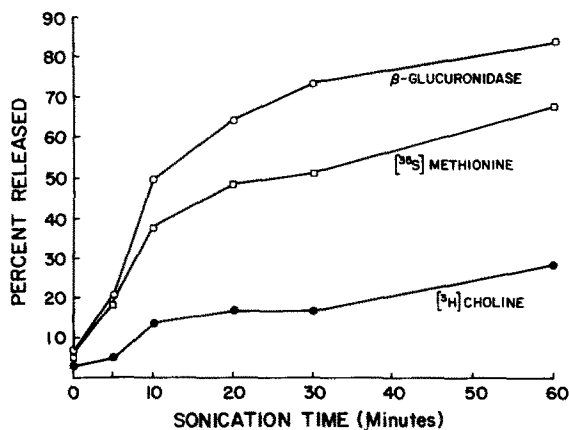


Figure 4. Release of microsomal β -glucuronidase by sonication. Liver microsomal vesicles, prepared from radiolabeled mice as in Fig. 2, were sonicated for increasing times at low power and 0°C. Unsolubilized membrane components were separated from solubilized components by centrifuging at 100,000 g for 30 min.

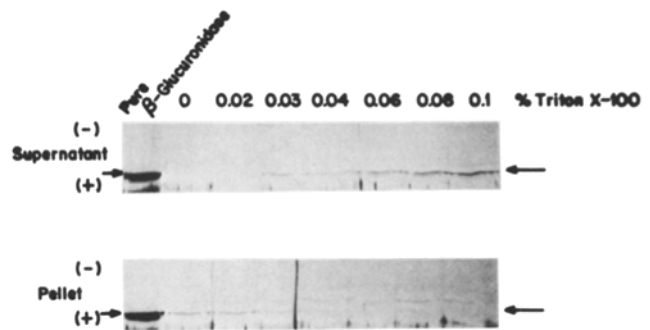


Figure 5. Release of immunoreactive kidney microsomal β -glucuronidase with Triton X-100. Microsomes from kidneys of female mice (DBA/2Ha), which were treated with testosterone to fully induce β -glucuronidase, were treated with the indicated concentrations of Triton X-100. The resulting pellet and supernatant fractions were subjected to electrophoresis on SDS gels, electrophoretically transferred to nitrocellulose membranes, and the membranes were probed with goat anti-mouse β -glucuronidase antibody followed by peroxidase-conjugated rabbit anti-goat antibody. Membranes were stained with 4-chloro-1-naphthol in the presence of H_2O_2 . The portion of the stained membrane near the electrophoretic migration region of β -glucuronidase is shown. β -Glucuronidase, which was purified from kidney microsomes by an immunoaffinity column (48), was electrophoresed in the first lane. The arrow indicates the electrophoretic migration position of microsomal β -glucuronidase.

fore by extension the β -glucuronidase-egasyn complex (see below) from microsomes by Triton X-100 remained the same when microsomes were suspended in low ionic strength buffer (0.25 M sucrose + 1 mM EDTA). Brada and Dubach (7) found this buffer causes association of glucosidase II and inosine-5'-diphosphatase with the microsomal membrane.

β -Glucuronidase Is Released as the Intact Complex with Egasyn

It was of interest to determine if the β -glucuronidase activity released from detergent-treated microsomes represented the intact glucuronidase complex (i.e., complexed with egasyn) or a dissociated form. This was assayed by taking advantage of the fact that the higher molecular mass M-form complexes are readily separated from the free β -glucuronidase X-form tetramer by acrylamide gel electrophoresis at pH 8.1 (44). The gel patterns indicated that intact M-form β -glucuronidase-egasyn complexes are released from microsomal vesicles (Fig. 6) at low Triton X-100 concentrations (50% release at 0.03–0.04%). Thus, release of β -glucuronidase from microsomal vesicles does not require prior dissociation of the β -glucuronidase-egasyn complex. Egasyn, in its complexed form, is a luminal component or is only very weakly attached to the inside of microsomal vesicles. Similar results have been obtained (not shown) with sonicated vesicles.

There is always a small amount of lysosomal L-form β -glucuronidase in microsomal vesicles. Form L may arise as part of maturation of microsomal X to lysosomal L in prelysosomal vesicles or from tightly adhering L form from lysosomal vesicles broken during homogenization. However, the proportion of this form does not increase in the supernatant of treated vesicles nor does X-form (microsomal β -glucuronidase dissociated from egasyn) (44) appear with these treatments.

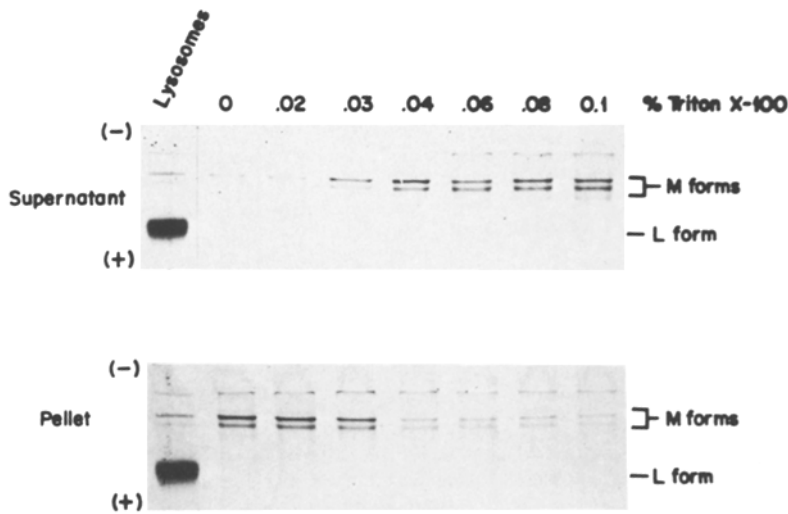


Figure 6. Microsomal luminal β -glucuronidase, released by Triton X-100 treatment, is complexed with egasyn. Liver microsomes were prepared and treated with Triton X-100 as in Fig. 2. Supernatant and pellet fractions derived by centrifugation after treatment with the indicated concentrations of Triton X-100 were analyzed on nondenaturing polyacrylamide gels and stained for β -glucuronidase activity. Lysosomal β -glucuronidase was derived from the osmotically sensitive (lysosomal) fraction of the osmotic shock procedure (9).

Free Egasyn Is a Luminal Component of Microsomes

It is known that only a small proportion (10%) of total microsomal egasyn is complexed with β -glucuronidase (24). The remaining 90% exists as a free monomer form, uncomplexed with other liver proteins (26). Also, recent experiments (26)

have established that both this free egasyn and egasyn complexed with β -glucuronidase have esterase activity. We have used (Fig. 7 A) the esterase activity of free egasyn to determine if it, like egasyn complexed with β -glucuronidase, is a luminal component. In addition, the enzymatic assay has

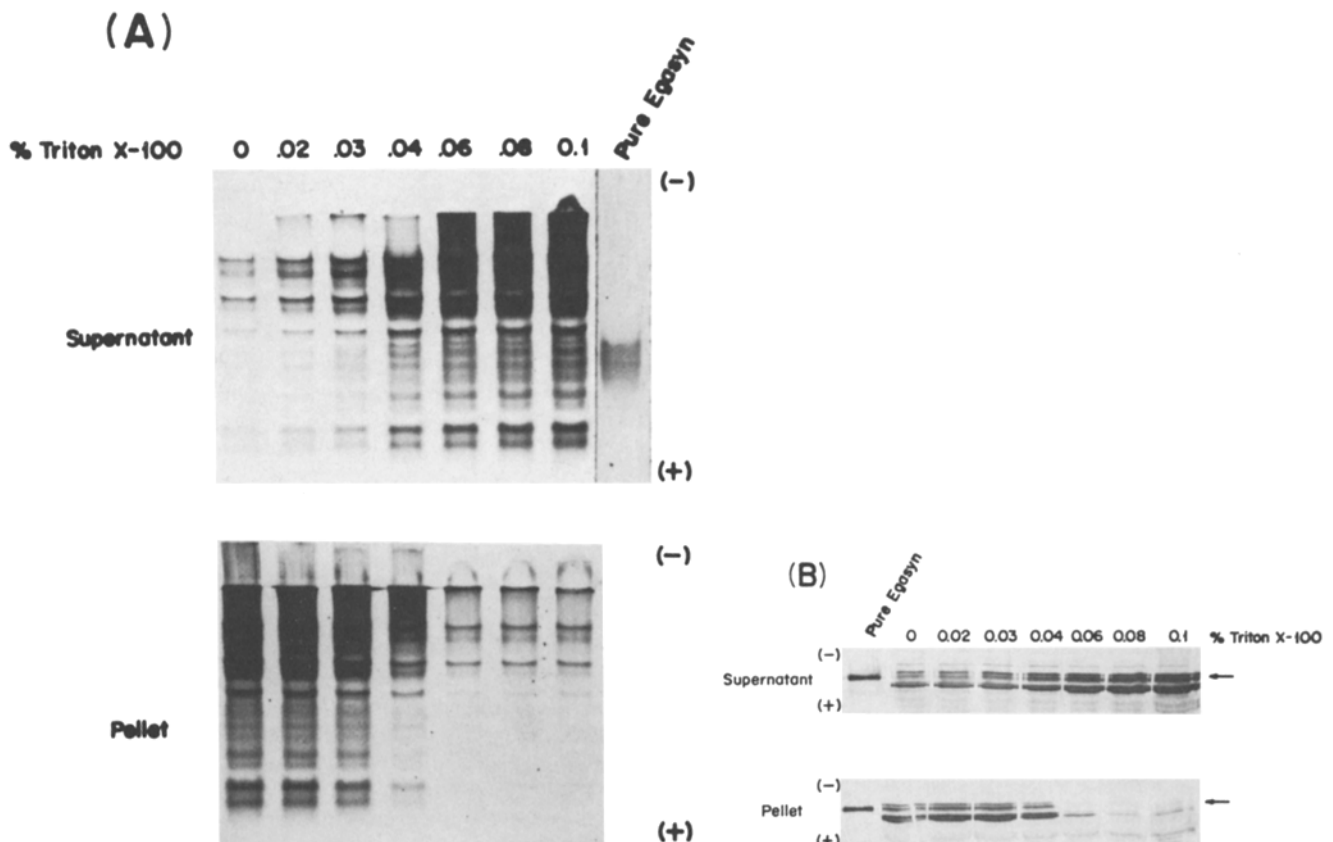


Figure 7. Release of free egasyn-esterase with Triton X-100. Liver microsomal pellets were isolated and treated with Triton X-100 as described in Fig. 2. Pure egasyn was prepared from the microsomal β -glucuronidase complex by precipitation of the complex with anti- β -glucuronidase followed by release of egasyn by heat treatment (23). (A) Gels were stained for esterase activity with alpha-naphthyl acetate (26). (B) Supernatant and pellet samples were subjected to electrophoresis on SDS gels and transferred to nitrocellulose membranes. Membranes were treated with goat anti-mouse egasyn antibody, followed by peroxidase-conjugated rabbit anti-goat antibody. Membranes were stained with 4-chloro-1-naphthol plus H_2O_2 . The portion of the stained membrane near the electrophoretic migration region of egasyn is shown. The arrow indicates the electrophoretic migration position of egasyn.

been supplemented by an immunoassay (Fig. 7 B) that recognizes the entire egasyn content of microsomes.

The enzymatically active esterase components of total liver microsomes are numerous (36) with >15 visible bands after acrylamide gel electrophoresis at pH 8.1 (Fig. 7 A). Purified egasyn migrates as three principal esterase components probably representing charge isomers (10, 27). All esterases, including egasyn, appear to be luminal components as gel scanning determined they were 50% released at 0.03–0.04% detergent similarly to luminal proteins (Fig. 2). The 10% of egasyn complexed with microsomal β -glucuronidase is, for technical reasons, not visible in this experiment. Triton X-100 causes other esterases to migrate in a diffuse fashion near the gel origin, possibly as a result of detergent binding, thus obscuring the complexed egasyn-esterase, which is visible in experiments that do not use detergents (26).

An immunoassay was performed after electrophoretic transfer of total SDS-denatured liver microsomal proteins to nitrocellulose membranes (Fig. 7 B). A component, recognized by the egasyn antibody, which comigrates with purified egasyn at 64 kD (26) was, like egasyn-esterase activity, 50% released from membranes at 0.03–0.04% Triton X-100. Three additional components, one of higher and two of lower molecular mass, were recognized by the antibody and were released in parallel with egasyn. It is likely that these components are structurally similar esterases that have been shown to share antigenic determinants with egasyn (40).

Tests of the subvesicular location of egasyn by proteolytic methods were inconclusive. The inhibitor phenylmethylsulfonyl fluoride, used to inhibit proteinase K, also completely inhibited egasyn-esterase activity. Also, other proteases, such as mixtures of trypsin and chymotrypsin, caused little or no digestion of egasyn even after microsomes were completely solubilized.

The release of glucuronidase and egasyn by Triton X-100 was repeated, including a cocktail of proteinase inhibitors in the homogenization buffer and throughout the Triton X-100 release steps to test if either component were artificially released from the membrane by proteolysis. The inhibitors and their final concentrations were EDTA (1 mM), chymostatin (5 μ g/ml), aprotinin (5 μ g/ml), leupeptin (5 μ M), and pepstatin (5 μ M). It was found that the proteinase inhibitors did not affect the results (not shown).

The Microsomal β -Glucuronidase Complex and Free Egasyn Are Hydrophilic

Phase separation of microsomal proteins using the nonionic detergent Triton X-114 (6) was performed to assess the hydrophobicity of the glucuronidase-egasyn complex. In this procedure, intrinsic membrane proteins, hydrophobic in nature, separate in the detergent phase while hydrophilic proteins separate in the aqueous phase. The microsomal β -glucuronidase-egasyn complex appeared in the aqueous phase, while no detectable activity was associated with the detergent phase (Fig. 8). Also, most esterases, including those identified as free egasyn, were associated with the aqueous phase (Fig. 8). Greater than 92% of egasyn-esterase was associated with the aqueous phase. In control experiments (not shown), BSA, a luminal secretory protein, separated in the aqueous phase while bacterial rhodopsin, an integral membrane protein, separated in the detergent phase. Pryde (38) has recently

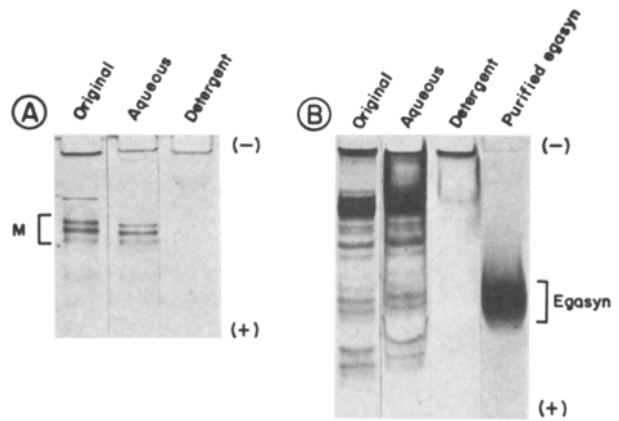


Figure 8. Phase separation of microsomal β -glucuronidase-egasyn complexes, esterases, and purified egasyn-esterase. The liver microsomal proteins from *M. m. molossinus* and *M. spretus* mice were subjected to phase separation with Triton X-114 as described in Materials and Methods. Aliquots of the original sample and of the aqueous and detergent phases were analyzed by nondenaturing PAGE at 4°C (26, 44). These mice were used since egasyn-esterase is more clearly separated from other liver esterases in them than in other common inbred strains (26, 27). (A) Gels were stained for β -glucuronidase activity. M forms are the β -glucuronidase-egasyn complexes from *M. m. molossinus*. (B) The liver microsomal proteins from *M. spretus* were subjected to nondenaturing electrophoresis and gels were stained for esterase activity. Control purified egasyn-esterase was purified from *M. spretus* by precipitation of the complex with anti- β -glucuronidase followed by heat treatment to dissociate egasyn from the glucuronidase-egasyn complex (25).

pointed out that a class of membrane proteins appear in the aqueous phase because of binding to residual detergent. If the residual detergent is removed from the aqueous phase by prolonged dialysis, these membrane proteins are precipitable by high speed centrifugation. The aqueous phase containing microsomal proteins was accordingly dialyzed and centrifuged. It was found that >70% of β -glucuronidase, whether complexed with egasyn or dissociated to the X form, and >90% of free egasyn remained in the supernatant aqueous phase after such treatment. These results indicated that microsomal β -glucuronidase, egasyn within the β -glucuronidase complex and free egasyn, are hydrophilic proteins.

Discussion

Egasyn has been thought of as an integral membrane component, which anchors β -glucuronidase within microsomal vesicles (24). This idea was based upon the location of egasyn and the egasyn- β -glucuronidase complex within microsomes and upon certain hydrophobic properties of egasyn. For example, egasyn is hydrophobic in behavior in that it is relatively soluble in certain organic solvents and appears to aggregate in the absence of detergents (24).

The present studies indicate to the contrary that the microsomal β -glucuronidase-egasyn complex and free (uncomplexed) egasyn are not integral membrane components. Rather, they are either within the vesicle lumen or are very weakly attached as peripheral proteins on the interior of microsomal vesicles. The evidence for these conclusions includes the results of proteinase treatments that showed that

microsomal β -glucuronidase is resistant to proteinases in intact but not in detergent-solubilized microsomal vesicles. Also, by both detergent-mediated and sonic perforation of microsomal vesicles, the complex is released with luminal secretory proteins rather than with integral membrane protein and lipid components. Similarly, free egasyn is released with luminal components after detergent treatment of microsomes. These conclusions held whether microsomal β -glucuronidase and egasyn were assayed by either enzymatic or immunological methods. The immunological tests revealed no evidence of pools of inactive β -glucuronidase or egasyn associated with the membrane as intrinsic components. Further evidence that the microsomal β -glucuronidase complex is not an intrinsic membrane component is the fact that both the intact complex and the free components β -glucuronidase and egasyn exhibit hydrophilic properties in phase partitioning experiments with the detergent Triton X-114. Egasyn, therefore, is more accurately described as a protein that maintains β -glucuronidase within the lumen of microsomal vesicles rather than one that anchors β -glucuronidase to membranes. Previous experiments (summarized in reference 24) did not adequately distinguish between a vesicle (which contains both membrane and luminal contents) and a true membrane location of the complex. Also, despite its reported solubility in certain organic solvents, egasyn is known not to have a high content of hydrophobic amino acids (24).

Nilsson and Dallner (32) likewise found that treatment of microsomes of another species, the rat, with low concentrations of the detergent deoxycholate caused release of most microsomal β -glucuronidase. Previous experiments (34) suggested that rat microsomal β -glucuronidase was not attached to an egasyn-like protein. However, we have recently found (29) that rat microsomal β -glucuronidase, like the mouse enzyme, exists in a complex with an egasyn-like protein in most inbred rat strains. In fact, like mouse β -glucuronidase (26), it exists as a complex with an esterase. Taken together, therefore, these results indicate that in both rats and mice the β -glucuronidase-egasyn complex is a luminal component of microsomes.

Although the egasyn- β -glucuronidase complex segregated with luminal secretory proteins in these *in vitro* studies, a fascinating *in vivo* difference is that while secretory proteins are rapidly released from the liver, the microsomal glucuronidase complex is stable within the lumen of the endoplasmic reticulum with a half-life of several days (43). One possible explanation of these findings is that secretory proteins and the β -glucuronidase complex are in separate vesicles and that the microsomal vesicle containing the β -glucuronidase complex is unusual in that it is not on the secretory pathway. Our experiments do not rule out the separate vesicle hypothesis since the microsomal vesicle fraction used in these experiments is a mixture of many organelles including endoplasmic reticulum, Golgi apparatus, plasma membrane, and others. Experiments in the closely related rat species, however, have clearly shown that liver microsomal β -glucuronidase is a component enzyme of the endoplasmic reticulum, codistributing in several types of sucrose gradient centrifugal separations with other markers of the endoplasmic reticulum including glucose-6-phosphatase, nucleoside diphosphatase, glucuronyl transferase, and esterase (2). The finding that liver esterases are endoplasmic reticulum enzymes is relevant to our studies since recent experiments

have shown that mouse (26) and rat (29) egasyns are esterases. Also, by electron microscopic histochemical techniques (12), mouse microsomal β -glucuronidase has been described as a component of the endoplasmic reticulum. Thus it is likely that secretory components and the β -glucuronidase-egasyn complex are residents of the same subcellular compartment, namely the endoplasmic reticulum.

A second possibility is that the complex is weakly bound to the luminal face of the endoplasmic reticulum. The nature of the putative weak binding site(s) on the membrane is unknown. However, the parallel release of the complex with secretory proteins after controlled disruption of membranes by both treatment with low concentrations of detergent and low intensity sonication under physiological salt and pH conditions suggests that such binding is extremely weak. It is possible that binding of the complex to the microsomal membrane is metabolically regulated as has been found for the binding of other proteins to other membranes (15) and this regulated binding is lost under our experimental conditions.

Munro and Pelham (31) have recently shown that a carboxyl-terminal signal, lys-asp-glu-leu, prevents secretion of three other proteins (BiP, grp94, and protein disulphide isomerase) from the lumen of the endoplasmic reticulum. In addition to the above mechanisms for retention of proteins in the lumen of the endoplasmic reticulum, they described a third possible mechanism. Namely the carboxyl-terminal signal of luminal proteins may be recognized by a receptor in or near the *cis*-Golgi region. This receptor-protein complex would recycle in reverse direction to the endoplasmic reticulum and release luminal proteins into the endoplasmic reticulum. This mechanism is consistent with the observation that β -glucuronidase of the endoplasmic reticulum has electronegative oligosaccharides of the high-mannose or hybrid type (46) shown by Mizuochi et al. (30) to include phosphodiesterases. Phosphodiesterases are added to lysosomal enzymes by the enzyme *N*-acetylglucosaminyl-transferase, which is thought to reside in the *cis*-Golgi (19).

Whatever the mechanism of maintenance of the complex within the microsomes, it is highly likely that this mechanism is mediated via egasyn rather than β -glucuronidase. The principal evidence for this statement is that mice without egasyn lack microsomal β -glucuronidase (14) despite the fact that precursor β -glucuronidase is synthesized at a normal rate and presumably is inserted into the endoplasmic reticulum during synthesis as in normal mice. Since both the 10% of egasyn which is bound to β -glucuronidase and the 90% which is the free egasyn monomer are vesicle associated (24, 26), egasyn is probably providing an intrinsic structure which serves to stabilize both it and the egasyn- β -glucuronidase complex within the endoplasmic reticulum. It is of interest that several esterases of the endoplasmic reticulum of rat liver have similarly been shown to be stable components of this organelle despite their apparent luminal location (1, 39). Presumably a structural feature(s) of rat and mouse esterases including egasyn-esterase (26, 27) stabilizes them and associated β -glucuronidase within the lumen of the endoplasmic reticulum. The recently described protein BiP (5) is similar to the microsomal esterases in that it is a resident of the endoplasmic reticulum and yet partitions into the hydrophilic phase during Triton X-114 detergent partitioning. Glucosidase II (7), inosine 5'-diphosphatase (7), protein disulfide-isomerase (13), several collagen hydroxylases (18),

and grp94 (31) are also apparently members of this class of proteins.

Although the mechanism by which egasyn maintains the complex within the endoplasmic reticulum is unknown, recent studies (28) indicate that β -glucuronidase is bound to egasyn by the egasyn-esterase active site.

The location of β -glucuronidase within the lumen of the endoplasmic reticulum may be metabolically important. Belinsky et al. (3) have presented evidence that microsomal β -glucuronidase is important in modulating the degree of glucuronidation of drugs and endogenous compounds. UDP glucuronyl transferase is thought to be on the luminal face of the endoplasmic reticulum (4) and the glucuronides it produces would be luminal and, thus, exposed to the action of microsomal β -glucuronidase.

We thank Cheryl Mrowczynski for expert secretarial assistance and Denise Devore-Carter for valuable technical aid.

This work was supported in part by National Institutes of Health grants GM-33559 and GM-19521.

Received for publication 24 July 1986, and in revised form 29 May 1987.

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