RELATIONSHIPS BETWEEN LEVELS OF MEMBRANE-BOUND GLUCURONIDASE AND THE ASSOCIATED PROTEIN EGASYN IN MOUSE TISSUES

ALDONS J. LUSIS and KENNETH PAIGEN

From The Department of Molecular Biology, Roswell Park Memorial Institute, Buffalo, New York 14263

ABSTRACT

Mouse β-glucuronidase has a dual intracellular localization, being present in both endoplasmic reticulum and lysosomes of several tissues. Previous studies demonstrated that the protein egasyn is complexed with microsomal but not lysosomal glucuronidase and that a mutant lacking egasyn is deficient in microsomal, but not lysosomal, glucuronidase. By means of a recently developed radioimmunoassay for egasyn, the relationship between microsomal glucuronidase levels and egasyn levels has been examined in various adult tissues, during postnatal development in liver, and after androgen induction of glucuronidase in kidney. The results indicate that the relative availability of egasyn determines the balance between glucuronidase incorporation into membranes and that into lysosomes.

Mouse glucuronidase is a useful system for studies of mechanisms bringing about the intracellular localization of enzymes. A body of genetic and biochemical information concerning the expression of glucuronidase is available (for reviews, see 3, 7, 27), and the enzyme is unusual in that it has a dual intracellular location, being present in both lysosomes and the membrane of the endoplasmic reticulum (microsomes) of several tissues (4, 5, 6, 9, 19, 26). The intracellular distribution of glucuronidase is tissue specific. All organs contain lysosomal glucuronidase but only some, including liver, kidney, and lung, contain microsomal glucuronidase (9, 10, 34). The enzyme at both sites is similar in thermal stability, antigenicity, and kinetic parameters, and by means of genetic mutants it has been shown that the enzyme at both subcellular sites is derived from the same structural gene, *Gus*, on chromosome 5 (8, 17, 18, 26, 28).

Evidence both biochemical and genetic suggests that the binding of mouse glucuronidase to endo- plasmic reticulum membrane is stabilized by the formation of noncovalent complexes with egasyn, a membrane-bound glycoprotein. Glucuronidase is present in lysosomes as a free tetramer, termed L, of molecular weight 280,000 (19, 20, 34, 37). The microsomal enzyme is extracted by means of the detergent Triton X-100 as a series of higher molecular weight aggregates, termed M1, M2, M3, and M4 (34). These consist of a glucuronidase tetramer core, termed X, complexed with, respectively, one, two, three or four molecules of egasyn, whose molecular weight is 64,000 (21, 34, 36). The two tetrameric forms of glucuronidase, X and L, appear to differ by covalent modification. At neutral pH, form L is slightly more negative than X, and there is a small difference in electrophoretic mobility in the presence of sodium dodecyl sulfate (SDS) (36).

The assembly of the M form complexes depends upon a genetic locus, *Eg*, on chromosome 8 (9, 10, 14, 34). A defect at this locus, termed Eg^0, is

present in the mouse strain YBR and results in the absence of glucuronidase activity in microsomes, although Eg^0 mice contain normal levels of the lysosomal enzyme (9, 10, 14, 34). Recently, it has been found that the primary defect in Eg^0 mice is a deficiency of egasyn (22).

We now report evidence that in normal mice it is the quantitative availability of egasyn which determines the distribution of glucuronidase between lysosomes and microsomal membranes. The results also suggest that the integration of glucuronidase into microsomal membranes is controlled, among cell types and during development, by the production of egasyn. These studies became possible with the availability of a specific radioimmunoassay for egasyn (21).

MATERIALS AND METHODS

Animals and Homogenates

The inbred mouse strains C57BL/6J and A/J were obtained from The Jackson Laboratory (Bar Harbor, Me.) and the inbred mouse strain YBR/WiCv (YBR) was raised in our own colony. At the time of dissection, mice were about 2 mo of age. Tissues were homogenized in 9 vol of ice-cold 0.02 M imidazole, 0.25 M sucrose, pH 7.4, in a Polytron (Brinkman Instruments Inc., Westbury, N.Y.) homogenizer, and were stored at −20°C. For studies involving quantitation of the microsomal (M forms) of glucuronidase, homogenates were used fresh or, at most, once frozen, since repeated freeze-thawing disrupts partially the M forms (34).

Glucuronidase Assay

A fluorimetric assay employing the substrate 4-methylumbelliferyl-β-D-glucuronide (Sigma Chemical Co., St. Louis, Mo.) was used. The sample was incubated at 37°C with 0.4 mM substrate and 0.1 M sodium acetate, pH 4.6, in a volume of 0.1 ml. The reaction was terminated by the addition of 1 ml of 0.1 M sodium carbonate. Fluorescence was determined with an Aminco 4-7439 fluorimeter (American Instrument Co., Travenol Labs Inc., Silver Spring, Md.) equipped with a Corning 7-60 excitation filter (Corning Glass Works, Science Products Div., Corning, N.Y.) and a Kodak 2A emission filter (Eastman Kodak Co., Rochester, N.Y.). One unit of activity is that amount of enzyme hydrolyzing 1 μmol of substrate per hour.

Polyacrylamide Gel Electrophoresis

Electrophoresis was performed essentially as has been described previously (34). The detergent Triton X-100 (Sigma) was added to homogenates to a final concentration of 2%, and the mixture was allowed to stand on ice for 30 min, when it was centrifuged at 100,000 g for 30 min. This method results in the complete solubilization of microsomal glucuronidase and preserves the M forms intact (34). Homogenate supernatant solutions (10 μl) were applied to 7% polyacrylamide gels at pH 8.1, prepared according to Clarke (1), and 300 v (constant voltage) were applied for 2.5 h. Glucuronidase activity was visualized with naphthol-AS-BI-glucuronide as substrate (11, 12). This method results in the clear separation of the M forms and the L form of glucuronidase. Gels were scanned at 550 nm with a Corning densitometer (Corning Medical, Corning Glass Works, Medfield, Mass.). Electrophoretic separation of microsomal and lysosomal forms, followed by scanning and peak integration, was used in estimating the fraction of glucuronidase present in microsomes The validity of this method has previously been demonstrated by showing that the M forms of mouse organs, including androgen-induced kidney, are exclusively microsomal and that 90–95% of the L form is lysosomal (34, 36).

Isolation of Egasyn and Preparation of Antibody

These procedures were carried out as described previously (21). Briefly, microsomal glucuronidase-egasyn complexes were released from the particulate fraction of mouse liver and kidney, and were precipitated by the addition of anti-mouse β-glucuronidase antibody, prepared in goats (36, 37). Egasyn was then selectively released from these immunoprecipitates by either treatment with 0.5% sodium deoxycholate or incubation for 5 min at 50°C in 0.02 M Tris, 0.15 M NaCl, pH 7.5 (21). The isolated egasyn was homogeneous as judged by polyacrylamide gel electrophoresis in the presence of SDS by the method of Laemmli (15). The yield of egasyn from 100 g of pooled livers and kidneys averaged 100 μg to 200 μg for most mouse strains.

Antiserum to egasyn was prepared in rabbits by using intradermal injections at multiple sites of a total of about 50 μg of egasyn emulsified in Freund's complete adjuvant. Injections were given at intervals of about 2 wk, and rabbits were bled at 8 wk. Antiserum to egasyn reacted with free egasyn and with the M forms of glucuronidase but was unreactive towards glucuronidase that was not complexed with egasyn (21). Preimmune serum was unreactive with egasyn.

Radioimmunoassay

This procedure was carried out as previously described (21). Briefly, egasyn was labeled with ^{125}I (Amersham-Searle Corp., Arlington Heights, Ill., carrier free) using chloramine-T essentially as described by Hunter (13). Samples to be assayed were first extracted with Triton X-100 at a final concentration of 2%. This procedure solubilized egasyn and egasyn-glucuronidase complexes (21). All dilutions for the assay were made in 0.02 M Tris, 0.15 M NaCl, 0.1% Triton X-100, 0.1% bovine serum albumin, pH 7.4. Assays were carried out

in 0.6- × 5-cm culture tubes (Kimble Products Div., Owens-Illinois, Inc., Toledo, Ohio). The sample was mixed with antiegasyn antibody in a volume of 100 μl in the presence of 1% sodium deoxycholate and incubated for 1 h at 37°C and 24 h at 4°C. The antiegasyn antibody added represented about a 500-fold dilution of antiserum and was enough to bind about 50% of the labeled egasyn, to be added at the next step. The presence of sodium deoxycholate was required to unmask antigenic sites of egasyn (21). Egasyn labeled with ^{125}I (4,000 cpm, in 50 μl) was then added, and the mixture was further incubated for 1 h at 37°C and for 72 h at 4°C. The free and antibody-bound egasyns were then separated by a double antibody precipitation method in which 30 μl of goat antibody to rabbit IgG and 40 μl of 4% control rabbit serum (as carrier) were added. This mixture was incubated for 1 h at 37°C and for 4 h at 4°C, when the immunoprecipitate was sedimented by centrifugation at 10,000 g for 10 min. The pellet was washed and the radioactivity in the pellet was determined with a gamma counter (Biogamma model, Beckman Instruments, Inc., Fullerton, Calif.). A preparation of purified egasyn was used as the standard in each experiment.

RESULTS

Tissue Distribution

Previous studies have shown that the intracellular distribution of mouse glucuronidase differs among tissues (10, 34). Although all organs contain lysosomal glucuronidase activity, only some, including liver, kidney, and lung, contain microsomal glucuronidase. Among tissues, the levels of egasyn show a strong correlation with the levels of glucuronidase in microsomes (Fig. 1 and Table I). Liver, the richest source of microsomal glucuronidase, contained about 56 μg of egasyn per gram of tissue, and kidney, with less microsomal enzyme, contained about 10 μg of egasyn per gram of tissue. Brain and spleen lacked both microsomal glucuronidase and egasyn, while lung contained only trace levels of each (Table I). Thus, the production of egasyn in each tissue appears to determine the proportion of total glucuronidase that is incorporated into microsomal membrane.

The lack of correlation between the total levels of glucuronidase and egasyn among tissues suggests that the synthesis of each protein is regulated independently. We have confirmed this by testing the response in kidney of both proteins after androgen administration. Androgens induce kidney glucuronidase 20-50 fold, depending upon the mouse strain (35). Egasyn, however, proved to be totally nonresponsive to testosterone (Table II). In both liver and kidney, no significant differences
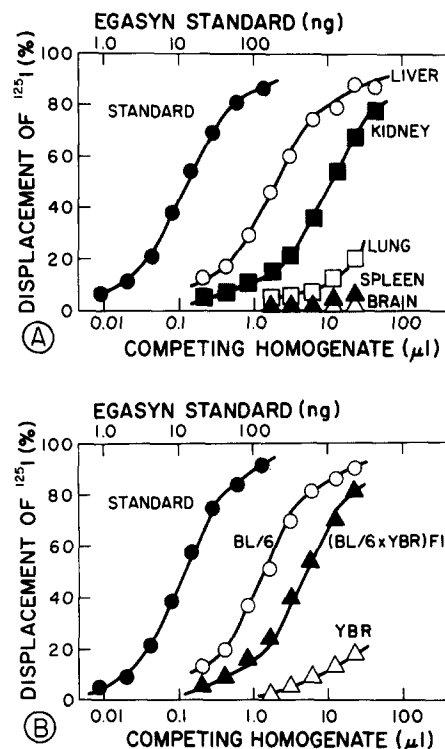

FIGURE 1 Radioimmunoassay of egasyn. Samples were prepared by homogenizing organs in 9 vol of 0.02 M imidazole, 0.25 M sucrose, pH 7.4, using a Polytron homogenizer (Brinkman Instruments). Antibody prepared against mouse egasyn was incubated first with sample and then with egasyn labeled with iodine-125, as described in Materials and Methods. The presence of egasyn was detected by the displacement of radioactivity from the antiegasyn antibody. Unlabeled, purified egasyn was used as the standard. A Distribution of egasyn in tissues of a typical C57BL/6J female. Egasyn standard (●–●), liver (O–O), kidney (■–■), lung (□–□), spleen (▲–▲) and brain (△–△). B Egasyn content of livers of strain YBR mice (which carry the defective Eg^0 allele) and strain C57BL/6J mice (which carry the normal Eg^+ allele). Egasyn standard (●–●), C57BL/6J (O–O), (C57BL/6J × YBR)F$_1$ (▲–▲), YBR (△–△).

in egasyn content were observed between male and female animals or between testosterone-treated and control females. Thus, egasyn and glucuronidase are under independent hormonal control.

Development

Preliminary observations of Swank[1] suggested that the fraction of glucuronidase present in mi-

[1] R. T. Swank. Personal communication.

TABLE I

Levels of Egasyn and of Microsomal Glucuronidase in Mouse Tissues

Strain	Tissue	Total glucuronidase mean ± S.E.	Microsomal glucuronidase mean ± S.E.	Egasyn mean ± S.E.
		U/g tissue	*U/g tissue*	*μg/g tissue*
C57BL/6	liver	32.3 ± 1.9	12.0 ± 0.5	56.1 ± 4.1
C57BL/6	kidney	8.4 ± 0.1	1.9 ± 0.1	9.1 ± 0.6
C57BL/6	lung	9.4 ± 1.5	1.2 ± 0.1	trace
C57BL/6	spleen	25.5 ± 1.9	not detectable	not detectable
C57BL/6	brain	2.2 ± 0.1	not detectable	not detectable
YBR	liver	21.1 ± 2.0	not detectable	not detectable
YBR	kidney	5.9 ± 0.3	not detectable	not detectable
(C57BL/6 × YBR)F$_1$	liver	27.8 ± 1.4	10.0 ± 0.6	25 ± 1.8
(C57BL/6 × YBR)F$_1$	kidney	6.7 ± 0.3	1.5 ± 0.1	—

Female mice, about 60 days of age, were used. The fraction of glucuronidase in microsomes was determined by electrophoretic separation of lysosomal and microsomal forms, as described in Materials and Methods.

TABLE II

Effect of Androgen upon the Levels of Egasyn and Glucuronidase in Kidney

Strain	Egasyn		Glucuronidase	
	Control	Induced	Control	Induced
	μg/g kidney		*U/g kidney*	
A/J	10.8	10.0	7.0	285
C57BL/6J	9.1	8.0	5.2	92

Female mice, about 60 days of age, were used. Testosterone pellets (Schering Corp.) were implanted subcutaneously, and mice were sacrificed about 2 wk after testosterone implantation. Control mice received no testosterone treatment. Assays were performed on the pooled kidneys of three or more mice.

crosomal membrane undergoes a large increase immediately after birth. As a further test of the hypothesis that the availability of egasyn determines the extent of membrane binding of glucuronidase egasyn concentrations and levels of microsomal glucuronidase were compared in mouse liver during postnatal development. Neither egasyn nor microsomal glucuronidase is present to an appreciable extent in liver at birth (Fig. 2). After birth, the levels of both egasyn and microsomal glucuronidase increase rapidly and then plateau at about 15 days of age (Fig. 2). Thus, the correlation between the levels of egasyn and microsomal glucuronidase applies not only to the different tissues but also during the development of a single tissue.

Eg^0/Eg$^+$ Heterozygotes

An apparent exception to the correlation be-

tween levels of egasyn and membrane binding of glucuronidase is the observation that, although F$_1$ heterozygotes of a cross between normal mice and the Eg^0 mutant contain only half-normal levels of egasyn (22), they nevertheless retain the same proportion of microsomal glucuronidase as their normal parents (Fig. 1 and Table I). This unexpected result may be related to the presence of an excess of egasyn in liver and kidney. We have previously estimated that only about 10% of the
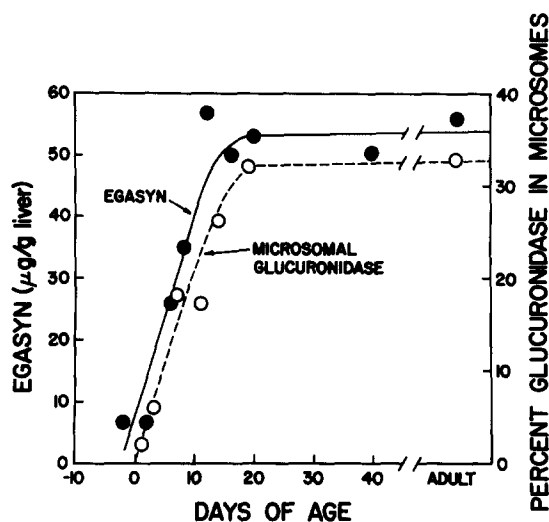

FIGURE 2 Egasyn levels in developing mouse liver. All mice were of strain C57BL/6J and were born on day 0. Each point represents the pooled organs of 3 or more mice. Livers from mice on day 14 of gestation, about 6 days before birth, contained no detectable egasyn. Egasyn (●—●), microsomal glucuronidase (○—○).

TABLE III
Effect of Androgen on the Levels of Egasyn Complexed with Glucuronidase in Kidney

	Total	Glucuronidase forms				
		L	M1	M2	M3	M4
Uninduced kidney						
glucuronidase (U/g kidney)	5.2	3.8	0.17	0.24	0.47	0.47
egasyn complexed with glu-curonidase (μg/g kidney)	0.50	0	0.02	0.06	0.18	0.24
Induced kidney						
glucuronidase (U/g kidney)	92.0	53.4	20.7	11.3	5.5	1.1
egasyn complexed with glu-curonidase (μg/g kidney)	8.1	0	2.6	2.9	2.1	0.5

Female C57BL/6J mice, about 60 days of age, were used. Mice were treated for 2 wk with testosterone as described in Table II. Kidneys were homogenized in 9 vol of 0.02 M imidazole, 0.25 M sucrose, pH 7.4, and the homogenates were extracted with 2% Triton X-100. The solutions were centrifuged at 100,000 $\times$ g for 60 min, and the supernatant solutions were electrophoresed and stained for glucuronidase activity as described in Materials and Methods. The gels were scanned at 550 nm and peak integration gave the glucuronidase activity present in each form. One unit of glucuronidase activity corresponds to 0.6 μg of glucuronidase[2] (35). Using this value, and the molecular weights of egasyn (64,000) and glucuronidase (75,000 per subunit), and assuming that M1, M2, M3 and M4 contain, respectively, 1, 2, 3, and 4 molecules of egasyn per glucuronidase tetramer, the amount of egasyn in each glucuronidase form was calculated.

egasyn in C57BL/6J liver is complexed with glucuronidase (21), and from the distribution of glucuronidase among its microsomal forms we estimate that only a small fraction of egasyn in uninduced female kidney is complexed with glucuronidase (Table III).

To test whether this explanation is correct, we have taken advantage of the androgen inducibility of glucuronidase, but not egasyn (Table II), to compare heterozygotes and their normal counterparts under conditions in which egasyn is not in excess. Swank and Paigen (34) have previously shown that there is a large increase in microsomal as well as lysosomal glucuronidase in kidney after androgen induction. Following induction of normal kidney, virtually all of the egasyn becomes complexed with glucuronidase (Table III). This is also reflected in a shift in the proportion of the M forms of glucuronidase toward the lower molecular weight forms containing fewer molecules of egasyn (Table III). In strains which have even higher inducibility of glucuronidase than the C57BL/6 strain used in these experiments, small amounts of uncomplexed X glucuronidase have been detected in normal induced kidney (34). These observations suggest that in normal induced kidney the supply of egasyn available for complexing with glucuronidase becomes depleted. When Eg^0/Eg^+ heterozygotes, which contain about half

as much egasyn as normal mice (22), were induced for glucuronidase, they contained significantly less of the microsomal forms of glucuronidase than did induced Eg^+/Eg^+ mice (Fig. 3). Also, the relative amounts of the microsomal forms of glucuronidase differed between the two types of mice; while normal homozygotes contained significant amounts of M2 and M3, the heterozygotes contained primarily M1 and the uncomplexed glucuronidase tetramer X (Fig. 3). Thus, Eg^0/Eg^+ heterozygotes that contain half-normal levels of egasyn also have reduced levels of microsomal glucuronidase when conditions are arranged so that egasyn is no longer in excess.

DISCUSSION

Our experimental observations indicate that the availability of egasyn is a primary factor in determining the partition of glucuronidase between lysosomes and endoplasmic reticulum. Levels of egasyn and microsomal glucuronidase are strongly correlated in different tissues and during the development of a single tissue. Also, previous observations show that phenobarbital is able to induce egasyn about two-fold, and that accompanying this induction is a significant shift in the subcellular distribution of glucuronidase from lysosomes to microsomes, although the total level of glucuronidase remains constant (25).

There is one anomaly in the quantitative relationship between egasyn levels and the membrane

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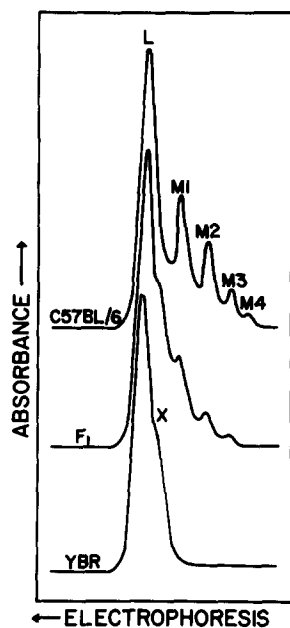[2] A. J. Lusis and K. Paigen, 1976. Unpublished results.

FIGURE 3 Polyacrylamide gel electrophoresis of glucuronidase from kidneys of androgen-induced mice. Female mice, about 60 days of age, were pelleted with testosterone as described in Table II and were sacrificed about 10 days after pelleting. Kidneys were homogenized in 0.02 M imidazole, 0.25 M sucrose, pH 7.4, and these were extracted with 2% Triton X-100 and centrifuged at 100,000 g for 30 min. Supernatant solutions (10 μl) were applied to polyacrylamide gels, and electrophoresis was performed as described in Materials and Methods. Gels were stained for glucuronidase activity and scanned at 550 nm. The separation of the X and L forms of glucuronidase was incomplete, and X is seen as a shoulder behind L.

binding of glucuronidase. Halving the egasyn level, by making animals genetically heterozygous (Eg^0/Eg^+), does not cause a significant shift in the subcellular localization of glucuronidase unless the animals are pushed to synthesize unusually large amounts of glucuronidase. However, doubling the egasyn level, via phenobarbital induction, is able to cause a significant shift in the subcellular localization of glucuronidase even when there is no change in enzyme activity (25). The explanation for this may be related to the fact that genetic manipulation to reduce egasyn levels has no effect on the synthesis of other membrane proteins, whereas phenobarbital causes a generalized proliferation of endoplasmic reticulum protein as well as an induction of egasyn. The additional membrane components induced by phenobarbital may

influence the interaction between glucuronidase and egasyn.

The syntheses of egasyn and glucuronidase appear to be regulated independently. There is no correlation between the total levels of egasyn and glucuronidase among tissues, and glucuronidase but not egasyn is induced by androgen in kidney. This suggests that the major factor controlling the integration of glucuronidase into membranes, among cell types and during development, is the regulation of egasyn synthesis. In relation to this, a particularly interesting question that remains to be answered is whether egasyn is complexed with proteins other than glucuronidase. In both liver and kidney, only a small fraction of the total egasyn is complexed with glucuronidase. That the remainder may well be associated with other proteins is suggested by the observation that antigenic sites of egasyn are unmasked under conditions that would dissociate egasyn-protein complexes (21). Thus, egasyn synthesis may regulate the membrane binding of a family of proteins, only one of which is glucuronidase.

Some evidence suggests that egasyn functions to stabilize the membrane binding of glucuronidase, once it has occurred, rather than to initiate the binding. First, under conditions of egasyn depletion, such as in androgen-induced kidneys of some mouse strains, small amounts of X glucuronidase, not complexed with egasyn, are present in membranes (34). Second, Ganschow and Smith[3], by following the incorporation of radioactively labeled amino acids into microsomal and lysosomal glucuronidase, have obtained evidence that Eg^0 mice synthesize approximately normal amounts of membrane-bound X glucuronidase but that in the absence of egasyn the membrane-bound glucuronidase is rapidly removed.

The intracellular forms of glucuronidase have been examined in rabbit and rat tissues as well as in mice (2). Livers of both rabbit and rat have large amounts of glucuronidase activity in microsomes as well as lysosomes, and the microsomal forms are extracted by Triton X-100 (3, 24). Biochemical studies suggest that, as in mice, the enzyme at both subcellular sites is derived from a common structural gene, although genetic evidence of this has not been obtained (2, 24). Microsomal glucuronidase and lysosomal glucuronidase in rat liver differ in charge, but no evidence of macromolecular complexes has been presented

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[3] R. Ganschow and K. Smith. Personal communication.

(24, 32). Rabbit liver, on the other hand, contains a series of glucuronidase molecular weight forms similar to those observed in mice; however, attempts to correlate these with different subcellular fractions have not yielded results which are as clear as those obtained with mouse tissues (3).

There is now considerable evidence in support of the original concept that the microsomal forms of glucuronidase exist complexed with egasyn in endoplasmic reticulum, and that these complexes are required for maintenance of glucuronidase in membranes. It is likely that this mechanism of membrane binding, involving noncovalent complexing with a specific integral membrane protein, is used widely among biological systems. Studies involving several "peripheral" membrane proteins, including mitochondrial ATPase (15, 23, 29), cytochrome c (30, 31), and spectrin (33), have been reviewed by Singer (30). In common with glucuronidase, each of these proteins appears to be anchored to membrane by specific noncovalent interaction with integral membrane proteins. Membrane binding by this mechanism may have special biological utility. Structurally, it provides a means of stabilizing the attachment of highly polar molecules, such as glucuronidase (37), to membranes, and it allows considerable exposure of the bound protein to the cytosol, which may be important for the functioning of certain enzymes. In terms of regulation, it allows independent control of the total activity and intracellular distribution of an enzyme, and provides a mechanism for coordinating the membrane binding of a set of enzymes. The studies with egasyn now provide evidence that integral anchor proteins such as egasyn may have a regulatory as well as a structural function.

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