Involvement of the Carboxyl-terminal Propeptide of β-Glucuronidase in Its Compartmentalization within the Endoplasmic Reticulum as Determined by a Synthetic Peptide Approach*

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Sukumar Medda†, Richard M. Chemelli‡, Jackie L. Martin¶, Lance R. Pohl‖, and Richard T. Swank§

From the †Roswell Park Memorial Institute, Department of Molecular and Cellular Biology, Buffalo, New York 14263, the ‡Department of Oral Biology, State University of New York at Buffalo, Buffalo, New York 14207, and the ¶Laboratory of Chemical Pharmacology, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20892

The proenzyme form of β-glucuronidase is compartmentalized in large quantities within the endoplasmic reticulum by binding to the esterase, egasyn. Also, the propeptide of the proenzyme form of β-glucuronidase is likely located at the carboxyl terminus. We have, therefore, tested if this carboxyl-terminal peptide is important in binding to egasyn. A polyclonal antibody to a 30-mer synthetic peptide, corresponding to the carboxyl-terminal 30 amino acids of pro-β-glucuronidase, provided evidence that egasyn binds to the carboxyl terminus of β-glucuronidase. This antibody interacted with proenzyme β-glucuronidase-egasyn complexes in which one, two, or three egasyn molecules were bound to the β-glucuronidase tetramer, but not with those complexes (M₄) which contained four egasyn molecules. We interpret these results as indicating that all available carboxyl termini of the β-glucuronidase proenzyme tetramer are shielded by egasyn in the M₄ complexes. The same antibody did not recognize the mature lysosomal form of β-glucuronidase, indicating that only the proenzyme form of microsomal β-glucuronidase contains the original carboxyl terminus. Also, the synthetic 30-mer was found to be a specific and potent inhibitor (50% inhibition at 1.3 μM) of the esterase activity of purified egasyn but exhibited little inhibitory activity toward other purified esterases including a rat trifluorocetylated esterase or egasyn esterase from another species. Together, these data describe a potent interaction of the exposed carboxyl terminus of precursor glucuronidase with the esterase catalytic site of egasyn, which in turn results in the specific localization of glucuronidase within the lumen of the endoplasmic reticulum.

β-Glucuronidase is maintained within the endoplasmic reticulum (ER) of cells of several organs including liver, kidney, and lung of murine species by complexation with the protein egasyn (1-3). Physiologically, it has been suggested that ER glucuronidase modulates the degree of glucuronidation of drugs and endogenous compounds (4-6). Egasyn is a member (2) of a group of liver carboxyl esterases which hydrolyze palmitoyl-CoA, palmitoyl carnitine, monoglycerides, and lysophospholipids as well as ester and amide-type drugs (7-10).

The glucuronidase-egasyn system thus serves as a model for the specific subcellular localization of a protein by binding to an accessory protein. Thirty to fifty percent of total hepatocyte glucuronidase is found in the ER rather than in the lysosome. Complexes of the two proteins are localized to the lumen of the endoplasmic reticulum (11). Egasyn, therefore, resembles in its subcellular location several recently described proteins including BiP, protein disulfide isomerase, grp94 protein, endoplasm and an M₅, 55 × 10³ protein (12, 13). Lumenal retention of these proteins requires the amino acid sequence KDEL at their carboxyl termini. Whether egasyn is similarly retained by a KDEL signal is unknown. However, the signal for retention of the glucuronidase-egasyn complex within the ER resides on egasyn, since mutants which lack egasyn also lack microsomal glucuronidase (1).

Some of the features by which glucuronidase and egasyn recognize each other and thus maintain glucuronidase within the ER are being clarified. First, the form of glucuronidase complexed to egasyn is the high molecular weight precursor form rather than the processed form found within lysosomes (14). Subunits of precursor glucuronidase are about 3000 Daltons larger, as determined by denaturing sodium dodecyl sulfate gel electrophoresis (14), than those of mature lysosomal glucuronidase which has undergone proteolytic processing (15, 16). Second, the region on egasyn which interacts with glucuronidase has been defined further. The finding that egasyn is actually a catalytically active esterase (2) combined with previous demonstrations (17-19) that liver microsomal glucuronidase is released into plasma by specific proteinase/esterase inhibitors suggested that the esterase active site of egasyn may be involved in binding glucuronidase. More direct tests (3) have confirmed this. A quite unique aspect of this system is, therefore, that the mechanism of subcellular compartmentalization of one protein involves the enzyme catalytic site of a second protein.

Although a major region of egasyn important in complex formation has been delineated, the region(s) of glucuronidase which specifically interact with egasyn and thus maintains glucuronidase within the endoplasmic reticulum is unknown. In this paper, we test the hypothesis that the carboxyl terminus of glucuronidase interacts with egasyn. The rationale for this hypothesis is: 1) only the high molecular weight precursor form of glucuronidase is bound to egasyn (14), and 2) there is evidence from another cell type, porcine kidney (16), that the proenzyme form of glucuronidase differs from the smaller lysosomal mature form by processing at the carboxyl terminus. These results are supported by the finding that precursor and mature forms of glucuronidase of mouse
macrophages have identical amino termini. It is possible, therefore, that the reason that egasyn is not complexed with mature lysosomal glucuronidase is that its attachment site has been removed. The recent publication of the complete sequences of cDNAs of glucuronidase from several species (20–24) has enabled the synthesis of defined peptide reagents which facilitate exploration of the region of glucuronidase which directly interacts with egasyn.

**MATERIALS AND METHODS**

**Peptide Synthesis—**Peptides were synthesized in a Biosense model 9500 peptide synthesizer by standard t-butoxycarbonyl chemistry (25) using a Merrifield resin with the carboxyl-terminal amino acid attached by a benzyol ester linkage. The 30-mer peptide sequence (NH2-Arg-Glu-Arg-Tyr-Glu-Arg-Ile-Ala-Asn-Glu-Thr-Arg-Gly-Tyr-Gly-Ser-Val-Pro-Arg-Thr-Gln-Cys-Met-Gly-Ser-Arg-Pro-Phe-Thr-Phe-COOH) corresponding to the carboxyl terminus of rat glucuronidase was derived from the nucleotide sequence of rat preputial gland β-glucuronidase cDNA (20) and rat liver β-glucuronidase cDNA (23). Peptides were cleaved from the resin support with hydrofluoric acid and desalted on a Bio-Gel P-2 gel filtration column in 5% acetic acid. The 30-mer was further purified by reverse-phase gradient HPLC on a Vydac C-4 column, using 0.1% trifluoroacetic acid for solvent A and 0.082% trifluoroacetic acid/70% acetonitrile for solvent B on a Waters chromatography system. The compositions of peptides were verified by a Waters F’ico-Tag amino acid analysis system. The insulin B chain of 30 amino acids was purchased from Sigma. An unrelated 11-mer peptide (NH2-Pro-Leu-Ala-Leu-Phe-Ala-Ala-Asn-Thr-Pro-COOH) was provided by Dr. Garth Anderson of Roswell Park Memorial Institute.

**Antibody Production—**To prepare antibody to the glucuronidase carboxyl terminus, 3 mg of the synthetic 30-mer peptide was diluted into 50 μl of 0.01 M acetic acid plus 3 ml of phosphate-buffered saline. 0.5 ml or 500 μg was mixed with 0.5 ml of Freund’s complete adjuvant and injected into multiple sites intradermally on the back of a New Zealand White rabbit. This was immediately followed with 0.5 ml of a 1:10 dilution of pertussis vaccine intramuscularly. This was followed by injections of 250 μg on days 21 and 42 as described above except that on day 42 half the dose was given intradermally and half intramuscularly. Serum was collected on day 49.

**Purification and Assay of Egasyn and a 59-kDa Trifluoroacylated Esterase—**Egasyn was purified by established immunological methods (26, 27), starting with extracts from livers of Sprague-Dawley SD rats or BALB/c ROS mice. Analysis of purified preparations on nondenaturing gels which were stained with α-naphthyl acetate was used as a test for the presence of esterases other than egasyn. A 59-kDa trifluoroacylated carboxylesterase was purified from microsomes of halothane-treated male Sprague-Dawley rats (160–180 g, Taconic Farms, Germantown, NY) by a procedure that was different from the method reported previously (28). Microsomes (800 mg, Taconic Farms, Germantown, NY) by a procedure that was different from the method reported previously (28). Microsomes (800 mg, Taconic Farms, Germantown, NY) by a procedure that was different from the method reported previously (28). Microsomes (800 g) were homogenized in ten volumes of 0.22 mM NaCl in buffer B, at a concentration of 125–145 mM. Purification was carried out by sodium decyl sulfate-polyacrylamide gel electrophoresis and immunoblotting with anti-59-kDa antibodies (28). The overall yield of the purification was 3 mg (0.38%). During halothane treatment, this carboxyl esterase is covalently modified by the reactive trifluoroacetylated halide metabolite of halothane, but esterase activity is retained (30). The trifluoroacylated esterase is an apparent trimer, is a high mannose glycoprotein, and is highly similar to a previously characterized rat microsomal serine-type carboxyl esterase (29).

**Esterase activities were assayed spectrophotometrically, measuring the rate of production of p-nitrophenol from p-nitrophenyl acetate at 405 nm as described by Heymann and Mentlein (30), except the final pH was adjusted to 7.2 to decrease the spontaneous substrate hydrolysis observed at higher pH values and to more closely approximate the physiological pH of the endoplasmic reticulum. Another change was that the substrate, p-nitrophenyl acetate, was dissolved in 0.75 ml of acetone rather than in acetonitrile and then made to 100 ml with water. 10 μl of egasyn (∼10−3 μM) in 0.05 M Tris, pH 8.0, or 10 μl of trifluoroacylated esterase (4 × 10−4 μM) plus 10 μl of peptide inhibitors (in 0.02 M acetic acid plus 0.2 mM Triton X-100) and 100 μl of buffer solution (0.5 M Tris-HCl at pH 7.2) were mixed and allowed to stand 3 min at room temperature. The reaction was started by adding 50 μl of water plus 800 μl of substrate solution. The rate of ester hydrolysis was monitored at each time point using a Waters 486 solvent A and 0.082% trifluoroacetic acid/70% acetonitrile for solvent B on a Waters chromatography system. The compositions of peptides were verified by a Waters F’ico-Tag amino acid analysis system. The insulin B chain of 30 amino acids was purchased from Sigma. An unrelated 11-mer peptide (NH2-Pro-Leu-Ala-Leu-Phe-Ala-Ala-Asn-Thr-Pro-COOH) was provided by Dr. Garth Anderson of Roswell Park Memorial Institute.

An antiserum was produced against the carboyx-terminal 30 amino acids of the proenzyme form of rat β-glucuronidase. We tested (Fig. 1A) the ability of this antiserum to interact with the various forms of liver glucuronidase, including microsomal glucuronidase tetramer forms M1, M2, M3, and M4 (which are glucuronidase tetramers containing one, two, three, and four molecules, respectively, of bound egasyn), microsomal glucuronidase form X (which is a glucuronidase tetramer containing no bound egasyn), and lysosomal glucuronidase tetramer form L (which also has no bound egasyn) (1, 31). Glucuronidase forms were detected by specific histochemical staining of nondeaturing gels with a specific glucuronidase substrate. Microsomal M and X forms contain the proenzyme form of glucuronidase, whereas lysosomal form L has the processed lower molecular weight subunits (14). All glucuronidase forms are tetramers containing four identical subunits. When this antiserum was reacted with extracts which had been pretreated at 56 °C (to dissociate egasyn from the M forms of microsomal glucuronidase) in vitro and thus to produce free X form glucuronidase). microsomal X form glucuronidase was removed from the extracts, whereas, in contrast, there was no apparent effect on lysosomal L form enzyme. Extracts were also heated at an intermediate temperature, 42 °C, to produce easily observable amounts of the partially dissociated M1, M2, and M3 forms. All three of these forms were readily removed from the extract by the antibody and again no effect on the lysosomal L form was observed. The most striking observation was that when antibody was reacted with untreated extracts, which have large amounts of microsomal form M4, in which all glucuronidase subunits are “coated” with egasyn, no removal of M4 occurred.

In control experiments, preimmune serum did not recognize any glucuronidase forms, and no glucuronidase components were visible in immune serum. Furthermore, the specificity of the immune recognition was confirmed in experiments (Fig. 1B) demonstrating that the carboxyl-terminal peptide effect-
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FIG. 1. Egasyn prevents the interaction of microsomal glucuronidase with antiserum to the carboxyl-terminal peptide of glucuronidase. A, antisemur (3 µl) to the carboxyl-terminal peptide of rat glucuronidase (+) or an equal quantity of preimmune serum (−) was mixed with 10% liver homogenates (20 µl) of male C57BL/6J mice. After overnight incubation at 0 °C, immune complexes were removed from homogenates by treating 1 h with 12 µl of protein A (Pansorbin, Calbiochem) followed by centrifugation. Homogenates were then electrophoresed on nondenaturing gels which were stained for glucuronidase activity as described in A. C57BL/6J mice. After overnight incubation at 0 °C, immune complexes, electrophoresed, and stained for glucuronidase activity as described in A. C57BL/6J mice. After overnight incubation at 0 °C, immune complexes were removed from homogenates by treating 1 h with 12 µl of protein A (Pansorbin, Calbiochem) followed by centrifugation. Homogenates were then electrophoresed on nondenaturing gels which were stained for glucuronidase activity. Some homogenates were pretreated 10 min at 42 °C to form easily visible amounts of the glucuronidase-egasyn complexes M1 and M2, containing one or two molecules of egasyn. Other homogenates were pretreated 10 min at 56 °C to form easily visible amounts of microsomal glucuronidase form X, which is not complexed with egasyn. The electrophoretic migration positions of microsomal glucuronidase forms X, M1, M2, M3, and M4, containing 0, 1, 2, 3, and 4 molecules, respectively, of egasyn and lysosomal glucuronidase form L are indicated. B, antiserum to the carboxyl-terminal peptide of glucuronidase was mixed with heated (56 °C) mouse liver homogenates as described in A. In the “plus peptide” case, excess (20 µg) of the 30-mer peptide corresponding to the carboxyl terminus of glucuronidase was added to the homogenate prior to addition of antibody. An equal volume of peptide solvent alone was added in the “minus peptide” case. Samples were incubated overnight at 0 °C, cleared of immune complexes, electrophoresed, and stained for glucuronidase activity as described in A. The positions of microsomal glucuronidase component X and lysosomal glucuronidase form L are indicated.

Egasyn esterase activity was compared in the presence and absence of various concentrations of different peptides in spectrophotometric assays using p-nitrophenyl acetate as substrate (Fig. 2). The 30-mer carboxyl-terminal peptide of glucuronidase was found to be a potent inhibitor of egasyn esterase, with 50% inhibition occurring at 1.3 µM levels. Approximately 75% inhibition occurred at 10 µM. It was not possible to test larger peptide concentrations, since the peptide became insoluble in the final assay mixture at concentrations greater than 10 µM.

The inhibition by the peptide is quite potent when it is considered that the substrate, p-nitrophenyl acetate, is present at 1.25 mM in these tests. The inhibition appears to be caused by the peptide itself rather than contaminating material, since the same inhibition curve was obtained whether crude peptide, obtained after HF cleavage, or HPLC-purified peptide was used.

In contrast, two unrelated peptides had little or no inhibitory activity. The insulin B chain, which is comparable in size to the 30-mer, did not inhibit even at 42 µM. Another peptide, an 11-mer of unrelated sequence, inhibited only slightly (20%) when used at very high (42 µM) levels. Pancreatic trypsin inhibitor, at 50 µM levels, did not inhibit (not shown).

Because of the high content (6 residues) of arginine within the 30-mer peptide and because of the possibility that residual protecting groups on these arginines could inhibit egasyn esterase, we also tested whether L-arginine alone or L-arginine with its protecting group (N′-butoxy-carbonyl-L-arginine) inhibited egasyn esterase activity. No inhibition was observed when either of these compounds was used up to very high concentrations (1030 and 420 µM, respectively).

To test if the 30-mer peptide is specific in regard to the type of esterase it inhibits, inhibition of purified rat and mouse egasyn and of purified trifluoroacetylated carboxyl esterase was compared (Fig. 3). It is apparent that despite a small (20%) inhibition of rat trifluoroacetylated esterase by the highest concentrations (11 µM) of 30-mer, the more potent inhibition by far was of rat egasyn esterase. Also, the 30-mer, derived from the rat glucuronidase sequence, much more efficiently inhibited rat than mouse egasyn-esterase. Although...
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50% inhibition of rat egasyn-esterase activity occurred at 1.3 μM peptide, less than 29% inhibition of mouse egasyn occurred even at much higher peptide levels (8.2 μM).

CONCLUSIONS

Two principal experimental results suggest that the carboxyl-terminal propeptide of microsomal glucuronidase is the region of the molecule which directly interacts with the associated protein, egasyn.

First, an antibody prepared against a synthetic 30-mer peptide corresponding in sequence to the carboxyl terminus of the proenzyme form of glucuronidase was able to recognize all microsomal glucuronidase forms containing at least one free carboxyl terminus, but did not interact with form M₀, which has all four subunits of the microsomal glucuronidase tetramer covered with egasyn. Egasyn apparently effectively physically shields at least the carboxyl terminal 30 amino acids of glucuronidase from interactions with an antibody which specifically interacts with this region of glucuronidase. The finding that glucuronidase interacts with egasyn through the esterase active site of egasyn and effectively inhibits the esterase activity of egasyn allowed a second test of the region(s) of glucuronidase which are important in interaction of these two proteins. The availability of the cloned sequence of rat glucuronidase (16, 20, 23) enabled the synthesis of peptides to defined regions of the molecule and the testing of the ability of these peptides to inhibit the esterase activity of purified egasyn.

It was found that the synthetic 30-mer carboxyl-terminal peptide is a potent and specific inhibitor of egasyn-esterase activity. Two unrelated peptides, in contrast, had negligible inhibitory activity. Also, there was esterase specificity in the action of the rat 30-mer peptide. Very little inhibition by the peptide of the esterase activity of purified rat liver trifluoroacetylated esterase was apparent. This type of specificity is to be expected, since among all the esterases, glucuronidase apparently binds only to egasyn (2). Also, although there is a certain degree of hydrolysis of common substrates by the various liver carboxyl esterases in in vitro assays, there is considerable substrate specificity (7, 9).

This specificity extended even to the presumably closely related egasyn-esterases of rat and mouse. The peptide readily inhibited the homologous rat egasyn-esterase but had little inhibitory activity toward mouse egasyn-esterase. Whether this specificity is due to differences in the structure of rat and mouse egasyns, glucuronidase carboxyl terminal peptides, or both is uncertain. That such differences in structure exist can be inferred from the finding that the stability of the rat egasyn-glucuronidase complex differs considerably from that of the mouse egasyn-glucuronidase complex when the complexes are dissociated by organophosphorous inhibitors of the egasyn-esterase catalytic site (3). It is also known that mouse and rat glucuronidase differ at four positions among the 30 amino acids at their carboxyl termini (16, 17, 20, 22–24) and one or more of these amino acids may be critical in interaction with egasyn.

A priori, the determination that egasyn interacts with the carboxyl terminus of glucuronidase, indicates that the interaction between these proteins is a post-translational rather than a co-translational event.

A search of the Bionet database revealed no significant homology of the sequence of the 30-mer carboxyl peptide with known sequences. This result is consistent with the fact that experiments designed to detect the possible binding of other proteins to egasyn have given negative results (2). The interaction between glucuronidase and egasyn thus involves regions which are very sequence-specific.

The structural features of the 36-mer propeptide region which are recognized by egasyn are uncertain. It contains extended regions of hydrophobic or hydrophilic structure. Analysis by the method of Chou and Fasman (32) revealed that there are no clusters of amino acids likely to form an a-helix. There are several regions of the peptide that could form a β sheet. An interesting feature of the peptide is that it has a high net positive charge (+4) at physiological pH because of the high arginine content. This feature is likewise present in the carboxyl-terminal peptide of glucuronidase of the mouse (22, 24) (another species with high levels of the glucuronidase-egasyn complex). However, neither high levels of arginine nor the protected form of arginine used in peptide synthesis were found to inhibit egasyn esterase activity.

Other proteins of the lumen of the ER are known, like the glucuronidase-egasyn system, to form complexes. For example, the protein BIP forms transient ATP-reversible complexes with immunoglobulin heavy chains and with incorrectly folded proteins (33). Whether the binding of BIP to other proteins involves the carboxyl terminus of either partner of the complex is unknown.

Other more indirect evidence is consistent with the finding that the carboxyl terminus of glucuronidase interacts with egasyn. For example, a variety of proteinases are known to readily convert the microsomal proenzyme form of glucuronidase to the processed lysosomal form in vitro, whereas the resulting lysosomal form is very resistant to further proteinase action (11, 34). This suggests that the carboxyl terminus exists in an extended conformation, readily accessible to modifying enzymes and to more permanent interaction with egasyn.

The finding that the antibody to the carboxyl terminus recognizes the microsomal proenzyme form of glucuronidase, but not the processed lysosomal glucuronidase form, indicates that the normal maturation of this lysosomal enzyme involves processing at the carboxyl terminus, i.e. that the propeptide region is at the carboxyl terminus. Erickson and Blobel (16) also found evidence, by carboxypeptidase digestions, for carboxyl-terminal processing of glucuronidase in porcine kidney cells. Glucuronidase is thus unusual among the examples of

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maturation of lysosomal enzymes (15) in that processing occurs at the carboxyl rather than the amino terminus.

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