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Membrane Topology of Recombinant Rat Liver Microsomal Glutathione Transferase Expressed in *E. coli*

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Rat liver microsomal glutathione transferase is a mammalian membrane protein that can be successfully expressed in *Escherichia coli* in an enzymatically active form. The protein does not form inclusion bodies and is recovered in the membrane fraction. The membrane topology of recombinant rat liver microsomal glutathione transferase expressed in *E. coli* was investigated by comparing the proteolytic cleavage products from intact and permeabilized spheroplasts. It was shown that lysine-4 of microsomal glutathione transferase is directed towards the outside, whereas lysine-41 faces the inside of the *E. coli* inner membrane. This shows that microsomal glutathione transferase has an inside-out orientation in *E. coli* spheroplasts as compared to liver microsomes. This fact enables us to make topology experiments that were previously not possible. Intact spheroplasts treated with pronase yielded a cleavage pattern consistent with two additional exposed segments closer to the C-terminus. Thus a polytopic model is suggested for the membrane association of microsomal glutathione transferase.

The glutathione transferases are a family of enzymes that conjugate numerous carcinogenic, mutagenic, toxic and pharmacologically active compounds with glutathione [1]. Microsomal glutathione transferase is a membrane-bound member of this family of enzymes [2-4]. Several studies have focused on structural and functional aspects of this trimeric membrane protein [5-7]. The membrane topology of microsomal glutathione transferase has been partly characterized in liver microsomes but would benefit greatly from the ability to produce inside-out microsomes which is currently not possible. Recently we have developed a heterologous expression system for microsomal glutathione transferase that allows expression of comparatively high amounts of functional enzyme in *E. coli* [8]. Here we show that spheroplasts from *E. coli*, that express the recombinant rat liver enzyme, can be regarded as inside-out microsomes with respect to the topology of microsomal glutathione transferase.

MATERIALS

Rat liver microsomal glutathione transferase cDNA isolation has been described earlier [9]. Suitable fragments were generated by PCR and ligated into the bacterial expression vector pSP19T7LT as described [10]. The construct was transformed into *E. coli* BL21 (DE3) (that harboured the plasmid pLys SL [11]). Microsomal glutathione transferase was purified as described previously [12].

Trypsin and pronase was purchased from Boehringer Mannheim, FRG. Trypsin inhibitor was from Sigma Chemicals, USA.

All other chemicals were of analytical purity and obtained from common commercial sources.

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Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; IPTG, isopropyl-β-D-thiogalactoside; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

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TABLE 1
Distribution of Microsomal Glutathione Transferase in *E. coli* Subcellular Fractions as Determined by CDNB Activity Measurements and Immunoblotting (Described under Methods)

	Specific activity (μmol/min mg)		Content (%)	
Fraction	Full length	Truncated (42–154)	Full length	Truncated (42–154)
5000 g pellet	N.D.	N.D.[a]	5–10	55
250,000 g pellet	2.0	N.D.	85–95	45
Supernatant	N.D.	N.D.	N.D.	N.D.

[a] N.D., not detectable.

METHODS

Bacterial expression of recombinant microsomal glutathione transferase was induced as follows: a small aliquot (5-10 μl) of bacterial glycerol stock was grown in 1.5 ml 2×YT overnight at 37°C. The culture was diluted 1:100 in TB (terrific broth [13]) and grown until the OD_{600} was 0.4-0.8. At this point expression was induced by the addition of 1 mM IPTG, the temperature was switched to 30°C and the culture allowed to grow for another 2-4 hours. All steps were performed in the presence of ampicillin (75 μg/ml) and chloramphenicol (10 μg/ml) with 240 rpm shaking.

For determination of the subcellular distribution of wild type and truncated microsomal glutathione transferase the cells were resuspended in 15 mM Tris-HCl, pH 8.0, 0.25 M sucrose, 0.1 mM EDTA, 1 mM GSH. The cells were lysed by sonication using four 30 s pulses from a MSE Soniprep 150 sonifier at 40-60% of maximum power. Cell debris (and inclusion bodies) were removed by centrifugation at 5000g for 10 min and resuspended in 10 mM potassium phosphate, pH 7.0, 20% glycerol, 0.1 mM EDTA, 1 mM GSH. The supernatant was centrifuged at 250000g for 60 min and the membrane pellet was resuspended as described above.

For determination of the proteolytic cleavage sites the cells (20 ml) were pelleted and resuspended in 5 ml 40% sucrose, 33 mM EDTA. 100 μg/ml of lysozyme was added and allowed to digest for 30 min on ice. The resulting spheroplasts were divided into 0.5 ml aliquots and treated with proteases (50 μg/ml) in the presence or absence of detergent (1% Triton X-100) at 22°C. When trypsin was used the reactions were stopped by addition of a three-fold excess of trypsin inhibitor and boiling in SDS-PAGE denaturing sample buffer. Pronase incubations were stopped by boiling in sample buffer (0-time incubations insured the effectiveness of this procedure).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (20% acrylamide [14]) was performed as described except that the concentration of Tris-HCl in the separation gel was doubled in order to improve resolution. Trypsin treated purified microsomal glutathione transferase and rainbow molecular weight markers (the Radiochemical Centre (Amersham, England)) served as standards.

Western blots [15] were employed to analyze the cleavage patterns and to quantitate the amounts of expressed microsomal glutathione transferase in the subcellular fractions. Quantitation of immunoreactive bands was performed using a Shimadzu scanner in the reflectance mode at 450 nm.

Enzyme activity assays utilizing CDNB were performed as described [12] except that the Triton X-100 concentration was raised to 1% with crude fractions in order to avoid an increase in turbidity.

Protein was determined by the method of Peterson [16].

RESULTS AND DISCUSSION

Microsomal glutathione transferase is a mammalian membrane protein that can be expressed in *E. coli.* The enzyme is inserted into the *E. coli* inner membrane in a functional form (Table 1). This allows for studies of the membrane topology of the enzyme in this heterologous system. Advantages include the possibility to perform in vitro mutagenesis and the fact that *E. coli* spheroplasts are well characterized as tools for membrane topology studies.

Confirming our earlier results, full-length and a truncated microsomal glutathione transferase could be successfully expressed in *E. coli* (Table 1) [8]. The truncated form lacking the first 41 amino acids (including a membrane spanning segment) is still partly recovered in the membrane fraction but is inactive (Table 1). Inclusion bodies contain the remainder and majority of the inactive protein in this case. Since the truncated form can be generated from

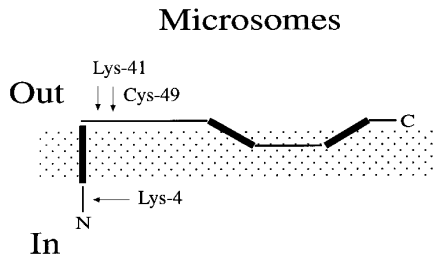

FIG. 1. Known determinants of the membrane topology of rat liver microsomal glutathione transferase [6].

the full-length purified protein by proteolysis in an enzymatically active form it appears that the first membrane spanning segment is required for correct folding and membrane insertion. Subseeding segments can also mediate some membrane insertion(association) of (in this case an inactive) microsomal glutathione transferase. The notion that membrane association is mediated by additional segments is supported by experiments showing that the enzyme is still firmly attached to the microsomal membrane when cleaved at lysine-41 [6]. Thus we arrive at the conclusion that microsomal glutathione transferase is attached to the membrane at multiple sites. However, we have not answered the question whether microsomal glutathione transferase is polytopic, i.e. whether the multiple sites of attachment are also membrane spanning.

Data gathered on the orientation of microsomal glutathione transferase in the endoplasmic reticulum is depicted in Figure 1. Trypsin, impermeable sulfhydryl reagents as well as phase separation in Triton X-114 aided in the partial definition of membrane topology [6]. Theoretical considerations suggest that spheroplasts would be equivalent to inside out microsomes regarding microsomal glutathione transferase topology. Would this be the case, lysine-4 and not lysine-41 (which is cleaved in intact rat liver microsomes) should be the target for trypsin in this case. These expectations were proven correct as can be seen in Figure 2. In Triton X-100 permeabilized spheroplasts the cleavage pattern was that of the isolated detergent-solubilized enzyme as expected (not shown). The positive inside rule correctly predicts the N-terminal orientation in both the endoplasmic reticulum and *E. coli* [17, 18].

Since trypsin predominantly cleaves at lysine-4 and -41 even with the purified enzyme [19] it cannot be used to probe the orientation of the C-terminal two thirds of the protein. Other proteases were therefore tested to generate additional fragments in spheroplasts. Pronase treatment yielded several fragments that could be visualized by Western blot (Figure 3). The size of the fragments were determined by comparison to standard proteins and the trypsin cleaved microsomal glutathione transferase. The major pronase cleavage products (a,d and f in Fig. 3)

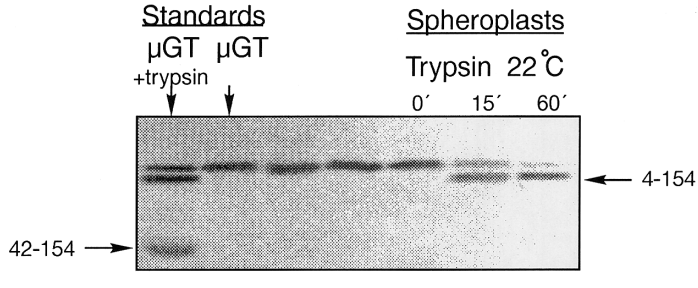

FIG. 2. SDS-PAGE (20 %) and immunoblot analysis of microsomal glutathione transferase cleaved with trypsin in intact *E. coli* spheroplasts. Experimental details are described under Methods.

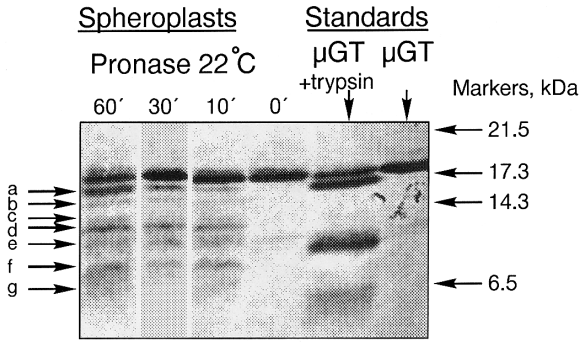

FIG. 3. SDS-PAGE (20 %) and immunoblot analysis of microsomal glutathione transferase cleaved with pronase in intact *E. coli* spheroplasts. Experimental details are described under Methods.

are interpreted to result from proteolysis around positions 4, 100 and 125 (Fig. 4). The fact that these products are major and arise early indicate that they result from single cuts. The pattern clearly shows the polytopic nature of microsomal glutathione transferase and cannot be explained by the model in figure 1. Positions of the fragments along the amino acid sequence and hence the position of cleavages were deduced by making one assumption: no cleavages can occur in the regions close to lysine-41 and cysteine-49 since these must be internal (vide supra). This effectively excludes alternate interpretations of the cleavage pattern obtained (for instance shifting fragment d or f towards the C-terminus) and indicates that the polypeptide does not cross the membrane until a sufficiently hydrophobic stretch is present (pos. 82-97). The cleavage sites are of course approximate since molecular weight determination of polypeptides on SDS-PAGE is not exact. Therefore, regions rather than exact proteolysis sites are to be inferred. Fainter bands (b,c,e and g) are not used to deduce topological information although (although fragment e could result from further cleavage of d and fragment g is the C-terminal counterpart of fragment f). When permeabilized spheroplasts were subject to proteolysis by pronase no bands could be visualized upon western blotting. This most likely results from destruction of immunogenic determinants and generation of smaller (undetectable) fragments. Thus pronase treatment served also as a control for the intactness of the spheroplasts.

It is tempting to accept the positioning of the pronase cleavage sites based also on the hydrophatic character of the protein since they correspond approximately to the end and beginning of hydrophobic segments 2 and 3 (Figures 4 and 5). The simplest possible model supported by the data thus is compatible with the model based upon theoretical considerations [17, 18] involving three membrane-spanning alpha-helices. However, although the membrane protein structures known today are either all alpha or all beta in the membrane environment

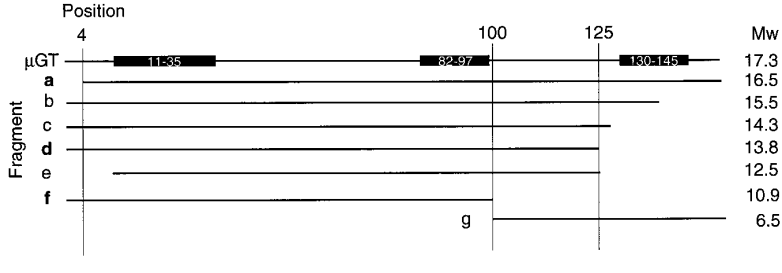

FIG. 4. Interpretation of the cleavage pattern obtained by pronase treatment of intact *E. coli* spheroplasts. Labels correspond to those used in Fig. 3.

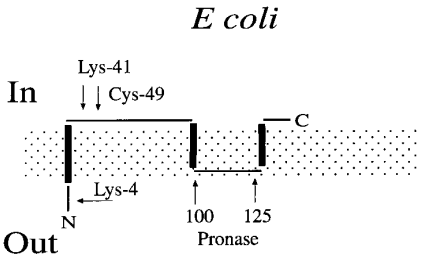

FIG. 5. Proposed membrane topology model of recombinant rat liver microsomal glutathione transferase expressed in *E. coli*. Trypsin cleavage sites are shown by arrows and deduced pronase cleavage sites are indicated by arrows with numbers.

[20-22], alternative arrangements cannot be excluded. For instance a mixed secondary structure membrane interaction has been proposed for the acetylcholine receptor [23]. A mixed secondary structure membrane interaction of microsomal glutathione transferase is also compatible with structural data obtained by electron crystallography ([24] and unpublished observations) as well as a possible structural homology to other glutathione binding proteins [25, 26].

The experimental system developed here clearly shows that microsomal glutathione transferase is a polytopic membrane protein. It also shows that the N-terminal part of the protein is necessary for correct folding and efficient membrane insertion in *E. coli.* Heterologous expression of a functional polytopic mammalian membrane protein can thus be accomplished in *E. coli,* a fact that inspires hope for increased insight into this group of proteins utilizing systematic in vitro mutagenesis.

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