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Expression of human liver epoxide hydrolase in *Saccharomyces pombe*

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Human liver microsomal epoxide hydrolase cDNA was inserted into the yeast expression vector pEVP11. The resulting recombinant plasmid was introduced into *Saccharomyces pombe*. The epoxide hydrolase protein and enzymic activity was subsequently expressed and identified in the 105000 *g* pellet after centrifugal fractionation of homogenized yeast cells. This method will provide a useful source of human liver epoxide hydrolase, avoiding the problems of obtaining human tissue.

INTRODUCTION

The metabolic oxidation of many xenobiotic compounds results in the formation of epoxides which may cause carcinogenic, mutagenic and cytotoxic effects [1,2]. However, a family of epoxide hydrolases catalyses the transformation of the reactive epoxides to dihydrodiols, which in general exhibit decreased biological activity [3,4].

The major microsomal xenobiotic metabolizing epoxide hydrolase (mEHb; EC 3.3.2.3) plays a key role in the detoxication of many compounds [3]. A rapid and simple method for purification of this isoenzyme from rat liver [4] has allowed the preparation of specific antibodies which also recognize the similar enzyme present in human liver microsomes [5]. The availabilities of these specific antibodies have facilitated the cloning and sequencing of human liver EHb cDNA from a human liver cDNA library in the expression vector λgt11 [6,7].

It was important to demonstrate that the cDNA isolated encoded a functional mEHb protein and that the sequence had not been altered during the cloning procedure; we have addressed this problem by expression of the cDNA in whole cells. Expression of cloned human liver epoxide hydrolases in yeast and eukaryotic cells in culture would allow further studies of the structure and metabolic function of the individual enzymes in whole cells rather than microsomal preparations. The expression of enzymically active cloned mEHb is also a preliminary step in determining the active site of this human enzyme by mutagenesis *in vitro*. In this paper we describe the introduction of human liver mEHb cDNA into yeast and the expression of the enzyme protein and catalytic activity towards styrene oxide in the transformed cells.

MATERIALS AND METHODS

Enzymes and chemicals

Restriction endonucleases and other DNA synthesizing and modifying enzymes and [^{3}H]styrene oxide (161 mCi/mmol) were obtained from Amersham International. Bluescript was from Stratagene and pUC18 was obtained from Boehringer. Affinity purified goat anti-(rabbit IgG)–horseradish peroxidase conjugate was from BioRad, and 4-chloro-1-naphthol was from Sigma. All other chemicals used to prepare reagents and media were the best grade available.

Construction of the recombinant plasmid for expression of human liver mEHb cDNA in *S. pombe*

A cDNA encoding human liver mEHb was isolated as a fragment from an *Eco*RI digest of a λgt11 recombinant and cloned into the *Eco*RI site of the plasmid Bluescript. A *Kpn*I-*Xba*I fragment containing mEHb cDNA was excised from the Bluescript recombinant and directionally subcloned into polylinker of the plasmic pUC18. The entire mEHb cDNA was then excised from the pUC18 recombinant using the flanking *Hin*dIII sites and ligated into the unique *Hin*dIII site in the yeast expression vector pEVP11. A recombinant plasmid pEVP EH where the mEHb cDNA is in the correct orientation with respect to transcription from the alcohol dehydrogenase promoter was identified by restriction mapping.

Transformation of *S. pombe* with the recombinant expression vector

This yeast/bacteria plasmid shuttle vector contains a selectable yeast marker *LEU2* along with the autonomous replication portion of the 2 μm circle and the β-lactamase gene conferring ampicillin resistance to bacterial transformants. The recombinant plasmid pEVP EH was used to transform the *leu*$^-$ *S. pombe* strain 972 [8] by the lithium acetate procedure [9]. Both the vector and the yeast strain were kindly provided by Professor Paul Nurse (University of Oxford).

Transformants were selected in *leu*$^-$ minimal plates and subsequently grown in minimal media without leucine at 30 °C [10].

Preparation of yeast 'microsome' fraction and assay of epoxide hydrolase activity

Exponential phase cultures of yeast cells (1 litre) were collected by centrifugation at 5000 *g* for 5 min and the pellets were resuspended in 30 ml of 0.25 M-sucrose/20 mM-Hepes, pH 7.4. Cells were broken with 5 × 15 s pulses of a Bead Beater (Biospec Products, Bartlesville, OK, U.S.A.). The resulting homogenate was centrifuged at

Abbreviations used: (m)EHb, (microsomal) epoxide hydrolase; UDPGT, UDP-glucuronosyltransferase.

10000 *g* for 10 min at 4 °C, and then the supernatant was decanted and centrifuged at 105000 *g* for 1 h at 4 °C. The 105000 *g* pellet was resuspended in the above buffer to a concentration of 15–20 mg of protein/ml as determined by the Lowry protein assay [11]. The yeast cell fractions were either assayed immediately for activity towards styrene oxide [12] or stored at −70 °C.

Immunoblot analysis of yeast subcellular fractions

Yeast cytosolic and microsomal proteins were separated by SDS/polyacrylamide-gel electrophoresis [13] using 7.5% gels. The proteins were transferred to nitrocellulose as described by Towbin *et al.* [14] and the blots analysed for mEH protein using a rabbit anti-(rat mEH) antibody (kindly provided by Dr. John Craft, Glasgow College of Technology) in conjunction with affinity-purified goat anti-(rabbit IgG) coupled to horseradish peroxidase and 4-chloro-1-naphthol as substrate.

RESULTS AND DISCUSSION

Measurement of epoxide hydrolase activity in recombinant yeast

Homogenate and subcellular fractions of wild type and recombinant yeast cells were prepared and assayed for epoxide hydrolase activity (see the Materials and methods section). Activity was easily measurable in homogenates of recombinant yeast, but not the wild type. Only 18% of this activity was recovered in the 105000 *g* pellet after subcellular fractionation. The remainder of the activity was retained in the 10000 *g* pellet, indicating problems with cell breakage or that the 'classical' subcellular fractionation procedure used [15] may not be optimized for fractionation of yeast cells. A more vigorous treatment of the cells in the Bead-Beater (5 × 1 min) only resulted in loss of enzyme activity from all fractions. No enzyme activity was detected in the 105000 *g* supernatant. Cell homogenization was difficult, but the results of the fractionation indicated that the expressed enzyme was present in the 105000 *g* pellet, which contains the microsomal fraction and possibly other small cellular vesicles, such as inclusion bodies.

Table 1 shows that the levels of epoxide hydrolase activity in 'microsomes' from wild type *S. pombe* and *S. pombe* transformed with the expression plasmid containing a cloned human liver UDPGT cDNA (pEVP GT) [16] were both very low. Thus transformation of yeast with expression plasmid containing another cDNA did not specifically allow expression of epoxide hydrolase.

The assay of the 105000 *g* pellet fraction from *S. pombe* transformed with the expression plasmid containing the human mEHb cDNA (pEVP EH) showed that the level of enzyme activity had increased from 0.01 units (in controls) to 1.24 units (Table 1). However, the specific activity of the yeast 105000 *g* fraction was calculated to be 8-fold lower than the activity in human liver microsomes (9.65 ± 3.0 nmol/min per mg of protein).

Immunoblot identification of the human epoxide hydrolase protein in microsomes from *S. pombe*

The presence of the human epoxide hydrolase protein in *S. pombe* transformed with human mEHb cDNA was assessed to confirm that the enzymic activities measured

Table 1. Assay of epoxide hydrolase activity in *S. pombe* 'microsome' fraction

The mean results and S.D. values were derived from the number of experiments indicated in parentheses.

S. pombe sample	Specific activity (nmol/min per mg of protein)
Wild type ($n = 2$)	0.01 ± 0.01
Transfected with UDPGT cDNA ($n = 2$)	0.01 ± 0.01
Transfected with human mEHb cDNA	1.24 ± 0.38

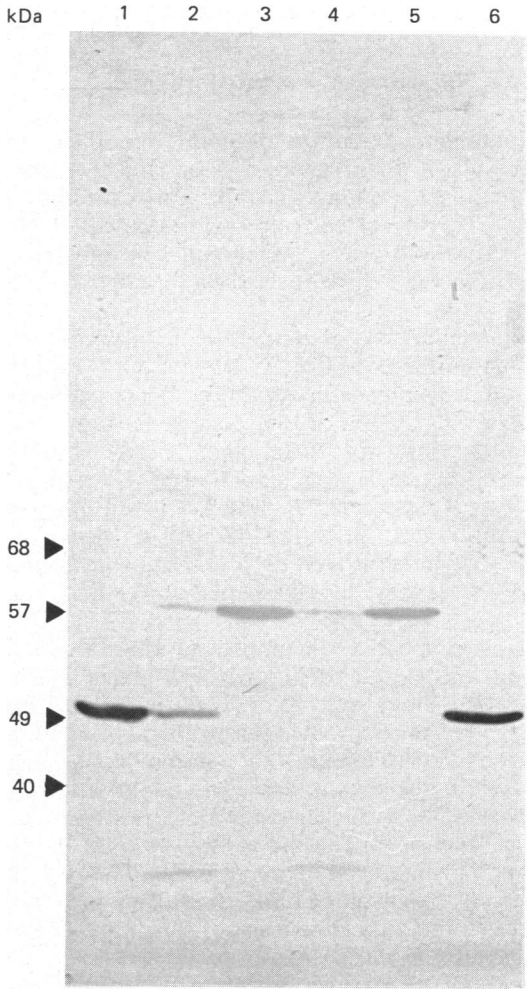

Fig. 1. Immunoblot analysis of microsomal and cytosolic fractions from transfected *S. pombe*

Proteins present in two yeast cell fractions and human liver microsomes were examined by immunoblotting as described in the Materials and methods section. Lanes 1 and 6, human liver microsomes; 'microsome' fraction (lane 2) and cytosol (lane 3) from yeast transformed with HmEHb cDNA; 'microsome' fraction (lane 4) and cytosol (lane 5) from yeast transformed with H25UDPGT cDNA. Equal amounts of protein (50 μg) were electrophoresed in each track. The mobilities of the molecular mass standards are indicated on the left; bovine serum albumin, 68 kDa; pyruvate kinase, 57 kDa; fumarase, 49 kDa; aldolase, 40 kDa.

could be associated with the expression of the correct protein. Proteins in microsomes from yeast transformed with pEVP EH or pEVP GT were separated by SDS/polyacrylamide-gel electrophoresis and analysed by immunoblotting with an anti-EH antibody. Fig. 1 shows the presence of an immunodetectable protein in microsomes from yeast transformed with pEVP EH which is absent from the control microsomes. In addition, Fig. 1 shows that this protein, with an approximate molecular mass of 50 kDa, exhibits the same mobility as epoxide hydrolase specifically immunoloabelled in human liver microsomes. Four other microsomal and one cytosolic yeast proteins were also stained when using this antibody, but as these proteins are also recognized in the cell fractions from the control transfected yeast, the staining observed can be attributed to non-specific cross-reactivity.

The level of expression of the cloned protein appears to be about 10% of that present in the human hepatic microsomes, based on a comparison of the amounts of immunodetectable protein observed in the same amount (50 μg of protein) of the different microsomes. These data would correlate well with the relative levels of activity measured in microsomes from the transformed yeast and human hepatic microsomes, which was approx. 8-fold higher in the liver microsomes.

This result indicated the protein product expressed was large enough to be enzymically active and apparently the protein was correctly folded in active conformation in the yeast. Epoxide hydrolase is recognised as a marker enzyme for rat liver smooth endoplasmic reticulum [17] and it is possible that the enzyme has been correctly inserted into yeast endoplasmic reticulum, although direct evidence of the insertion of the protein into the correct membrane could only be obtained by an electron microscopic analysis. It would seem unlikely that this membrane protein would be correctly folded and enzymically active in inclusion bodies. Another endoplasmic reticulum protein expressed in yeast (*S. cerevisiae*), a cytochrome *P*-450, was apparently correctly directed to the microsomes where interaction with the endogenous microsomal NADPH–cytochrome *P*-450 reductase allowed detection of the mono-oxygenase activity [18]. It is possible that the mechanisms required for insertion of this protein into the correct membrane may be conserved between yeast and man. Both the epoxide hydrolase and the cytochrome *P*-450 contain an *N*-terminal signal sequence which are not proteolytically cleaved and these two proteins are not extensively glycosylated during post-translational processing [7,18,19]. This latter aspect is perhaps fortunate for the heterologous expression of these two eukaryotic proteins in yeast, as a variety of problems have been encountered in the glycosylation of foreign proteins [20].

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