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EXPRESSION OF HUMAN LYSOSOMAL β-HEXOSAMINIDASE IN YEAST VACUOLES

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Abstract: The yeast *Saccharomyces cerevisiae* was tested as a recipient for functional expression of a mammalian lysosomal enzyme. The β chain of human β-hexosaminidase formed active dimeric enzyme, HexB, in transformants. HexB activity was localized to the vacuole, the yeast counterpart to the lysosome. A simple *in situ* enzyme assay was developed, which could be useful in expressing other lysosomal cDNAs.

In lysosomal storage diseases, undegraded substrates accumulate in lysosomes, with severe medical consequences. The study of the biochemical basis for these defects is advanced by isolating cDNAs encoding lysosomal enzymes. Since sensitive fluorogenic assays exist for some lysosomal enzymes, it is possible to screen a cDNA expression library or test candidate cDNAs for the ability to produce functional enzyme in recipient cells. The yeast *Saccharomyces cerevisiae* is an ideal host, because it is efficiently transformed, and lacks many of the acid hydrolases found in mammalian cells, so that background levels of enzyme activity are minimal. In the cases where yeast have homologous enzymes, the mammalian version can be isolated by complementation of yeast mutants. Proteins which are localized to the vacuole, the acidic degradative organelle of yeast, share many features with lysosomal enzymes of higher eukaryotes (1,2). Vacuolar proteins, like mammalian lysosomal proteins, are translocated into the lumen of the ER, where signal sequence cleavage and Asn-linked glycosylation occur. Carbohydrate sidechains are modified in the Golgi, and can be phosphorylated, although this is not necessary for transport to the vacuole, as it is for lysosomal enzyme trafficking (3). Finally, in the vacuole, propieces are cleaved off (4).

Abbreviations used in this paper: HexB, β-hexosaminidase B; ER, endoplasmic reticulum; Asn, asparagine; CPY, carboxypeptidase Y; kb, kilobasepair(s); bp, basepairs; 4-MU, 4-methylumbelliferyl; 4-MU-GlcNAc, 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside.

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The β-chain of human β-hexosaminidase was tested in yeast for expression and location. The β-chain was chosen as the test cDNA because others had demonstrated the formation of active dimeric enzyme, HexB, by *in vitro* translation of its mRNA in the presence of microsomes (5). Since the early stages of the eukaryotic secretory pathway are well-conserved (6), β-chain subunits, by virtue of the signal sequence, should be translocated into the ER lumen of yeast, where active dimers could associate. In initial experiments, the HexB produced in transformed yeast was detected in cell lysates, and an *in situ* filter assay was developed. While this result was promising, other lysosomal proteins, unlike HexB, might require post-ER modifications (phosphorylation, proteolytic cleavage, folding in an acidic environment) for enzymatic activity. Thus, the location of HexB in an organelle downstream of the ER in the yeast secretory pathway could bode well for the expression of other lysosomal enzymes. Although with the plasmid used in these studies, the level of HexB expression was too low for biosynthetic experiments, the location of the enzyme could be determined by the highly sensitive fluorogenic assay. Cell fractionation experiments demonstrated that human HexB is co-localized to the yeast vacuole with the marker enzyme carboxypeptidase Y, in a Ficoll step gradient (1). While plant lysosomal phytohemagglutinin had been shown to be localized to the vacuole (7), the vacuolar localization of HexB is the first reported case of a human lysosomal enzyme being transported to the yeast counterpart organelle.

METHODS

Plasmid Constructions. A 685 basepair EcoRI-BamHI restriction fragment containing the *gal1,10* bidirectional promoter (8) was inserted into the pUC12 polylinker region of pCF35 (a gift from G. Payne). pCF35 is the same as pSEY8 (9), except for the polylinker, and has selectable markers and the ColEl and 2μ replication origins for propagation in *E. coli* and yeast, respectively. Into the resultant plasmid, pTPGal, the cDNA encoding the β-chain of human β-hexosaminidase was inserted at the BamHI site adjacent to the galactose-inducible promoter. The β-chain cDNA was isolated from the plasmid pSP64β1 (5) as a SalI-SacI restriction fragment of about 1.7 kb, which includes 35 bp and 189 bp of pBR322 DNA at the 5' and 3' ends of the β-chain cDNA, respectively. After the ends were made flush, BamHI-XmnI adaptors were ligated to the cDNA fragment to allow ligation into the BamHI site of pTPGal, resulting in the plasmid pTPGalβ. DNA was manipulated according to Maniatis *et al.* (10), and Haymerle *et al.* (11). Adaptors were from New England Biolabs; restriction and DNA modifying enzymes were from New England Biolabs, Promega Biotec, or Boehringer Mannheim Biochemicals.

Strains and Growth Media. The bacterial strain MC1061 (12) was grown in LB medium with 50 μg/ml ampicillin for plasmid preparations (10). The yeast strain TP7hL (called 1-7a *hem1::Leu2* in ref. #13) was the recipient for transformations (14) with the plasmids pTPGal and pTPGalβ. Yeast were grown in YEP or minimal media (SD) for plasmid selection, with 2% glucose or galactose and required growth supplements (15). Reagents were from Difco and Sigma.

Filter Assay of β-Hexosaminidase. TP7hL/pTPGalβ transformants were visualized in a filter replica assay using the fluorogenic substrate 4-MU-GlcNAc. About 200 control cells (TP7hL/pTPGal) were plated onto SD galactose with required supplements, and then TP7hL/pTPGalβ cells were spotted at a density of 10-20 cells per 0.5 μl drop in a pattern that would display a "β" symbol. The colonies grew to the same size by 3 days at 30°C, and replicas were imprinted on Whatman #50 circles. The filters were frozen and thawed three times by submerging in liquid nitrogen for 10 sec, and thawing at room temperature. The permeabilized colonies were incubated with 0.6 ml of substrate mix (16) in plastic petri dishes, and incubated at 37°C for 25 min. After production of 4-methylumbelliferone, seen with long-wave UV light, the filters were refrigerated until photography was performed. Non-permeabilized colonies were not treated with the freeze/thaw regimen. Assay filters were placed on top of a UV transilluminator box, and photographed with Polaroid 55 film, with a yellow filter and 20 sec exposure time.

Lysates. Yeast transformants were grown to mid-logarithmic phase in SD glucose or SD galactose with necessary growth supplements. After centrifugation of the cells, the medium was saved, and the cell pellets were resuspended in 0.9% saline. Equal volumes of cell suspensions were lysed by vortexing 40 sec with 0.45 μm glass beads, or left intact.

Enzyme Assays. β-Hexosaminidase (HexB) was assayed with 1 mM 4-MU-GlcNAc (16) overnight at 37°C. α-Glucosidase assays used 1 mM 4-MU-α-D-glucopyranoside in 5 mM TES:NaOH, pH 6.8, 1% Triton X-100, for 25 min at 37°C (protocol of M. Marnell for determination of the fluorescent product, unpublished). Both reactions were stopped by addition of 1 ml 0.17 M glycine, 0.17 mM Na_2CO_3, pH 10. CPY activity was determined at 37°C, 40 min for the first reaction, and 45 min for the second reaction (17). NADPH cytochrome *c* reductase was assayed at 30°C for 33 min (18). Protein was assayed according to Lowry *et al.* (19). The 4-MU substrates were purchased from CalBiochem.

Vacuole Flotation Gradients. Yeast transformants were grown in supplemented SD galactose to early logarithmic phase. About 130-140 A_{600} units of cells were spheroplasted (20), then gently sheared by 4-5 passes through a 22-gauge syringe. The lysate was resuspended in 5 ml of 15% Ficoll lysis buffer; 0.5 ml was saved as the lysate control and 4.5 ml was applied to the bottom of a Ficoll step gradient consisting of 7 ml of 8% and 3.5 ml of 0% Ficoll lysis buffers (1). After centrifuging 1.5 hr at 100,000 x g in a Beckman SW28.1 rotor, 18 fractions of 0.8 ml each were collected with a Haake/Buchler fraction collector. Enzyme activities loaded on the gradient were approximately 28 nmol/hr of HexB, 22,900 nmol/hr of α-glucosidase, 2.3 mmol/min of CPY, and 1200 nmol/min of NADPH cytochrome c reductase. 80-90% of the enzyme activities were recovered in the 100,000 x g pellet; the rest remained soluble in the gradient.

RESULTS

HexB expression

The cDNA encoding the β-chain of the human lysosomal enzyme β-hexosaminidase (5) was placed under the transcriptional control of the bidirectional *gal1,10* promoter (8) of the yeast *Saccharomyces cerevisiae* in the plasmid pTPGalβ. pTPGal, which lacks the β-hex cDNA insert, was used as a negative control to detect background levels (if any) of HexB activity.

Initially, yeast transformants harboring the plasmids pTPGalβ and pTPGal were grown on SD galactose and tested for expression of HexB in a filter replica assay similar

to that of Kuranda and Robbins (21), except that instead of making *in situ* spheroplasts, whole cells were permeabilized by freeze/thawing the filter replicas in liquid nitrogen. For ease of visualization, the pTPGalβ transformants were plated in an array that would show a "β" pattern among randomly plated colonies containing the pTPGal control plasmid. Figure 1 shows that the pTPGalβ transformants, and not the pTPGal colonies, are able to hydrolyze the 4-MU-GlcNAc substrate *in situ* when the colonies are permeabilized. Cells not subjected to freezing and thawing had much lower activity, indicating that most of the HexB activity was intracellular.

The HexB activity of the pTPGalβ transformants was quantitated in glass bead lysates of logarithmically grown cells. The specific activities in Table 1 show that HexB activity in pTPGalβ transformants was induced by galactose 160-fold over background levels in glucose-grown cells, as expected for a cDNA under the transcriptional control of this galactose-inducible promoter. Control cells grown in galactose (TP7hL/pTPGal) did not have HexB activity. Thus, the HexB activity seen in the filter assays and in the lysates was due to expression of the human β-chain cDNA.

The specific activity of 1.6 nmol 4-MU-GlcNAc hydrolyzed per hour per mg protein corresponds to about 4 molecules of active HexB per cell (16). Although enzyme activity could be depleted from the extract with antisera directed against human HexB (not shown), attempts to visualize the β-hexosaminidase subunits synthesized in yeast by immunoprecipitations or Western blots were unsuccessful, due to the low level of expression of HexB.

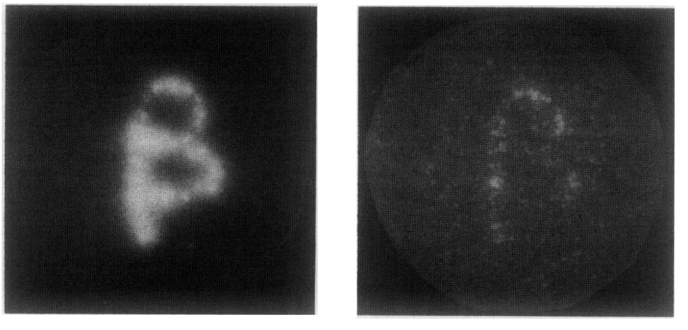

Figure 1. ***In situ*** **assay of HexB in yeast transformants.**
Yeast cells containing the plasmid pTPGalβ were plated in a "β" array on SD galactose. Control cells containing the plasmid pTPGal are present, but do not exhibit HexB activity. Replica filter assay is described in Methods. (left) with permeabilization, (right) without permeabilization.

Table 1. Galactose-inducible Activity of Human HexB in Yeast

	Carbon source	
Plasmid	Glucose	Galactose
pTPGalβ	0.01	1.6
pTPGal	0.005	0.01

Specific activity of HexB in extracts of yeast transformants, containing the β-chain cDNA under the control of the galactose-inducible *gal1* promoter. Activity is expressed as nmol 4-MU-GlcNAc hydrolyzed per hour per mg protein.

HexB location

To determine what proportion of the HexB was intracellular or secreted, enzyme activity was assayed in lysates (total enzyme), intact cells (periplasmic enzyme plus intracellular enzyme accessible to substrate), and medium (secreted enzyme). Liquid cultures were lysed or left intact and assayed for HexB and for α-glucosidase, a cytoplasmic marker enzyme. Both enzyme assays use similar 4-MU-sugar substrates. On the assumption that the cells are equally permeable to the two substrates, nearly 90% of the HexB activity was intracellular. As can be seen in Table 2, about 13% of HexB activity was accessible to substrate without permeabilization, as compared to 2.6% of α-glucosidase. This result agrees with the need for permeabilization of the colonies in the filter replica assay of HexB. Others also found that with three other 4-MU-sugar substrates assayed in yeast, intracellular enzyme required cell lysis for detection of a fluorescent signal, while periplasmic enzyme did not (21).

The intracellular location of HexB was examined by applying spheroplast lysates of galactose-grown cells at the bottom of a Ficoll step gradient, in which vacuoles float to the 0/8% Ficoll interface (1). Fractions were tested for HexB activity and the marker enzymes for vacuoles (CPY) (17), ER (NADPH cytochrome *c* reductase) (18), and cytoplasm (α-glucosidase). The results are shown in Figure 2, top. The peak of HexB activity coincided

Table 2. Intracellular Location of Human HexB Activity in Yeast

Enzyme	Lysate	Intact Cells	Medium
HexB	1.6 (100%)	0.21 (13%)	0.0043
α-Glucosidase	232.5 (100%)	6.12 (2.6%)	0.00

pTPGalβ transformants were grown in SD galactose. Equal volumes of cells were lysed or left intact for enzyme assays, as described in Methods. Activity is expressed as nmol substrate hydrolyzed per hour per mg protein.

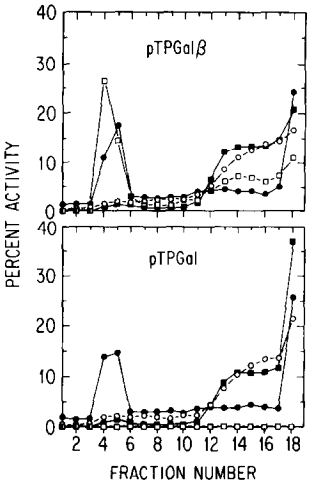

Figure 2. Fractionation of HexB in vacuole flotation gradient.
Spheroplast lysates of yeast transformants containing the plasmids pTPGalβ (top) and pTPGal (bottom) were applied to a discontinuous Ficoll gradient. Fraction 1 is from the top of the gradient. Vacuoles float to fractions 4 and 5. (■ α-glucosidase, □ HexB, ● CPY, o NADPH cytochrome *c* reductase.)

with that of the vacuolar marker enzyme CPY, in fractions 4 and 5. These fractions were not enriched for enzymes located in the cytoplasm or the ER. By normalizing to protein in each fraction, the HexB specific activity was enriched 33-fold, and that of CPY 22-fold in the pooled fractions 4 and 5, compared to the activity in the rest of the gradient. Of the total activity which remained soluble in the gradient, 53% of the HexB and 45% of the CPY were found in the vacuole fractions.

Although lyticase, the enzyme preparation used to make spheroplasts, has activity toward 4-MU-GlcNAc, the substrate for HexB, the control gradient in Figure 2, bottom (TPThL/pTPGal), had no HexB activity. Thus, the lyticase was not endocytosed by the spheroplasts, and the HexB activity in the pTPGalβ gradient (Figure 2, top) was indeed due to expression of the human β-chain cDNA in yeast.

DISCUSSION

The β-chain of the human lysosomal enzyme β-hexosaminidase has been expressed in the yeast *Saccharomyces cerevisiae*. By assaying HexB activity *in situ* in a filter replica assay and in cell lysates, the bulk of the active enzyme was found to be intracellular. Cell fractionation experiments showed that HexB is localized to the vacuole, the acidic degradative organelle of yeast, which is often referred to as the yeast counterpart to the lysosome of mammalian cells. These results are encouraging for the eventual isolation and expression of cDNAs encoding other lysosomal enzymes.

Although it is not surprising that active enzyme can be made in yeast cells, the localization of mature enzyme to the vacuole was not necessarily expected. While yeast and mammalian cells share the secretory pathway from the ER through the Golgi, where degradative enzymes are sorted from secreted proteins by a saturable, pH-dependent process (i.e. receptor-mediated) (1,2,22-25), the determinants recognized by the transport systems differ. Transport of lysosomal enzymes requires a mannose-6-phosphate marker (26). Yeast vacuolar proteins can contain phosphorylated mannose in their carbohydrate sidechains, but glycosylation is not necessary for proper localization (3). The yeast vacuolar targeting signal resides instead in a 3-dimensional structure in the propiece (27,28).

Perhaps the sorting systems in the Golgi of higher eukaryotes and yeast recognize conserved structures in degradative enzymes. Yeast proteinase A, which is 46% homologous to lysosomal cathepsin D, can be recognized by the *Xenopus* Golgi GlcNAc-phosphotransferase (29). This enzyme phosphorylates carbohydrate sidechains in lysosomal enzymes in a conformation-dependent manner (30,31). The localization of HexB to the yeast vacuole represents the reciprocal experiment, in which features of a lysosomal enzyme are recognized in yeast. Whatever the mechanism, the expression and vacuolar localization of human HexB demonstrates the potential utility of expressing lysosomal enzymes in yeast.

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