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OCH1 encodes a novel membrane bound mannosyltransferase: outer chain elongation of asparagine-linked oligosaccharides

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The *Saccharomyces cerevisiae och1* mutant shows a deficiency in the mannose outer chain elongation at the non-permissive temperature. We have cloned the *OCH1* gene by complementation of temperature sensitive (*ts*) phenotype for growth. The integrant of *OCH1* gene in the yeast chromosome can complement the *ts* phenotype and shows the same mapping position as that of the *och1* mutation, indicating that the cloned gene is the true gene for mutation. The *OCH1* gene disruptant is not lethal but *ts* for cell growth, and lacks mannose outer chains. The *OCH1* gene sequence predicts a 55 kDa protein consisting of 480 amino acids. It contains four potential asparagine-linked (N-linked) glycosylation sites and a single transmembrane region near the N-terminus. *In vitro* translation/translocation analysis revealed that the large C-terminal region of the OCH1 protein is located at the lumenal side of microsomal membranes with some sugar modification, indicating a type II membrane topology. The OCH1 protein was detected in yeast membrane fractions as four forms of 58 - 66 kDa, which correspond to the size of a glycoprotein containing four N-linked sugar chains the length of which is almost the same or slightly larger than the inner core ($Man_8GlcNAc_2$) formed in the endoplasmic reticulum (ER). Finally, the *OCH1* gene was found to encode a novel mannosyltransferase which specifically transfers [^{14}C]mannose to the unique acceptor, the core-like oligosaccharide of cell wall mannan accumulated in the *och1* disruptant.

Key words: mannosyltransferase/membrane spanning protein/outer chain elongation/protein glycosylation/yeast

Introduction

The asparagine-linked (N-linked) glycosylation pathways have been well studied in both mammalian and yeast cells. These pathways are similar in yeast and mammalian cells up to the oligosaccharide assembly on dolichol pyrophosphate (Dol-PP) in the endoplasmic reticulum (ER); they are different, however, in the later stage of oligosaccharide chain processing (Kornfeld and Kornfeld, 1985). In yeast the oligosaccharide chain can be larger than in higher eukaryotes, consisting of both inner core ($Man_8GlcNAc_2$) formed in the ER and mannose outer chain (Man_{15-100}) formed in the Golgi (Kukuruzinska *et al.*, 1987). In mammalian cells, several genes encoding glycosyltransferases, such as *N*-acetylglucosaminyltransferase (Kumar *et al.*, 1990), galactosyltransferase (Nakazawa *et al.*, 1988; Shaper *et al.*, 1988; D'Agostaro *et al.*, 1989) and sialyltransferase (Weinstein *et al.*, 1987), catalyzing part of the sequential reactions to form a complex type oligosaccharide from $Man_3GlcNAc_2$, were cloned and their common topological features were pointed out according to their amino acid sequences (Paulson and Colley, 1989).

In contrast, studies on glycosyltransferases in yeast are few. Although several genes concerned with part of the glycosylation pathway have been isolated, only two genes are known to encode a glycosyltransferase. The *ALG7* gene encodes the first enzyme in the lipid-linked oligosaccharide synthesis, UDP-*N*-acetyl-D-glucosamine:dolichyl-phosphate *N*-acetyl-D-glucosamine phosphotransferase (Rine *et al.*, 1983; Hartog and Bishop, 1987). Albright and Robbins (1990) cloned the *ALG1* gene which complements the temperature sensitive (*ts*) phenotype of the *alg1* mutant; this shows a deficiency in core oligosaccharide synthesis in the ER at the non-permissive temperature. They found that the *ALG1* gene encodes a mannosyltransferase which catalyzes the formation of Dol-PP-$GlcNAc_2Man$ from GDP-Man and Dol-PP-$GlcNAc_2$. Both gene products are responsible for the formation of inner core oligosaccharide in the ER. However, there are no reports on yeast glycosyltransferase genes whose product is functional in outer chain elongation.

In a previous paper we reported the isolation of an *och1* mutant which shows a deficiency in mannose outer chain elongation in yeast (Nagasu *et al.*, 1992). Here we report the *OCH1* gene cloning, DNA sequence, and the topological feature of OCH1 protein, together with its function in yeast. The OCH1 protein is an integral type II membrane protein whose C-terminal major region residues in the lumenal side, and contains four N-linked oligosaccharide chains. The overproduction of OCH1 protein shows an increase of an unknown mannosyltransferase activity which specifically transfers mannose to the core-like oligosaccharide acceptor accumulated in the *och1* disruptant. This suggests that the OCH1 protein is a novel mannosyltransferase which is functional in mannose outer chain elongation in yeast Golgi membranes.

Results

Cloning of OCH1 gene

The wild type *OCH1* gene was isolated by complementation of *ts* growth phenotype in the *och1* mutant. EHF-2C (*leu2 och1*) was transformed with a yeast genomic DNA library on the multi-copy plasmid pTN3200, which carries the *LEU2* gene as a selectable marker. Among a population of 2×10^4 transformants, four were able to grow at the non-permissive temperature (36°C). This phenotype was plasmid dependent and all the transformants contained the same DNA fragment. One of the plasmids designated as pM1 was analyzed in more detail. The restriction map of pM1 revealed

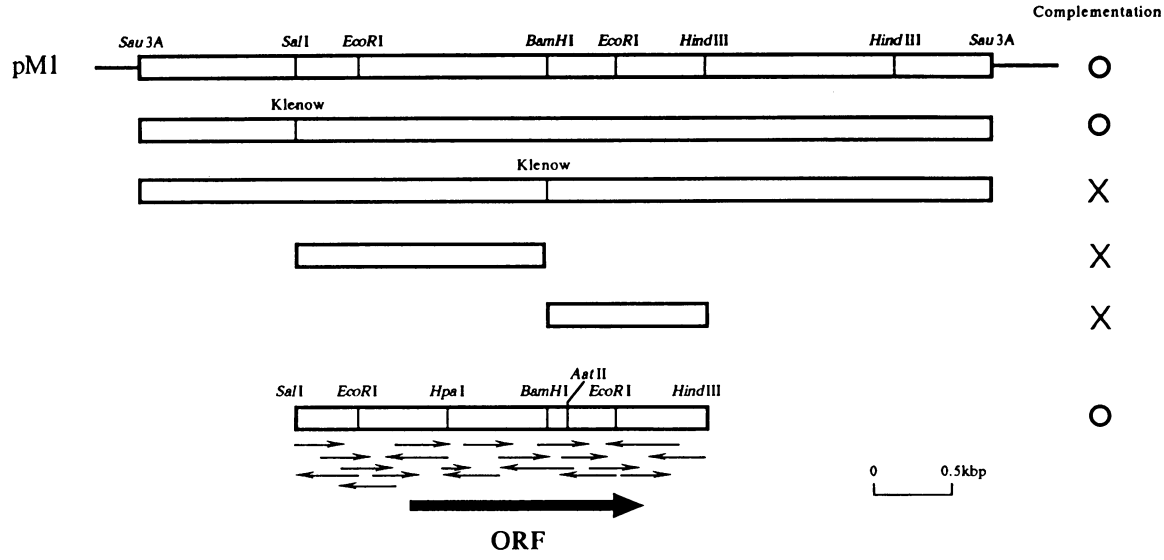

Fig. 1. Restriction map of isolated DNA and DNA sequencing strategy. Cloned *OCH1* DNA was derived from plasmid pM1. Open areas and straight lines indicate cloned yeast DNA and vector DNA, respectively. In order to cause the frame shift, *Sal*I digested pM1 and *Bam*HI digested pM1 were blunt-ended by Klenow fragment and ligated. Complementation O and X show that DNA fragments can and cannot complement the *ts* phenotype of the *och1* mutant, respectively. Thin arrows below the map represent the direction and extent of sequences obtained from a given M13 clone. The thick arrow denotes the long open reading frame (ORF) proposed to encode the *OCH1* gene product.

the presence of a 5.4 kbp DNA insert (Figure 1). The *Sal*I−*Hin*dIII 2.6 kbp fragment within this insert was found to complement the *ts* phenotype. To confirm that the 2.6 kbp fragment encodes the *OCH1* gene itself, the plasmid DNA was integrated on the yeast chromosome with the *LEU2* gene as a marker. The strain EHF-2C (*MAT***a** *leu2 och1*) was transformed with a linearized plasmid DNA (pTN-OCH1) containing the *LEU2* and *OCH1* genes, and leucine prototrophic transformants which can grow at 36°C were isolated. This transformant was crossed to YS37-4C (*MATα leu2 cyh2*) to determine the integrated gene locus. All spores derived from 31 asci examined showed ts^+ phenotype and no progeny showed *ts* phenotype. Segregation of parental *och1 OCH1*−*LEU2* and *OCH1 leu2* alleles through meiosis indicates that the *LEU2* gene is integrated at the *och1* gene locus. The genetic linkages between integrated gene locus and neighbouring markers were also examined. The ratios of parental ditype (PD):tetra type (T):non-parental ditype (NPD) were 31:0:0 for ts^+−*LEU2* and 11:20:0 for *LEU2*−*cyh2*. It was clearly indicated that the integration occurred at the *och1* locus because the distance between ts^+ and *LEU2* was calculated as 0 cM. The mapping position of the integrated *OCH1* gene as calculated from the distance between *LEU2* and *cyh2* (32.3 cM proximal from the *cyh2* gene on the left arm of Chr VII) was almost the same as that reported previously for the *och1* mutation (32.4 cM proximal from *cyh2*) (Nagasu *et al.*, 1992). The results indicated that the cloned gene is the true gene responsible for the *och1* mutation, and not a multi-copy suppressor.

Sequence analysis of the OCH1 gene

The nucleotide sequence of the *OCH1* gene was determined by the dideoxy method of Sanger *et al.* (1977). As shown in Figure 2, the unique open reading frame (ORF) of 1443 bp was found within the *Sal*I−*Hin*dIII fragment of the *OCH1* gene. This ORF encodes a protein of 480 amino acids with a molecular weight of 55 155 Daltons. A hydropathy profile of the predicted OCH1 protein indicated a membrane spanning protein with a single hydrophobic region near the N-terminus and a hydrophilic reigon at the C-terminal side. The hydrophobic region will serve as a membrane anchor but not as a signal peptide, because this region contains several positively charged amino acids immediately outside of both the N- and C-terminal ends but no typical cleavage site for signal peptidase (von Heijne, 1986). Four potential N-linked glycosylation sites (Asn-X-Ser/Thr) were found in the large C-terminal region. The amino acid sequence of OCH1 was compared with the sequence in the EMBL and GenBank databases, however, no significant homology was found with other sequences reported so far, including those of any glycosyltransferase.

Disruption of OCH1 gene

The DNA fragment having a disrupted *och1* gene, in which more than half of the ORF was deleted and replaced with the *LEU2* gene instead, was introduced into wild-type diploid cells (Figure 3A). After confirming by genomic Southern blot that one copy of *OCH1* gene is replaced with the disrupted gene (Figure 3B, lane 3), the diploid cells were tetrad dissected. Surprisingly, all four spores in the asci could germinate and make colonies (Figure 3C), indicating that the *och1* gene disruption is not lethal for cell growth.

All *och1* disrupted colonies (*LEU2* colonies) were *ts* for cell growth (Figure 4A). Thus, the *OCH1* gene is not essential for cell growth but necessary for tolerance at higher temperature. Further, all the *och1* disruptants examined showed the outer chain deficiency at the permissive temperature (Figure 4B).

In vitro translation and translocation of OCH1 protein

To analyze the properties of the *OCH1* gene product, *OCH1* mRNA prepared by SP6 transcription system was translated *in vitro* in the rabbit reticulocyte lysate in the presence of [^{35}S]methionine. Further, to examine the membrane topology of the OCH1 protein, the above *in vitro* translation was performed in the presence or absence of canine

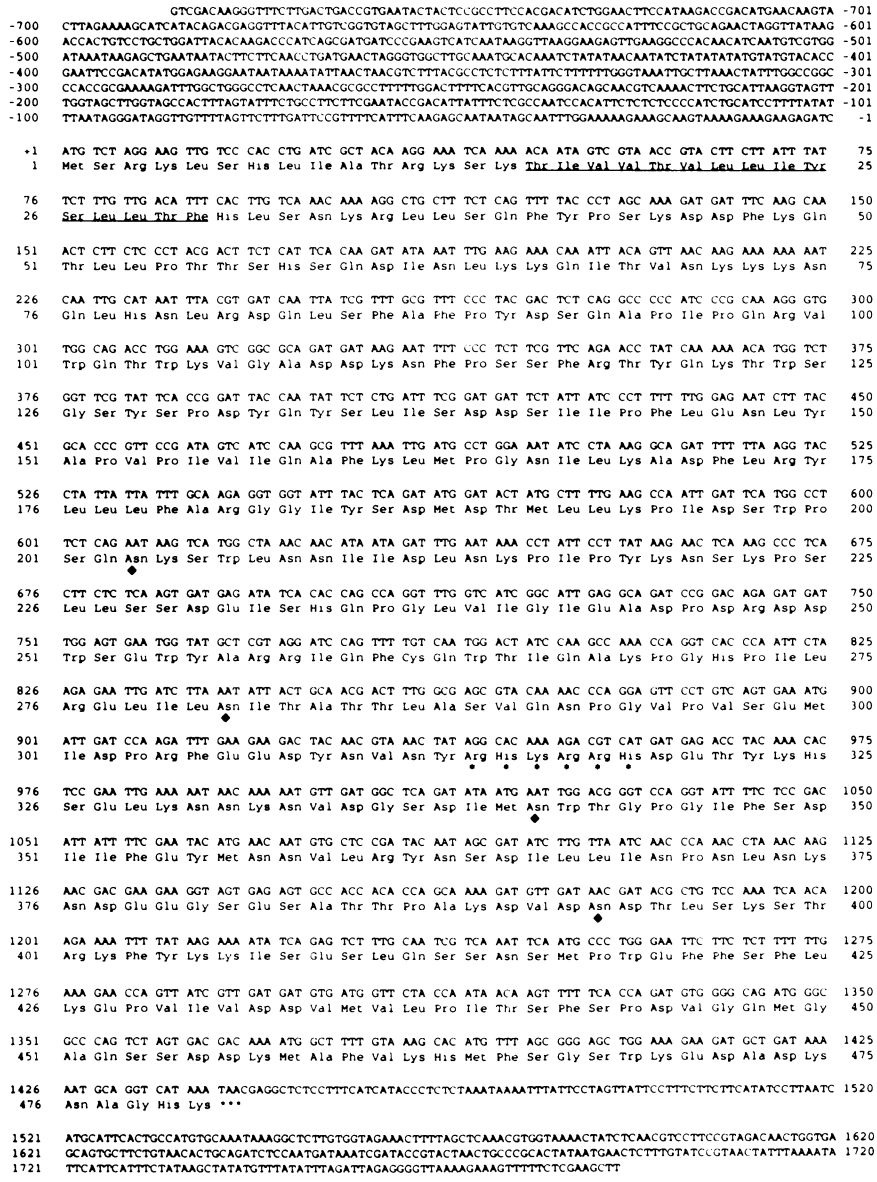

Fig. 2. Nucleotide and deduced amino acid sequences of the *OCH1* gene. Amino acids are abbreviated with the three letter code. The putative transmembrane domain is underlined. Rhombuses are located below the four potential N-glycosylation sites. Asterisks show the basic amino acids cluster. The sequence data is available from EMBL/GenBank/DDBJ under accession number D11095.

pancreatic microsomal membranes. While the translation product in the absence of membranes showed the 55 kDa band (Figure 5, lane 1), which is identical to the predicted molecular weight of the OCH1 protein, four discrete bands >55 kDa were observed in the presence of membranes. The molecular weight of each band corresponds to 57, 60, 63 and 66 kDa (Figure 5, lane 2). These bands were protected from proteinase K digestion, indicating that the large C-terminal region of the OCH1 protein was located in the lumenal side, with some modification (Figure 5, lane 3). When the OCH1 protein which was translated and modified *in vitro*, was treated with endoglycosidase H (endo H), a band corresponding to 55 kDa was observed (Figure 5, lane 5), confirming that the OCH1 protein is a glycoprotein containing four N-linked sugar chains.

Expression of OCH1 gene in yeast

We analyzed the localization and properties of the OCH1 protein in yeast cells using the anti-peptide antibody. The yeast strains, KK4 (*OCH1* wild type), YS52-1-1B (*och1* disruptant derived from KK4) and a KK4 transformant harboring the plasmid YEp51-OCH1 (OCH1 protein overproducer), were grown in the SG + 0.5% glucose medium at the permissive temperature (25°C). The whole cell extracts were subjected to Western blot analysis. The KK4 transformant showed a 6-fold higher amount of OCH1 protein ranging from 58 to 66 kDa than that of the KK4 wild type cell, while the corresponding bands were not observed in the *och1* disruptant (Figure 6A). Further, the cell extract of the KK4 transformant was separated into cytosolic and membrane fractions. It is reported that the ER membrane is mainly recovered in low speed pellet (LSP) (65−76%) and the Golgi membrane is separated into LSP (45%) and high speed pellet (HSP) (40%) (Nakano *et al.*, 1988). The 58−66 kDa OCH1 protein bands were observed in the LSP and HSP fractions (Figure 6B, lanes 2 and 3), while no specific bands were observed in the high speed supernatant (HSS) fraction (Figure 6B, lane 4). The above data may

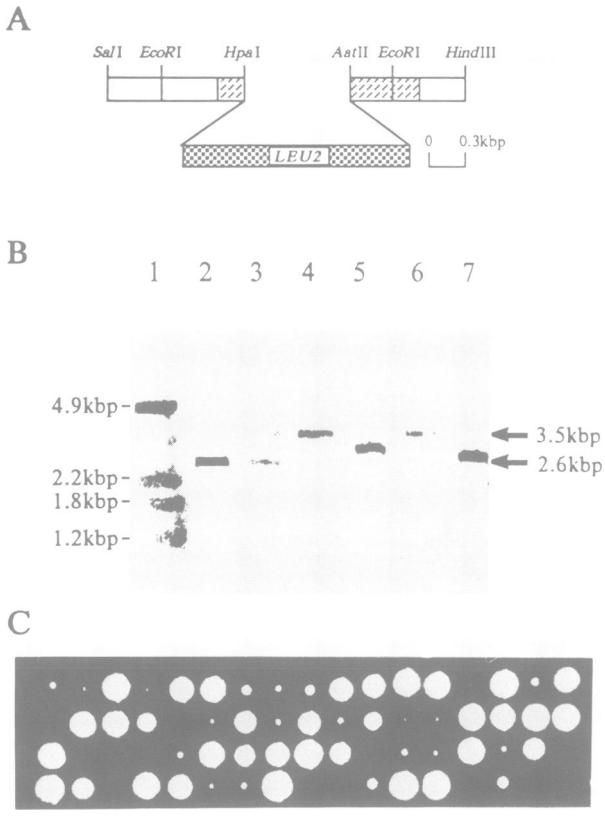

Fig. 3. Disruption of the *och1* gene. The wild type diploid strain YS57 was transformed with the *och1* disrupted DNA fragment in which the central half of the ORF was deleted and replaced with the *LEU2* gene instead, and tetrad dissected after sporulation. (**A**) Construction of the *och1*::*LEU2* disrupted DNA fragment. The 742 bp of the *Hpa*I−*Aat*II fragment in the ORF of *och1* was deleted and the *LEU2* fragment was inserted instead. The resulting 3.5 kb *och1*::*LEU2* fragment was introduced into the wild type diploid strain by one-step replacement. (**B**) Southern blot analysis. The *Sal*I−*Hin*dIII fragment of the wild type strain gives a 2.6 kb band, and that of the *och1* disrupted strain gives a 3.5 kb band. Lane 1, size marker; lane 2, the wild type diploid; lane 3, the *och1*::*LEU2* fragment introduced diploid strain; lanes 4−7, meiotic progenies of the disrupted diploid. In lanes 5 and 7, the 2.6 kbp band was slightly shifted to the slower migration position, because salt concentrations in these samples were higher than other samples. (**C**) Tetrad dissection of *och1* disrupted diploid. Most of the segregants showed two large and two small colonies.

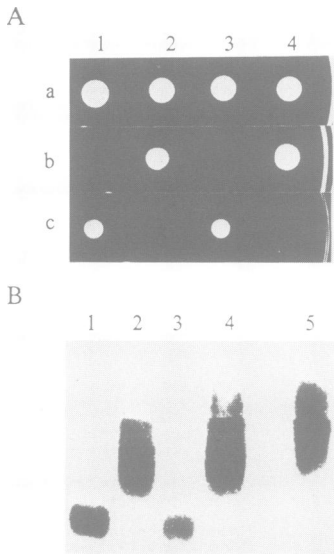

Fig. 4. Growth and glycosylation phenotype of the *och1* disruptant. (**A**) A set of meiotic progeny, (1) YS57-5A, (2) YS57-5B, (3) YS57-5C and (4) YS57-5D, were grown on (a) YPD at 25°C, (b) YPD at 37°C, and (c) SD without leucine. (**B**) Active staining of invertase. Mobility of invertase from meiotic progeny was analyzed by 8% SDS−PAGE, lanes 1−5 are YS57-5A, YS57-5B, YS57-5C, YS57-5D and wild type cell YS54-6B, respectively. Invertase active staining was carried out as described in Materials and methods.

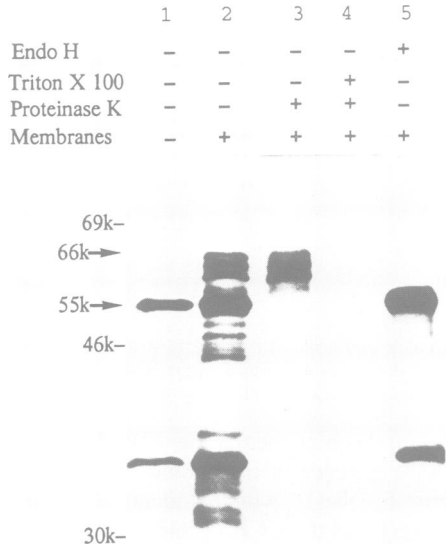

Fig. 5. *In vitro* translation and translocation of the *OCH1* gene product. The *OCH1* mRNA which was transcribed under the SP6 promoter and RNA polymerase system was translated in rabbit reticulocyte lysate in the presence (lanes 2 and 3) or absence (lane 1) of canine pancreatic membranes. After 90 min of translation in the presence of canine pancreatic membranes, the samples were either incubated with proteinase K in the presence (lane 4) or absence (lane 3) of 1% Triton X-100 or treated with endoglycosidase H (lane 5). The positions shown on the left side indicate the ^{14}C-labeled molecular weight standards (Amersham), bovine serum albumin (69 kDa), ovalbumin (46 kDa) and carbonic anhydrase (30 kDa). The arrows show the OCH1 protein with (66 kDa) and without (55 kDa) oligosaccharide chains. The reaction products were resolved on 7.5% SDS−PAGE and [^{35}S]methionine labeled OCH1 protein was visualized by autoradiography.

suggest that the OCH1 protein is a type II membrane protein which resides in the ER and/or Golgi membranes *in vivo*.

The HSP fraction solubilized with 2% Triton X-100 was treated with endo H and analyzed by Western blotting. Most of the OCH1 protein bands were shifted from 58−66 kDa to 55 kDa but two minor bands were also observed at 58 and 60 kDa, indicating incomplete endo H digestion (Figure 6C, lane 2). The results indicate that the OCH1 protein probably has four oligosaccharide chains and the length of each oligosaccharide is relatively short and equivalent to 2−3 kDa. This is in accordance with the results of *in vitro* translation/translocation analysis that demonstrated the presence of four larger endo H sensitive bands (57, 60, 63 and 66 kDa) and the unglycosylated OCH1 protein band (55 kDa). The length of the oligosaccharide chain produced in yeast was almost the same or slightly larger than the inner core size formed in the ER because the sizes of the OCH1 protein estimated by *in vitro* translation/translocation and Western blotting were almost identical. This result also

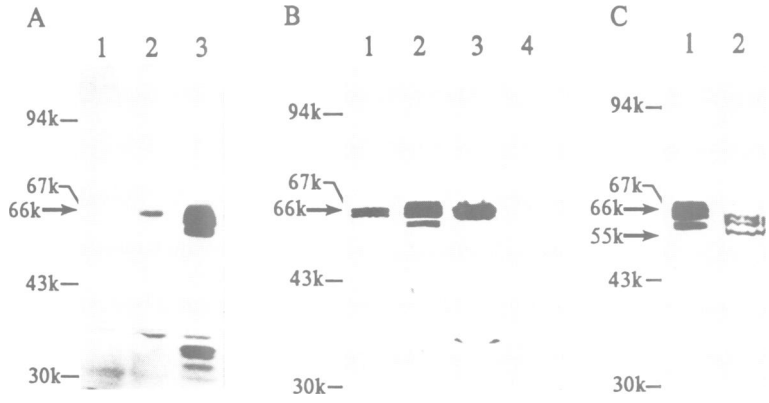

Fig. 6. Western blot analysis of the OCH1 protein. Polyclonal anti-OCH1 antibody (anti-OCH1-C) was prepared using the synthetic peptide corresponding to the amino acid sequence of the C-terminal region (465–480 amino acids) of the OCH1 protein. (**A**) Whole cell lysate. The *och1* disruptant cells (lane 1), wild type haploid cells (KK4) (lane 2) and KK4 transformant cells harboring a multi-copy plasmid YEp51-OCH1 (KK4/YEp51-OCH1) (lane 3), were disrupted by glass beads and the cell lysates were electrophoresed on 4–20% gradient SDS–PAGE and transferred to a polyvinylidene difluoride membrane filter. OCH1 proteins were detected with anti-OCH1-C antiserum and alkaline phosphatase conjugated second antibody. (**B**) Subcellular fractionation and OCH1 protein localization. The whole cell lysates from KK4/YEp51-OCH1 (lane 1) were fractionated into low speed pellet (lane 2) and low speed supernatant by centrifugation (10 000 × *g*, 10 min). The latter was further fractionated into high speed pellet (HSP) (lane 3) and high speed supernatant (lane 4) by centrifugation (100 000 *g*, 60 min). Electrophoresis and detection method are the same as above. (**C**) Endoglycosidase H treatment. HSP fraction was solubilized by 2% Triton X-100 and treated with endo H. Lane 1, solubilized HSP; lane 2, endo H treated sample.

Table I. Assay of mannosyltransferase activity

Acceptor	Mannosyltransferase activity [pmol mannose/h/mg (ratio)]		
	och1 disruptant	KK4	KK4(YEp51-OCH1)
α-methyl-mannoside (2 mM)	241(0.72)	334(1.00)	315(0.94)
α-1,6-mannobiose (2 mM)	233(0.91)	255(1.00)	277(1.09)
mannotetraose(α-1,3-α-1,2-α-1,2) (2 mM)	5(0.32)	15(1.00)	10(0.68)
$Man_8GlcNAc$ (0.06 mM)	8(0.44)	18(1.00)	17(0.93)
Δ*och1* mannan (0.03 mM)[a]	4(0.36)	12(1.00)	58(4.79)

[a]The molar concentration was calculated on the basis of the average molecular weight (70 kDa) of *och1* disruptant mannan.

suggests that the OCH1 protein may exist in the ER and/or Golgi membranes.

Assay of mannosyltransferase activity

Since it is most likely that the OCH1 protein may function in the Golgi as a mannosyltransferase itself or its modifier protein to control enzyme activity, several mannosyltransferase activities were measured and compared among the cells of *och1* disruptant, KK4 transformant and KK4 wild type. The small oligosaccharides, methyl-α-mannoside and α-1,6-mannobiose, are known as good acceptors for two different α-1,2-mannosyltransferases (Lewis and Ballou, 1991), and α-1,3-α-1,2-α-1,2-mannotetraose is an acceptor for the conventional α-1,6 mannosyltransferase (Nakajima and Ballou, 1975). However, no significant differences were observed in these enzyme activities among the above cells. In contrast, when the core-like oligosaccharide of cell wall mannoprotein which accumulated in the *och1* disruptant cells, was used as an acceptor, a 4.8-fold higher amount of [^{14}C]mannose transfer from GDP-Man to the acceptor molecule was observed in the KK4 transformant than in the control cells (Table I). It is noteworthy that no differences are observed between the cells when examined for mannose transfer activity from GDP-Man to $Man_8GlcNAc$, which is derived from the endo H treated inner core oligosaccharide accumulated in the ER.

The addition of EDTA (Figure 7A) and the immunoprecipitation of OCH1 protein by the antibody against OCH1 peptide (Figure 7B) caused a reduction of the above mannose transfer reaction in a dose dependent manner. Since the Mn^{2+} requirement is a common feature of glycosyltransferase (Paulson and Colley, 1989) but not of protein kinase which can modify the glycosyltransferase activity (Bunnell *et al.*, 1990), the above data indicate that the OCH1 protein functions as a novel mannosyltransferase which specifically recognizes a unique core-like oligosaccharide structure as an acceptor molecule.

Discussion

We have succeeded in cloning the *OCH1* gene which complements *ts* growth phenotype of *och1* mutant, and have shown that this DNA encodes a membrane spanning protein with 480 amino acid residues (Figure 2). The *in vitro* and *in vivo* analyses suggest that the OCH1 protein is a type II membrane protein containing four N-linked sugar chains. The OCH1 protein shows common properties with a group of mammalian glycosyltransferases existing in the Golgi

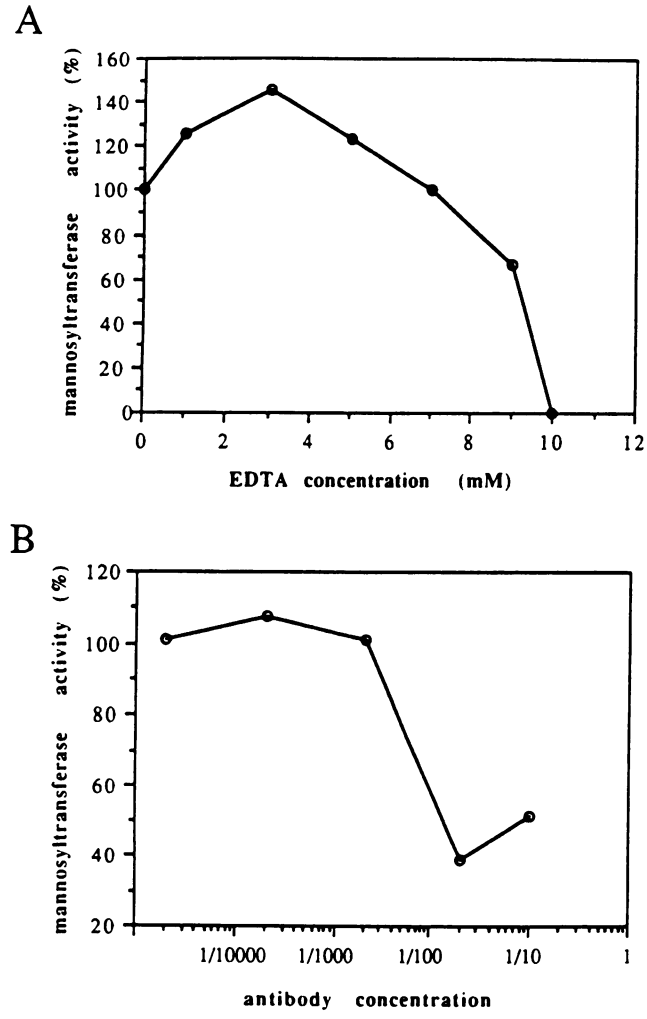

Fig. 7. Inhibition by EDTA and decrease by immunoprecipitation of mannosyltransferase activity. The mannosyltransferase activity of high speed pellet fraction from KK4 transformant was measured as described in Materials and methods using the *och1* disruptant mannan as an acceptor. (**A**) Inhibition of mannosyltransferase activity by the addition of EDTA. Assay of mannosyltransferase activity was performed in 0, 1, 3, 5, 7, 9 and 10 mM EDTA, respectively. (**B**) Decrease of mannosyltransferase activity by immunoprecipitation of OCH1 protein. The OCH1 protein was precipitated by antibody against OCH1-C peptide before the measurement of mannosyltransferase activity. The antibody concentrations were 1/10, 1/50, 1/500, 1/5000 and 1/50000.

membranes. They all have a short N-terminal cytoplasmic tail, a 16–20 amino acid membrane anchor domain, and an extended stem region which is followed by the large C-terminal catalytic domain (Paulson and Colley, 1989). The length of oligosaccharides ($Man_{9-13}GlcNAc_2$) of invertase accumulated in the *och1* mutant (Nagasu *et al.*, 1992), is very close to that of the *mnn9* mutant, which is reported to accumulate the $Man_{10-13}GlcNac_2$ oligosaccharide that contains one additional α-1,6 mannose residue, together with a few other α-1,2 and/or α-1,3 mannose residues, into the inner core ($Man_8GlcNAc_2$) (Hernandez *et al.*, 1989). Although the *mnn9* mutation affects the expression of mannosyltransferase activities, all the evidence suggests that the mutation is not in the structural genes for such enzymes (Gopal and Ballou, 1987; Ballou *et al.*, 1989; Devlin and Ballou, 1990).

Clear evidence for the OCH1 protein function was obtained by the measurement of several mannosyltransferase activities. By using the core-like oligosaccharide formed in the *och1* disruptant as an acceptor, the OCH1 protein overproducing cells showed a 4.8-fold higher activity of mannose transfer than the control wild type cells, whereas the *och1* disruptant showed a reduced activity (36%) of control cells. This residual activity in the *och1* disruptant may be contributed by the other α-1,2 or α-1,3 mannosyltransferase which can transfer mannose from GDP-Man to the above core-like acceptor. The OCH1 protein dependence for the reaction and inhibition of the reaction by EDTA are also supporting evidence for the demonstration of mannosyltransferase function of the *OCH1* gene product. It is noteworthy that neither the endo H treated inner core oligosaccharide ($Man_8GlcNAc$) nor the smaller oligosaccharides which are commonly used to measure α-1,2 or α-1,6 mannosyltransferase serve as an acceptor. We did not measure the α-1,3 mannosyltransferase activity, because this reaction takes place within the side chain of the α-1,6 mannose backbone (Nakajima and Ballou, 1975) and is not concerned with the outer chain elongation which is deficient in the *och1* disruptant.

In yeast cells the α-1,6 mannose should be added to the inner core ($Man_8GlcNAc_2$) to form $Man_9GlcNAc_2$ by the initiation specific α-1,6 mannosyltransferase (Romero and Herscovics, 1988; Reason *et al.*, 1991). Ballou *et al.* (1990) proposed that the mannose outer chain elongation will be started by the different elongation specific α-1,6 mannosyltransferase. In this step, if the α-1,2 mannose instead of α-1,6 mannose is added to $Man_9GlcNAc_2$ by the termination specific α-1,2 mannosyltransferase, mannose outer chain elongation will be stopped (Ballou *et al.*, 1990). It is noteworthy that $Man_8GlcNAc$ does not serve as an acceptor of mannose transfer by OCH1 protein, whereas the mannose oligosaccharide prepared from the *och1* disruptant does (Table I). It is confirmed that the oligosaccharide of invertase prepared from the *och1* disruptant consists of $Man_{9-13}GlcNAc_2$ (data not shown), which is the same as that preparaed from the *och1* mutant grown at the non-permissive temperature (Nagasu *et al.*, 1992). It is most likely that $Man_{10-13}GlcNAc_2$ is formed by the addition of mannose to the side chain of $Man_9GlcNAc_2$ by the α-1,2 and/or α-1,3 specific mannosyltransferase. Therefore $Man_9GlcNAc_2$ will be the true substrate accumulated in the *och1* mutant and disruptant. The OCH1 protein may be either the elongation specific α-1,6 mannosyltransferase or the termination specific α-1,2 mannosyltransferase, both of which use $Man_9GlcNAc_2$ as an acceptor. However, the latter possibility looks unlikely, because the lack of α-1,2 mannosyltransferase does not show the deficiency in mannose outer chain elongation observed in the *och1* mutant and disruptant. Therefore, it is most likely that the OCH1 protein is the novel α-1,6 mannosyltransferase which initiates the elongation of $Man_9GlcNAc_2$. The detailed structure of the acceptor molecule for OCH1 protein dependent mannose transfer should be elucidated in future.

Materials and methods

Strains and media

Escherichia coli strain XL1-Blue [*endA1 hsdR17* (r_k^-,m_k^-) *supE44 thi-1* λ^- *recA1 gyrA96 relA1* Δ(*lac*) (*F' proAB lacI*^{q}ZΔM15 Tn*10* (tet^R)] (Frischauf *et al.*, 1983) was used for preparation of plasmids and single strand DNA. *Saccharomyces cerevisiae* strain EHF-2C (*MAT***a** *och1 leu2-3*

leu2-112 pep4-3) (Nagasu *et al.*, 1992) was used for cloning the complementary gene and integration of the *OCH1* gene. KK4 (*MATα ura3 his1* or *his3 trp1 leu2 gal80*) (Nogi *et al.*, 1984) was used for preparation of microsomal membranes, overproduction of OCH1 protein and *OCH1* gene disruption. EHA-1C (*MAT***a** *leu2-3 leu2-112 pep4-3*) was used for *OCH1* gene disruption. YS37-4C (*MATα leu2cyh2*) was used for tetrad analysis. EG1-103 (*MAT***a** *ura3 leu2 trp1*) obtained from Dr B.Hall, University of Washington, was used for the construction of the yeast genomic DNA library. *Escherichia coli* cells were grown in LB-broth (1% Bacto-tryptone, 0.5% yeast extract, 0.5% NaCl and 0.2% glucose). *Saccharomyces cerevisiae* cells were grown in YPD (2% Bacto peptone, 1% yeast extract and 2% glucose), SD −leu (0.67% Bacto-yeast nitrogen base w/o amino acids, 2% glucose and 20−400 μg/ml amino acids mixture lacking leucine) (Sherman *et al.*, 1986a) and SG + 0.5% glucose (0.67% Bacto-yeast nitrogen base w/o amino acids, 2% galactose, 0.5% glucose and 20−400 μg/ml amino acids mixture).

Materials

Plasmids pUC19, M13mp18 and 19 were purchased from Toyobo Co. pSP65 was obtained from Amersham. Bluescript SK+, Phagescript SK and Exo/Mung Deletion kit were from Stratagene. The dye primers for DNA sequencing, reagents for DNA and peptide synthesis were obtained from Applied Biosystems Inc. pUC12 and dATP, dCTP, dTTP were purchased from Pharmacia Co. 7-deaza-dGTP, dideoxy-NTP, DIG-RNA labeling and detection kits and 1-deoxy-mannojirimycin were purchased from Boehringer-Mannheim. Sequenase Ver.2 (Tabor and Richardson, 1989) was from United States Biochemical Co. Restriction endonucleases were from Toyobo Co. Mutagene *in vitro* mutagenesis kit (Kunkel, 1985), Dowex 1-X8 and Bio-Gel P2 were obtained from Bio-Rad Laboratories. SP6 System (*in vitro* transcription kit) (Melton *et al.*, 1984), rabbit reticulocyte lysate and canine pancreatic microsomal membranes were purchased from Amersham. L-[^{35}S]methionine (specific activity, 37 TBq/mmol) and GDP-[^{14}C]mannose (specific activity, 8.8 GBq/mmol) were from Du-Pont−New England Nuclear. *m*-Maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS), proteinase K and phenylmethylsulfonyl fluoride (PMSF) were from Sigma. Endoglycosidase H and zymolyase 100T were from Seikagaku Kogyo Co. Polyvinylidene difluoride (PVDF) membrane filter was purchased from Millipore. Keyhole limpet hemocyanin (KLH) was from Calbiochem Co. Goat anti-rabbit IgG alkaline phosphatase conjugate was from Cappel. Antipain, chymostatin, leupeptin and pepstatin A were purchased from Peptide Institute Inc. BCA protein assay reagent was from Pierce chemical company. Clear-sol II and α-methyl-mannoside were obtained from Nakarai tesque. $Man_8GlcNAc$ was purchased from Genzyme corporation. α-1,2-mannobiose was kindly provided by Dr T.Nakajima (Tohoku University, Japan).

Plasmid construction

The *LEU2* gene was excised from pCV13 by digestion with *Acc*I and *Hpa*I. The fragment overhang was made blunt-ended by Klenow fragment of DNA polymerase I. pTN0103 was constructed by inserting this *LEU2* fragment into the *Sma*I site of pUC12. Yeast 2 μ DNA was digested from YEp51 (Broach *et al.*, 1983) with *Pst*I and *Xba*I, and the 1.3 kbp fragment containing *REP3* and replication origin was inserted into pTN0103 *Pst*I−*Xba*I sites. This plasmid (pTN3200) was used for construction of the yeast genomic DNA library. The yeast genomic library was constructed by inserting the *Sau*3A partially digested genomic DNA isolated from EG1-103 strain into the *Bam*HI site of pTN3200 (Nakayama *et al.*, 1985). Plasmid pM1 was isolated from the yeast genomic DNA library by complementation of the *och1 ts* mutant. It carried the *OCH1* 5.4 kbp fragment and was digested with *Sal*I and *Hin*dIII. This 2.6 kbp *Sal*I−*Hin*dIII *OCH1* fragment was inserted into *Sal*I/*Hin*dIII sites of pTN0103, Bluescript SK+ and M13mp19, respectively. The *OCH1* inserted pTN0103 (pTN-OCH1) was used for chromosomal integration of the *OCH1* gene to the *och1* strain, and the *OCH1* inserted M13mp19 was used for site directed mutagenesis.

The *OCH1* gene was mutagenized to make a *Sph*I site at ATG by Mutagene *in vitro* mutagenesis (Kunkel, 1985). The mutagenized *OCH1* gene was digested with *Sph*I and *Hin*dIII, and the 1.8 kbp fragment was inserted into pUC19 *Sph*I/*Hin*dIII sites. This plasmid was cut by *Sal*I and *Hin*dIII and the 1.8 kbp fragment was inserted into *Sal*I/*Hin*dIII sites of pSP65 (Melton *et al.*, 1984) and of YEp51. These plasmids (pSP-OCH1 and YEp51-OCH1) were used for *in vitro* transcription and overproduction of OCH1 protein in yeast, respectively.

The *OCH1* gene disruption was carried out by replacing a central part of *OCH1* gene with *LEU2* gene. The *LEU2* gene was excised from plasmid pTN0103 by digestion with *Sac*I and *Bam*HI. The fragment overhangs were blunt-ended by digestion with Mung Bean nuclease. The blunt-ended *LEU2* fragment was inserted into the *Hpa*I−*Aat*II site of the *OCH1* gene that was located in Bluescript SK+. This plasmid (pBL-Δoch1) in which the 0.7 kbp *Hpa*I−*Aat*II *OCH1* fragment was replaced with the 1.6 kbp *LEU2* gene was used for disruption of the *OCH1* gene.

Cloning of the OCH1 gene

EHF-2C (*och1*) was transformed with a yeast genomic DNA library in pTN3200 by the lithium acetate procedure (Ito *et al.*, 1983). The *LEU2* transformants were grown on a SD −leu plate and *OCH1* transformants were isolated by complementation of *ts* phenotype at 36°C. The plasmids were isolated from these transformants by the yeast minipreparation method (Sherman *et al.*, 1986b) and used to transform competent *E.coli* XL1-Blue cells from which plasmids were recovered by the alkali method (Maniatis *et al.*, 1982).

DNA sequencing

Restriction fragments containing a portion of the *OCH1* gene were subcloned into M13mp18, 19 or Phagescript. Deletions were made of the DNA inserts in Phagescript using the Exo/Mung deletion system. The clones were sequenced by the dideoxy method (Sanger *et al.*, 1977) with dye primers using ABI DNA sequencer (model 370A).

Integration and disruption of the OCH1 gene

The targeted *OCH1* gene integration was according to Rothstein (1991). EHF-2C (*MAT***a** *och1*) cells were transformed with *Hpa*I digested pTN-OCH1 by lithium acetate method. One of the transformants (*LEU2 OCH1*) was crossed to YS37-4C (*MAT***a** *leu2 cyh2*) and 31 tetrads of integrant were analyzed by standard genetic methods (Sherman and Hicks, 1991) to determine the integration site.

One step gene disruption was performed according to Rothstein (1991). Plasmid pBL-Δoch1 was digested by *Sal*I and *Hin*dIII and *och1*::*LEU2* DNA was excised. The 3.5 kbp linear DNA fragment was used to transform the EHA-1C/KK4 diploid cells, which require leucine for growth. *LEU* transformants were selected on SD −leu medium and these transformants were allowed to sporulate to make disruptants. Gene disruption was confirmed by Southern blot analysis and one of the meiotic disruptants designated as YS52-1-1B was used for mannosyltransferase assay.

Southern blot analysis

Total chromosomal DNA was isolated from the EHA-1C/KK4 diploid, from one of the *LEU2*$^+$ transformants derived from the above diploid, and from a set of segregants after tetrad dissection. These chromosomal DNAs were digested with *Sal*I and *Hin*dIII, and separated by 0.8% agarose gel electrophoresis. After electrophoresis, digested DNAs were transferred to Nylon membranes using electro-blotter (Nippon eido, Japan). The *OCH1* probe was made by the DIG-RNA labeling method and detection was carried out by the DIG-detection system (Dooley *et al.*, 1988) according to the supplier's protocol.

In vitro translation/translocation

The plasmid pSP-OCH1 (5 μg) was linearized by digestion with *Hin*dIII and transcribed *in vitro* by SP6 RNA polymerase system containing m^7GpppG in 50 μl reaction mixture (Melton *et al.*, 1984). The reaction mixture was ethanol precipitated to remove nucleotides and was dissolved in 10 μl of deionized water. *In vitro* translation was performed at 30°C for 60 min in solution containing 40 μl of rabbit reticulocyte lysate, 7 μl of [^{35}S]methionine and 1 μl of the above reaction mixture as a source of transcribed mRNA according to Pelham and Jackson (1976). *In vitro* translation/translocation was carried out at 30°C for 90 min as described by Walter and Blobel (1983) in solution containing 20 μl of rabbit reticulocyte lysate, 8 μl of [^{35}S]methionine, 20 μl of canine pancreatic microsomal membranes, 1 μl of transcribed mRNA and 11 μl of H_2O. Proteinase K and endo H digestion were carried out by the method of Hansen *et al.* (1986).

Preparation of polyclonal antibodies against chemically synthesized peptides

Three peptides ranging from 16 to 17 amino acids, which were chosen as a representative of N-terminal, central and C-terminal domains of the OCH1 protein, were synthesized by peptide synthesizer ABI model 470A. These synthetic peptides (Met1−Thr16, Val295−Val311 and Phe465−Lys480), were coupled to keyhole limpet hemocyanin (KLH) through the cysteine residue of the peptides by the method of Lerner *et al.* (1981). These conjugates were injected into rabbit to obtain anti-peptide antibodies. Among three synthetic peptides only the C-terminal peptide (OCH1-C, Phe465−Lys480) was successful in preparing the polyclonal antibody which was able to cross react with the OCH1 protein. This anti-OCH1-C antibody was purified on a C-terminal peptide column (Hunt *et al.*, 1985).

Western blot analysis

The yeast KK4 was transformed with YEp51-OCH1 by the lithium acetate method. YS52-1-1B (*och1* disruptant), KK4 (wild type), KK4 harboring the YEp-OCH1 (KK4 transformant) were grown in 250 ml of SG + 0.5% glucose, in the presence or absence of leucine to mid-logarithmic phase at 25°C. After confirming that the cells had exhausted glucose and *OCH1* gene expression under the *GAL10* promoter was induced by galactose, the cells were collected by centrifugation at 3000 *g* for 5 min, washed with 1% KCl and resuspended in 5 ml of 50 mM Tris−HCl (pH 7.5), 10 mM $MnCl_2$, 1 mM PMSF, 5% glycerol and 2 μg/ml each of proteinase inhibitor (antipain, chymostatin, leupeptin and pepstatin A). Glass beads (0.45−0.50 mm) were added to half of the cell suspension volume and homogenized with B. Brown homogenizer for 1 min × 3 times at 4°C. Homogenates (WCL; whole cell lysate) were filtered by G1 glass filter and centrifuged at 10 000 *g* for 20 min. Pellet was collected as LSP (low speed pellet), and supernatant was further centrifuged at 100 000 *g* for 1 h. Pellet (HSP, high speed pellet), and supernatant (HSS, high speed supernatant) were collected. LSP and HSP were resuspended in a buffer containing 50 mM Tris−HCl (pH 7.5), 10 mM $MnCl_2$, 1 mM PMSF, 5% glycerol and proteinase inhibitor mixture (2 μg/ml each). Protein concentration was determined by BCA protein assay reagent.

HSP fraction from KK4 transformants was solubilized with 2% Triton X-100 by sitting the HSP on ice for 2 h. Endo H treatment of HSP solubilized with 2% Triton X-100 was carried out by the method of Hansen *et al.* (1986).

A 170 μg protein of each cell fraction was separated by SDS−PAGE and proteins were transferred to PVDF membrane filter by electro-blotter (Atto, Japan) at room temperature under the constant current (0.4 A) for 1 h. After 1 h incubation in a blocking buffer containing 3% gelatin, 20 mM Tris−HCl (pH 7.5) and 500 mM NaCl, this filter was transferred to 10 ml of antibody buffer containing 1% gelatin, 20 mM Tris−HCl (pH 7.5), 500 mM NaCl, 0.05% Tween 20 and affinity purified anti-OCH1-C antibody at a dilution of 1:5000. The membrane filter was incubated overnight at room temperature, washed three times with washing buffer containing 20 mM Tris−HCl (pH 7.5), 500 mM NaCl, 0.5% Tween 20 for 10 min each, and then incubated for 1 h with anti-rabbit IgG alkaline phosphatase conjugate at a dilution of 1:1000 in 10 ml of antibody buffer. The membrane filter was washed three times with washing buffer for 10 min each, and incubated with 10 ml of 100 mM Tris−HCl (pH 9.5), 100 mM NaCl, 50 mM $MgCl_2$ containing nitroblue tetrazolium salt (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (X-phosphate). Coloring reaction was stopped by washing the membrane filter for 5 min with 50 ml of 10 mM Tris−HCl (pH 8.0), 1 mM EDTA.

Active staining of invertase

Active staining of invertase was carried out by the method of Gabriel and Wang (1969). The yeast strains YS57-5A, YS57-5B, YS57-5C, YS57-5D and YS54-6B were grown to mid-logarithmic phase in YPD at 25°C. A 5 ml aliquot of culture was centrifuged, washed with water and resuspended in 5 ml of YP containing 0.05% glucose. The cells were cultured for 2 h at 25°C, centrifuged and resuspended in 50 mM Tris−HCl (pH 6.8), 1 mM PMSF, 1 mM DTT, 2% SDS and 10% glycerol. Glass beads (0.45−0.50 mm) were added up to the surface of cell suspension, and vortexed for 1 min with cooling down on ice. After adding an equal volume of 50 mM Tris−HCl (pH 6.8), 1 mM PMSF, 1 mM DTT, 2% SDS and 10% glycerol and spinning in centrifuge for 5 min, supernatant was loaded on an 8% SDS−polyacrylamide gel and electrophoresis was performed. After the SDS−PAGE, the gel was bathed in 0.1 M sucrose, 0.1 M sodium acetate (pH 5.1) at 37°C for 20 min. The gel was washed with water, placed in 0.1% 2,3,5-triphenyltetrazolium chloride, 0.5 M NaOH and boiled to detect red bands.

Mannosyltransferase assay

Mannosyltransferase activity was measured by a modification of Nakajima and Ballou (1975). A 200 μg protein of HSP was incubated in 50 μl of 50 mM Tris−HCl (pH 7.5), 10 mM $MnCl_2$, 0−2 mM acceptor, 0.6% Triton X-100, GDP-[^{14}C]mannose (740 Bq), 0.5 mM 1-deoxy-mannojirimycin at 25°C for 30−120 min. 1-Deoxy-mannojirimycin was used as an inhibitor of α-mannosidase in yeast (Jelinek-Kelly *et al.*, 1985). Excess GDP-[^{14}C]mannose was removed by passing the solution through a Dowex 1-X8 column (1 ml). The neutral products were eluted with 1 ml of water and radioactivity was counted in 10 ml of clear-sol II. In this assay, α-methyl-mannoside, α-1,6-mannobiose, mannotetraose (α-1,3-α-1,2-α-1,2), $Man_8GlcNAc$, *OCH1* disruption mannan were used as acceptors.

The EDTA was added to the assay mixture containing HSP from the KK4 transformant and mannan from the *och1* disruptant, until the final concentration of 0−10 mM. Immunoprecipitation of OCH1 protein using antibody against OCH1-C peptide was performed as follows. A 200 μg protein of HSP (from the KK4 transformant) was incubated in 15 μl of 2% Triton X-100 and anti-OCH1-C antibody added to final concentrations of 1/10, 1/50, 1/500, 1/5000, 1/50000. After incubation for 1 h on ice, 15 μl of 20% protein A−sepharose (Pharmacia) was added and incubated for 1 h on ice. The sample was centrifuged and the supernatant used for the mannosyltransferase assay.

Yeast KK4 (*OCH1*) and YS52-1-1B (*Δoch1*) mannans were isolated by Peat *et al.* (1965). KK4 mannan was hydrolyzed by the acetolysis method (Kocourek and Ballou, 1969) and mannotetraose (α-1,3-α-1,2-α-1,1) was isolated by Bio-Gel P2 column (2.6 × 200 cm) according to the method of Raschke *et al.* (1973).

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