Mol Gen Genet (1997) 255:400–409

ORIGINAL PAPER

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The *MBR1* gene from *Saccharomyces cerevisiae* is activated by and required for growth under sub-optimal conditions

Received: 17 January 1997 / Accepted: 20 March 1997

Abstract The *MBR1* gene was isolated as a multicopy suppressor of the phenotype on glycerol medium of a *Saccharomyces cerevisiae* strain mutant for the Hap2/3/4/5 transactivator complex. In this paper, we show that Mbr1p is a limiting factor for growth on glycerol medium under the following sub-optimal culture conditions: in late growth phase, at low temperature, at high external pH or in the presence of 1,10-phenanthroline. Moreover, deletion of *MBR1* protects cells against stress, whilst overexpression of this gene has the opposite effect. *MBR1* expression is induced in the late growth phase and is negatively controlled by the cAMP-dependent protein kinase A (PKA). Both activation of PKA or overexpression of *SOK1* or *SCH9* – two genes isolated as multicopy suppressors of a PKA null mutant – suppress the *mbr1* growth defect. Our results indicate that Mbr1p is not an essential element of any one of these pathways. Deletion of *SAC1*, a gene implicated in vesicular transport, in association with *MBR1* deletion, causes synthetic lethality. A possible role of Mbr1p in intracellular trafficking is discussed.

Key words Stress · cAMP · *SAC1* · *SOK1* · *SCH9*

Introduction

The CCAAT-binding transactivator in *Saccharomyces cerevisiae* contains at least four subunits encoded by the *HAP2*, *HAP3*, *HAP4* and *HAP5* genes (McNabb et al. 1995). In response to glucose limitation, this yeast complex controls the expression of numerous genes involved in the utilisation of alternative carbon sources (De Winde and Grivell 1993; Dang et al. 1994; Dang 1996).

Deletion of the *HAP* genes leads to absence of growth on medium containing lactate as a carbon source (Pinkham and Guarente 1985; Olesen et al. 1987; Forsburg and Guarente 1989) and to slow growth on glycerol medium (Daignan-Fornier et al. 1994). Two genes, *MBR1* and *MBR3*, have been isolated as multicopy suppressors of this slow growth phenotype (Daignan-Fornier et al. 1994). Mbr1p and Mbr3p do not show significant similarity to any characterised protein and their functions are unknown. The N-terminal domain of Mbr1p contains two groups of cysteine residues that resemble a previously described metal-binding structure (reviewed in Berg 1990). This domain is acidic and seems to function as a transactivator when fused to the Gal4p DNA-binding domain (P. Reisdorf, unpublished results). In a previous paper we suggested that Mbr1p might act as a transcription activator that partially compensates for the absence of the CCAAT-binding transactivator function (Daignan-Fornier et al. 1994). In order to test this hypothesis and to search other possible functions of Mbr1p, we have studied (i) the subcellular localisation of Mbr1p; (ii) the phenotypes associated with *MBR1* deletion or overexpression; (iii) the regulation of *MBR1* expression and (iv) gene products functionally related to Mbr1p.

Communicated by C. P. Hollenberg

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Materials and methods

Yeast strains and media

Yeast strains used in this study are listed in Table 1. Complete glucose medium (YPD) contains 1% yeast extract, 2% Bacto peptone and 2% glucose. Other rich media contained 1% yeast extract, 1% Bacto peptone and 2% either glycerol (YPGly), ethanol or galactose. Buffered glycerol medium (YPGlypH) contains

Table 1 Yeast strains used

Strain	Genotype	Source reference
CY1260	*MAT***a** *ade2 ade3 his3 leu2 trp1 ura3*	K. Arndt
1260C	Same as CY1260 except *MATα*	This work[a]
1260CR1	Same as 1260C except *mbr1-2*	This work[b]
W775-2C	*MATα ade2 his3 leu2 trp1 ura3*	B. Daignan-Fornier
2CR1A	Same as W775-2C except *mbr1-2*	This work[b]
2CR4	Same as W775-2C except *sok1*::*URA3*	This work[c]
2CR1AR4	Same as 2CR1A except *sok1*::*URA3*	This work[c]
OL556	*MAT***a**/*MATα cdc25-5*/*cdc25-5 his3*/*his3 leu2*/*leu2 rca1*/*rca1 trp1*/*TRP1 ura3*/*ura3*	Boy-Marcotte et al. (1996)
DBY-1829	*MATα his3 leu2 lys2 trp1 ura3*	G. Faye
1829-2	Same as DBY-1829 except *mbr1-2*	This work[b]
1829-2C2	Same as DBY-1829 except *MAT***a**	This work[a]
1829-2C28	Same as 1829-2C2 except *mbr1*::*TRP1*	This work[d]
W303	*MAT***a**/*MATα ade2*/*ade2 his3*/*his3 leu2*/*leu2 trp1*/*trp1 ura3*/*ura3*	C. Cullin
W303-1Bα	*MATα ade2 his3 leu2 trp1 ura3*	E. Boy-Marcotte
W303-1BS	Same as W303-1Bα except *sac1*::*HIS3*	This work[e]

[a] Mating locus conversion was done according to Herskowitz and Jensen (1990)
[b] *MBR1* disruption was done as described in Daignan-Fornier et al. (1994)
[c] See Materials and methods for the disruption of *SOK1*
[d] *mbr1*::*TRP1* is identical to *mbr1-2* except that *TRP1* replaces *HIS3*
[e] See Materials and methods for the disruption of *SAC1*

0.05 M KH_2PO_4/K_2HPO_4 (adjusted to the required pH). Lactate, pyruvate or acetate medium consisted of 0.75% yeast extract, 0.75% bacto peptone, 0.4% NaOH, 4.1% potassium phtalate and either 0.41% lactate, pyruvate or acetate, respectively. Yeast minimal medium contains 0.17% yeast nitrogen base, 0.5% ammonium sulphate, 2% glucose and appropriate requirements at the concentration recommended by Sherman (1991). When cells were replica-plated, growth was scored after 1 week.

Plasmids

pII11 and p7-181 are yeast multicopy vectors (derivatives of YEp*lac*181; Gietz and Sugino 1988) carrying, respectively, the 4-kb *Sal*I DNA fragment containing *SOK1* and *Bam*Hl-*Hin*dIII DNA fragment containing *SCH9*. pIIIras, pT111 and pX111 are yeast centromeric vectors (derivatives of YCp*lac*111; Gietz and Sugino 1988) containing, respectively, the $RAS2^{val19}$ allele and its promoter, the 2.7-kb *Bgl*II DNA fragment containing *MBR1* and the 2.4-kb *Xba*I DNA fragment containing *SAC1*. pGM51 is a YEp13 derivative plasmid carrying *MBR1* (Daignan-Fornier et al. 1994). pT33 derives from YCp*lac*33 (Gietz and Sugino 1988) and carries the 2.7-kb *Bgl*II fragment containing *MBR1*.

Construction of an *MBR1-lacZ* fusion and assay of β-galactosidase

The *MBR1-lacZ* fusion used in this study is expressed via the *MBR1* promoter and retains the first 337 codons of this gene. A *Nsi*I-*Bgl*II DNA fragment containing the 700bp upstream of the ATG and the first 1012 bp of the *MBR1* coding sequence, followed by the GGGAACAAAAACTTATTTCTGAAGATCT linker, was cloned in the *Pst*I-*Bam*HI sites of either YEp367R or YIp367R (Myers et al. 1986). These manipulations generate an in-frame fusion with *lacZ*. The resulting plasmids are termed, respectively, pR1Z and pR1Zi. The strain 1260CR1Z used to study the expression of *MBR1* was obtained by transforming 1260C with pR1Zi, linearized at the unique *Xho*I restriction site in the *MBR1* promoter. Correct single-copy insertion of the plasmid at the *MBR1* locus was verified by Southern blot analysis (data not shown). In each experiment, β-galactosidase (β-Gal) assays were performed as described by Ruby et al. (1983), in triplicate on independent cultures. Protein concentrations were determined by using the Bio-Rad Protein Micro Assay System, with bovine serum albumin as a reference. The variation between assays was $< 20\%$.

Plasmid manipulations and genomic libraries

Standard procedures were performed according to Sambrook et al. (1989). Yeast transformation was performed as described in Fukuda et al. (1993). Plasmid extraction from yeast was performed according to Hoffman and Winston (1987). The yeast multicopy genomic library was described in Daignan-Fornier et al. (1994), and the yeast centromeric genomic library was described in Rose et al. (1987).

SOK1 and *SAC1* disruptions

A null allele of *SOK1* was constructed as follows: the 4-kb *Sac*I-*Sal*I DNA fragment containing *SOK1* was cloned into pKS (Stratagene) digested with *Sac*I and *Sal*I, leading to pD11. This plasmid was digested with *Eco*RI and *Hin*dIII to remove the sequence encoding amino acids 5–529 of Sok1p, and recircularized using a *Bgl*II adaptor, leading to pD11BII. The *Bam*HI *URA3* fragment from Ydp-*U* (Berben et al. 1991) was cloned into the *Bgl*II site of pD11BII, leading to p11U. The *Sac*I-*Sal*I fragment containing *sok1*::*URA3* from p11U was used for one-step gene disruption (Rothstein 1983).

A null allele of *SAC1* was constructed as follows: the 2.4-kb *Xba*I fragment containing *SAC*1 was cloned at the *Xba*I site of pUC1318 (Kay and McPherson 1987), leading to pS26. This plasmid was digested with *Eco*RI and *Xho*I to remove the central part of *SAC1*, and recircularized with a *Eco*RI-*Sal*I DNA fragment containing *HIS3* from YDp-*H* (Berben et al. 1991), leading to pS26H. The *Xba*I fragment containing *sac1*::*HIS3* from pS26H was used for one-step gene disruption (Rothstein 1983).

Estimation of cell viability

Cells were cooled on ice and sonicated to disrupt cell aggregates as described in Plesset et al. (1987). After appropriate dilution into cold YPD, cells were plated in duplicate onto YPD, incubated at 28° C for 3 to 5 days and colonies were counted.

Estimation of cell concentration and percentage of cells budding

Cells were fixed by addition of 0.1 volume of 37% formaldehyde for 2 h, sonicated (Plesset et al. 1987) and diluted in water; at least 300 cells were counted under a microscope. In each case, at least two independent experiments were done.

Protein localization by immunofluorescence

The procedure was performed according to Girard et al. (1992) with the following modification: after fixation onto polylysine slides and washing with PBS the protoplasts were immersed for 5 min in methanol (−20° C), then for 30 s in acetone (−20° C). Cells were dried and rehydrated in PBS before adding antibody. Both protocols lead to the same result for the proteins localised in this work. The Mbr1p-β-GaI chimera was detected using an anti-β-GaI antibody from Promega.

Isolation of *slm* mutants (Synthetic Lethal with *MBR1*)

Isolation of *slm* mutants is based on the 5-fluoro-orotic acid (5-FOA) plasmid shuffling procedure (Mazzoni et al. 1993). Strain 1829-2C28 (see Table 1) was transformed with the centromeric plasmid pT33 containing both *MBR1* and *URA3* and grown to early log phase in rich glucose medium. Cells were washed with H_2O, resuspended at 10^8 cells/ml in 0.1 M sterile phosphate buffer at pH 8. 1.3 ml of cell suspension was mutagenized with 104 µl of pure ethyl methyl sulfonate (EMS) and 200 µl was removed after 15 min and diluted with 800 µl of 6.25% sterile sodium thiosulfate to inactive EMS. These conditions resulted in approximately 35% survival. The mutagenized cells were plated on rich glucose medium at a cell density of 300 cells per plate. Colonies were grown for 2 days at 28° C and replica plated on YPD, YPGlypH6.8 and on YPGlypH6.8 containing 1 g/l 5-FOA. Plates were incubated at 36° C to screen for colonies capable of growing on YPGlypH6.8 but not on YPGlypH6.8 containing 5-FOA. Candidates were purified from the original YPD plates and cured of the plasmid pT33 containing *MBR1*.

Results

Mbr1p does not behave as a classical transcription factor

To test whether Mbr1p might be a transactivator, we localised this protein in the cell. This was done using an *MBR1-lacZ* fusion encoding the first 337 amino acids of MBR1 fused to LacZ. We have verified that the Mbr1p-β-Gal chimera has properties indistinguishable from the wild type Mbr1p: this fusion carried on a centromeric plasmid is able to complement the *mbr1* growth defect; in addition, it suppresses the *hap* phenotype when carried on a multicopy plasmid. These results indicate that a significant fraction of the chimera is correctly localized. Since we did not succeed in obtaining a signal on Western blots or with in situ immunofluorescence using a centromeric plasmid, the cellular localization experiments were carried out with the β-Gal fusion expressed from the natural *MBR1* promoter on a multicopy plasmid. We checked by Western blot analysis that the β-Gal part of the chimera was not cleaved from the Mbr1p moiety (data not shown). We observed, with a specific monoclonal anti-β-Gal antibody, that the Mbr1p chimera is predominantly found at the periphery of the nucleus (Fig. 1). This localisation suggests that Mbr1p is not a diffusible cytoplasmic protein and is largely associated with the perinuclear part of the endoplasmic reticulum (ER), as shown by its similarity to the staining obtained with the ER protein marker Kar2p (Rose et al. 1989). The N-terminal domain of Mbr1p contains two groups of cysteine residues that resemble a previously described metal-binding domain (reviewed in Berg 1990) present in some transactivators. Alteration of either of two cysteines in the putative zinc finger domain (either in position 46 or in position 71) does not affect the function of Mbr1p (data not shown).

Phenotypes associated with *MBR1* overexpression or deletion

The growth of *mbr1* null mutant and isogenic wild-type strains was compared. *MBR1* deletion leads to a leaky cold-sensitive phenotype on YPGly and to a complete growth defect on YPGlypH7.2 at all temperatures (Fig. 2a). On other carbon sources (glucose, galactose, ethanol, lactate, pyruvate, acetate) no mutant phenotype was observed (data not shown). The growth of *mbr1* was also analysed in liquid YPGly medium at 23° C. Mutant and wild-type cells have the same generation time (Fig. 2b, c). In the log phase, the percentage of budding cells (Fig. 2b) seems to be lower in the mutant (25%)

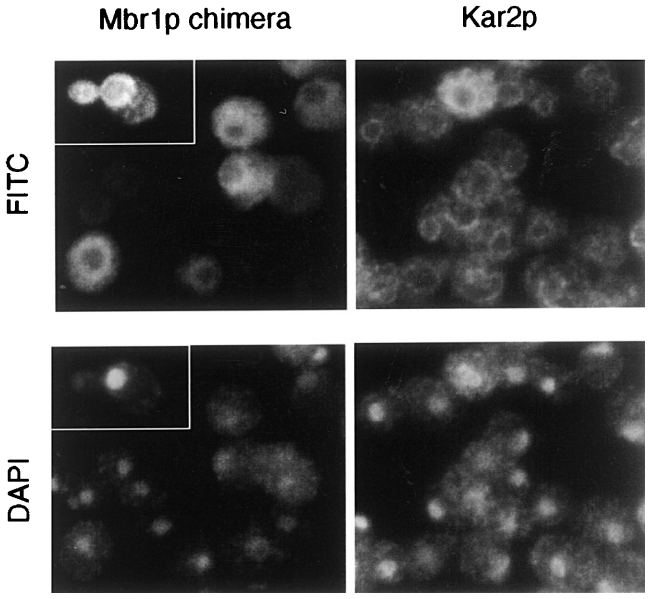

Fig. 1 Immunolocalisation of Mbr1p-β-Gal fusion chimera. The diploid W303 strain was transformed with pR1Z and grown on liquid YPGly at 28° C to an OD_{600} of 4 (this value was chosen because optimal expression of *MBR1* occurs under these conditions; see Fig. 4). Cells were prepared for immunofluorescence analysis as described in Materials and methods. DNA was stained with 4′-6′diamino-2-phenylindol (DAPI). A fraction of the cells was treated so as to stain the ER with an anti-Kar2p antibody (provided by M. Rose). Magnification 1400X

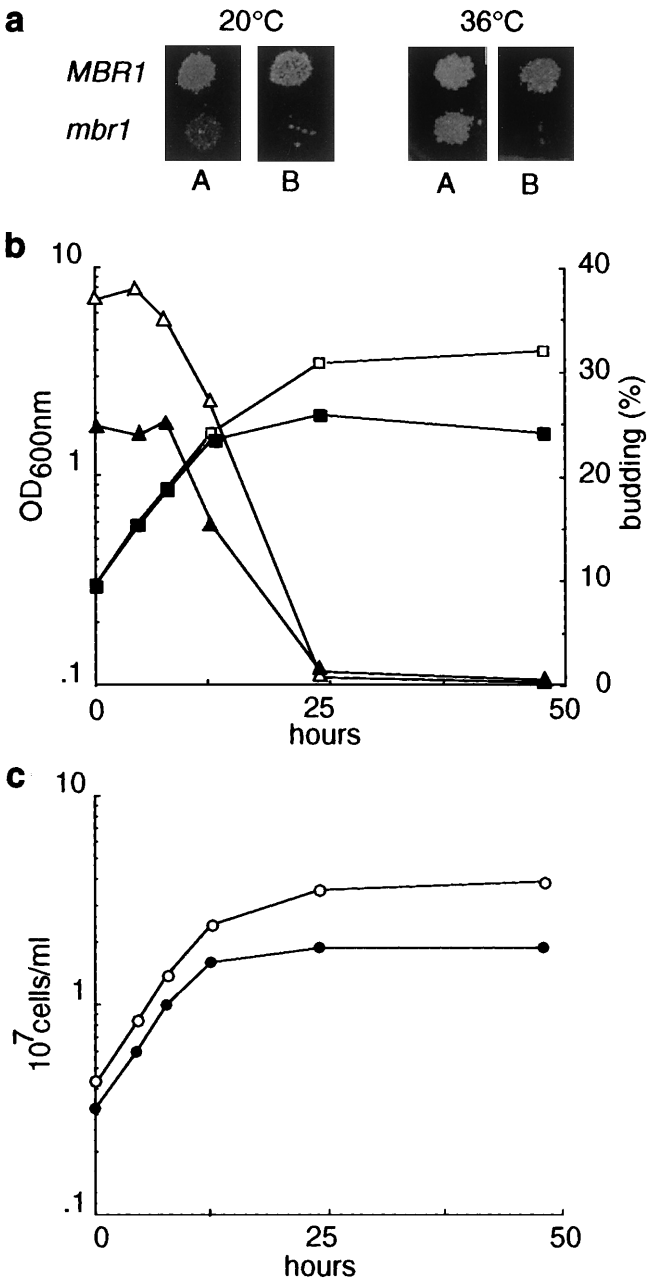

Fig. 2a–c *mbr1-2* growth phenotype. **a** Isogenic 1260C (*MBR1*) and 1260CR1 (*mbr1-2*) strains were patched on YPD and replica-plated on YPGly (A) or YPGlypH7.2 (B) and incubated at 20 or 36° C. **b**, **c** Strain 1260C (*empty symbols*) and 1260CR1 (*filled symbols*) were pregrown on YPD at saturation and diluted 1000-fold in YPGly. The cultures were then incubated at 23° C. Progression of the cultures was monitored by measuring the OD_{600} with a Zeiss PMAII spectrophotometer (*squares*) or by estimating the percentage of budding cells (*triangles*) and the cell concentration (*circles*)

than the wild type (40%). At any given OD_{600} value, mutant cells are 1.6 times less numerous than the wild-type cells. Microscopic observation of the cells does not indicate a change in shape. The increase in the turbidity coefficient may therefore reflect a small difference in size, which was not detected visually. The biomass reached at the stationary phase, as estimated by the turbidity of the culture (Fig. 2b), the cell concentration (Fig. 2c) and the protein concentration (data not shown) is three times lower in the mutant compared to the wild type. We can thus conclude that the *mbr1* mutant has a reduced ability to grow on glycerol when the nutrient becomes limiting.

Since 1,10-phenantroline is known to arrest cell division and to drive the cells to enter G_0 (Barnes et al. 1990), resistance to this compound was tested in the mutant, wild-type and in cells overexpressing *MBR1* (Fig. 3a). The mutant cells are sensitive to lower concentration of the drug (7.5 μg/ml at 36° C or 12.5 μg/ml at 28° C) than wild type (10 μg/ml at 36° C or 15 μg/ml at 28° C). Conversely, overexpression of *MBR1* increases resistance to this compound, especially at 36° C.

The effect of *MBR1* deletion or overexpression on cell sensitivity to heat shock was also measured. Compared to the wild-type strain, cells overexpressing *MBR1* are ten times more sensitive and *mbr1* cells are ten times more resistant (Fig. 3b). We have also observed that the *mbr1* cells are one hundred times more resistant to prolonged storage at 4° C (after growth in YPGly) than wild-type cells (Fig. 3c). Thus, the deletion of *MBR1* leads to an increased resistance to various critical conditions, such as heat treatment or maintenance at stationary phase, overexpression of *MBR1* having the opposite effect.

Expression of *MBR1* is induced in late growth phase

Using the *MBR1-lacZ* fusion integrated at the *MBR1* locus, we have examined the expression of *MBR1* during the different phases of growth on YPGly (Fig. 4). During the early growth phase (OD_{600} below 2–3), the fusion is expressed at a low basal level. When the culture reaches late growth phase (OD_{600} above 3), expression of the fusion is increased four- to fivefold. Thus *MBR1* expression is induced concomitantly with the fall in the growth rate. The activation of the RAS-cAMP pathway is known to impair the expression of a number of genes induced by nutrient starvation and various stresses (Tanaka et al. 1988; Boorstein and Craig 1990; François et al. 1992; Marchler et al. 1993; Klein and Struhl 1994). We have measured *MBR1* expression in the presence of the dominant allele $RAS2^{val19}$, which encodes a constitutively activated Ras2p, leading to high constitutive levels of cAMP in the cell (Toda et al. 1985). Under these conditions, the level of *MBR1-lacZ* expression is decreased four- to fivefold (Fig. 4).

The effect of cAMP on the accumulation of *MBR1* mRNA was analysed during growth on YPD in the strain OL556, which allows modulation of the intracellular cAMP level by addition of exogenous cAMP (Boy-Marcotte et al. 1996). From the results in Fig. 4, it appears that *MBR1* mRNA is not detectable during exponential growth and is dramatically induced when cell division ceases due to glucose exhaustion (Boy-Marcotte et al. 1996). When exogenous cAMP is present, this induction is prevented.

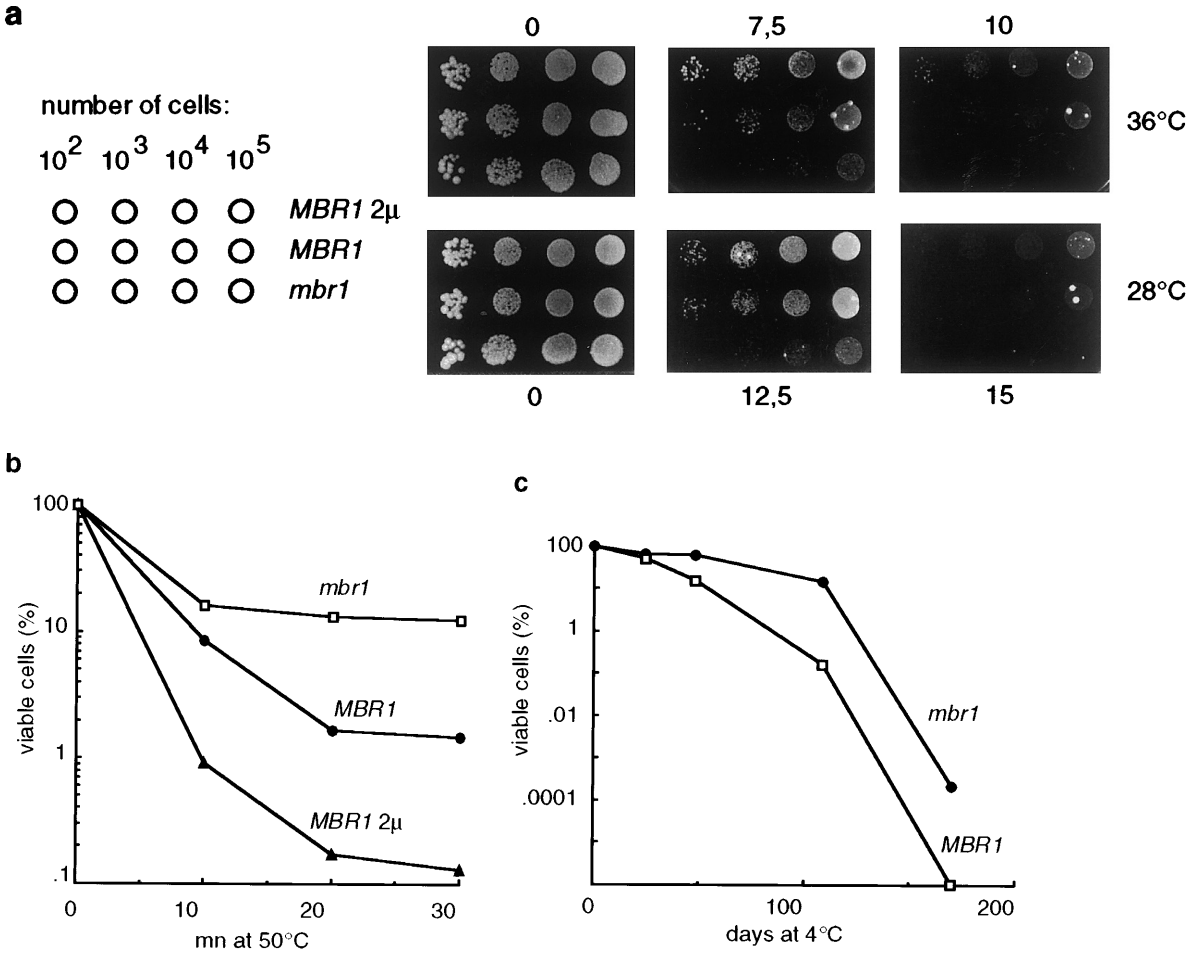

Fig. 3a–c Effect of *MBR1* gene dosage on cellular response to various physiological conditions. **a** Strain 1260C was transformed with pGM51 (*MBR1* 2μ cells) or with YEp13 (*MBR1* cells) and 1260CR1 was transformed with YEp13 (*mbr1* cells). Strains were pregrown in glucose minimal medium, selecting for the presence of the plasmids. Drops of serial dilutions were spotted on YPGly (as shown on the left) containing 1,10-phenanthroline. Drug concentrations (μg/ml) are indicated above and below the photographs. Experiments were carried out at 28 and 36° C as indicated. **b** The same transformants as used in **a** were grown in YPGly medium at 28° C. Exponentially growing cells (OD_{600} about 0.5) were transferred to 50° C and samples were taken at different times to estimate the percentage of viable cells by plating on YPD. Similar results were obtained when YPD was replaced by the minimal medium selective for the presence of the plasmids (data not shown). **c** Strains 1260C and 1260CR1 were grown in YPGly at 28° C until stationary phase and stored at 4° C. Aliquots were taken at different times and the percentage of viable cells was determined

Thus, the expression of *MBR1* is induced when cellular growth is limited, and is negatively controlled by the RAS-cAMP pathway.

Activation of the RAS-cAMP pathway and overexpression of *SOK1* or *SCH9* suppress the *mbr1* phenotype

Genes from a yeast multicopy genomic library were selected for their ability to suppress the *mbr1* growth defect on YPGlypH7.2 at 36° C. In addition to *MBR1*, one set of plasmids containing overlapping DNA inserts was shown to suppress the *mbr1* phenotype. The region required for suppression was delimited by insertional mutagenesis with the mini-Mu transposon method (Daignan-Fornier and Bolotin-Fukahara 1988) and was shown to contain the *SOK1* gene. The suppression phenotype is shown in Fig. 5a. *SOK1* has been identified as a multicopy growth suppressor of a mutant defective in PKA activity (Toda et al. 1988; Ward and Garrett 1994). Since *SCH9* presents the same suppression property, we tested whether its overexpression or the activation of the RAS-cAMP pathway also suppresses the *mbr1* growth defect. Indeed the *mbr1* phenotype is completely suppressed by the $RAS2^{val19}$ allele and partially suppressed by overexpression of *SCH9* (Fig. 5a).

Epistatic relations between *MBR1* and *SOK1* were then investigated. On YPGly medium, the double mutant presents a more pronounced phenotype than either single mutant (Fig. 5b). This indicates that the products of these genes do not participate in the same linear pathway.

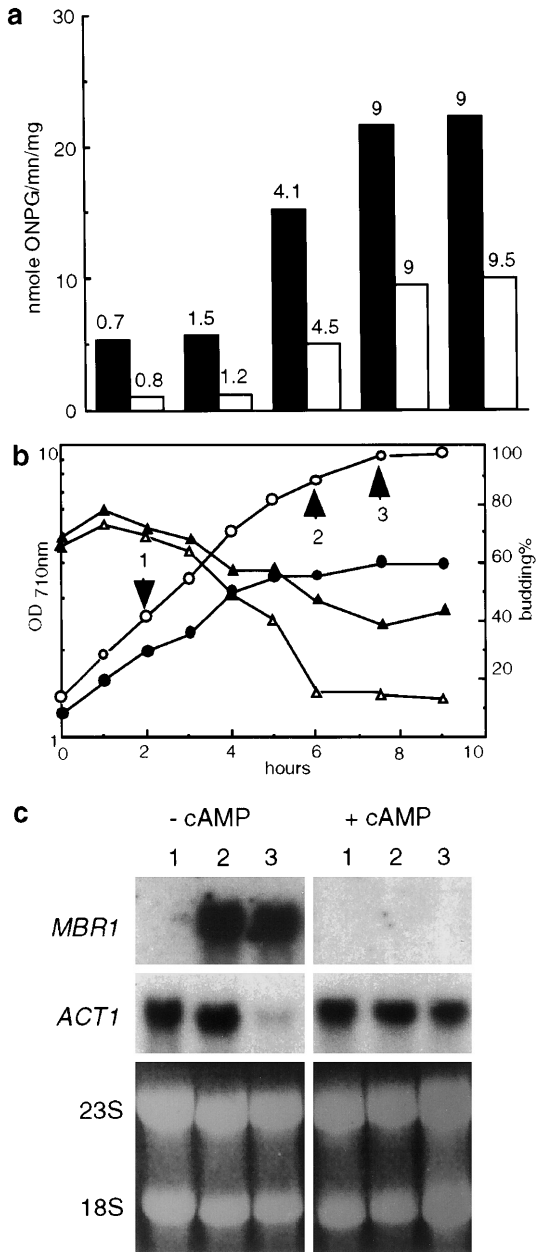

Fig. 4a–c Regulation of *MBR1* expression. **a** Strains 1260CR1Z (*filled boxes*) and 1260CR1Z *RAS2*^{val19} (1260CR1Z transformed with pIIIras, *empty boxes*) were grown on liquid YPGly at 28° C. Aliquots were withdrawn at the OD_{600} values indicated above the bars and the specific activity of β-galactosidase was measured. Plasmid loss experiments have shown that 98% of the 1260CR1Z *RAS2*^{val19} cells contained the pIIIras plasmid at the end of the culture (data not shown). **b** Strain OL556 was grown in YPD at 30° C in absence (*empty symbols*) or presence (*filled symbols*) of 3 mM cAMP. The cell concentration in this particular experiment was estimated by measuring turbidity at 710 nm with a Jenway 6061 colorimeter (*circles*). The percentage of budded cells (*triangles*) was estimated as described in Materials and methods. Arrows 1–3 indicate times at which samples were withdrawn for extraction of total RNA (Schmitt et al. 1990). **c** Northern blot analysis was performed with 10-μg samples of RNA (Sambrook et al. 1989). The ribosomal RNAs (stained by ethidium bromide) were used to monitor equal loading of the lanes. The probes used were internal DNA fragments of *MBR1* (1011 bp) and *ACT1* (570 bp). Hybridization was performed as described (Boy-Marcotte et al. 1996) with the *MBR1* probe. After removal of the *MBR1* probe the membrane was rehybridized with the *ACT1* probe. The sizes of the mRNAs are 1.45 kb for *ACT1* and 1.4 kb for *MBR1*

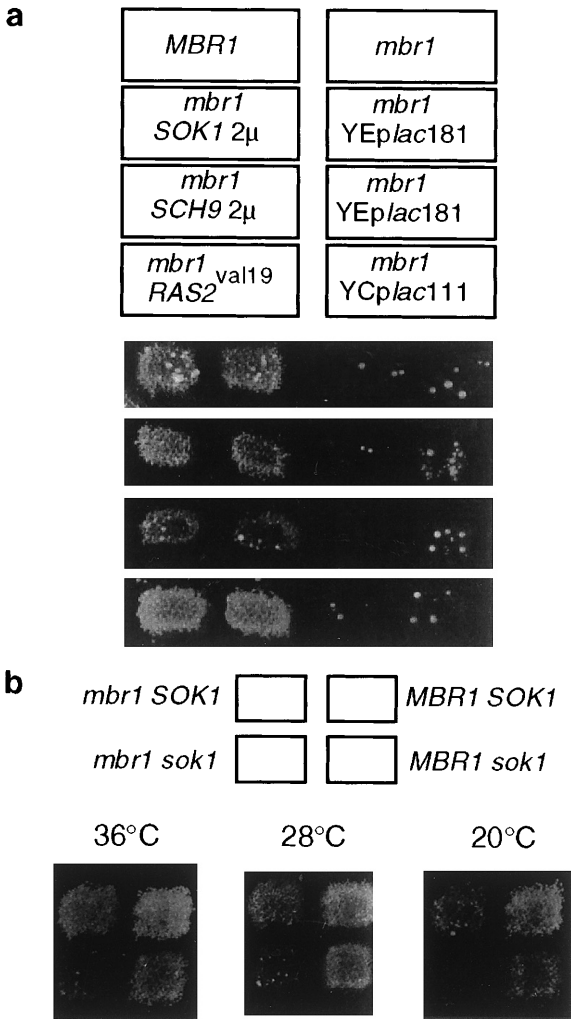

Fig. 5a, b Interactions between *MBR1*, *SOK1*, *SCH9* and the RAS-cAMP pathway. **a** The *mbr1* strain 1260CR1 was transformed with the yeast episomal plasmids pII11, p7-181 and the yeast centromeric plasmid pIIIras containing, respectively, *SOK1*, *SCH9* and *RAS2*^{val19}. Transformation was also performed with the control vectors (YEp*lac*181 and YCp*lac*111). Strains 1260C (*MBR1*) and 1260CR1 (*mbr1*), and two independent subclones of each transformant strain, were spotted on glucose medium (as shown at the top of the Figure), replicated on YPGlypH7.2 and incubated at 36° C. Suppression phenotypes were also detectable at 28 and 20° C (data not shown). **b** The four isogenic combinations of wild type, *mbr1* and *sok1* null alleles (strains W775-2C, 2CR1A, 2CR4 and 2CR1AR4) were spotted on YPD as shown in the diagram. This master plate was replicated on YPGlypH6.4 and incubated at 20, 28 or 36° C

Sac1 is synthetic lethal in combination with *mbr1*

In the genetic context of the 1829-2C28 strain, *mbr1* deletion does not lead to a marked growth defect on YPGlypH6.8 at 36° C (Fig. 6, compare lanes 4 and 5). Mutants which show *MBR1* dependence for growth in these conditions were isolated (*slm*; mutants see Materials and methods). One of them, called 1829-*slm9*, shows a strong synthetic lethal phenotype (Fig. 6, compare lanes 2–4). This mutant also presents a cold-sensitive phenotype that is independent of *MBR1* (data not shown). To verify that the lethal phenotype and the

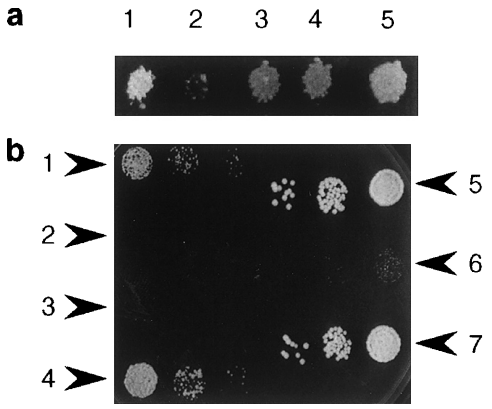

Fig. 6a, b Synthetic lethal phenotype obtained by combination of *sac1* with *mbr1*. **a** Isogenic strains were patched on YPD, replica-plated on YPGlypH6.8 and incubated at 36° C. 1, *slm9 mbr1-2* + *MBR1* ARS-CEN (1829-*slm9* + pT111); 2, *slm9 mbr1-2* (1829-*slm9* + YCp*lac*111); 3, *slm9 mbr1-2* + *SAC1* ARS-CEN (1829-*slm9* + pX111); 4, *SLM9 mbr1-2* (1829-2C28 + YCp*lac*111); 5, *SLM9 MBR1* (1829-2C2 + YCp*lac*111). **b** Strains were first grown in YPD and 10-µl aliquots of serial dilutions (containing, respectively, 10^5, 10^4 and 10^3 cells/ml) were spotted on YPD and incubated for 14 days at 11° C. Haploid strains are: 1, 1829-2C2 (*MAT***a** *SLM9*); 2, 1829-*slm9* (*MAT* **a** *slm9*); 3, W303-1BS (*MAT***a** *sac1*); 4, W303-1B**a** (*MAT***a** *SAC1*); and diploid strains are: 5, W303-1B**a** crossed with 1829-*slm9*; 6, W303-1BS crossed with 1829-*slm9*; 7, W303-1BS crossed with 1829-2C2

cold-sensitive phenotypes are due to the same mutation, the mutant 1829-*slm9* (*MATα slm9 mbr1*) was crossed with the isogenic strain 1829-2 (*MATα slm9 mbr1*). Ten complete tetrads of the resulting diploid strain were analysed. In each case the two phenotypes co-segregate, indicating that both phenotypes are linked to a single locus. Genes from a yeast centromeric genomic library were isolated by their ability to suppress both the synthetic lethal and the cold-sensitive phenotypes of the mutant 1829-*slm9*. All the isolated plasmids contain *SAC1*, a gene implicated in secretion (Cleves et al. 1989; Whitters et al. 1993; Mayinger et al. 1995). An *Xba*I DNA fragment carrying only *SAC1* (Cleves et al. 1989) on a centromeric vector is able to suppress the synthetic lethal phenotype (Fig. 6, compare lanes 2 and 3) and the cold-sensitive phenotype of the *slm9 mbr1* mutant. As in the case of *slm9*, disruption of *SAC1* leads to a cold-sensitive phenotype (Cleves et al. 1989; Novick et al. 1989). To verify that *slm9* is allelic to *SAC1*, the *MATα* sac1 strain (W303-1BS) was constructed (as described in Materials and methods). It shows a cold-sensitive phenotype. Strain W303-1BS (*MATα sac1*) or W303-1Bα (*MATα SAC1*) were crossed with 1829-2C2 (*MAT***a** *SLM9*) or 1829-*slm9* (*MAT***a** *slm9*). The diploid strain resulting from the cross between the *sac1* and the *slm9* mutants has a cold-sensitive phenotype (see Fig. 6b). Thus, *slm9* and *sac1* mutations are recessive and they do not complement. Since the diploid double mutant strain is unable to sporulate, the segregation of the cold-sensitive phenotype was not examined. From the results presented above, we conclude that *slm9* is allelic to *SAC1*.

Discussion

Does Mbr1p act as a transactivator?

The *MBR1* gene has been previously isolated as a multicopy suppressor of the yeast CCAAT-binding transactivator mutant phenotype on glycerol medium (Daignan-Fornier et al. 1994). Mbr1p contains an acidic domain and a potential zinc finger motif in its N-terminal moiety. In a previous paper (Daignan-Fornier et al. 1994), we proposed that Mbr1p might be a transactivator having targets in common with the yeast CCAAT-binding complex Hap2p/3p/4p/5p. Here we observed a perinuclear localization of an MBR1p-*β*-Gal chimera. Although we cannot completely exclude a mislocalization due to the use of the *β*-Gal fusion, its functionality argues for the correct localization of a significant fraction of the protein fusion. Nevertheless, this result as such is not sufficient to eliminate a nuclear function for Mbr1p. Indeed it is possible that only a small fraction of the protein, which would not be detectable, is located in the nucleus. Alternatively, the activity of this putative transcriptional factor may be controlled by nuclear translocation under specific physiological conditions. However, the mutational analysis of this protein argues against such a function, since replacement of the two cysteines in the putative zinc finger domain does not alter Mbr1p function.

Mbr1p function is required under sub-optimal growth conditions

The *mbr1* growth defect is observed only when glycerol is used as the sole carbon source. In these conditions, an *mbr1* culture does not reach the final wild-type biomass, although the doubling time during exponential growth phase is not affected. We have identified three factors that enhanced the *mbr1* growth phenotype: (i) low temperature, (ii) high external pH and (iii) addition of 1,10-phenantroline (a drug which forces the cells to enter a non-proliferating state; Barnes et al. 1990). The exact mechanism by which these factors influence the requirement for Mbr1p is not clear, but they are all known to reduce cell growth, which suggests that Mbr1p is required for growth under sub-optimal conditions.

We observed (data not shown) that overexpression of *MBR1* improves growth on glycerol medium not only of the *hap* mutants, as already described (Daignan-Fornier et al. 1994), but also of a large variety of conditional mutants (affected in the cell cycle, mitochondrial metabolism, secretion and actin function) which show a leaky growth phenotype under semi-permissive conditions. This growth improvement is also observed for some wild-type strains whose growth is reduced on glycerol medium. This growth stimulatory effect of *MBR1* overexpression is only observed after 2–3 days of culture and is more obvious when cells are replica-plated

onto solid medium, compared to growth in liquid medium. On solid medium, the nutrients diffuse in limiting amounts from the periphery of the plate to the growth area. This diffusion allows an extended phase of growth in sub-optimal conditions. This phenomenon does not exist in liquid medium since the concentration of nutrients is homogeneous. Thus, it seems that cells overexpressing *MBR1* are able to perform some additional cell cycles when the conditions become limiting. This effect is not dependent on a particular type of genetic background since it is manifest in a large variety of mutant or wild-type strains. From the growth phenotype associated with either *MBR1* deletion or *MBR1* overexpression, we can deduce that *MBR1* gene dosage has a strong effect on growth when conditions become sub-optimal.

In addition to the growth-deficient phenotype, deletion of *MBR1* protects the cells against stress. Mutant cells are more resistant to heat shock and to long-term storage at 4° C. Conversely, overexpression of *MBR1* sensitizes the cells to heat shock. Thus the resistance of the cell to stress seems to be inversely correlated with the level of Mbr1p in the cell. The negative effect of Mbr1p on cellular stress response and its positive effect on growth could be the result of a single function. This function could be related to the control of intracellular pH, since it is known that growth capacity and stress resistance are sensitive to pH (Coote et al. 1991; Simon et al. 1994). Indeed the *mbr1* growth defect is enhanced by high pH of the medium.

MBR1 is induced in late growth phase and is repressed by PKA activity

MBR1 expression was studied in cells cultivated on glucose or on glycerol medium. Our data indicate that *MBR1* mRNA is not detectable in glucose medium and is strongly induced when glucose approaches exhaustion. On glycerol medium, the Mbr1p-β-Gal chimera is expressed at a basal level during the exponential phase and is induced four- to fivefold in the late growth phase. In both media *MBR1* expression is induced when the cells leave the exponential growth phase. Activation of PKA, either by addition of cAMP to the culture medium or by the presence of an activated allele of *RAS2*, decreases *MBR1* expression. These data suggest that *MBR1* belongs to the set of genes whose prototype is *CTT1* (Marchler et al. 1993). Expression of these genes is negatively regulated by cAMP through the STRE (Stress Response Element) consensus sequence AGGGG (Marchler et al. 1993) and induced through this element by the Msn2p/Msn4p transactivating factors in response to carbon starvation and to various stresses (Martinez-Pastor et al. 1996; Schmitt and McEntee 1996). In agreement with this interpretation, two AGGGG sequences are found in the upstream region of *MBR1*, at positions −305 and −1215, and *MBR1* expression is reduced in a *msn2 msn4* mutant strain (D. Tadi, personal communication). This suggests a role for this transcription factor in the expression of *MBR1*. It is worth noting though, that while most of the genes which are regulated through the STRE element are involved in cellular accommodation to the non-growing state and stress resistance (Ruis and Schüller 1995), the function of *MBR1*, in contrast, has the opposite effect as shown by its growth stimulating effect.

What is the function of Mbr1p?

From the results presented above two questions arise. First, what are the links between Mbr1p and cell growth regulation? Second, what is the biochemical function of Mbr1p? We have found that the *mbr1* growth defect is suppressed either by the activation of the RAS-cAMP pathway or by the overexpression of *SOK1* or *SCH9*. In yeast, the RAS-cAMP pathway regulates the level of the PKA activity. This kinase is implicated in a large variety of biological processes and is a key regulator of cell growth in response to environmental modifications (see Broach and Deschenes 1990 for a review). *SOK1* and *SCH9* have been isolated as multicopy suppressors of a PKA null mutant growth defect (Toda et al. 1988; Ward and Garrett 1994). Genetic studies have shown that the products of these two genes are likely to be elements of two distinct pathways which act in parallel with the RAS-cAMP pathway to regulate cell growth (Ward and Garrett 1994; Hartley et al. 1994). We have shown that the activation of any of these pathways suppresses the *mbr1* growth deficient phenotype. Formally, two models can explain this suppression effect. In the first one, Mbr1p could act as a structural or a regulatory element of one of these pathways. Our experimental data indicate that Mbr1p is certainly not a structural element of any of these pathways: (i) unlike Mbr1p, the RAS-cAMP and the Sch9p pathways are necessary for normal growth on glucose medium (Toda et al. 1987, 1988) and (ii) we have shown that *mbr1* and *sok1* phenotypes are at least additive, indicating that the products of these two genes do not act in a linear sequence. In the second model, a target of these pathways could replace or compensate for the Mbr1p function. Mbr1p in this case would act independently of these pathways. One candidate for such a target is Mbr3p. The *MBR3* gene was isolated in the same screen as *MBR1* (Daignan-Fornier et al. 1994). Its overexpression suppresses *mbr1* growth defects (data not shown) and it presents a synthetic phenotype with *mbr1* (Daignan-Fornier et al. 1994). Furthermore, Mbr3p has a functional domain in common with Mbr1p (Daignan-Fornier et al. 1994) and the same cellular localisation (data not shown). More work on the Mbr1p function may determine if this protein is a regulator of these pathways or if one of their targets replaces or modulates the Mbr1p requirement for growth.

On glycerol medium *mbr1* presents a synthetic lethal phenotype with *sac1*, which is observed when pH is high. Sac1p is involved in secretion; it is a membrane protein

that mediates adenosine triphosphate transport into the ER (Whitters et al. 1993; Mayinger et al. 1995). The synthetic lethal phenotype observed with *mbr1* and *sac1* suggests that the products of these two genes are involved in the same cellular process. The possible colocalization of Mbr1p with the perinuclear part of the ER is consistent with the hypothesis that Mbr1p is involved in intracellular trafficking.

Acknowledgements The authors thank Dr. M. Rose for the anti-Kar2p antibody. We acknowledge Dr. F. Kepes and Dr. H. Riezman for their advice on Mbr1p localisation and Dr. J. G. Valay and Dr. B. Daignan-Fornier for critical discussion in the course of this work. We thank Dr. H. Fukuhara and Dr. M. Jacquet for critical reading of the manuscript. This work was supported by an EEC "Human Capital and Mobility" grant (ERBCHRXCT940520). P.R. was supported by fellowships from the Ministère de la Recherche et de la Technologie and the Association pour la Recherche Contre le Cancer.

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