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Molecular cloning of Rab-related genes in the yeast *Yarrowia lipolytica*. Analysis of *RYL1*, an essential gene encoding a SEC4 homologue

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Abstract Small GTP-binding proteins of the Rab family are involved in the vesicular traffic inside eukaryotic cells. A gene library from the yeast *Yarrowia lipolytica* was screened with an oligonucleotide deduced from a highly conserved sequence in the Rab family. Four different genes were isolated. One of them, *RYL1*, was shown to be essential for cell viability. RYL1p displayed a high similarity with and tight phylogenetic relationships to SEC4p. When placed under the control of the *GAL10* promoter, *RYL1* was able to specifically relieve the thermosensitivity of a *sec4-8* mutant of *Saccharomyces cerevisiae*. Therefore, it is proposed that *RYL1* is a functional homologue of the *S. cerevisiae SEC4* gene and is involved in the fusion of secretory vesicles with the plasma membrane in the general protein secretion pathway.

Key words: Yeast · *Yarrowia lipolytica* · Protein secretion · Rab protein

Introduction

The Rab protein family is a subdivision of the Ras protein superfamily initially described in a rat brain cDNA library by Touchot et al. (1987). It consists of tightly related small GTP-binding proteins sharing common features (for reviews, see Bourne et al. 1990; Grand and Owen 1991; Valencia et al. 1991), including a size of about 21 kDa, conserved blocks involved in GTP binding, and two cysteines at the C-terminal end which are post-translationally modified to interact with membranes (Rossi et al. 1991). This Rab family is widespread among eukaryotes and is implicated in a variety of membrane traffic processes (Rothman and Orci 1992) from the endocytic pathway (rab5) to the transport of synaptic microvesicles (rab6). This family could be subdivided in several groups of more tightly related sequences: the *SEC4* subfamily, the *YPT1* subfamily, and the *RAB4* subfamily. It is supposed that these subfamilies share functional similarity. This hypothesis was supported by experiments of heterologous complementation and by intracellular localization in common compartments. For instance, *YPT2* of *Schizosaccharomyces pombe* was able to complement a *sec4* mutant of *Saccharomyces cerevisiae* (Haubruck et al. 1990), and *RAB1* from mouse cells was able to complement the *YPT1* deficiency in *S. cerevisiae* (Haubruck et al. 1989). At least, two components of the protein secretion pathway were shown to belong to this protein family in the yeast *S. cerevisiae*: Sec4p is involved in the targeting of the secretory vesicles to the plasma membrane (Goud et al. 1988; Walworth et al. 1989); Ypt1p is implicated in the fusion of endoplasmic reticulum-derived microvesicles with the Golgi apparatus (Segev et al. 1988; Bacon et al. 1989).

The site of action and the function of these two Rab proteins were assessed on conditional mutants by a combination of electron microscopy, immunofluorescence microscopy and cell fractionation. These studies showed that these proteins could control the vectorial flow of the vesicular transport process according to the model of Bourne (1988). The Rab proteins are associated with the cytoplasmic side of the carrier vesicle. In their GTP-bound form, they may interact with a receptor on the cytoplasmic side of the downstream compartment, thus targeting the vesicle. GTP hydrolysis allows recycling to the upstream compartment. Interaction with this compartment triggers nucleotide exchange from GDP to GTP. It is thought that every vesicle fusion step is controlled by such a mechanism through different types of GTP-binding proteins like members of the Rab or the Arf subfamily (Rothman and Orci 1992).

In order to identify genetic markers along the protein secretion pathway of the yeast *Yarrowia lipolytica*, a screening for members of the Rab family was undertaken. Four sequences called *RYL* (for Rab-related gene in *Y. lip-*

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olytica) were selected and among them *RYL1* was shown to be closely related to *SEC4/YPT1*. This gene is essential and a thorough sequence analysis led us to postulate that Ryl1p could be the homologue of Sec4p. In order to confirm this hypothesis, the *RYL1* coding sequence was placed under the control of the *GAL10* promoter in a *S. cerevisiae* expression vector and was shown to specifically complement the *sec4-8* growth defect.

Materials and methods

Strains, media, and general molecular biology techniques. Standard media were used to grow *Escherichia coli*, *S. cerevisiae* and *Y. lipolytica* (Sherman et al. 1983; Sambrook et al. 1989). The strains and plasmids used in this work are listed in Table 1. 5-FOA selection was performed on minimal medium (1% glucose, 0.17% Difco yeast nitrogen base without ammonium sulphate, 0.1% proline as nitrogen source, supplemented as required) containing 10 mg/l of uracil and 1.25 g/l of 5-fluoroorotic acid (5-FOA; PCR Inc., Gainesville, Florida, USA). YPgal was 1% yeast extract, 1% peptone, 2% galactose. All restrictions, ligations and hybridization experiments were performed according to standard protocols (Sambrook et al. 1989). Labelled compounds were supplied by Amersham-France. Transformation techniques for *Y. lipolytica* have already been described (Beckerich et al. 1994). Plasmid stability tests were done as described by Fournier et al. (1991).

Plasmid construction. The inserts from the *Y. lipolytica* gene library were subcloned as *Acc*I fragments in the *Sma*I site from the Bluescript SK$^-$ plasmid (Stratagene). Plasmids pINA609 and pINA610 are Bluescript derivatives carrying *RYL1* cloned in the two possible orientations with regard to the polylinker. To construct the disruption vector pINA845, pINA610 was partially digested by *Sph*I and completely with *Nco*I, thus excising 121 bp upstream of the ATG of *RYL1* and 250 bp of the coding sequence, and ligated to a 1.65-kb *Nco*I-*Sph*I fragment carrying the *URA3* gene marker from pINA300. Alternatively, the same digest of pINA610 was blunted with T_4 DNA polymerase and re-circularized on itself, to generate the deleted vector pINA847. To construct the replicative plasmids pINA841 and pINA844, carrying *RYL*1 and the *LEU2* or *URA3* gene markers, a 2.1-kb *Bam*HI-*Cla*I fragment from pINA610 was cloned in the replicative vectors pINA237 and pINA398 cut by *Bam*HI and *Cla*I, respectively. Use of the *S. cerevisiae* expression plasmid Yep52′ has been reported previously (Beckerich et al. 1994). First, a 1-kb *Xba*I-*Xba*I fragment carrying the whole *RYL1* coding sequence starting 9 bp upstream of the initiator ATG was gel purified and inserted in the correct orientation in YEp52′ opened by *Xba*I, downstream from the *GAL10* promoter. Second the *LEU2* gene marker of YEp52′ was substituted by *URA3*: a *Cla*I-*Fsp*I *URA3* fragment from YIp5 was inserted in YEp52′ opened by *Cla*I and *Eco*RV in the *LEU2* region. The resulting plasmid pINA871 carrying the *URA3* gene marker and *RYL1* was used to transform the Sec$^-$ Ura$^-$ mutants. A control plas-

Table 1 List of strains and plasmids

Strain/plasmid	Genotype/description	Source
E. coli		
HB101	*hsd*R$^-$, *hsd*M$^-$, *rec*A13, *SupE*44, *lacZ*4, *leuB*6, *proA*2, *thi*-1, *Sm*^{R}	B. Bachmann
TG1	F$^-$, *rec*A1, *end*A1, *gyr*A96, *thi*, *hsd*R17 (r_k^-, m_k^+), *supE*44, *rel*A1	Stratagene
CJ236	*dut-1*, *ung-1*, *thi-1*, *relA-1*; pCJ105 (Cm^r)	Stratagene
Y. lipolytica		
W29	Reference wild-type strain	Our collection
E129	MatA, *lys11-23*, *ura3-302*, *xpr2-322*, *leu2-270*	Our collection
E146	MatB, *his1-1*, *ura3-302*, *leu2-270*	Our collection
111-27	MatA, *lys11-23*, *ura3-302*, *xpr2-322*, *leu2-270*, *ryl1 : : URA3*, [pINA841]	This work
135995	MatA, *lys11-23*, *ura3-302*, *xpr2-322*, *leu2-270*, *ryl1 delta*, [pINA844]	This work
S. cerevisiae		
NY405	Mata, *ura3-52*, *sec4-8*	B. Goud et al. (1988)
Sec1	Matα, *ura3-52*, *sec1-1*, *leu2-3-112*, *SUC2*	R. Schekman
Sec12	Mata, *ura3-52*, *sec12-1*, *leu2-3-112*, *his314*, *trp1-2891*	R. Schekman
Plasmids		
pINA237	pBR322 with *LEU2* of *Y. lipolytica* and *ARS*18 inserted in the *Eco*RI site	AM Ribet (personal communication)
pINA300	pBR322 with *URA*3 of *Y. lipolytica* inserted in *Sal*1	P. Fournier (personal communication)
pINA398	pBR322 with *URA*3 of *Y. lipolytica* and *ARS18*	P. Fournier (personal communication)
pINA591	Initial *RYL4* plasmid derived from pINA62	This work
pINA594	Inititial *RYL3* plasmid derived from pINA62	This work
pINA596	Initial *RYL2* plasmid derived from pINA62	This work
pINA600	Initial *RYL1* plasmid derived from pINA62	This work
pINA609	2.1-kb *Acc*I fragment from pINA600 cloned at the *Sma*I site of Bluescript™	This work
pINA610	2.1-kb *Acc*I fragment from pINA600 cloned at the *Sma*I site of Bluescript™	This work
pINA841	2.1-kb *Cla*I-*Bam*HI fragment of pINA610 inserted in pINA237	This work
pINA844	2.1-kb *Cla*I-*Bam*HI fragment of pINA610 inserted in pINA398	This work
pINA845	*URA3* fragment inserted into *Sph*I-*Nco*I of pINA610 disrupting the *RYL1* gene	This work
pINA847	*Sph*I-*Nco*I deletion of pINA610	This work
YEp52′	Derivative of YEp52 with the polylinker of M13 tg131 downstream from the *GAL10* promoter	P. Durrens (personal communication)
pINA871	*RYL1* inserted in *Xba*I-*Xba*I of YEp52′, the insertion of *URA3* in the *Cla*I-*Eco*RV deletion	This work
pINA872	*Bam*HI-*Bam*HI deletion of *RYL1* gene in pINA871	This work