ESCR-T-I components of the endocytic machinery are required for Rim101-dependent ambient pH regulation in the yeast Yarrowia lipolytica

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INTRODUCTION

Yarrowia lipolytica is a non-pathogenic saprophytic yeast that colonizes various food products and natural environments (Barth & Gaillardin, 1997). A recent genomic survey confirmed that it is very distantly related to Saccharomyces cerevisiae and Candida albicans (Dujon et al., 2004). Like C. albicans and many other fungi, it secretes proteases whose expression is tightly controlled by a combination of environmental stimuli, including nutrient availability and ambient pH (Ogrydziak et al., 1977, 1993). Y. lipolytica secretes either an acidic protease encoded by the AXPI gene at acidic pH, or an alkaline protease encoded by the XPR2 gene at neutral/alkaline pH. Previous work showed that pH-dependent expression of the XPR2 gene was achieved through activation of a conserved signalling pathway regulating the expression of many pH-dependent genes (Gonzalez-Lopez et al., 2002; Lambert et al., 1997; Tréton et al., 2000). This pathway, called Pal in filamentous fungi and Rim in yeasts, was initially described as a pH-response pathway in Aspergillus nidulans (Caddick et al., 1986), and was later identified in Y. lipolytica (Lambert et al., 1997), and several other ascomycetes including C. albicans (Ramon et al., 1999), S. cerevisiae (Lamb et al., 2001) and other fungi (Peñalva & Arst, 2002, 2004). The current model states that at alkaline pH, a cascade of six PAL or five RIM genes activates the zinc finger transcriptional factor PacC/Rim101p through a complex C-terminal proteolytic processing event involving the PalB/Rim13p protease. The PacC/Rim101p truncated form is able to activate alkaline-PH-responsive genes and to repress acid-induced genes (for a review see Peñalva & Arst, 2002, 2004). Direct interaction of Rim13p/PalB and Rim101p/PacC could not, however, be observed, and the only direct interactions detected in two-hybrid screens of Pal/Rim components were between Rim20p/PalA and Rim101p/PacC (Vincent et al., 2003; Xu & Mitchell, 2001).

In Y. lipolytica, screening of an insertional library for altered expression of either acidic or alkaline protease genes, or both, revealed besides components of the Rim pathway, other potential regulators of protease expression, in particular YIVps28p (Gonzalez-Lopez et al., 2002). Mutations in YIVPS28 conferred an Xpr2− phenotype like Ylrim mutations. But, in contrast to Ylrim mutations, they clearly interfered with the capacity of the strains to execute the yeast–hyphae transition (Gonzalez-Lopez et al., 2002).

In S. cerevisiae, Vps28p is a class E component of the endosomal-sorting complex ESCR-T-I (Katzmann et al., 2001). This complex participates in the recognition of...
both ubiquitinated endocytic and biosynthetic cargos that are targeted to the multivesicular body (MVB) vesicles to be ultimately delivered to the lumen of the vacuole when the MVB fuses with this organelle (Futter et al., 1996). In contrast, transmembrane proteins that remain in the limiting membrane of the MVB are either delivered to the limiting membrane of the vacuole, or recycled to the plasma membrane or the Golgi (Lemmon & Traub, 2000). The MVB-sorting pathway thus affects both endocytosis and exocytosis of membrane-anchored proteins. It is conserved from yeast to higher eukaryotes, and is required for a growing list of cellular functions that include downregulation of receptors and transporters, regulation of the immune response and even the budding of certain viruses like human immunodeficiency virus (for a review see Katzmann et al., 2002).

Therefore, the question was whether Ylvsps28 mutations affected expression of protease genes through an indirect effect on protein expression, or through a specific interference with the Rim signalling pathway. This last hypothesis was particularly attractive since interactions had been detected during a whole-genome screen of two-hybrid interactants in S. cerevisiae, between Rim20p and two other Vps components, Snf7p and Vps4p, and Rim13p and Snf7p (Ito et al., 2001). Snf7p is a component of the ESCRT-III complex (Babst et al., 2002a), which acts downstream from ESCRT-I and ESCRT-II complexes in the MVB pathway, whereas the Vps4p/End13p AAA ATPase acts at the end of the endocytic cycle to dissociate the ESCRT-III complex from the endosomal membrane (Babst et al., 1998).

Subsequent observations in A nidulans, S. cerevisiae and C. albicans confirmed that Snf7p indeed interacted physically with Rim20p/PalA (Bowers et al., 2004; Ito et al., 2001; Vincent et al., 2003; Xu et al., 2004). Analysis of mutations affecting the interaction domain of Rim20p showed that both Rim20p–Rim101p and Rim20p–Snf7p interactions were required for Rim101p processing (Xu et al., 2004). The interaction between Rim20p and Vps4p was also observed in another two-hybrid analysis (Bowers et al., 2004), but its in vivo significance was later questioned since Vps4p does not appear to be required for Rim101p processing in S. cerevisiae (Kullas et al., 2004). Indeed, both Snf7p and Vps4p were shown to interact with Bro1p/Vps31p (Gavin et al., 2002), a Rim20p/PalA parologue, which is another soluble class E factor required for MVB sorting but not for pH sensing (Odorizzi et al., 2003). The interaction between Rim13p and Snf7p has not been confirmed by other studies. Finally, a systematic phenotypic analysis of a nearly complete collection of gene-deletion mutants of S. cerevisiae indicated that several vps mutant strains exhibited impaired growth at alkaline pH or in the presence of high concentrations of monovalent cations (Giaever et al., 2002), two phenotypes that are characteristic of rim mutants in this organism (Lamb et al., 2001).

More recently, and while our work was in progress, definite evidence for a functional link between the Rim and Vps pathways was obtained in the yeasts S. cerevisiae and C. albicans. In C. albicans, a mutant strain inactivated for the CaSNF7 gene displayed all the phenotypes expected for a Carim mutant strain (pH and ion sensitivity during growth, and the absence of alkaline-induced filamentation and Rim101p processing) and was suppressed by a constitutively active form of CaRim101p (Kullas et al., 2004). Other vps mutants affecting different ESCRT-I, -II and -III components were shown, in a separate study, to exhibit morphological defects characteristic of rim mutants (Xu et al., 2004). In S. cerevisiae, mutations in several ESCRT-I, -II and -III components affected processing of the transcriptional activator Rim101p, as would do, for example, a rim20 mutation (Xu et al., 2004). Interestingly in this last study, not all ESCRT-encoding genes were required for the processing of Rim101p, and none of the non-ESCRIT vps genes were. The theory that not the whole endocytic pathway was involved in pH signalling was further confirmed by the observation that Vps4p defects had no drastic effect on Rim101p processing either in S. cerevisiae or in C. albicans (Kullas et al., 2004; Xu et al., 2004).

This paper reports on the characterization of vps mutant strains in Y. lipolytica. It confirms the conservation of a functional link between the components of the endocytic pathway and pH signalling, and the absence of an involvement of Vps4p, over the full range of ascomycetous yeasts. We further show that SNF7 is an essential gene in Y. lipolytica. This last observation may explain why ESCRT components were not easily, or not at all, identified in exhaustive screens for genes affecting pH signalling in Y. lipolytica and A. nidulans.

METHODS

Strains, sequence data and gene designation. The bacterial strains used for transformation and amplification of recombinant DNA were Escherichia coli DH5βa and SURE (Stratagene). Yeast strains are described in Table 1. Previously reported Y. lipolytica mutations in pal1 (pal1-26) and pal4 (pal4-24) (Lambert et al., 1997) were shown to be specifically complemented by YIRIM13 and YIRIM20, respectively (data not shown), and have been designated rim13-26 and rim20-24 mutations, respectively.

Unless otherwise stated, all sequence data were obtained from the complete genome sequence of Y. lipolytica (http://cbi.labri.fr/ Genolevures/; Dujon et al., 2004). Systematic gene nomenclature referring to this project is given in the text where appropriate.

Culture media and phenotypic tests. Complete YPD medium, minimal YNB-glucose medium, derepressing (Y) medium and protease-inducing medium (YPDm) have been described previously (Blanchin-Roland et al., 1994; Lambert et al., 1997). Solid and liquid media were buffered at pH 4.0 (with 0.2 M sodium citrate buffer), pH 7.0 (with 0.2 M sodium phosphate buffer) or pH 8.0 (with 25 mM Tris/HCl) and supplemented with 0.1 g uracil l⁻¹. The pH of liquid media was stable within 0.3 pH units during growth. The Lac⁺ phenotype was screened on Y medium buffered at pH 7.0, as previously described (Gonzalez-Lopez et al., 2002).

Growth at various pHs was assayed by spotting serial threefold dilutions of exponential-phase cultures in YPD liquid medium, on plates buffered either with 0.2 M citrate-phosphate buffer at pH 3.5, or with 0.2 M MOPS for assays at pH 7.0-8.5. Crossing of compatible
**Table 1. Strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>AM3</td>
<td>MAT A, ura3-302, leu2-270, lys11-23, XPR2, LEU2, XPR2: lacZ</td>
<td>Lambert et al. (1997)</td>
</tr>
<tr>
<td>AM4</td>
<td>MAT B, ura3-302, leu2-270, his-1, XPR2, LEU2, XPR2: lacZ</td>
<td>Lambert et al. (1997)</td>
</tr>
<tr>
<td>E122</td>
<td>MAT A, ura3-302, leu2-270, lys11-23</td>
<td>E. Fabre*</td>
</tr>
<tr>
<td>LAM26-03</td>
<td>MAT A, ura3-302, leu2-270, XPR2, LEU2, XPR2: lacZ, pal1-26/rim13-26</td>
<td>This study</td>
</tr>
<tr>
<td>SBR116</td>
<td>MAT A, ura3-302, leu2-270, XPR2, LEU2, XPR2: lacZ, pal1-26/rim13-26, RIM101-1119</td>
<td>This study</td>
</tr>
<tr>
<td>CGL-U15u</td>
<td>MAT A, ura3-302, leu2-270, lys11-23, XPR2, LEU2, AXPI: gusC, XPR2: lacZ, vps23-U15</td>
<td>This study</td>
</tr>
<tr>
<td>SBR117</td>
<td>MAT A, ura3-302, leu2-270, lys11-23, XPR2, LEU2, AXPI: gusC, XPR2: lacZ, vps23-U15, RIM101-1119</td>
<td>This study</td>
</tr>
<tr>
<td>CGL-Y2u</td>
<td>MAT A, ura3-302, leu2-270, lys11-23, XPR2, LEU2, AXPI: gusC, XPR2: lacZ, vps28-Y2</td>
<td>This study</td>
</tr>
<tr>
<td>SBR118</td>
<td>MAT A, ura3-302, leu2-270, lys11-23, XPR2, LEU2, AXPI: gusC, XPR2: lacZ, vps28-Y2, RIM101-1119</td>
<td>This study</td>
</tr>
<tr>
<td>SBR114u</td>
<td>MAT A, ura3-302, leu2-270, lys11-23, XPR2, LEU2, AXPI: gusC, XPR2: lacZ, vps4A14</td>
<td>This study</td>
</tr>
<tr>
<td>SBR119</td>
<td>MAT A, ura3-302, leu2-270, lys11-23, XPR2, LEU2, AXPI: gusC, XPR2: lacZ, vps4A14, RIM101-1119</td>
<td>This study</td>
</tr>
<tr>
<td>SY1364</td>
<td>MAT A, ura3-302, leu2-270, lys11-23, XPR2, LEU2, AXPI: gusC, XPR2: lacZ, vps4A172A, URA3, vps4A14</td>
<td>This study</td>
</tr>
</tbody>
</table>


**Y. lipolytica** auxotrophs, diploid sporulation and spore isolation was done as described previously (Gonzalez-Lopez et al., 2002). Dilutions of spore suspensions were plated on complete medium and colonies were tested on appropriate media.

**DNA and RNA techniques.** Standard recombinant DNA techniques were performed essentially as previously described (Gonzalez-Lopez et al., 2002). Sequences were obtained from the DNA sequencing department of Eurogentec. They were assembled and annotated using the GCG package. All transformation events were checked by colony PCR using PfuRe Taq Ready-To-Go PCR beads (Amersham) and confirmed by Southern blotting.

Gene expression was determined by real-time quantitative RT-PCR using a LightCycler (Roche Molecular Biochemicals). Cells were grown at 28°C in Y medium buffered either at pH 4 or 8, and harvested at the exponential phase (OD<sub>600</sub> about 0.6). Total RNA was extracted with the RNeasy mini-kit from Qiagen and then treated by DNase I (Qiagen). The cDNA was synthesized from 1 μg total RNA according to the manufacturer’s protocol of the SuperScript II RNase H<sup>−</sup> reverse transcriptase kit (Invitrogen). Primer sets for the genes were as follows: ACT20-ACT21r for ACT1, XPR20-XPR21r for XPR2, PHR1-12-PHR113r for PHR1, RIM104-RIM105r for RIM101 and PHR203-PHR204r for PHR2 (Table 2). The PCR parameters were 95°C for 8 min, followed by 45 cycles at 95°C for 10 s, 55°C for 7 s, and 72°C for 10 s. A negative control with sterile water was performed for each primer set. The threshold cycle (Cp) was determined as the cycle above which the fluorescence signal produced by the SYBRGreen I dye, reached a baseline level. The expression levels of the genes were determined relative to the expression of the ACT1 gene, which was used as an internal control in all instances. The Student’s t-test was used to determine statistical significance.

**Deletion of YIVPS4 and YISNF7.** To create null versions of YIVPS4 and YISNF7, a disruption cassette was constructed according to Fickers et al. (2003). First, the promoter and the terminator regions of each gene were amplified separately using the SY12 genomic DNA as template. The promoter and terminator regions of YIVPS4 were amplified with the primers V4BP1/VPS4P2 and VPS4T1/V4XT2, as ~0-99 and ~0-89 kb fragments, respectively (Table 2). For YISNF7, the promoter and terminator regions were amplified with the primers V32XP1/VPS32P2 and VPS32T1/V32BT2, as ~1-00 and ~1-15 kb fragments, respectively. Primers VPS4P2 and VPS4T1, and primers VPS32P2 and VPS32T1 all contain the meganuclease I-SceI recognition sequence. For each gene, the resulting promoter-I-SceI and I-SceI-terminator fragments were then annealed and further amplified by using primers V4BP1/V4XT2 or V32XP1/V32BT2. The resulting promoter-I-SceI-terminator cassettes were then digested by BamHI/XbaI and cloned into pBluescript KS+, digested by the same restriction enzymes.
enzymes, to generate plasmids pINA1344 (promoter-terminator of VPS4) and pINA1346 (promoter-terminator of SNF7). The loxR-URA3-loxP module was rescued from JMP113 (Fickers et al., 2003), by I-SceI digestion, and cloned into pINA1344 and pINA1346 at the I-SceI site, generating plasmids pINA1345 and pINA1347, respectively. The disruption cassettes, PUTV4 and PUTV32, were generated by PCR amplification using the primers V4BP1/V4XT2 with the flanking primers to generate a full-length PCR product containing the internal mutation. This PCR product was digested by BglII and then inserted into the replicative LEU2 plasmid pINA240 digested by NrdI/Nhel to give pINA1355, which was checked by DNA sequencing.

**Introduction of the \(YIRIM101-1119\) truncated allele into \(Yl\) \(vps23-U15\) mutant strains.** \(YlURA3\) was excised from strains CGL-U15 (\(Yl\)vps23-U15 allele), CGL-Y2 (\(Yl\)vps2-Y2 allele) and SBR114 (\(Yl\)vps4-14 allele), by using plasmid pUB4-CRE as previously described (Fickers et al., 2003) to give strains CGL-U15u, CGL-Y2u and SBR114u, respectively. The DraIII-digested pINA1119 (Lambert et al., 1997) was targeted to the terminator of \(YIRIM101\) in these resulting Ura- strains, and in Lam26-03 strain (rim3-16 allele). Secondary Ura- clones were selected on 5-fluoroorotic acid medium, and recombination events leaving the \(YIRIM101-1119\) truncation were selected (strains SBR116–SBR119, see Table 1).

**Staining with FM4-64.** FM4-64 localization experiments were done using a modification of the protocol described by Vida & Emr (1995). Yeast cells were grown in YPD to an OD 600 of about 0.6. Cells (5 mI culture volume) were harvested, incubated in 150 μl YPD containing 40 μM FM4-64 (Molecular Probes) for 30 min at 0°C, washed three times on ice, and further incubated in 200 μl YPD for 20 min at 18°C. An aliquot of cells was centrifuged, resuspended in water and visualized by differential interference contrast (DIC) and fluorescence microscopy using an Olympus U-RFL-T microscope with a CoolSNAP camera.

**RESULTS**

**Isolation of the \(Yl\)vps23-U15 mutant**

To identify potential regulators affecting the expression of either acidic (Axp1p) or alkaline (Xpr2p) proteases, or both, in \(Y.\) \(lipolytica\), mutant strains had previously been isolated from the \(Yl\) strain mutated by a library of genomic
fragments transposed by the mTnY11-transposon (Gonzalez-Lopez et al., 2002). Characterization of mutations affecting the expression of XPR2 alone, or both XPR2 and AXP1, identified five YIRIM and five non-RIM genes. Fourteen additional mutations resulted from integrations at other loci and were characterized only by in silico analysis of the sequences flanking the transposon (Gonzalez-Lopez et al., 2002). PCR analysis, carried out as previously described (Gonzalez-Lopez et al., 2002), showed that homologous integration of the transposed genomic DNA fragments had occurred in three of these mutant strains. Only one of these showed a genetic linkage between the protease phenotype and the URA3-labelled transposon, as checked by mating against the SY12 isogenic derivative AM4 (data not shown). The complete sequence of the disrupted ORF was assembled from overlapping flanking regions and by primer walking (accession no. AJ509167); it was identical to that of YALI0F19272g later found in the complete genome assembly (Dujon et al., 2004).

This mutant strain was affected in a gene orthologous to S. cerevisiae VPS23, which encodes a component of the ESCRT-I complex required for sorting membrane proteins into the MVB pathway (Katzmann et al., 2001), as was YIVPS28 previously identified in the same screening (Gonzalez-Lopez et al., 2002). YIVPS23 encodes a predicted protein of 378 aa (accession no. AJ509167) displaying 36% identity with Homo sapiens TSG101 (accession no. AAC52083), 34% with Drosohila melanogaster TSG101 (accession no. AF315343), 28% with S. cerevisiae Vps23p/ Sgt22p (Li et al., 1999) and with C. albicans CA2343/orf19.2343 (http://genolist.pasteur.fr/CandidaDB/). Comparison of the Y. lipolytica amino acid sequence with its homologues showed a conservation of the domain structure predicted for ScVps23p and TSG101 (Katzmann et al., 2001): a N-terminal domain homologous to ubiquitin-conjugating enzymes, but unlikely to catalyse ubiquitination because of the substitution for the C residue that binds ubiquitin, a conserved C-terminal portion. The YlmTn1 insertion truncates YIVps23p at aa 150 (YIVps23-U15).

**Role of YIVPS23 and YIVPS28 in alkaline- or acidic-pH responses**

To establish whether YIVPS genes are involved in ambient pH signalling, we assessed the effects of YIVPS mutations on the transcription of different pH-regulated genes by real-time quantitative PCR on total RNA extracted from cultures grown in Y medium at pH 4.0 and 8.0, using actin transcript as a reference. We chose the alleles YIVps23-U15 (the only mutation available affecting this gene) and YIVps28-Y2, which truncates Vps28p at aa 87. As ambient alkaline-pH responding genes, we chose, as previously, XPR2 and YIPHR1 (accession no. YALI0D04851g) (Gonzalez-Lopez et al., 2002) plus YIRIM101. Since expression of the acidic-protease gene AXP1 was shown to be independent of the Rim pathway, we chose as acidic pH-responsible gene YIPHR2 (accession no. YALI0D06039g), a homologue of CaPHR2 known to be expressed at acidic pH only in C. albicans (Muhlschlegel & Fonzi, 1997). As a control of the effect of rim mutations on the expression of these genes, we chose one YIRIM mutant, carrying the YIRIM13-26 mutation (see Methods) that displays a typical Rim phenotype (Gonzalez-Lopez et al., 2002).

At pH 8.0, both Ylvps23-U15 and Ylvps28-Y2 mutations significantly decreased YIRIM101 expression (more than 2 times compared to wild-type, P values ≪ 0.01), reduced YIPHR1 expression more than 20 times and nearly abolished XPR2 transcription (Fig. 1a). These observations were fully confirmed by measuring the expression of the XPR2: lacZ fusion integrated at the XPR2 locus of these strains and by Northern blotting (data not shown). At pH 4.0, no or very little transcription of these alkaline reporter genes could be detected (Fig. 1b). Thus, like YIRIM13 and other YIRIM genes (Gonzalez-Lopez et al., 2002; Lambert et al., 1997), YIVPS23 and YIVPS28 are absolutely required for transcriptional activation of alkaline-induced genes at pH 8.0. In both Ylvps mutant strains, as in the YIRIM13-26 mutation strain, at alkaline pH, expression of the acid-induced gene YIPHR2 derepressed more than 20 times over the wild-type level at the same pH, reaching its level at acidic pH in the reference strain (Fig. 1a, b).

YIRIM mutants induce a characteristic growth defect at alkaline pH and have no effect on growth at acidic pH (Gonzalez-Lopez et al., 2002; Fig. 2a). The growth of the Ylvps mutant strains was slightly affected at acidic pH and drastically affected at alkaline pH, even more than that of the YIRIM13-26 mutant (Fig. 2a). All these results strongly suggest that YIVPS23 and YIVPS28 genes are, like YIRIM genes, required for sensing ambient pH and adjusting cellular responses to it.

**A C-terminal truncated allele of YIRIM101 suppresses Ylvps23 and Ylvps28 mutations at both pHs**

In order to test whether pH effects observed in the Ylvps mutants were mediated through the Rim pathway, we checked whether a dominant, constitutively active, truncated form of YRIM101p was able to suppress Ylvps mutations for their defects in alkaline and acidic responses. To address this question, we replaced the wild-type YIRIM101 allele by the most truncated allele, YIRIM101-1119 (Lambert et al., 1997), in the Ylvps23-U15 and Ylvps28-Y2 mutants and in the YIRIM13-26 mutant (see Methods). The VPS+ AM319AC strain (Lambert et al., 1997), where YIRIM101-1119 replaces the wild-type YIRIM101 allele, was used as a control. As shown on Fig. 1, expression of the YIRIM101-1119 allele efficiently suppressed the defective alkaline-induced gene expression observed in the YIRIM13-26 and Ylvps mutations, and resulted in expression levels not significantly different from those observed in the RimSL control strain (F2 = 0.99). At both alkaline and acidic pH, the two alkaline-induced genes, XPR2 and PHR1, were induced more than 10 times over the levels measured in the
unsuppressed mutants at the same pH. At both pHs, RIM101 itself was induced 2–5 times over the mutant levels at the same pH ($P \leq 0.01$). The acid-induced gene PHR2 was repressed more than 10 times at alkaline pH, and about 3 times ($P \leq 4 \times 10^4$) at acidic pH, relative to its level in the vps mutants (Fig. 1a, b). Taken together, these results indicate that Ylvps mutations, like Ylrim mutations, can be bypassed by expressing a constitutively active form of YlRim101p.

Expression of the YlRIM101-1119 allele rescues the alkaline-sensitive growth phenotype of the Ylrim13-26 mutant and generates a mild acid-sensitive phenotype (Fig. 2a, RimSL).
or rim13+RimSL). Both effects were observed when this activated form was expressed in Ylves28-Y2 and Ylves23-U15, the acid-sensitive phenotype being exacerbated in these strains compared to Ylrim13-26 (Fig. 2a).

All these results confirmed that (i) like Ylrim mutations, Ylves23 and Ylves28 mutations are suppressed by a constitutively active form of YlRim101p, suggesting that their effects at alkaline pH are mediated through YlRim101p, (ii) unlike Ylrim mutant strains, Ylves mutant strains display a strong alkaline-sensitive growth phenotype and an acid-sensitive phenotype that is exacerbated when the alkaline response is turned on at acidic pH, suggesting that their response to ambient pH is affected both in Rim-dependent and in Rim-independent ways.

**YISNF7 is an essential gene in *Y. lipolytica***

In *S. cerevisiae*, Vps23p and Vps28p are both components of the ESCRT-I complex (Katzmann et al., 2001). We wondered whether Snf7p, which is known to interact with Rim20p in *S. cerevisiae*, *A. nidulans* and *C. albicans* (see Introduction), was also involved in pH regulation. The YALI0C16027g sequence encodes a predicted protein of 215 aa that matches putative orthologous gene products in other species, with the following identity scores: 49 % with *S. cerevisiae* Snf7p, 50 % with *C. albicans* Snf7p (CA4903 http://genolist.pasteur.fr/CandidaDB/), 43 % with *Schizosaccharomyces pombe* Spac-1142-07c (CA117014.1), 47 % with *Neurospora crassa* hypothetical protein (CAD70846+1) and 41 % with *H. sapiens* CHMP4b (CAC14088.1). Like its homologues, YlSnf7p presents several putative coiled-coil regions (positions 14–48, 53–85 and 149–173).

As SNF7 is non-essential in *S. cerevisiae* and *C. albicans*, we first tried to delete the *YISNF7* coding sequence in the haploid strain SY12 (see Methods). We got only two disruptants from several transformation assays, most trans-formants resulting from ectopic integrations. The two clones were not affected in endocytosis and displayed no pH-response defect (data not shown). In order to rule out that a functionally redundant protein may compensate for the loss of YlSnf7p, we searched for possible YlSnf7p homologues within the *Y. lipolytica* genome. Only two distant paralogues YlVps20p (28 % identity) and Mos10p/Vps60p (33 % identity) were identified, as would be the case in *S. cerevisiae* for a search of ScSnf7p paralogues. We wondered if the presence of suppressing mutation(s) in the *YIRIM101* gene may account for the lack of phenotypes for YlSnf7Δ strains. The sequence of the *YIRIM101* locus was checked entirely in these strains, but no mutation could be detected. We thus assumed that *YISNF7* might be essential. Therefore, two strategies were developed to disrupt this gene. (i) One *YISNF7* allele of the diploid strain AM3/AM4 was disrupted as before, with selection for Ura + isolates. Deletion of one *YISNF7* allele was confirmed by PCR and Southern blotting for several transformants. Two independent diploids were sporulated and their progeny was studied by random spore analysis. Among a total of 153 fast-growing auxotrophic segregants, none were Ura + (i.e. disrupted for *YISNF7*), whereas the other auxotrophic markers segregated normally. Twelve very slow-growing colonies were later observed on the plates; they were all Ura +, and upon a PCR test were shown to be disomic, carrying both a normal and a deleted copy of *YISNF7* (data not shown). We concluded, therefore, that the *YISNF7* gene was essential for spore germination and/or vegetative growth. (ii) The plasmid pINA1356, carrying an internal deletion of the whole *YISNF7* coding sequence, was integrated by a single crossing-over event into the promoter of the *YISNF7* gene in the haploid strain E122, with selection for Ura +. This created a wild-type copy and a deleted copy integrated in tandem in the genome and flanking the URA3 marker. The resulting strain was then transformed with the Leu + replicative plasmid pINA1355 carrying the wild-type *YISNF7* gene, with selection for unstable Leu + clones. Secondary Ura + clones were selected on 5-fluoroorotic acid medium and screened for the occurrence of recombination events leaving the YlSnf7-1356 deletion. Six Ura− Leu + transformants, carrying the expected deletion of the genomic *YISNF7* allele and a free pINA1355 plasmid (as shown by *E. coli* transformation), were tested for the stability of the Leu + marker. No loss of the replicative plasmid was observed among more than 600 clones tested, after 30 generations on non-selective medium, thus confirming that the *YISNF7* gene was essential for the viability of the cells during vegetative growth.

**YIVPS4 does not appear to be required for the Rim101-dependent pH response***

We wondered whether Vps4p (Babst et al., 1998), which acts downstream from the three ESCRT complexes and has been reported to interact with Rim20p (Bowers et al., 2004), was also involved in pH regulation in *Y. lipolytica*. A putative homologue (accession no. YALI0B16368g) of this gene was identified in the *Y. lipolytica* genome. The predicted 428 aa YlVps4p protein matches putative homologues in other species with the following identity scores: 77 % with *C. albicans* Vps4p (CA1340; http://genolist.pasteur.fr/CandidaDB/), 73 % with *S. cerevisiae* Vps4p, 67 % with *A. nidulans* hypothetical protein AN3061.2 (EAA63632.1), 63 % with *Schizosaccharomyces pombe* Spac-2G11.06 (CAA91171.1), 61 % with *H. sapiens* Vps4-2 (AAD3022.1) and 60 % with *Mus musculus* Skd1p (AAD47570). Like its homologues, YlVps4p displays the three-domain structure postulated for ScVps4p (Babst et al., 1998): the N-terminal domain with a putative coiled-coil motif (position 51–80), one central AAA domain containing the AAA-protein family signature (position 263–281), two consensus ATP-binding sites consisting of a Walker A box motif (position 172–179) and a version of the Walker B motif called the DEAD box (position 229–242) and the highly charged C-terminal domain.

To assess the effects of *YIVPS4* on pH response, the phenotype of the SBR114 strain, carrying the null mutation YlVps4-14, was established (see Methods). The effects of the *YIVPS4* deletion on the expression of alkaline-
acid-induced genes were assessed by real-time quantitative PCR, at pH 4·0 and 8·0 (Fig. 1). At alkaline pH, in YlVps4Δ-14, the transcript levels of XPR2, YIPHR1, YIRIM101 and YIPHR2 varied by a factor of less than two compared to the wild-type strain, less than observed in the rim13, vps23 and vps28 mutants (see above). At acidic pH, XPR2 and YIPHR1 showed significantly more expression than in these mutants (P < 0·01), whereas the level of expression of the acid-induced gene YIPHR2 was barely affected (less than twofold, 5 × 10^{-3}). These results indicate that the YlVps4Δ mutation affects the pH response much less severely than the Ylrim13, Ylvps23 and Ylvps28 mutations at alkaline pH, and affects the pH response in an opposite way with respect to alkaline-induced genes at acidic pH. These complex effects might partly relate to the slow growth of this mutant. Indeed, the deletion of YIVPS4 conferred a pronounced growth defect on cells grown on YPD medium at all pH values tested (Fig. 2b).

Next, we checked whether expression of the constitutively active, truncated form YIRIM101-1119 was able to suppress the defects of the YlVps4Δ-14 mutant, as it did for the rim13, vps23 and vps28 mutants. To address this question, we replaced the wild-type YIRIM101 allele by the most truncated allele, YIRIM101-1119 (Lambert et al., 1997), in the YlVps4Δ-14 mutant strain (see Methods). As shown in Fig. 2(b), expression of the YIRIM101-1119 allele did not rescue the growth defects of YlVps4Δ-14, at all pH values tested. At both alkaline and acidic pHs, the YIRIM101-1119 allele failed to restore the expression of the two alkaline-induced genes, XPR2 and PHR1, in the YlVps4Δ-14 mutant (Fig. 1a, b).

Vps4p exhibits an ATPase activity, which is required in S. cerevisiae for the dissociation of ESCRT complexes at the end of the MVB cycle (Babst et al., 1998), and has been proposed as an interactant of Rim20p (see Introduction). In order to check whether the growth defect of YlVps4Δ-14 was due to the loss of the ATPase activity or to the loss of the protein itself, we constructed the SY1364 strain (see Methods; Table 1) where a K172A mutation specifically destroys the ATP-binding site of Vps4p. Correct expression of the mutated allele, which is expected to display a dominant negative phenotype on endocytosis (Babst et al., 1997), was checked by its ability to block endocytosis in a YlVps4Δ-14 background, as checked by FM4-64 staining (data not shown). As shown in Fig. 2(b), the growth of the YlVps4Δ-K172A mutant was as severely affected as the growth of YlVps4Δ-14 at all pH values tested. Taken together, these results suggest that effects of the YlVps4Δ-14 mutation mostly result from an endocytic Rim-independent defect on cell physiology.

**YlVps mutants exhibit endocytic defects not suppressed by the C-terminal truncated allele of RIM101**

In order to confirm that the vps mutations indeed affected the MVB pathway in *Y. lipolytica*, the endocytic defects of YlVps4Δ-14, Ylvps23-U15 and Ylvps28-Y2 mutant strains were visualized with the fluorescent dye FM4-64, a lipophilic dye that binds to the plasma membrane, is internalized and then delivered to the vacuolar membrane (Vida & Emr, 1995). Cells were labelled with FM4-64 at 0 °C, then warmed to 18 °C and assessed for the distribution of FM4-64. By 20 min, FM4-64 could be seen on the vacuole limiting membrane in the wild-type cells. In contrast, in all the mutant strain cells, the bulk of FM4-64 remained in small compartments adjacent to the vacuole (Fig. 3). These structures are highly reminiscent of class E compartments, which accumulate proteins destined to the vacuole in class E vps mutants in *S. cerevisiae* (Rieder et al., 1996; Vida & Emr, 1995). When the constitutively active YIRIM101-1119 allele was expressed in YlVps4Δ-14, Ylvps23-U15 and Ylvps28-Y2 cells, the bulk of FM4-64 remained in small compartments adjacent to the vacuole (Fig. 3), as in the parental mutant strains. These results show that mutations in YlVPS4, YlVPS23, YlVPS28 indeed lead to a membrane-trafficking defect that, as expected, is independent of the status of the Rim pathway.

**DISCUSSION**

At the late endosome, the MVB pathway sorts endosomal proteins destined for the lumen of the vacuole (resident hydrolases for proper localization and endocytosed activated cell-surface proteins for downregulation) away from proteins destined for the limiting membrane of the vacuole or to be recycled back to the plasma membrane or Golgi complex. The MVB pathway involves several class E Vps factors, some of them organized in three distinct endosome-associated protein complexes called ESCRT complexes (Babst et al., 2002a, b; Bowers et al., 2004; Katzmann et al., 2001). Previous reports pointed toward possible interactions between components of the MVB pathway and of the Rim pathway devoted to pH sensing in ascomycetes. The interactions of Snf7p with Rim20p homologues are conserved across evolution from *S. cerevisiae* Rim20p to *A. nidulans* PaA and to human AIP1/Alix homologues (Bowers et al., 2004; Ito et al., 2001; Vincent et al., 2003).

Here we report on the characterization of YlVps mutations affecting the ESCRT-I complex of the MVB pathway in *Y. lipolytica*. We show that YlVps23 and YlVps28 mutations not only affect the endocytic pathway, as expected from the function of the proteins encoded by their homologues in *S. cerevisiae* and *C. albicans* (Rieder et al., 1996; Vida & Emr, 1995; Kullas et al., 2004; Cornet et al., to be published), but also that the mutant strains have a bona fide Rim phenotype, as described in these two other yeasts (Kullas et al., 2004; Xu et al., 2004; unpublished data). The proteins are required for growth at alkaline pH (Fig. 2), activation of alkaline-induced genes and repression of an acid-induced gene, like YIPHR2 (Fig. 1). All the defects in the alkaline response are suppressed at least partially by the constitutively active RIM101-1119 allele expressing a truncated form of the downstream transcriptional activator Rim101p. The suppressive effect of the truncated form of Rim101p suggests

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that these MVB components act upstream from Rim101p. In agreement with findings reported in \textit{S. cerevisiae} and \textit{C. albicans}, we showed that \textit{Ylvps4} does not appear to be required for activation of the Rim pathway in \textit{Y. lipolytica}. This suggests that part of the MVB pathway only is required for ambient pH signalling in all ascomycetous yeasts. An attractive hypothesis would be that early steps of endocytosis including formation, but not dissociation, of the ESCRT complexes mediate signalling by the postulated membrane receptors Rim21p/PalH and/or Rim9p/Pall (Peñalva & Arst, 2004), and lead to recruitment by Snf7p of the cytoplasmic Rim machinery (Kullas \textit{et al.}, 2004; Xu \textit{et al.}, 2004).

During this work, we observed that \textit{YlPHR2} behaved as expected for a bona fide acid-induced gene. \textit{YlPHR2} was derepressed at alkaline pH in \textit{YlRim13}, \textit{Ylvps23} or \textit{Ylvps28} mutant strains to identical levels, close to those observed at pH 4.0 in the wild-type strain (Fig. 1). It was repressed in these three backgrounds to similar levels at pH 4.0 upon expression of the artificially truncated form of \textit{YlRim101p}. This repression was incomplete in all cases, suggesting that our construct \textit{YlRim101p-1119p} does not perfectly mimic the physiologically active form of \textit{YlRim101p}, as previously reported (Gonzalez-Lopez \textit{et al.}, 2002; Lambert \textit{et al.}, 1997). These results show that, like \textit{C. albicans}, \textit{Y. lipolytica} expresses two isoforms of the surface proteins Phr1p/Phr2p with inverted pH-regulation patterns, both \textit{YlRim101p}-dependent (Muhlischlegel & Fonzi, 1997). A search in the promoters of \textit{YlPHR1} and \textit{YlPHR2} for GCCARG, the putative consensus binding site for \textit{YlRim101p} (Blanchin-Roland \textit{et al.}, 1994; Lambert \textit{et al.}, 1997), identified one such site in \textit{YlPHR1} (position −139 from ATG) and three in \textit{YlPHR2} (positions −302, −296 and −224 from ATG). Further work is needed to confirm the role of the putative \textit{YlRim101p}-binding sites identified so far in the promoters of these acid- and alkaline-induced genes.

Besides effects on endocytosis and alkaline-pH sensing, our results evidenced other effects associated with mutations in \textit{VPS} genes in \textit{Y. lipolytica}. \textit{Ylvps} mutants appear more sensitive to acidic and alkaline pH than \textit{Ylrim} mutants (Fig. 2), and the induction of an alkaline response, by a constitutively active form of the \textit{Rim101p} regulator, is more deleterious at acidic pH for \textit{Ylvps23} and \textit{Ylvps28} than for \textit{Ylrim} mutants (Fig. 2). This exacerbated defect could be explained by the fact that the growth of \textit{Ylvps} mutant strain was already affected at acidic pH, whereas the growth of \textit{Ylrim} mutants was not. These defects suggest that ambient pH might influence MVB pathway activity \textit{in vivo}, as has been suggested by Xu \textit{et al.} (2004).

A puzzling observation is that only two ESCRT-I component encoding genes were identified in our initial mutagenesis. This may seem surprising in view of the different \textit{VPS} genes reported in \textit{S. cerevisiae}, from the screening of a haploid deletion-strain library, as being required for growth at mild alkaline pH (Serrano \textit{et al.}, 2004), or functioning in the \textit{Rim101p} pathway (Barwell \textit{et al.}, 2005). This last exhaustive screening revealed the genes already shown to be involved in \textit{Rim101p} processing (Xu \textit{et al.}, 2004), as
encoding components of either the ESCRT-I (Vps23, Vps28, Vps37), the ESCRT-II (Vps22, Vps25, Vps36) or the ESCRT-III A sub-complex (Vps20, Vps32). Although our screen was not exhaustive, it identified most (i.e. five out of six) of the RIM genes, but only two out of the eight VPS genes, so far identified in S. cerevisiae as affecting Rim101p processing. It is even more surprising to observe that no vps mutant at all was identified in A. nidulans, in exhaustive screens for defects in pH signalling (Peñaalva & Arst, 2004). Recent reports suggest that fungi do have homologues of the VPS genes (Read & Kalkman, 2003). The fact that YISNF7 was found to be essential, the YlVps4Δ mutant strain grows very poorly, and YlVps23 and YlVps28 mutations mildly affect growth at all pH conditions tested (Fig. 2), even at pH 7-0, may offer a general clue to this apparent paradox. A likely hypothesis is that most, if not all, VPS genes are essential, or are required to avoid major growth defects in A. nidulans and to a lesser extent in Y. lipolytica. In S. cerevisiae, although vps mutant strains were reported to be viable, a careful analysis shows that many, including vps23 and snf7, may present severe growth defects on several types of media, including complete medium, after 20 or more generations (Giaever et al., 2002; see also http://www.yeastgenome.org/). In C. albicans, the growth of the snf7Δ/snf7Δ mutant is significantly reduced on complete medium and drastically impaired at alkaline pH (Kullas et al., 2004). Since pH signalling is not an essential function in fungi under most circumstances, it might be that endocytosis is required for cellular growth.

In Y. lipolytica, transition from unicellular yeast-type growth to filamentous hyphal growth is not pH dependent, contrary to what is observed in C. albicans (Fonzi, 2002). We notice that all YlVps mutants tested show a strong defect in hyphal formation not suppressed by expression of the truncated, active form of YlRim101p, contrary to Yrim mutants, which display normal morphogenesis at all pH conditions tested (Gonzalez-Lopez et al., 2002; data not shown). In contrast to this, all C. albicans rim or vps mutant strains present defects in hyphal formation and these defects are either totally, in Carim mutants, or at least partially, in Cavps mutants, bypassed by the expression of the constitutive form of CaRim101p (Kullas et al., 2004; Xu et al., 2004). Taken together, these results suggest that VPS genes might be required directly for hyphal growth in Y. lipolytica and probably also in other fungi, but that this function has been taken over by the Rim pathway in C. albicans. This may further explain why VPS genes were not identified during hunts for mutant strains defective in pH response in A. nidulans. Further work is thus needed to assess the involvement of VPS genes in pH sensing and control of cellular morphology in diverse fungal species.

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