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A conserved function for Inp2 in peroxisome inheritance

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ABSTRACT

In budding yeast *Saccharomyces cerevisiae*, the peroxisomal protein Inp2 is required for inheritance of peroxisomes to the bud, by connecting the organelles to the motor protein Myo2 and the actin cytoskeleton. Recent data suggested that the function of Inp2 may not be conserved in other yeast species. Using *in silico* analyses we have identified a weakly conserved Inp2-related protein in 18 species of budding yeast and analyzed the role of the identified protein in the methylotrophic yeast *Hansenula polymorpha* in peroxisome inheritance. Our data show that *H. polymorpha* Inp2 locates to peroxisomes, interacts with Myo2, and is essential for peroxisome inheritance.

1. Introduction

Peroxisomes are ubiquitous organelles and component of various eukaryotes. The organelles display a wide range of functions that varies with the cell/organism in which they occur, their developmental stage and environmental conditions [1]. Key functions of peroxisomes are hydrogen peroxide metabolism and oxidation of fatty acids [1,2].

In yeast species, peroxisomes are predominantly involved in the metabolism of various unusual carbon sources, i.e. fatty acids, alkanes, methanol, purines and D-amino acids. Cultivation of these organisms on either one of these compounds is associated with proliferation of peroxisomes that are crucial for growth, as they contain the key enzymes involved in the metabolism of these carbon sources [3]. In wild type (WT) *Saccharomyces cerevisiae* and *Hansenula polymorpha* cells, peroxisomes have been shown to predominantly multiply by fission from pre-existing organelles [4,5]. However, the organelles may also form *de novo* from the endoplasmic reticulum (ER) at conditions wherein the cells lack peroxisomes, e.g. due to a failure in inheritance [6]. During normal growth of WT yeast cells at peroxisome-inducing conditions, *de novo* synthesis most likely does not contribute significantly to the total organelle population [4,5], although exceptions may occur i.e. in *Yarrowia lipolytica* [7]. In contrast, in plants and mammals, *de novo* peroxisome formation appears to be a more prominent process [8,9]. As during vegetative reproduction of WT yeast cells new peroxisomes derive by fission, the organelle population must be contained during multiple rounds of budding. Upon division, part of the organelle population is administered to the bud. In the methylotrophic yeast *H. polymorpha*, this is accompanied by asymmetrical peroxisome fission and subsequent migration of the newly formed, small organelle to the developing bud. The number of organelles migrating to the bud is dependent on the cultivation conditions [10].

In *S. cerevisiae*, peroxisome inheritance requires the function of Inp1, Inp2, the class V myosin motor Myo2 and the actin cytoskeleton [11–13]. Recently, a function in inheritance was also attributed to Pex3 [14] and in *H. polymorpha* to Pex19 [6] and Pex11 [15]. *H. polymorpha* and *S. cerevisiae* cells lacking Pex3 or Pex19 are devoid of any peroxisomal remnants [16–18]. In contrast, overproduction of Pex3 leads to a dramatic increase in peroxisomal structures [19]. Of the proteins involved in inheritance, Inp1 is directly involved in organelle retention in the mother cell, whereas the integral membrane protein Inp2 attaches the globular tail of Myo2 to the peroxisome to enable its transport to the bud. In a recent study, Chang et al. [20] suggested that Inp2 is unique for *S. cerevisiae* and most likely not present in other organisms. These authors demonstrated that in *Y. lipolytica* a Pex3 paralog, designated Pex3B [21], played a crucial role in organelle inheritance through direct interaction with the Myo2 globular tail. A similar role was suggested for Pex3 in *Y. lipolytica* and *S. cerevisiae*, implying that Pex3 family members may be more relevant in organelle inheritance than Inp2.

This led us to investigate whether in *H. polymorpha* a similar mechanism is responsible for peroxisome inheritance. Our data show that this organism contains a *bona fide* Inp2 homolog that locates to

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peroxisomes, interacts with Myo2, and is essential for peroxisome inheritance.

2. Materials and methods

2.1. Strains and growth conditions

Yeast strains used in this study are listed in Table 1. Plasmids and primers are listed in Supplementary tables 1 and 2, respectively. Yeast cultures were grown at 37 °C on (i) YPD media containing 1% yeast extract, 1% peptone and 1% glucose, (ii) selective media containing 0.67 % yeast nitrogen base without amino acids, supplemented with 1% glucose (YND) or 0.5% methanol (YNM), or (iii) mineral media (MM) [22] supplemented with 0.5% glucose or 0.5% methanol as carbon sources and 0.25% ammonium sulphate as a nitrogen source. When required, amino acids and nucleotides were added to a final concentration of 20 μg/ml (histidine and adenine) or 30 μg/ml (leucine, lysine, and uracil). For growth on agar plates the medium was solidified with 2% agar. For the selection of dominant markers, YPD plates containing 100 μg/ml nourseothricin or zeocin (Invitrogen, Breda, The Netherlands) were used. For cloning purposes *Escherichia coli* DH5α was used. *E. coli* cells were grown at 37 °C in LB media supplemented with 50 μg/ml kanamycin or 100 μg/ml ampicillin, when required.

2.2. Molecular techniques

Standard recombinant DNA techniques were carried out according to Sambrook et al [23]. Transformation of *H. polymorpha* cells [24] and site specific integration in the *H. polymorpha* genome [24] were performed as described. DNA modifying enzymes were used as recommended by the suppliers (Fermentas, St. Leon-Rot, Germany and Roche Diagnostics, Mannheim, Germany). *Pwo* polymerase was used for polymerase chain reactions (PCR). Oligonucleotides were synthesized by MWG Operon (Ebersberg, Germany). For DNA sequence analysis, the Clone Manager 5 program (Scientific and Educational Software, Durham, USA) was used.

2.3. Construction of an H. polymorpha INP2 null mutant

An *inp2* deletion strain was constructed by replacing the genomic region of *INP2* comprising nucleotides +1 to +1914 by the *H. polymorpha URA3* marker as follows: first, a 630 bp 5′ flanking fragment and a 690 bp 3′flanking fragment of *INP2* were amplified from *H. polymorpha* WT genomic DNA using the primer combinations INP2-5UTR-FW + INP2-5UTR-RV and INP2-3UTR-FW + INP2-3UTR-RV, respectively. The fragments were digested with Asp718I + SalI and SpeI + NotI, respectively, and cloned into pBluescript II SK+ digested with the same enzyme combinations, yielding pBS-INP2-5UTR and pBS-INP2-3UTR, respectively. Subsequently, the 663 bp Asp718I–BamHI fragment of pBS-INP2-5UTR was cloned into Asp718I + BamHI-digested pBS-INP2-3UTR, yielding pBS-INP2-5&3UTRs. Finally, a 1573 bp SalI–SpeI fragment from plasmid pSK81 containing *H. polymorpha URA3* was inserted between the SalI and SpeI sites of pBS-INP2-5&3UTRs, yielding pBS-INP2-del-ODC1.

For deletion of *INP2*, the deletion cassette was excised from pBS-INP2-del-ODC1 by Asp718I + NotI-digestion and transformed into *H. polymorpha* NCYC495 *leu1.1 ura3* cells. Uracil-prototrophic transformants were selected. Correct deletion was confirmed by PCR and Southern blot analysis (data not shown) and the resulting strain was designated as *inp2*. For visualization of peroxisomes, SphI-linearized plasmid pHIPZ4-GFP-SKL, allowing production of the peroxisome-located GFP•SKL protein, was integrated at the P_{AOX} region of the *H. polymorpha inp2* genome.

2.4. Localization of Inp2 in H. polymorpha

To determine the subcellular location of Inp2 in *H. polymorpha*, a plasmid was constructed carrying an in-frame *INP2•mGFP* fusion gene under the control of the endogenous *INP2* promoter. First, a 746 bp fragment comprising mGFP was obtained by PCR with primers mGFP-fw and mGFP-rev using plasmid pCGCN-FAA4 as template. The PCR product was digested with BglII and SalI and cloned between the BglII and SalI sites of plasmid pANL31 resulting in pSNA10. Subsequently, an 805 bp fragment containing the *INP2* gene lacking a stop codon was amplified with primers Inp2-GFPforward and Inp2-GFPreverse using *H. polymorpha* WT genomic DNA as template. The PCR product was digested with BamHI and cloned into plasmid pSNA10, digested with HindIII (blunted by Klenow treatment) and BglII. This resulted in plasmid pSNA11.

To simultaneously mark peroxisomes with a red fluorescent marker protein, SacII-linearized plasmid pSNA03, producing peroxisome-located DsRed•SKL protein, was transformed into *H. polymorpha* NCYC495 *leu1.1 ura3* cells. Uracil-prototrophic transformants were selected. Correct integration at the *H. polymorpha AOX* locus was confirmed by PCR and fluorescence (data not shown). This yielded strain *H. polymorpha* DsRed•SKL. Subsequently, plasmid pSNA11 was linearized with ApaI and integrated at the *INP2* locus of the genome of *H. polymorpha* DsRed•SKL cells. Zeocin-resistant transformants were selected. Correct integration was confirmed by PCR (data not shown).

2.5. Yeast 2-hybrid analysis

The LexA system was used for screening interactions between *H. polymorpha* proteins using derivatives of the reporter strain *S. cerevisiae* L-40.

For *INP2* and *MYO2*, a 1935 bp DNA fragment comprising the entire *INP2* coding sequence (CDS; GenBank accession number GU591963) and a 1379 bp DNA fragment encoding the C-terminal globular domain of *H. polymorpha* Myo2 (aa 1083 to 1535; GenBank accession number GU591964) were amplified with primer combinations RSAinp2fw-bamhi + RSAinp2revecori and RSAmyo2fwbamhi + RSAmyo2revecori, respectively, using genomic *H. polymorpha* WT DNA as template. PCR fragments were digested with BamHI and EcoRI and inserted between the BamHI and EcoRI sites of the vectors pBTM116-C and pVP16-C, respectively. This yielded plasmids pBTM116-INP2, pVP16-INP2, pBTM116-MYO2 and pVP16-MYO2, respectively.

For *PEX19*, a DNA fragment of 887 bp, comprising the entire CDS of the gene, was obtained by PCR with primers RB16 and RB17 using genomic *H. polymorpha* WT DNA as template, digested with BamHI

Table 1
Yeast strains used in this study.

Strain	Description	Source/ Reference
H. polymorpha		
NCYC495	Wild type, *ura3 leu1.1*	[34]
HF246	NCYC495 with one copy integration of plasmid pHI-GFP-SKL, *leu1.1*	[35]
inp2	NCYC495 with deletion of *INP2* deletion, *leu1.1*	This study
inp2 GFP•SKL	*inp2* with integration of plasmid pHIPZ4-GFP-SKL, zeo^R	This study
DsRed•SKL	NCYC495 with integration of plasmid pSNA03, *leu1.1*	This study
DsRed•SKL INP2•GFP	DsRed•SKL with integration of plasmid pSNA11, *leu1.1* zeo^R	This study
S. cerevisiae		
L-40	MATa *leu2 his3 trp1 ade2 GAL4 gal80 LYS2::(lexAop)$_4$-HIS3 URA3::(lexAop)$_s$-lacZ*	Clontech
L-40-*Hp*PEX19	*L-40* with integration of plasmid pEXP-Met25-PEX19-Tcyc, nat^R	This study

nat^R, nourseothricin resistant; zeo^R, zeocin resistant.