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Obstruction of polyubiquitination affects PTS1 peroxisomal matrix protein import

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Abstract

Pex4p is an ubiquitin-conjugating enzyme that functions at a late stage of peroxisomal matrix protein import. Here we show that in the methylotrophic yeast *Hansenula polymorpha* production of a mutant form of ubiquitin (Ub^{K48R}) has a dramatic effect on PTS1 matrix protein import. This effect was not observed in cells lacking Pex4p, in which the peroxisome biogenesis defect was largely suppressed. These findings provide the first indication that the function of Pex4p in matrix protein import involves polyubiquitination. We also demonstrate that the production of Ub^{K48R} in *H. polymorpha* results in enhanced Pex5p degradation. A similar observation was made in cells in which the *PEX4* gene was deleted. We demonstrate that in both strains Pex5p degradation was due to ubiquitination and subsequent degradation by the proteasome. This process appeared to be dependent on a conserved lysine residue in the N-terminus of Pex5p (Lys21) and was prevented in a $Pex5p^{K21R}$ mutant. We speculate that the degradation of Pex5p by the proteasome is important to remove receptor molecules that are stuck at a late stage of the Pex5p-mediated protein import pathway.
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Keywords: Peroxisome; Protein translocation; PTS1 protein import; Ubiquitin; Yeast; *Hansenula polymorpha*

1. Introduction

Peroxisomal matrix proteins are synthesized in the cytosol and post-translationally translocated across the peroxisomal membrane. Most matrix proteins are sorted via a peroxisomal targeting signal type 1 (PTS1), which is present at the extreme C-terminus of the protein and consists of the tripeptide–Ser–Lys–Leu–COOH or a conserved variant thereof (reviewed by [1]). The PTS1 protein sorting process starts with recognition of newly synthesized PTS1 proteins by the PTS1 receptor Pex5p in the cytosol. This receptor–cargo complex is thought to associate with the peroxisomal membrane by binding to a proteinaceous docking machinery that consists of three interacting proteins, the peroxins Pex13p, Pex14p and Pex17p [1]. A putative translocation complex, comprising three RING-finger-containing peroxisomal membrane proteins (Pex2p, Pex10p and Pex12p), appears to be closely connected to the docking complex and is supposed to play a role in the translocation process. Both complexes are held tightly together via protein–protein interactions presumably via the peroxins Pex3p or Pex8p [2,3]. Recent evidence suggest that the entire receptor–cargo complex may actually be translocated across the peroxisomal membrane ("the extended shuttle model" of import [4]). It is believed that inside the organellar matrix Pex5p interacts with Pex8p, resulting in the dissociation of the cargo from the receptor [5,6]. The final step of the PTS1 protein import pathway is the recycling of Pex5p to the cytosol to enable a new import cycle. Peroxins thought to be involved in this stage of the process [7] are two AAA-type ATPases, Pex1p and Pex6p, and Pex4p, an ubiquitin-conjugating enzyme [8–10] that is anchored to the peroxisomal membrane via the peroxisomal membrane protein Pex22p [11].

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Ubiquitin-conjugating enzymes are involved in the modification of protein substrates by the covalent binding of ubiquitin (Ub), a peptide of 76 amino acids. Ub-conjugation involves three consecutive steps. In the first step Ub is activated in an ATP-dependent way by an Ub-activating enzyme, also designated E1. This enzyme transfers the activated Ub to an Ub-conjugating enzyme (E2), which finally acts in conjunction with an accessory Ub ligase (E3) that is involved in the recognition of the target protein (reviewed in [12,13]). This results in the formation of an isopeptide bond between the C-terminal glycine residue of Ub and the ε-amine group of a lysine residue in the substrate molecule. Often poly-Ub chains become conjugated to a substrate protein. Such chains are elongated by subsequent conjugation of additional Ub molecules to an internal lysine residue (mostly Lys48, occasionally another lysine residue of Ub [13]) in the previous Ub moiety that was already attached to the substrate protein.

Ubiquitination plays a role in several biological processes (e.g. the cell cycle, endocytosis, transcription, DNA repair, proteasomal degradation; see [13] and references therein). In some of these processes only a single Ub molecule is conjugated to a substrate molecule, whereas other functions involve polyubiquitination.

As yet it is still an enigma whether the sole E2 enzyme involved in peroxisome biogenesis, Pex4p, mono- or polyubiquitinates its substrate. The data presented in this paper provide the first indication that in the methylotrophic yeast *Hansenula polymorpha* the function of Pex4p in PTS1 protein import involves polyubiquitination. Additionally, also evidence is provided that defects in Pex4p functioning result in Pex5p degradation, a process that appears to involve a Pex4p-independent ubiquitination of Pex5p. We propose that the physiological function of this process is the removal of non-functional Pex5p molecules from the peroxisomal translocation machinery.

2. Materials and methods

2.1. Organisms and growth conditions

The *H. polymorpha* strains used in this study are all derivatives of NCYC495 and are listed in Table 1. The *pex4 pex5* double mutant was obtained by crossing the *pex4* (*leu1.1*) and *pex5* (*ura3*) mutants according to Gleeson and Sudbery [14]. Diploids were subjected to random spore analysis, and prototrophic segregants were subjected to complementation analysis to determine their genotypes.

Yeast cells were cultivated in batch cultures at 37 °C on (i) rich media, containing 1% yeast extract, 1% peptone and 1% glucose (YPD), (ii) selective minimal media, containing 0.67% Yeast Nitrogen Base without amino acids (DIFCO) supplemented with 1% glucose, or (iii) minimal medium [15], using glucose (0.5%), glycerol (0.5%), glycerol/methanol (0.1%+0.5%, respectively) or methanol (0.5%) as carbon sources, and methylamine (0.25%) or ammonium sulfate (0.25%) as nitrogen sources. When required, amino acids or uracil was added to a final concentration of 30 μg/ml. For growth on agar plates the media were supplemented with 1.5% agar. For the selection of zeocin-resistant transformants YPD plates containing 100 μg/ml zeocin (Invitrogen) were used.

Table 1
H. polymorpha strains used in this study

Strains	Characteristics	Reference
NCYC495 (*leu1.1*)	Wild type	[14]
NCYC495	Wild type, prototrophic	[14]
pex3 (*leu1.1*)	NCYC495 with deletion in *PEX3*	[34]
pex4 (*leu1.1*)	NCYC495 with deletion in *PEX4*	[10]
pex5 (*leu1.1*)	NCYC495 with deletion in *PEX5*	[33]
pex5 (*ura3*)	NCYC495 with deletion in *PEX5*	[35]
pex7 (*leu1.1*)	NCYC495 with deletion in *PEX7*	A. Koek et al., unpublished
pex8 (*leu1.1*)	NCYC495 with deletion in *PEX8*	R.J.S. Baerends et al., unpublished
pex4 pex5	Segregant of cross between *pex5* (*ura3*) and *pex4* (*leu1.1*)	This study
Ub.K48R	NCYC495 (*leu1.1*) with 2 copies of plasmid pX4-Ub.K48R integrated	This study
Myc-Ub.K48R	NCYC495 with 2 copies of plasmid pZ15-MycUb.K48R integrated	This study
pex3-Ub.K48R	*pex3* (*leu1.1*) with 2 copies of plasmid pX4-Ub.K48R integrated	This study
pex7-Ub.K48R	*pex7* (*leu1.1*) with 2 copies of plasmid pX4-Ub.K48R integrated	This study
pex8-Ub.K48R	*pex8* (*leu1.1*) with 2 copies of plasmid pX4-Ub.K48R integrated	This study
pex4-MycUb	*pex4* (*leu1.1*) with multiple copies of plasmid pX4-MycUb integrated	This study
PEX5.K21R	*pex5* (*leu1.1*) with 1 copy of plasmid pX12-PEX5.K21R integrated	This study
pex4 PEX5.K21R	*pex4 pex5* with 1 copy of plasmid pZ12-PEX5.K21R integrated	This study
PEX5.K21R MycUb.K48R	PEX5.K21R with 2 copies of plasmid pZ15-MycUb.K48R integrated	This study
pex4::P_{AOX}*PEX5*^{4c}	*pex4* overexpressing the *PEX5* gene under control of the *AOX* promoter	[10]
pex4::P_{AOX}*PEX5* ::MycUb	*pex4*::P_{AOX}*PEX5*^{4c} with 2 copies of plasmid pZ15-MycUb integrated	This study
pex4::P_{AOX}*PEX5* ::MycUb.K48R	*pex4*::P_{AOX}*PEX5*^{4c} with 2 copies of plasmid pZ15-MycUb.K48R integrated	This study
Ub-Leu-β-gal	NCYC495 with one copy of plasmid pZ5-Ub-Leu-β-gal integrated	This study
Ub.K48R Ub-Leu-β-gal	Ub.K48R with one copy of plasmid pZ5-Ub-Leu-β-gal integrated	This study

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