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Identification of PEX33, a novel component of the peroxisomal docking complex in the filamentous fungus *Neurospora crassa*

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ABSTRACT

The docking complex of peroxisomal matrix protein import is composed of PEX13 and PEX14 in all species analyzed so far, whereas only yeast appears to possess an additional component, PEX17. In this report we isolated PEX14 complexes of *Neurospora crassa*. Among the complex constituents, one protein designated as PEX33 possessed homology to PEX14 but only in a short N-terminal domain. The PEX14/PEX33 interaction was verified by means of two-hybrid analysis. Moreover, PEX33 was shown to interact with itself and the PTS1-receptor PEX5. Localization studies demonstrated that PEX33 constitutes a glyoxysomal protein. Growth tests of the *pex33* deletion strain revealed a defect of this strain in the biogenesis of glyoxysomes and Woronin bodies. As the function of PEX33 was not redundant to that of PEX14, it is a genuine novel peroxin. Based on our experimental data, the function of PEX33 seems to resemble that of yeast PEX17 despite clear structural differences.

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Introduction

Peroxisomes are a class of structurally and functionally related organelles virtually found in all eukaryotic cells that also comprise glyoxysomes of plants and fungi, glycosomes of Trypanosomes, and Woronin bodies of filamentous fungi. With the exception of Woronin bodies, whose sole function is to plug septal pores in case of hyphal injury (Reichle and Alexander, 1965; Wergin, 1973; Würtz et al., 2009), microbodies fulfil a variety of biochemical functions; foremost of these is fatty acid β-oxidation (Poirier et al., 2006). A single organism may house different types of microbodies in a temporary manner. In plants, for instance, glyoxysomes transform during organism development to peroxisomes and back again in senescing tissue (Hayashi et al., 2000). In contrast to this finding, *Neurospora crassa* and several other filamentous ascomycetes are unique in that they usually possess two distinct types of microbodies within a single cell at the same time, glyoxysomes and Woronin bodies (Soundararajan et al., 2004; Wanner and Theimer, 1982).

The maintenance of peroxisomes depends on the formation of the peroxisomal membrane and the subsequent import of both membrane and matrix proteins (Schrader and Fahimi, 2007). Without exception, the peroxisomal matrix proteins are nuclear encoded, synthesized on free ribosomes and subsequently imported post-translationally (Lazarow and Fujiki, 1985). Two peroxisomal-targeting signals (PTSs), termed PTS1 and PTS2, account for the transport of most proteins into the peroxisomal matrix (Lanyon-Hogg et al., 2010). The PTS1 is located at the extreme C-terminus and recognized by the import receptor PEX5 (Brocard et al., 1994; Gould et al., 1989; Terlecky et al., 1995). In contrast, the PTS2 is present in the N-terminus of cargo proteins and bound by PEX7 (Lazarow, 2006; Rachubinski and Subramani, 1995; Rehling et al., 1996). According to the model of cycling receptors (Dodt and Gould, 1996; Marzioch et al., 1994), subsequent to cargo recognition in the cytosol, the receptor-cargo complex binds to the docking complex at the peroxisomal membrane. This complex is constituted by PEX13 and PEX14, to which PEX17 is added to in yeast (Brown and Baker, 2008). Recently, it was demonstrated that the PTS1-receptor PEX5 forms together with its docking component PEX14 a gated ion-conducting channel with striking dynamics, which can be opened to a diameter of about 9 nm by the soluble receptor-cargo complex (Meinecke et al., 2010). This pore most likely facilitates the entry of cargo proteins into the peroxisomal matrix where the receptor-cargo complex dissociates by a so far unknown mechanism (Ma et al., 2009; Meinecke et al., 2010). Until

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now PEX17 has only been identified in yeast and its actual function in the receptor docking is still enigmatic. Although PEX17 is part of the docking complex, it does not significantly contribute to the structural integrity of the PEX14/PEX5 sub-complex (Agne et al., 2003; Meinecke et al., 2010). Likewise, the role of PEX13 remains elusive. This peroxin is a major constituent of the docking complex and it is suggested that it serves as an initial binding site for the PTS2 pre-import complex (Grunau et al., 2009).

The final step in the receptor cycle is the release of the receptor from the peroxisomal membrane to the cytosol. With respect to PEX5, detailed *in vitro* studies revealed that its binding and translocation is ATP-independent while the export of PEX5 back to the cytosol requires ATP (Miyata and Fujiki, 2005; Oliveira et al., 2003). The corresponding ATPases were identified by *in vitro* systems in *Saccharomyces cerevisiae* (Platta et al., 2005) and human fibroblast cells (Miyata and Fujiki, 2005) as the peroxisomal AAA (ATPase associated with various cellular activities) ATPases PEX1 and PEX6. These act as the motor proteins for PEX5 export, a process for which it turned out that ubiquitination of the receptor is a prerequisite (Carvalho et al., 2007; Platta et al., 2007). Once released, the receptor either performs another round of import or is degraded by the proteasome.

In the last years, it became more and more obvious that the general mechanism of matrix protein import and thus the proteins involved are strongly conserved among species. However, there are some examples for seemingly species-specific components. PEX15 and PEX26 have only a low sequence similarity but nonetheless exhibit the same topology and fulfil the same function in yeast and man (Birschmann et al., 2003; Matsumoto et al., 2003). The PTS2 receptor PEX7 needs assistance by a protein with co-receptor function. In fungi like *Yarrowia lipolytica, Pichia pastoris*, *N. crassa*, or *S. cerevisiae* this function is performed by PEX20 or its functionally redundant orthologues PEX18 and PEX21 (Schliebs and Kunau, 2006). In contrast, humans and plants are lacking additional co-receptors but express a longer splice variant of the PTS1-receptor PEX5 which contains a PEX7 binding site, thereby fulfilling co-receptor function (Dodt et al., 2001; Matsumura et al., 2000). The ubiquitin-conjugating enzyme PEX4 and its membrane receptor PEX22 were identified in yeast and in plants, but not in mammals. There, this function is carried out by the cytosolic UbcH5a/b/c-family, which serves as the functional counterpart of yeast PEX4 (Grou et al., 2008). Whether a mammalian PEX22 ortholog exists is currently unclear. Finally, PEX17 as part of the docking complex in yeast appears to be absent from any other organism. Interestingly, an *in silico* approach predicted the existence in filamentous fungi of a chimeric protein consisting of an N-terminal PEX14-like domain and a C-terminal PEX17-like domain (Kiel et al., 2006).

Here we analyzed the composition of the docking complex of *N. crassa* and addressed whether it indeed contains components in addition to PEX14. A His-tagged version of PEX14 was purified by a two-step procedure and the co-purified components were identified by mass spectrometry. One interesting candidate protein was scrutinized for its role in glyoxysome (peroxisome) biogenesis and its possible interplay with PEX14. The protein's function will be discussed in terms of similarities and dissimilarities to yeast PEX17.

Materials and methods

Strains and culture conditions

N. crassa strains were obtained from the Fungal Genetics Stock Center (Kansas City, KS, USA). Strain St. Lawrence 74-OR8-1a (FGSC#988, mat a) was used as wild-type; strains FGSC#12728 (*pex33::hph*, mat a) and FGSC#11305 (*pex14::hph*, mat a) were generated in the course of the *Neurospora* gene Knockout Project (Colot et al., 2006); FGSC#6103 (*his-3* mat A) was used for the integration of pDM37 into the *his3* locus. The generation of the His_6-PEX14 overexpression strain was described previously (Managadze et al., 2007).

Manipulations of *N. crassa* hyphae were carried out according to standard techniques (Davies, 2000). Growth tests with various *N. crassa* strains were performed as described (Managadze et al., 2007). For growth in liquid culture, the wild-type strain was inoculated with 10^5 conidia/ml in VMM and shaken (100 rpm) at 30 °C for 24 h. The knock-out strains were inoculated with small pieces of mycelia. Glyoxysomes were induced by shifting the harvested mycelia to VMM containing 3 mM oleic acid and 0.1% (w/v) Tween-40 and shaken for an additional 12 h.

Two-hybrid analysis was carried out with strain PJ69-4a (*trp1-901, leu2-3, his3-200, gal4, gal80, LYS2::GAL1-HIS3*, and *GAL2-ADE2*) (James et al., 1996). Standard media for the cultivation of *S. cerevisiae* strains were prepared as described (Sambrook et al., 1989; Stein et al., 2002).

Plasmids and cloning procedures

All plasmids and oligonucleotides used are listed in Supplementary Table 2. Genomic DNA of *PEX33* (NCU01535) was amplified by PCR using DNA isolated from wild-type strain 74-OR8-1a as a template. The cDNA of *PEX13* (NCU02618), *PEX14* (NCU03901) and *PEX33* were amplified from a *N. crassa* cDNA library. All amplification products were cloned into the various plasmids using the restriction sites and primer pairs indicated in Supplementary Table 2. The FLAG-tag and the His6-tag were introduced using the appropriate oligonucleotides.

Generation and usage of antisera

Antibodies against *N. crassa* FOX2 (Thieringer and Kunau, 1991), HEX1 (Schliebs et al., 2006), PEX14 (Managadze et al., 2007), TIM23 (Mokranjac et al., 2003), *A. gossypii* ICL1 (Maeting et al., 1999), *S. cerevisiae* PEX14 (Albertini et al., 1997), and GFP (BD Biosciences, Franklin Lakes, NJ, USA) have been described. Recombinant highly purified PEX33 protein was used to generate antibodies through immunization of rabbits. *E. coli* was transformed with the expression vector pMSC88 for heterologous production of PEX33. After 4 h of induction with 1 mM IPTG at 37 °C, cells were solubilized in buffer containing 8 M urea. The lysate was loaded onto a Ni-NTA column and non-specific compounds were removed in a single wash step. Bound protein was eluted by a linear shift to acidic pH (4.0). Selected elution samples were pooled and used for antibody production (Pineda, Berlin, Germany).

SDS-PAGE and immuno-blotting were conducted according to standard protocols (Sambrook et al., 1989). The ECL™ system from GE Healthcare (München, Germany), in combination with horseradish peroxidase-coupled anti-rabbit and anti-mouse IgG, was used to visualize immunoreactive complexes.

Purification of the N. crassa PEX14 membrane complex

Purification of the *N. crassa* docking complex from a His_6-PEX14 overexpression strain was achieved by a two-step procedure. In the first step, His-tagged PEX14 was isolated from digitonin-solubilized membranes by affinity chromatography on a Ni-NTA column (Qiagen, Hilden, Germany) as described (Managadze et al., 2007). The resulting Ni-NTA eluate was further processed by a gel filtration step. For this, 0.5 ml of the eluate were loaded onto a Superdex200 10/300GL column (GE Healthcare, München, Germany) and run at a flow rate of 0.5 ml/min, using a buffer containing 20 mM Tris pH 8, 50 mM NaCl, and 0.1% digitonin. 44 fractions (0.5 ml each) were collected, precipitated by TCA, separated by SDS-PAGE and stained

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