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Identification of Six Loci in Which Mutations Partially Restore Peroxisome Biogenesis and/or Alleviate the Metabolic Defect of *pex2* Mutants in Podospora

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ABSTRACT

Peroxins (PEX) are proteins required for peroxisome biogenesis. Mutations in *PEX* genes cause lethal diseases in humans, metabolic defects in yeasts, and developmental disfunctions in plants and filamentous fungi. Here we describe the first large-scale screening for suppressors of a *pex* mutation. In *Podospora anserina*, *pex2* mutants exhibit a metabolic defect [inability to grow on medium containing oleic acid (OA medium) as sole carbon source] and a developmental defect (inability to differentiate asci in homozygous crosses). Sixty-three mutations able to restore growth of *pex2* mutants on OA medium have been analyzed. They fall in six loci (*suo1* to *suo6*) and act as dominant, allele-nonspecific suppressors. Most *suo* mutations have pleiotropic effects in a $pex2^+$ background: formation of unripe ascospores (all loci except *suo5* and *suo6*), impaired growth on OA medium (all loci except *suo4* and *suo6*), or sexual defects (*suo4*). Using immunofluorescence and GFP staining, we show that peroxisome biogenesis is partially restored along with a low level of ascus differentiation in *pex2* mutant strains carrying either the *suo5* or the *suo6* mutations. The data are discussed with respect to β-oxidation of fatty acids, peroxisome biogenesis, and cell differentiation.

PEROXISOMES are near-ubiquitous organelles characterized by the presence of oxidase(s) and catalase, respectively responsible for production and degradation of hydrogen peroxide (H_2O_2; de Duve and Baudhuin 1966). In most eukaryotes, peroxisomes are involved in β-oxidation of long-chain fatty acids but they also perform specialized functions. For instance, in mammals, they are implicated in a variety of pathways, such as biosynthesis of plasmalogens (membrane phospholipids), cholesterol, and bile acids (see van den Bosch *et al.* 1992 for a review). In some yeasts, they are required for assimilation of a number of growth substrates (see van der Klei and Veenhuis 1997 for a review). In the filamentous fungus *Penicillium chrysogenum*, the last step of penicillin biosynthesis occurs in peroxisomes (Müller *et al.* 1991). As demonstrated by peroxisome biogenesis disorders, the lack of functional peroxisomes is lethal in humans (see Gould and Valle 2000 for a recent review). In contrast, in unicellular yeasts, especially in *Saccharomyces cerevisiae* (Gurwitz *et al.* 1998) and *Pichia pastoris* (Waterham *et al.* 1996), it seems that lack of peroxisomes results only in metabolic defects. However, in plants and in some fungi, peroxisomes or some of the proteins required for peroxisome biogenesis [called peroxins (PEX); Distel *et al.* 1996] play key roles in specific developmental programs. In the plant *Arabidopsis thaliana*, mutations in the gene encoding PEX16 impair formation of storage organelles in maturing seeds (Lin *et al.* 1999). With respect to fungi, it was first reported that, in the filamentous fungus *Podospora anserina*, mutations in the *pex2* gene (formerly *car1*) cause a block at a specific stage of sexual development (Berteaux-Lecellier *et al.* 1995). It was then shown that several peroxins (including PEX2, PEX6, and PEX16) are involved in the dimorphic transition from yeast to mycelial forms in *Yarrowia lipolytica* (Titorenko *et al.* 1997). More recently, Kimura *et al.* (2001) reported that PEX6 is required in *Colletotrichum lagenarium* for proper development of the apressorium, a fungal structure involved in plant infection. Peroxisomes may also play a role in the development of trap cells in the nematophagous fungus *Arthrobotrys oligospora*: These specialized cells are filled with microbodies that differ in morphology from those present in vegetative cells (Veenhuis *et al.* 1984). It was also unexpected to discover that the Woronin body, described almost 150 years ago and necessary for maintenance of cellular integrity in filamentous ascomycetes, is a particular class of peroxisomes (Jedd and Chua 2000; Tenney *et al.* 2000). Overall, these recent data clearly demonstrate that the functions of peroxisomes remain poorly understood.

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Their precise roles in developmental pathways are still unclear.

PEX2 is a peroxisomal integral protein with a RING finger motif (C3HC4 zinc-binding domain) in its C-terminal region. It was the first peroxin found to be involved in a peroxisome biogenesis disorder (Shimozawa *et al.* 1992). Its first (nonmammalian) ortholog was serendipitously discovered in *P. anserina* among mutants impaired in sexual development (Berteaux-Lecellier *et al.* 1995). This was soon followed by characterization of its yeast orthologs in *P. pastoris* (Waterham *et al.* 1996) and *Y. lipolytica* (Eitzen *et al.* 1996), both identified among mutants impaired in peroxisome metabolism. However, the precise function of PEX2 in peroxisome biogenesis remains elusive. In *P. anserina*, two mutants were described: *pex2-1* and *pex2-3* (formerly *car1-1* and *car1-3*) carrying a nonsense and a missense mutation, respectively (Berteaux-Lecellier *et al.* 1995). Both strains exhibit the same four features. First, they are unable to grow on a medium containing a long-chain fatty acid (*e.g.*, oleic acid) as sole carbon source. Second, two staining procedures failed to reveal peroxisomes in these strains. Third, homozygous mutant × mutant crosses are sterile because the dikaryotic cells (which contain one copy of each parental nucleus after fertilization) are unable to differentiate into asci; instead, these cells continue to divide mitotically. Fourth, mutant ascospores (issued from heterozygous mutant × wild type crosses) are unripe and display a low germination rate (Berteaux-Lecellier *et al.* 1995).

To shed light on the role of peroxisomes (and/or PEX2 *per se*) in development, we used a powerful approach: a systematic search for suppressors of *pex2* mutant defects. Sixty-three extragenic suppressors were obtained with a positive selection procedure, *i.e.*, restoration of growth on oleic acid as sole carbon source. They act as dominant, allele-nonspecific suppressors and fall in six loci (*suo*). The *pex2 suo* and *pex2*$^+$ *suo* strains have been subjected to extensive analyses, combining genetic, physiological, and cytological approaches. The data obtained contribute to a better comprehension of the metabolic defect of the *pex2* mutants and identify two loci in which mutations can partially restore both peroxisome biogenesis and ascus differentiation in a *pex2* mutant background.

MATERIALS AND METHODS

***P. anserina* strains and media:** *P. anserina* is a filamentous ascomycete whose life cycle and general methods of genetic analysis have been described (Rizet and Engelmann 1949). All strains are derived from the wild-type S strain. Analyses of the *pex2-1* and *pex2-3* mutants (previously called *car1-1* and *car1-3*) have been reported (Berteaux-Lecellier *et al.* 1995). The culture and the spore germination media have been reviewed by these authors, especially the minimal synthetic medium containing either dextrin (0.5%, M2 medium) or oleic acid (0.05% plus 0.2% Tween 40, OA medium) as carbon sources. In this study, we also used media in which dextrin was replaced by maltose (0.5%, M medium), by lauric acid (0.02% plus 0.2% Tween 40, L medium) or by both carbon sources (ML medium). The effect of 3-amino-1,2,4,-triazol (3-AT) was tested in the range of 10–50 mm in the M and ML media supplemented with 20 μg/ml histidine (AT medium).

Isolation of *pex2* revertants: Initially, we sought to obtain mutations able to restore ascospore formation in strains homokaryotic for the *pex2-1* or *pex2-3* mutations and heterokaryotic for mating-type information and thus able to self-fertilize. The presence of the *pex2* mutations leads to barren perithecia (fruiting bodies) in which no ascospores are formed due to a block at the dikaryotic stage. Four hundred thalli homokaryotic for *pex2-1* and 300 thalli homokarytoic for *pex2-3* were subjected to ultraviolet mutageneses (300–900 J/m^2). Each thallus produced >1000 perithecia, which all remained barren. Very seldom, green ascospores were recovered, which either did not germinate or yielded strains that gave barren perithecia when crossed with a *pex2* mutant strain. This extremely sparse production of mutant ascospores was due to a very low leakiness of the *pex2* mutants. We then sought revertants of the *pex2* mutants, as growing sectors on OA medium after ultraviolet mutagenesis (300 J/m^2). One hundred thalli of *pex2-1* and 170 thalli of *pex2-3* were subjected to this procedure, giving rise, respectively, to 54 and 99 independent growing sectors on OA medium (only 1 sector per culture was collected to ensure the independent origin of the revertants). Thirty-two *pex2-1* and 42 *pex2-3* revertants were then analyzed. Extragenic suppressors were called *suo* (*su*ppressors on *o*leic acid).

Complementation and recombination tests between *suo* mutants: Isolation of *pex2*$^+$ *suo* strains (through crosses of the revertants with the wild-type strain) revealed that some of the *suo* mutations led to recessive phenotypic defects. Crosses between these *suo* strains were thus performed to obtain complementation and recombination data. In *P. anserina*, uninucleate and binucleate ascospores are formed after meiosis, allowing the recovery of homokaryotic and heterokaryotic strains after germination. Examination of uninucleate ascospores can reveal recombination while examination of binucleate ascospores may reveal either complementation or recombination between the two *suo* mutations involved in a cross. Therefore, we performed crosses between strains bearing *suo* mutations, which lead to green ascospores. When a cross gave only green (mutant) ascospores, we concluded that neither complementation nor recombination occurred. If, in addition to green ascospores, uninucleate black (*suo*$^+$) ascospores were recovered, we concluded that recombination had occurred. Last, when dikaryotic black ascospores were recovered, analysis of their progeny allowed us to determine if their wild-type phenotype was due to recombination (presence of a *suo*$^+$ nucleus) or complementation. With *suo* strains displaying no phenotypic defects, recombination tests required the presence of a *pex2* mutation in either one of the two partners. We thus performed crosses between *pex2* mutant strains bearing a given *suo* mutation and *pex2*$^+$ strains bearing another *suo* mutation and sought *pex2 suo*$^+$ strains, issued from green ascospores and unable to grow on OA medium. In one case, we used a simpler procedure since the *suo1* mutations led to green ascospores while the *suo5* mutant exhibited a slow growth rate on OA medium. Crosses between *suo1-11* and *suo5-17* yielded green ascospores (*suo1-11*), of which one-half appeared to be double mutant (poor growth on oleic acid), and black ascospores (*suo1*$^+$) of which one-half appeared to be wild type (normal growth on OA medium). These data demonstrated that the *suo1* and *suo5* loci were genetically independent. In most analyses (except when specified in the text), one mutation of each locus was used, respectively *suo1-11*, *suo2-9*, *suo3-1* (the only *suo3* mutant available), *suo4-1*, and *suo5-17*

(the only *suo5* mutant available), except for the *suo6* locus of which the two mutations were systematically studied (*i.e*, *suo6-11* and *suo6-20*).

Construction of a peroxisome-targeted green fluorescent protein: Initially, a sequence encoding the SKL tripeptide (the peroxisomal targeting signal 1) was added at the end of the green fluorescent protein (*gfp*) open reading frame by PCR. The *gfp* gene from the pEGFP-1 vector (CLONTECH, Palo Alto, CA) was amplified with a primer located just upstream from the *gfp* start codon (5′-CTGCAGTCGACGGTACCGCG GGCC-3′) and with a 3′ primer (5′-GCATGGACGAGCTGTA CAAGAGCAAGCTCTAAGGTACCTAGAGCTCGCCC-3′) encompassing the 3′ end of the *gfp* open reading frame. In the latter, the sequence encoding the SKL tripeptide was inserted before the stop codon, which is followed by a sequence including a *Sac*I restriction site. The amplified fragment was cut by *Nco*I and *Sac*I and cloned in the pCBGPAH1 plasmid (Arnaise *et al.* 2001), also digested by *Nco*I and *Sac*I. The plasmid pCBGPAH1 is a PUC18 plasmid containing the strong *P. anserina gpd* promoter (Ridder and Osiewacz 1992) in fusion with the *pah1* gene. Thus the resulting plasmid consists of a pGPD::GFP-SKL fusion in a PUC18 context. Second, a *Eco*RI/*Hind*III 1-kb fragment containing the phleomycin resistance gene (*ble*) from pPaBle (Coppin and Debuchy 2000) was introduced in the vector. The pGPD::GFP-SKL vector was digested by *Sac*I and *Pst*I and both vector and fragment were blunt ended before ligation. The resulting plasmid is completely devoid of the *pah1* gene sequence. The pGPD::GFP-SKL transformant was submitted to genetic analysis. This allowed us to conclude that there was a single integration site. The copy number of the construct was not checked by Southern blotting. We inferred that it was adequate from its analysis in wild-type and *pex2* mutant backgrounds: In the first case, the labeling was limited to microbodies while, in the second case, it was cytosolic.

Cytological analyses: Processing of cells for immunofluorescence and meiocyte staining were described previously (Berteaux-Lecellier *et al.* 1995). Strains expressing GFP-SKL were observed with a Zeiss Axioplan photomicroscope. Fluorescence images were captured by a CCD Princeton camera system.

Enzyme assays: Crude extracts were obtained as follows: Mycelia were harvested from liquid cultures after 36 hr of growth and crushed in liquid nitrogen. The resulting powder was suspended in homogenization buffer (10 mm Tris pH 7.5/1 mm EDTA/76 mm glycin). After centrifugation at 11,000 rpm at 4° for 10 min, the supernatant was kept on ice. Acyl-CoA oxidase activity was determined as described (Vamecq 1990) with a Kontron spectrofluorimeter.

RESULTS

The extragenic suppressors of *pex2* mutations cause phenotypic defects in a *pex2*$^+$ context: Two screening procedures could be used to obtain mutations able to alleviate the *pex2* mutant defects: first, a search for mutations restoring ascospore production in perithecia (fruiting bodies) homozygous for a *pex2* mutation and second, a search for mutations restoring growth of the *pex2* mutants on a medium with oleic acid as sole carbon source (OA medium). Although extensively brought into play, the first strategy has so far failed (see materials and methods). In contrast, many *pex2* revertants have been obtained with the metabolic screen (materials and methods; Table 1). Initially, these strains were crossed to wild type. Intragenic suppressors (or back mutations) were expected to produce homogeneous progeny exhibiting a wild-type phenotype. This result was obtained for 11 *pex2-1* revertants. Sequencing of the *pex2* gene from these strains revealed that all mutations occurred in the stop codon, restoring a sense codon (Berteaux-Lecellier *et al.* 1995). The presence of extragenic suppressors was revealed by the recovery of *pex2* mutant ascospores in the progeny of revertants × wild-type crosses. These crosses also yielded ascospores bearing the suppressor mutations (*suo*) in a *pex2*$^+$ context.

Analyses of these *pex2*$^+$ *suo* strains revealed that most of them displayed phenotypic defects (Table 1). All mutants appeared recessive with respect to these defects. This offered the opportunity to perform complementation, which, along with recombination tests, allowed the definition of six loci called *suo1* to *suo6* (see next section). The mutants belonging to the *suo1*, *suo2*, *suo3*, and *suo5* loci and the *suo1 suo6* double-mutant strains showed a more or less pronounced defect when grown on OA medium (Figure 1, A and B). Mutants of the *suo4* gene grew normally on OA medium but all, except *suo4-32*, showed a sexual defect in homozygous crosses (Table 1). Homozygous crosses for *suo1*, *suo2*, and *suo3* produced green (unripe) ascospores. Mutant ascospores obtained from heterozygous crosses involving *suo4* mutations (with the exception of *suo4-30* and *suo4-32*) also exhibited unripe ascospores (Table 1). All green ascospores showed a reduced rate of germination as compared to wild-type, black ascospores (Table 2). Crosses of these mutants to wild type demonstrated, interestingly, that most exhibited a nonautonomous expression. This feature is exemplified by the *suo3-1* mutant, which, in homozygous crosses, yields green ascospores unable to germinate while, in heterozygous crosses, mutant ascospores are black and have a 100% germination rate (Table 2). Ascus analysis nonetheless confirmed that the *suo3-1*/*suo3*$^+$ alleles segregated 2:2 in heterozygous crosses. The nonautonomous expression of the other mutants was observed only for the germination rates. These rates increased (in homozygous *vs.* heterozygous crosses) from 0 to 25% and 0 to 12% for the *suo1* and *suo4* mutants, respectively (Table 2). The data suggest that a substance, under the control of the wild-type alleles of these *suo* genes, is able to diffuse inside the asci, either before or after ascospore formation, and acts in mutant ascospores during their maturation. In contrast, the *suo2* mutant tested exhibits an autonomous expression.

The *suo* mutations fall in six loci and are not allele-specific suppressors: First, on the basis of production of green (*vs.* black) ascospores and sexual defects (barren perithecia) in homozygous crosses, complementation tests defined four groups, *suo1* to *suo4*. Sexual defects were observed only in the *suo4* group (Table 1). Second, growth on OA medium of the relevant heterokaryotic

TABLE 1

Origin and phenotypic properties of the *pex2* suppressors

	Origin		Phenotypic properties of the $pex2^+$ *suo* strains		
Revertants[a]	*pex2-1*	*pex2-3*	Ascospores[b]	Growth on OA[c]	Sexual defect
Intragenic suppressors	11	0	—	—	—
Extragenic suppressors	21	42	—	—	—
suo1	18	16	Green	+++[d]	No
suo2	3	6	Green	++	No
suo3	0	1	Green (NA)[e]	++	No
suo4	0	14	Green	+++	Yes
(*suo4-30*)	—	1	Black	+++	Yes[f]
(*suo4-32*)	—	1	Black	+++	No
suo5	0	1	Black	++	No
suo6	0	2	Black	+++	No

[a] Genetic analysis has shown that the extragenic suppressors lie in six loci (*suo1* to *suo6*).
[b] In addition to impaired pigmentation, green ascospores show defects in germination efficiency.
[c] OA, medium containing oleic acid (0.05%) as sole carbon source; +++, wild type; ++, slow growth (see Figure 1).
[d] The mutant strains exhibit a flimsy aspect on OA medium (see Figure 1).
[e] NA, nonautonomous: *suo3* ascospores are green when issued from a mutant × mutant cross while they are black when issued from a mutant × wild-type cross.
[f] The *suo4-30* mutant exhibits a slight defect: Homozygous mutant perithecia expel ascospores later than wild-type perithecia.

strains confirmed that the *suo3* mutant did not belong to the *suo2* group and showed that the *suo5* mutant complemented both *suo2* and *suo3* mutants. Third, recombination tests (see MATERIALS AND METHODS) demonstrated that the *suo5* mutation was not linked either to the *suo4* (Table 3, first two crosses) or to the *suo1* loci (MATERIALS AND METHODS). These tests also led to the conclusion that a sixth locus (Table 3, crosses 3–11)

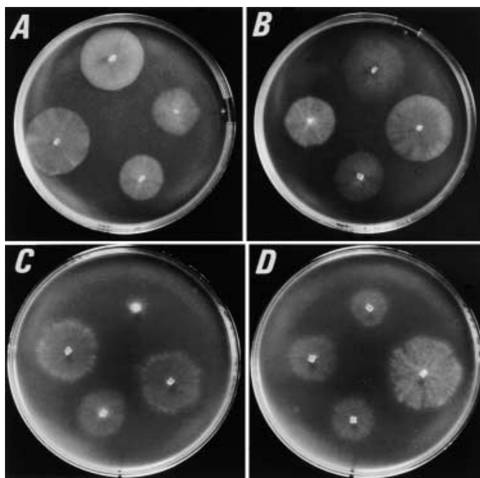

FIGURE 1.—Growth phenotypes on OA medium of strains used. Genotypes are as follows, clockwise from top left: (A) Wild type, *suo2-9*, *suo3-1*, *suo4-1*; (B) *suo1-11*, *suo6-20*, *suo1-11 suo6-20*, *suo5-17*; (C) *pex2-3*, *pex2-3 suo1-11*, *pex2-3 suo2-9*, *pex2-3 suo3-1*; (D) *pex2-3 suo4-32*, *pex2-3 suo5-17*, *pex2-3 suo6-11*, *pex2-3 suo6-20*. A and B, 2 days of growth; C and D, 3 days of growth.

was defined by two linked mutations, *suo6-11* and *suo6-20*, which cause no phenotypic defect (Table 1). During the recombination tests, we observed that the *suo1-11 suo6-20* double-mutant strains grew poorly on OA medium while the two single-mutant strains showed a near wild-type phenotype on this medium (Figure 1B). This observation prompted us to perform a cross between *suo1-11* and *suo6-11*. The green ascospores able to germinate grew either almost normally (*suo1-11*) or poorly (*suo1-11 suo6-11*) on OA medium. The growth defect of the double-mutant strains appeared to be recessive: *suo1-11* $suo6^+$/*suo1-11 suo6* heterokaryotic strains grew as well as the *suo1-11* single mutant on OA medium. This permitted complementation tests between the two *suo6* mutants, demonstrating that they are allelic. Finally, recombination data showed that the last mutation without phenotypic consequences (*suo4-32*, Table 1) was linked to the *suo4* locus (Table 3, last two crosses). Further analyses (see DISCUSSION) proved that the *suo4-32* mutation is located in the *suo4* gene.

Mutations in the *suo3* to the *suo6* loci were identified only among revertants of the *pex2-3* (missense) mutant. This prompted us to introduce these mutations, through crosses, in the *pex2-1* (nonsense) background. With respect to *suo3* and *suo5*, we tested the only mutations available, *i.e.*, *suo3-1* and *suo5-17*. Only 1 of the 2 *suo6* mutations (*suo6-20*) was tested, while 7 of the 16 *suo4* mutations were analyzed, including two leaky mutations (*suo4-30* and *suo4-39*) and the *suo4-32* mutation, which has no visible defect. In all cases, the metabolic defect of the *pex2-1* mutant was alleviated by the

TABLE 2

Nonautonomous expression of *suo* mutations in ascospores

Mutations	Homozygous crosses		Heterozygous crosses	
	Pigmentation	Germination (%)	Pigmentation	Germination (%)
suo1-11	Green	0	Green	25
suo2-9	Green	50	Green	50
suo3-1	Green	0	Black	100
suo4-39	Green	0	Green	12

Mutant ascospores, issued from either homozygous or heterozygous crosses involving the *suo4-30*, *suo4-32*, *suo5-17*, *suo6-11*, *suo6-20* mutations, are black and show a 100% germination rate. The *suo4-39* is a leaky mutant that produces few ascospores in homozygous crosses. The *suo4* mutations leading to a complete sporulation deficiency when homozygous in a cross (Table 1) can be tested only in heterozygous crosses: The green (mutant) ascospores germinate in the range of 10% as shown above for the *suo4-39* mutant. A total inability to germinate (0%) was ascertained on the following numbers of ascospores: 400 (*suo1*), 60 (*suo3*), and 75 (*suo4*).

suo mutations. We thus conclude that none of these suppressors is allele specific. Otherwise, all the *suo* mutations appear to be dominant suppressors: *pex2 suo*/*pex2 suo*$^+$ heterokaryotic strains grow on OA medium as do *pex2 suo* homokaryotic strains.

The *suo5* and *suo6* mutations are weak suppressors of the developmental defect of *pex2* mutants: The *suo* mutations were recovered due to their ability to restore growth of *pex2* mutants on OA medium. Although this restoration is far from total (Figure 1, C and D), it was interesting to know if they were able to alleviate the *pex2* developmental defects. *pex2* mutant ascospores (issued from heterozygous crosses) are green, show poor germination levels, and, when germinated, yield flimsy and tiny thalli on germination medium (Berteaux-Lecellier *et al.* 1995). The *suo* mutations did not restore either normal pigmentation or increased germination rates of the *pex2* mutant ascospores. However, germinating thalli of *pex2 suo5* and *pex2 suo6* (both alleles) appeared "healthy" and similar in size to those of wild-type ascospores. Perithecia homozygous for a *pex2* mutation are barren: They are blocked before differentiation of meiocytes (Figure 2A). The dikaryotic cells (croziers) are unable to differentiate like wild type (see Figure 2B) into asci, in which karyogamy, meiosis, and ascospore formation normally occur. Instead, they maintain a mitotic proliferative state (Berteaux-Lecellier *et al.* 1995). Perithecia obtained from *pex2 suo* × *pex2 suo* or *pex2 suo* × *pex2 suo*$^+$ crosses were not different from those obtained from *pex2* × *pex2* crosses: They were mainly barren with 1–5 asci among 100 perithecia, while each wild-type perithecium contains >100 asci. A slight but clear-cut increase in ascus formation (1–5 asci among 10 perithecia) was observed, but only when either the *suo5* or the *suo6* mutations were present in the crosses (Figure 2C). The restoration of ascus formation was more efficient with *suo6-11* than with the two other mutations. Furthermore, in addition to these rare asci, almost all *pex2-3 suo6-11* homozygous perithecia contained numerous elongated croziers that seemed in a prekaryogamy state, a figure rarely observed in *pex2* mutants (compare Figure 2D with 2A).

The *suo5* and the *suo6* mutations partly restore peroxisome biogenesis in the *pex2* mutant context: As previously described (Berteaux-Lecellier *et al.* 1995), an

TABLE 3

Recombination data between *suo* mutations

Crosses	Uninucleate green ascospores		
	Recovered	Germinated	*pex2 suo*$^+$
pex2-3 suo5-17 × *pex2*$^+$ *suo4-30*	34	18	4
pex2-3 suo5-17 × *pex2*$^+$ *suo4-32*	39	27	8
pex2-3 suo5-17 × *pex2*$^+$ *suo6-11*	79	61	13
pex2-3 suo5-17 × *pex2*$^+$ *suo6-20*	35	24	6
pex2-3 suo6-20 × *pex2*$^+$ *suo1-11*	71	33	12
pex2-3 suo6-20 × *pex2*$^+$ *suo2-9*	70	24	2
pex2-3 suo6-20 × *pex2*$^+$ *suo3-1*	58	36	6
pex2-3 suo6-20 × *pex2*$^+$ *suo4-1*	71	18	1
pex2-3 suo6-20 × *pex2*$^+$ *suo4-30*	110	41	3
pex2-3 suo6-20 × *pex2*$^+$ *suo4-32*	66	38	7
pex2-3 suo6-20 × *pex2*$^+$ *suo6-11*	86	61	0
pex2-3 suo4-30 × *pex2*$^+$ *suo4-32*	91	37	0
pex2-3 suo4-32 × *pex2*$^+$ *suo4-1*	39	24	0

The *pex2 suo*$^+$ ascospores are green and yield mycelia unable to grow on OA medium (see materials and methods for details).

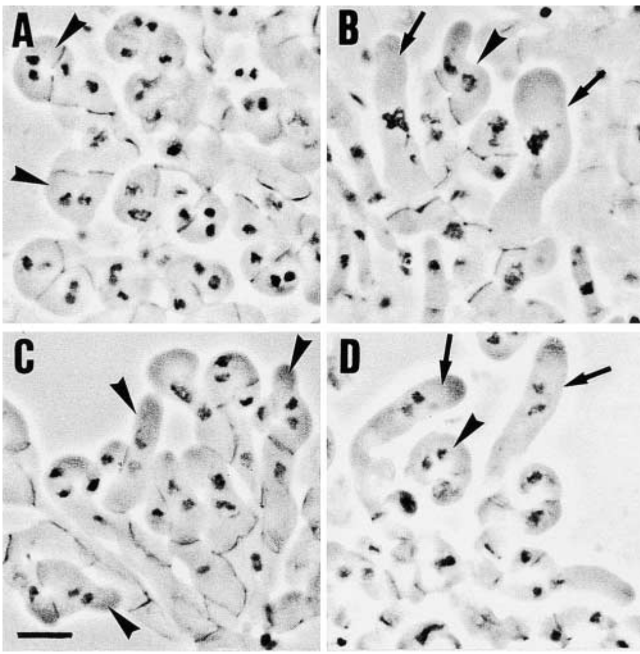

FIGURE 2.—Comparison between wild-type and mutant fruiting bodies. (A) *pex2-3* perithecia contain only croziers. Arrowheads point to the upper cell of two of the croziers; they show and keep the typical round shape of young croziers and contain two nuclei of opposite mating type. (B) Wild-type young perithecia are filled with a mixture of croziers (arrowhead) and asci (arrows). (C) *pex2-3 suo5-17* perithecia also contain mainly croziers. However, some croziers show elongated upper cells (arrowheads, compare with A). (D) In the *pex2-3 suo6-11* perithecia, croziers either are round (arrowhead) or show elongated upper cells as in *pex2-3 suo5-17*. Parts of the elongated upper cells are as long as young asci (arrows, compare with B) but their nuclei fuse only rarely (see text for further comments). All nuclei are stained by iron-hematoxylin. Bars, 5 μm.

antibody against the trifunctional peroxisomal FOX2 enzyme of *Neurospora crassa* (Fossa *et al.* 1995) clearly stained peroxisomes in the wild-type perithecial tissues, while no organelles were observed in the *pex2* mutant perithecia (see Figure 3, A and B). We used the same antibody to determine whether peroxisomes were visible in the single- (*pex2*$^+$ *suo*) and double-mutant (*pex2 suo*) strains. The single-mutant tests were especially important, because most of the *suo* mutations led to phenotypic defects more or less similar to those observed in the *pex2* mutants: green ascospores with poor germination efficiency, altered growth on OA medium, or sexual defects. These assays were performed with perithecia from homozygous crosses for one mutation of each locus (*suo1* to *suo6*). All homozygous *pex2*$^+$ *suo* × *pex2*$^+$ *suo* perithecia observed showed peroxisomes that did not differ in size and number from those observed in wild-type perithecia (data not shown). In contrast, no peroxisomes were visible in perithecia issued from crosses homozygous for both *pex2* and *suo* mutations (one mutation tested for each locus) except in the cases of *pex2-3 suo5-17* and *pex2-3 suo6-11* (Table 4). In *pex2-3 suo5-17* perithecia, peroxisomes are very rare: They are absent in sexual tissues (croziers) and could be seen only in vegetative tissues (paraphysae) where they were less numerous than in wild-type paraphysae (Figure 3C). In *pex2-3 suo6-11* perithecia, peroxisomes were observed in paraphysae, croziers, and asci. However, they appeared smaller and rounder than those observed in wild-type perithecia (compare Figure 3A and 3D). When the second mutation of *suo6* was tested, no peroxisomes were detected in the *pex2-3 suo6-20* × *pex2-3 suo6-20* perithecia.

In an attempt to understand the discrepancy between the two *suo6* alleles, we used a complementary approach. Most *pex* mutants, including *pex2* (Chang *et al.* 1999 and references therein) can form peroxisomes but these peroxisomal remnants are unable to import peroxisomal matrix proteins. Thus, the lack of peroxisomes stained with the anti-FOX2 antibody shows merely that the organelles are unable to import this type of protein. FOX2 (in *N. crassa* and probably in *P. anserina*) does not contain the peroxisome-targeting signal 1 (PTS1) motif (Fossa *et al.* 1995). We thus used a reporter system composed of the GFP protein, to which a PTS1 motif (SKL tripeptide) was added at the C terminus (materials and methods). The construct, under the control of the *P. anserina* pGPD constitutive promoter (Ridder and Osiewacz 1992), was introduced by transformation in a wild-type strain and then transferred into the *pex2-3* and all the *pex2-3 suo* and *pex2*$^+$ *suo* contexts through crosses. In all cases, the same integration site of the construct was used, to avoid differences due to position effects on its expression level and to allow clear comparisons between strains. Wild-type mycelia contain numerous fluorescent bodies that are mainly round (Figure 4A) or show rod forms. In contrast, in the *pex2-3* strain, the fluorescence was observed homogeneously in the cytosol (Figure 4B). Peroxisomes observed in the *pex2*$^+$ *suo* strains did not differ from wild-type peroxisomes in their shape, number, or distribution. Data obtained from *pex2-3 suo* strains are summarized in Table 4. In all cases, the fluorescence was mainly cytosolic. However, in some cases a few tiny bright bodies were observed on the green, homogeneous GFP background. Peroxisomal-like structures were observed only in *pex2-3* strains bearing one of the *suo6* mutations (Figure 4, C and D). These bodies appeared more numerous in the *pex2-3 suo6-11* cells than in the *pex2-3 suo6-20* cells, but their number was rather low compared to those in wild type, and they were seen mainly in the apical cells. Finally, the organelles seen in these double-mutant strains are more heterogeneous in shape than those of their wild-type counterparts: In addition to round and rod-shaped bodies, giant, snake-like structures were observed, especially in the *pex2-3 suo6-11* context (Figure 4D).

The metabolic defect of *pex2* mutants revisited: the H_2O_2 hypothesis: The *suo* mutations were screened for

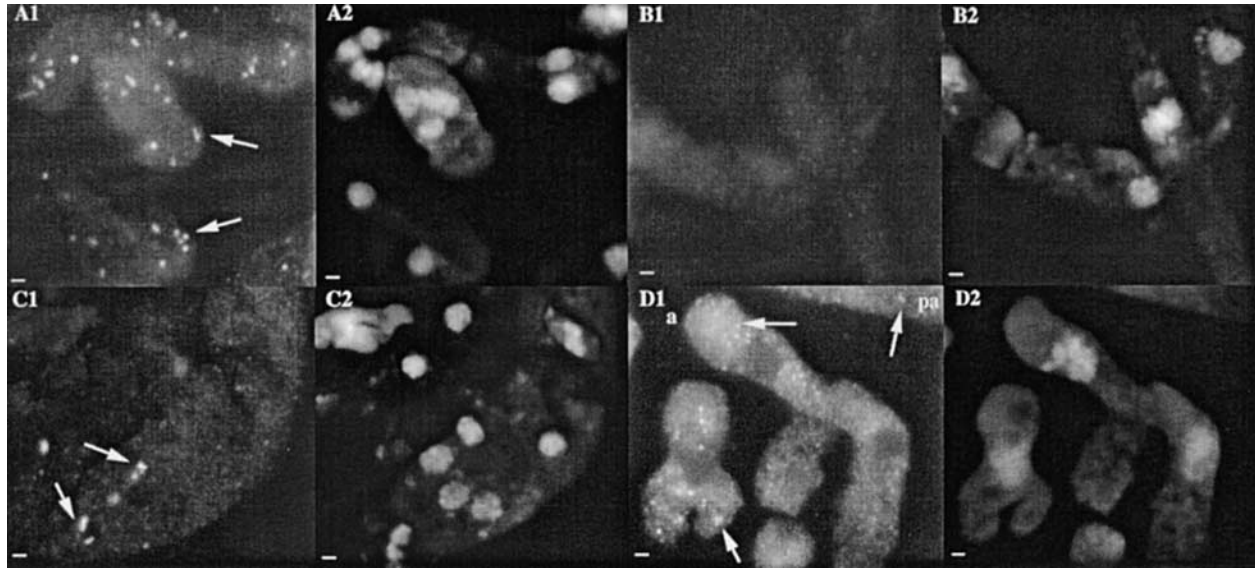

Figure 3.—Visualization of peroxisomes with anti-FOX2 antibody in perithecial tissues from homozygous crosses (immunofluorescence in photos numbered 1 and corresponding DAPI staining in photos numbered 2). (A) Wild type: The paraphysae (vegetative cells) contain many bodies (arrows). (B) *pex2-3*: No bodies are stained in these cells. (C) *pex2-3 suo5-17*: The cells exhibit a few bodies (arrows). (D) *pex2-3 suo6-11*: Bodies can be seen in paraphysae (pa) and in asci (a) but they seem smaller than those observed in wild-type cells (compare to A). Bars, 1 μm.

their ability to restore growth of *pex2* mutants on OA medium. Therefore, the fact that some of them exhibited a growth defect on this medium was puzzling. Two hypotheses could explain the inability of *pex2* mutants to grow on OA medium. First, oleic acid could not be a carbon source for these mutants because β-oxidation could not occur or would be greatly impaired in the cytosol. Second, β-oxidation would occur but the consequent production of H_2O_2 would be toxic for the cells if catalase activities were unable to efficiently detoxify this compound in the cytosolic compartment. It was previously observed that oleic acid was toxic to the *pex2* mutants, especially in the absence of another efficient carbon source, *e.g.*, glucose or maltose (Berteaux-Lecellier *et al.* 1995). This fact could (albeit weakly) support the second hypothesis, which was reinforced in two ways. First, acyl-CoA oxidase activity (the peroxisomal enzyme that produces H_2O_2) was detected in the *pex2* mutants (data not shown). Second, we tested a procedure previously described in *S. cerevisiae* for the isolation of peroxisomal mutants, using a positive selection based

TABLE 4

Relationships between ascus differentiation and peroxisome biogenesis in wild-type, *pex2-3*, and *pex2-3 suo* strains

		Visualization of peroxisomes		
		Anti-FOX2		
Strains	Ascus formation	Paraphysae	Croziers	GFP-SKL
pex2$^+$	+++	++	+	+++
pex2-3	−	−	−	−
pex2-3 suo1-11	−	−	−	±[a]
pex2-3 suo2-9	−	−	−	−
pex2-3 suo3-1	−	−	−	ε[a]
pex2-3 suo4-32	−	−	−	±[a]
pex2-3 suo5-17	±	±	−	−
pex2-3 suo6-20	±	−	−	+[b]
pex2-3 suo6-11	+	+	+	+[b]

−, undetectable; ε, very rare; ± to +++, from very low to high amounts, respectively. These results reflect rough but reproducible estimates, not precise countings.

[a] Tiny bright spots.

[b] Heterogeneous shapes including giant structures rarely observed in the wild-type cells.

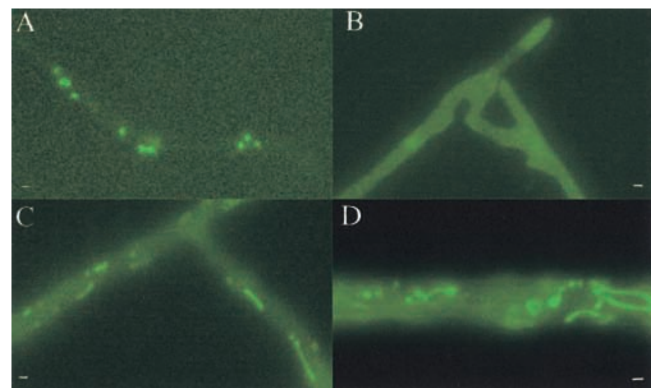

Figure 4.—Localization of the GFP-SKL protein in growing filaments. (A) Wild type. (B) *pex2-3*. (C) *pex2-3 suo6-20*. (D) *pex2-3 suo6-11*.

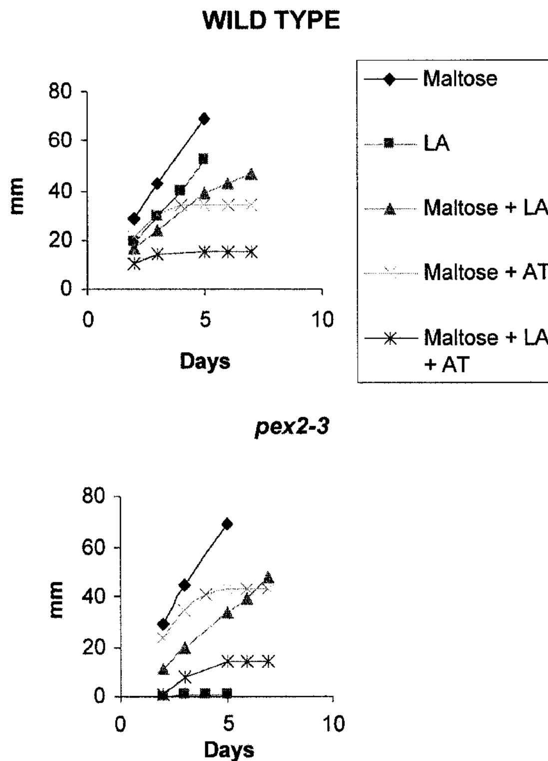

FIGURE 5.—Growth curves of the wild-type and *pex2-3* strains on different media. Numbers at each time point represent the diameters of the thalli after 3 days on the relevant media (average of six cultures for each strain). LA, lauric acid; AT, aminotriazol (25 mM). See MATERIALS AND METHODS for details on the media.

on the potential lethality of H_2O_2 produced during β-oxidation (VAN DER LEIJ *et al.* 1992). These authors used a medium on which the wild-type cells died, while mutants unable to perform β-oxidation could survive. This medium contained two carbon sources, lauric acid (requiring β-oxidation) and maltose, and was supplemented with 3-AT, a potent inhibitor of catalase. Thus, cells able to perform β-oxidation of lauric acid accumulate H_2O_2 and die while mutants impaired in this process should survive and grow (using maltose). With this procedure, VAN DER LEIJ *et al.* (1992) isolated peroxisome biogenesis mutants falling in 12 complementation groups. We thus compared the growth abilities of the wild-type and *pex2-3* mutant strains on this selective medium (MATERIALS AND METHODS). As shown in Figure 5, the *pex2-3* mutant does not grow on lauric acid as sole carbon source. It shows a reduced growth rate, with a 24-hr lag time, when lauric acid is added to maltose. This reduced growth rate, but not the lag time, is also observed for the wild-type strain grown on maltose plus lauric acid. More interestingly, the mutant appears as sensitive as the wild type when this medium is supplemented with 3-AT. The fact that both strains are resistant to 3-AT when maltose is the sole carbon source demonstrates that their extreme sensitivity to the drug in the presence of lauric acid is linked to β-oxidation. Therefore, at least some of the enzymes involved in β-oxidation (especially those acting upstream of H_2O_2 production) must function in the *pex2* mutants, efficiently enough to produce a lethal threshold of H_2O_2.

DISCUSSION

Genetics of peroxisomes: the suppressor caveat: Since the pioneer studies of Kunau and his co-workers (ERDMAN *et al.* 1989), an impressive collection of *pex* mutants affected in peroxisome biogenesis has been obtained in yeasts. Multiple strategies have been used to isolate these mutants (reviewed in SUBRAMANI 1998). Studies of *pex* mutants at the molecular, biochemical, and cytological levels have provided important insights into peroxisome biogenesis (see TABAK *et al.* 1999; BAERENDS *et al.* 2000; SACKSTEDER and GOULD 2000; SUBRAMANI *et al.* 2000; TITORENKO and RACHUBINSKI 2001a for recent reviews). However, as stressed in most reviews, a number of questions remain unanswered. With respect to the genetic approach, it is amazing to note that there has been no systematic search for suppressors of *pex* mutants, although their metabolic defects offer a positive selection for suppressor screening. We know of only three examples of suppression of a *pex* mutation by overexpression of another gene. Furthermore, the data were not obtained through an extensive search for multicopy suppressors but mainly by chance or through a targeted rationale using a small number of known *PEX* genes. In the first example, suppression is allele specific and characteristic of interacting proteins (FABER *et al.* 1998; GEISBRECHT *et al.* 1998). The second example illustrates how the function impaired in mutant strains can be bypassed by overexpression of another gene (VAN DER KLEI *et al.* 1998; SALOMONS *et al.* 2000). The third case shows that peroxisome biogenesis can be restored in *pex2* mutant cell lines by overproduction of either one of the peroxisomal ATP-binding cassette (ABC) transporters, PMP70 or ALDP (GÄRTNER *et al.* 1994, 1998; BRAITERMAN *et al.* 1998). Like PEX2, these two proteins are integral membrane proteins (MOSSER *et al.* 1993; IMANAKA *et al.* 1999) but they are not required for peroxisome biogenesis. In fact, the actual functions of the peroxisomal ABC transporters are still a matter of debate. In contrast to the other examples of multicopy suppressors cited above, this case remains unexplained and emphasizes our ignorance of the role(s) played by PEX2 in peroxisome biogenesis.

The same three types of functional suppression (due to interacting proteins, bypass of an impaired function, restoration of this function) can be obtained by either loss-of-function or gain-of-function mutations. Thus, in *P. anserina*, we used mutagenesis and searched for *pex2* suppressors. Here we report this large-scale screening, using a positive selection procedure, *i.e.*, restoration of growth of the *pex2* mutants on a medium containing

oleic acid as sole carbon source (OA medium). Genetic analysis of 63 extragenic suppressors demonstrated that they fall in six loci with a strongly biased distribution of mutations: *suo1* (34 mutations), *suo2* (9), *suo3* (1), *suo4* (16), *suo5* (1), and *suo6* (2). Thus, this genetic screen seems far from being saturated. The *suo* mutations act as allele nonspecific, dominant suppressors of *pex2* mutations and they cause recessive phenotypic defects in a *pex2*$^+$ background. The fact that all *suo* tested are allele nonspecific suppressors (they act on both a missense and a nonsense mutation) argues against direct interactions between PEX2 and either of the *suo* products. The recessivity of *suo* with respect to their own defects suggests that these defects are caused by loss-of-function mutations. Therefore, their dominance as suppressors is probably a consequence of a delicate gene dosage effect as recently described for mutants impaired in β-oxidation in *A. thaliana* (Hayashi *et al.* 1998). With respect to the *suo* mutations of *P. anserina*, one can assume that, in heterokaryotic *suo/suo*$^+$ strains, reduced β-oxidation would be sufficient to allow normal growth on OA medium while in *pex2 suo/pex2 suo*$^+$ strains, this reduction would lead to H_2O_2 production below the lethal threshold. The mechanisms that may underlie *pex2* suppression by *suo* mutations are discussed with respect to β-oxidation, peroxisome biogenesis, and cell differentiation.

***pex2* and *pex2 suo*: β-oxidation:** In this study, we show that *P. anserina pex2* mutants are as sensitive as the wild type to the catalase inhibitor 3-AT when a fatty acid metabolized through β-oxidation is present in the culture medium. This observation suggests that β-oxidation is efficient enough in the mutant cytosol to produce a lethal threshold of H_2O_2. There are three ways to alleviate the toxic effect of β-oxidation in *pex2* mutants: first, a decrease in H_2O_2 production; second, an increase in detoxification efficiency; and third, restoration of a functional peroxisome compartment.

The first way can be achieved by mutations in genes encoding the β-oxidation enzymes acting upstream of H_2O_2 production, namely long-chain acyl-CoA synthetase, transporter, and oxidase (Figure 6, steps 1–3). Thus, leaky mutations in these genes, decreasing (but not abolishing) the relevant activities, should reduce H_2O_2 production and allow a limited growth of *pex2* mutants on OA medium. The ability of *pex2 suo* strains to grow (albeit poorly) on this medium means that the β-oxidation enzymes acting downstream of H_2O_2 production (Figure 6) are also at least partly active in the *pex2* cytosol. In addition to the structural genes (encoding the proteins quoted above), *suo* mutations might also fall in regulatory genes whose products would be required for optimal expression of these structural genes, in particular in the presence of β-oxidation substrates. Such regulatory genes have been described in *S. cerevisiae* (Simon *et al.* 1991, 1992; Chelstowska and Butow 1995; Rottensteiner *et al.* 1996; Karpichev *et al.* 1997). Mutations in most of these genes impair but do not completely prevent oleic acid utilization. Thus, the *suo1*, *suo2*, *suo3*, and *suo5* loci, in which mutations cause a reduced growth on OA medium (Table 1 and Figure 1), are candidates for structural or regulatory genes involved in the β-oxidation pathway.

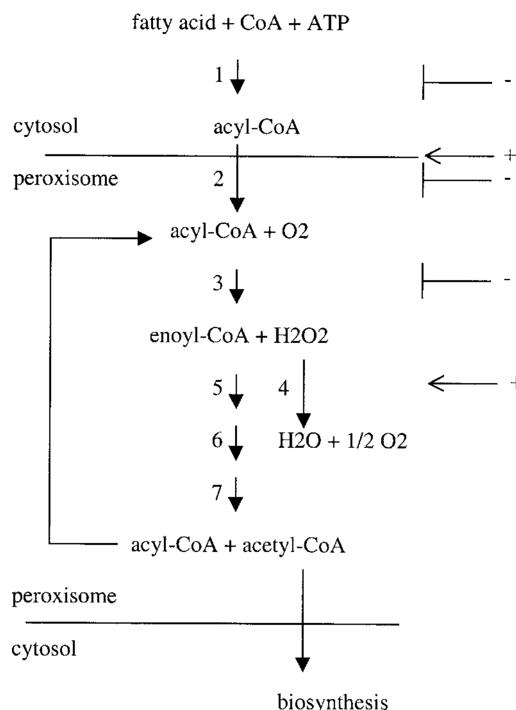

Figure 6.—Possible positions of *pex2* metabolic suppressors along the β-oxidation pathway. The minimum information required for the present purpose is given. 1, long-chain acyl-coA synthetase; 2, acyl-CoA transporter; 3, acyl-CoA oxidase; 4, catalase. Each cycle of oxidation shortens the chain length of the fatty acids by two carbons. The suppressor mutations are expected to alleviate the detrimental production of H_2O_2, which is assumed to occur in the cytosol of the *pex2* mutants. − and + refer, respectively, to loss- and gain-of-function mutations that can occur in either structural or regulatory genes. The arrow drawn at the level of peroxisome membrane implies restoration of peroxisome biogenesis (see discussion for further comments).

The second way for alleviating β-oxidation toxicity in *pex2* mutants implies a greater efficiency of H_2O_2 detoxification, through (for instance) an increased catalase activity (Figure 6, step 4). Interestingly, defects in some of the regulatory genes required for optimal induction of β-oxidation enzymes in *S. cerevisiae* have a much stronger effect on acyl-CoA oxidase than on the peroxisomal catalase (Kal *et al.* 1999). One report even shows that catalase expression is not affected in these mutants (Karpichev *et al.* 1997). Such mutations would thus cause a decrease in H_2O_2 production along with a maintenance of H_2O_2 detoxification. However, mutations that specifically increase catalase activity would not be expected to cause a growth defect on OA medium.

The only mutants exhibiting a normal growth on this medium are the *suo4* and the *suo6* mutants (Table 1 and Figure 1). In fact, *suo4* is the only gene we have cloned. Surprisingly, it encodes a mitochondrial citrate synthase. It was ascertained that the 16 mutations fell in this gene, including *suo4-32*, which causes no visible defect (Table 1). This unexpected case of suppression has been explained by an indirect effect of the *suo4* mutations upon catalase activity, which is indeed increased in these mutant contexts (G. Ruprich-Robert, D. Zickler, V. Berteaux-Leceller, C. Vélot and M. Picard, unpublished results).

***pex2* and *pex2 suo*: peroxisome biogenesis and cell differentiation:** The third way for suppression of the *pex2* metabolic defect involves restoration of peroxisome biogenesis (Figure 6). In *P. anserina*, occurrence of peroxisomes has been investigated through three procedures: electron microscopy with the conventional DAB procedure, which reveals catalase-containing structures; immunofluorescence with an antibody against the peroxisomal FOX2 enzyme (Berteaux-Lecellier *et al.* 1995); and *in vivo* staining with a GFP construct targeted to peroxisomes through a PTS1 (SKL) motif (this article). Peroxisomes were detected in the wild-type cells with the three procedures. In addition, a drastic increase of their number was observed in the sexual lineage when the croziers develop into asci. In contrast, *pex2* mutant strains do not show any peroxisomes and their croziers maintain a proliferative state instead of differentiating into meiocytes (Berteaux-Lecellier *et al.* 1995). Data obtained with immunofluorescence and GFP-SKL staining of *pex2 suo* strains along with observation of ascus formation are summarized in Table 4. They allow us to conclude (especially in the case of *suo6-11*) that partial restoration of peroxisome biogenesis is linked to a weak recovery of ascus differentiation.

In fact, the *suo* loci belong to two classes. The first class includes *suo1* to *suo4*, of which mutations do not restore ascus differentiation in a *pex2* mutant context. The anti-FOX2 antibody does not reveal any peroxisomes in these *pex2 suo* strains. However, with the exception of *pex2 suo2*, they exhibit few tiny bright spots with the GFP-SKL staining. Their size and shape make questionable whether these spots are related to peroxisomes. According to the hypotheses discussed above, suppression of the metabolic defect of *pex2* mutants would be due to either a decrease in H_2O_2 production (*suo1*, *suo2*, *suo3*) or an increase in catalase activity (*suo4*). The fact that the *pex2* strains containing the *suo1, suo3*, and *suo4* mutations exhibit these bodies remains unexplained. The second class of *suo* loci includes *suo5* and *suo6* in which mutations weakly restore ascus differentiation (Figure 2). Anti-FOX2 (but not GFP-SKL) staining reveals few peroxisomes in the perithecia of *pex2 suo5* (Figure 3). Peroxisomes are clearly observed with the GFP-SKL procedure in the *pex2* strains bearing either one of the *suo6* mutations (Figure 4). This partial restoration of peroxisome biogenesis, especially in the growing (apical) cells, should be sufficient to allow a limited growth of the strains on OA medium. With respect to cell differentiation, the amount of asci formed (albeit very low) is higher with *suo6-11* than with *suo6-20* and *suo5-17*. This correlates with the fact that peroxisomes of the *pex2-3 suo6-11* strains appear able to import both FOX2 and GFP-SKL (Table 4; Figures 3 and 4). However, their rather low numbers and their abnormal shape suggest that these organelles lack the ability to proliferate normally and/or still have import deficiencies. This may explain why the sexual cells of these *pex2 suo* strains, which seem able to enter into the differentiation pathway, do not complete the whole developmental program. In either case, to our knowledge, *suo5* and *suo6* are the first examples of genes of which mutations restore peroxisome biogenesis in a *pex* mutant.

As stressed in the Introduction of this article (see also Titorenko and Rachubinski 2001b) a new area in peroxisome studies has been opened with the discoveries linking these organelles to some developmental programs in plants and fungi (including pathogenic species). This study shows that the suppressor approach has been fruitful to better understand the defects of *pex2* mutants and to ascertain the link between peroxisomes and differentiation in *P. anserina*. Molecular characterization of these loci will give new insights into this process. Beyond this fungus, one can expect that such a systematic search for suppressors of *pex* mutants in other model systems would also illuminate some aspects of peroxisome biogenesis that remain obscure.

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