Expression and targeting of a 47 kDa integral peroxisomal membrane protein of *Candida boidinii* in wild type and a peroxisome-deficient mutant of *Hansenula polymorpha*

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A 47 kDa integral peroxisomal membrane protein (PMP47) of *Candida boidinii* was expressed in wild type (WT) and a temperature-sensitive (Ts6) peroxisome-deficient (*per*) mutant of *Hansenula polymorpha*. The subcellular location of PMP47 appeared to be dependent on the level of expression. At low expression levels PMP47 was sorted to the peroxisomal membrane; however, in Ts6 cells grown at restrictive temperatures (which lack intact peroxisomes) PMP47 was solely located in small cytosolic aggregates, together with homologous *H polymorpha* PMP's. At enhanced expression levels, however, part of the protein also became incorporated into mitochondria, both in transformed WT and Ts6 cells.

Yeast; *Hansenula polymorpha*; *Candida boidinii*; Peroxisome; Peroxisomal membrane protein; Peroxisome-deficient mutant; Protein targeting

1. INTRODUCTION

Microbodies (peroxisomes) are ubiquitous organelles of eukaryotic cells. Yeasts are attractive model organisms to study peroxisome biogenesis; in these organisms both the induction and enzyme composition of peroxisomes can be prescribed by manipulating the growth conditions [1]. Upon induction, yeast peroxisomes develop by growth and fission of already existing organelles [1]. Matrix proteins are synthesized on free polysomes which are posttranslationally imported without detectable modification. Topogenic signals, directing these proteins to their target organelles, are highly conserved and predominantly located at the extreme carboxy terminus of these proteins [2], although exceptions have been encountered [3].

In contrast, detailed information on sorting of peroxisomal membrane proteins is still lacking. Goodman and co-workers studied targeting of a major specific peroxisomal membrane protein (PMP47) of *Candida boidinii* in more detail and showed that, after heterologous expression in *Saccharomyces cerevisiae*, PMP47 is correctly sorted to the peroxisomal membrane [4], indicating that also for peroxisomal membrane proteins general targeting signals may exist. In order to obtain further information on this topic, we have studied the fate of PMP47 after heterologous expression in both wild type and a conditional (Ts) peroxisome-deficient

(*per*) mutant [5] of *Hansenula polymorpha*. The results of these studies are presented in this paper.

2. MATERIALS AND METHODS

2.1. Microorganisms

Wild type (WT) *H. polymorpha* de Morais et Maya CBS 4732, a temperature-sensitive (Ts) peroxisome-deficient (*per*) mutant (Ts6, Ura 3-1) derived from this strain [5] and *C. boidinii* ATCC 32195 were grown in batch cultures in mineral medium [6]. PMP47 was expressed under control of the homologous, substrate inducible alcohol oxidase (MOX) promotor of *H. polymorpha* (for plasmid construction see below). To obtain different levels of PMP47 expression, cells were grown on various carbon sources namely 0.5% (w/v) glucose, 0.5% (v/v) glycerol or 0.5% (v/v) methanol as carbon source. *H. polymorpha* strains were further grown in carbon-limited chemostat cultures on 0.25% (w/v) glucose/0.1% (v/v) methanol mixtures [6] for maximal expression.

2.2. Plasmid constructions and yeast transformation

A *H. polymorpha* – *E. coli* shuttle plasmid (YEpMOX47) was constructed, containing the 47 kDa PMP of *C. boidinii* behind the MOX-promoter of *H. polymorpha* (Fig. 1). By digestion of plasmid pMEX [7] with the restriction enzymes *Sal*I and *Eco*RI a 1.5-kb fragment (MOX-promoter) was obtained which was ligated into the *Eco*RI/ *Xho*I site (PGK-promoter; 400 bp) of plasmid YEpPGK47, creating plasmid YEpMOX47 (9.9 kb).

Transformation of the plasmid in WT *H. polymorpha* (Ura 3-1) and the Ts6 [5] was performed according to [8]. This method often results in chromosomal integration due to non-homologous recombination events. Integrants were selected by growth of transformants for at least 40 generations on non-selective media.

2.3. Biochemical methods

Crude extracts were prepared as described previously [9]. Protein concentrations [10] and activities of alcohol oxidase [9] and cytochrome c oxidase [11] were assayed as described. Enzyme activities are expressed as μmol substrate consumed or product formed/min · ml.

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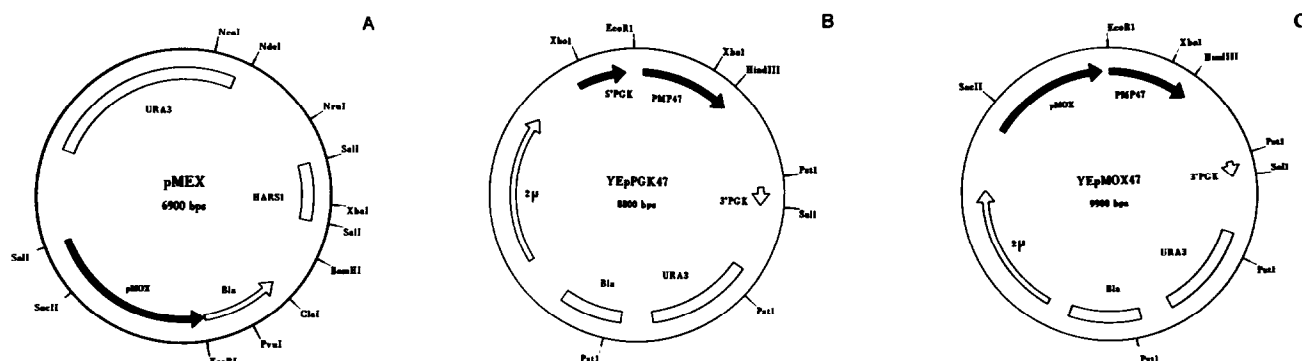
Fig. 1. Schematic representation of the plasmids, pMEX, YEpPGK47 and YEpMOX47. Sizes are indicated; the plasmids are not drawn to scale with respect to each other and only relevant restriction enzyme sites are shown.

Peroxisomes and mitochondria were purified by differential and sucrose density centrifugation of homogenized protoplasts [12]. The polypeptide composition of crude extracts and subcellular fractions was studied on 12.5% SDS-polyacrylamide gels [13]. Western blotting was performed using the protoblot immunoblotting system (Promega Biotec).

Monoclonal antibodies against PMP47 of *C. boidinii* [14] and polyclonal antibodies against a specific 68 kDa PMP of *H polymorpha* [15] were used throughout all immunological experiments.

2.4. Electron microscopy

Intact cells were fixed in 1.5% (w/v) KMnO$_4$ [12]. Immunolabelling was performed on ultrathin sections of Lowicryl K$_4$M embedded cells by the protein A/gold method [16].

3. RESULTS AND DISCUSSION

Transformants of WT *H. polymorpha*, containing YEpMOX47 integrated into the genome, had maintained the capacity to grow on methanol as sole carbon source, although at a slightly reduced rate (t_d = 5.5 h) compared to WT control cells (t_d = 4 h). Western blot analysis performed on crude extracts of variously grown transformed cells indicated that PMP47 was solely expressed during methylotrophic growth conditions. As expected, highest levels, comparable to the expression levels of PMP47 in WT *C. boidinii*, were detected in cells from a carbon-limited chemostaton glucose/methanol mixtures (Fig. 2, lane A,B); comparatively low expression levels were observed in cells from batch cultures on glycerol (Fig. 2, lane D) whereas PMP47 was undetectable during growth of cells on glucose (Fig. 2, lane E) and in untransformed WT control cells (Fig. 2, lane F).

Electron microscopically, in ultrathin sections of KMnO$_4$-fixed chemostat-grown WT transformants of *H. polymorpha* no significant alterations were observed on either the average number and size of intact peroxisomes or the structure of the peroxisomal membrane (Fig. 3A), compared to identically grown WT controls (not shown).

The intracellular localization of PMP47 in transformed WT *H. polymorpha* was studied by conventional cell fractionation methods. Two samples were analyzed displaying either high (from glucose/methanol chemostat cultures) or low to moderate expression levels (from batch cultures on glycerol) of PMP47. After sucrose density gradient centrifugation of the 30,000 × g pellet, obtained after differential centrifugation of homogenates of transformed WT cells, grown in batch cultures on glycerol, highly purified fractions of mitochondria (located at 45% (v/v) sucrose) and peroxisomes (located at 53% (v/v) sucrose) were obtained (Fig. 4B). The assignment of organelles was based on the distribution of their respective marker enzymes cytochrome C oxidase and alcohol oxidase (AO). Western blot analysis of the organellar peak fractions indicated that PMP47 cosedimented with AO, indicating its peroxisomal location (Fig. 4A). In identical experiments, performed on fully derepressed chemostat-grown cells, a major part of PMP47 cosedimented in the gradient with AO (Fig. 5A); however, in addition a distinct amount of PMP47 was also detected in the mitochondrial peak fraction (Fig. 5B).

The biochemical results on the location of PMP47 were confirmed immunocytochemically; using monoclonal antibodies against PMP47 and protein A/gold,
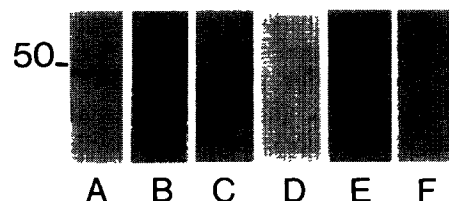
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A   B   C   D   E   F

Fig. 2. Western blots, using monoclonal antibodies against PMP47 of *C. boidinii*, of crude extracts prepared from *C. boidinii* and *H. polymorpha* transformants (containing YEpMOX47) and control cells, showing the induction of PMP47 at different growth conditions. Lane A, *C. boidinii*, batch culture on methanol; lane B, transformed WT *H. polymorpha*, chemostat on glucose/methanol; lane C, transformed Ts6, chemostat on glucose/methanol at the restrictive temperature (43°C); lane D, transformed WT *H polymorpha*, batch culture on glycerol; lane E, transformed WT *H. polymorpha* batch culture on glucose; lane F, control WT *H. polymorpha*, batch culture on methanol 20 μg of protein was loaded on each lane.
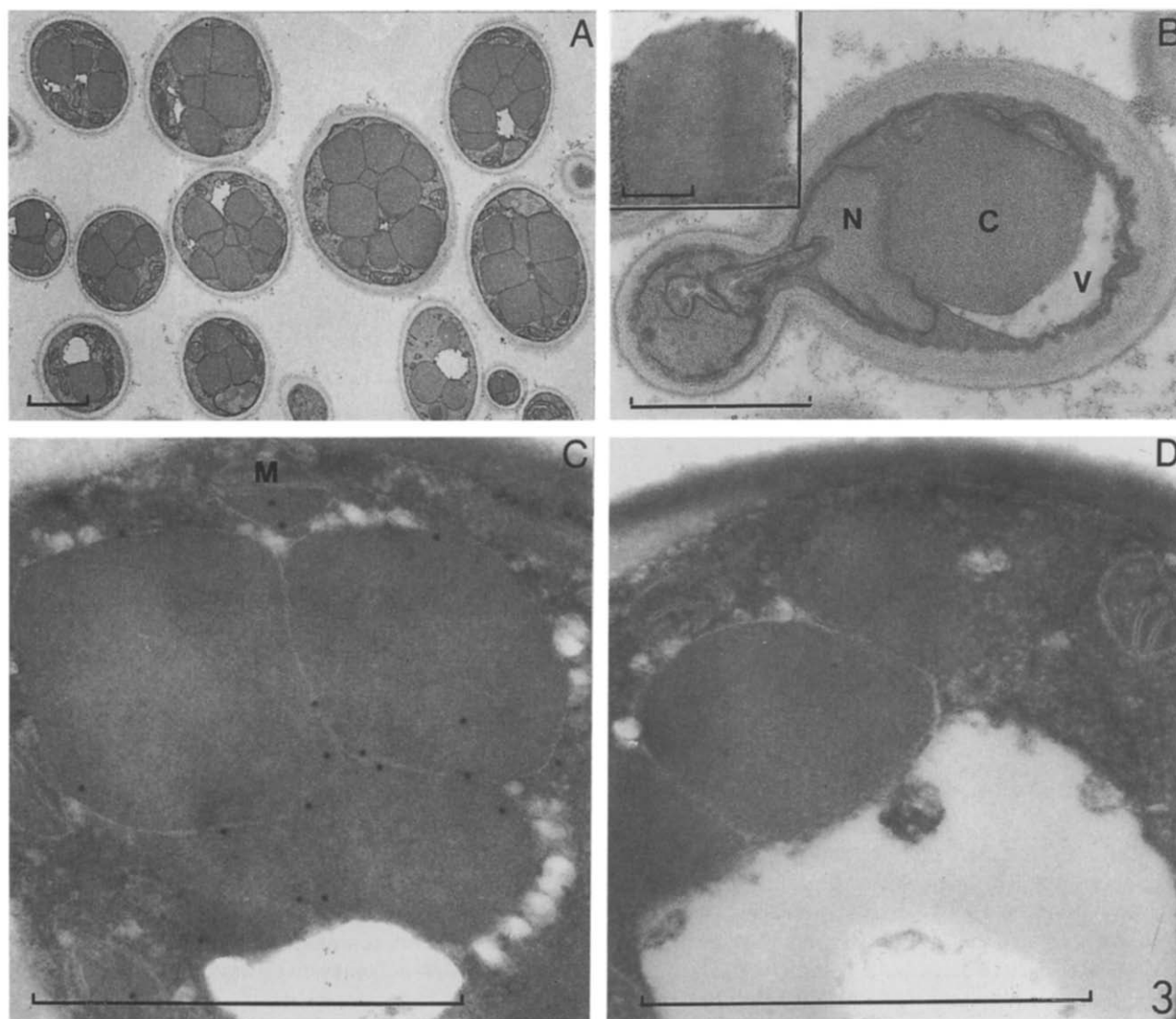
Fig. 3. Overall morphology of WT transformants of *H. polymorpha*, containing numerous peroxisomes during growth in a chemostat culture on a glucose/methanol (A) peroxisomes are lacking in cells of the transformed Ts6 mutant grown at restrictive temperature (43°C; B). In the latter cells a large cytosolic AO crystalloid is present; this structure, which is poorly preserved after KMnO$_4$-fixation (B), is clearly resolved in glutaraldehyde-fixed protoplasts of the organism (B, inset; bar = 0.5 μm). Immunocytochemical experiments, using specific antibodies against PMP47 and protein A/gold, performed on methanol-grown transformed WT cells showed labeling of both the peroxisomal membranes and mitochondria (C). Specific labeling was not observed in identical experiments on WT control cells (D). Electron micrographs are taken of KMnO$_4$-fixed cells unless otherwise indicated. C, cytosolic alcohol oxidase crystalloid; M, mitochondrion; N, nucleus; P, peroxisome; V, vacuole. The bar represents 1.0 μm unless otherwise stated.

specific labeling was confined to the peroxisomal membrane of transformed WT cells from batch cultures on glycerol (not shown; compare Fig. 7A). However, in methanol-grown WT transformants in addition labeling was observed on mitochondrial profiles (Fig. 3C); untransformed WT control cells invariably showed no labeling (Fig. 3D).

An identical series of experiments was performed on a transformed temperature-sensitive peroxisome-deficient mutant (Ts6) of *H. polymorpha*. When grown at permissive temperatures (30°C), Ts6 showed properties identical to the transformed WT strain in that (i) it contained intact peroxisomes and was able to grow on

methanol, (ii) the levels of PMP47 expression varied with the growth conditions (e.g. the composition of the growth medium), and (iii) PMP47 sedimented in the peroxisomal peak fractions from glycerol-grown cells as was evident from cell fractionation studies (results not shown). However, at restrictive temperatures (43°C) Ts6 lacks peroxisomes and consequently is not able to grow on methanol [17]. Western blot analysis indicated that the level of PMP47 expression in Ts6 was not significantly affected by the temperature and identical in cells, grown at either the permissive temperature (30°C; not shown) or the restrictive temperature (43°C; Fig. 2, lane C). Fully derepressed cells of Ts6 from a continu-
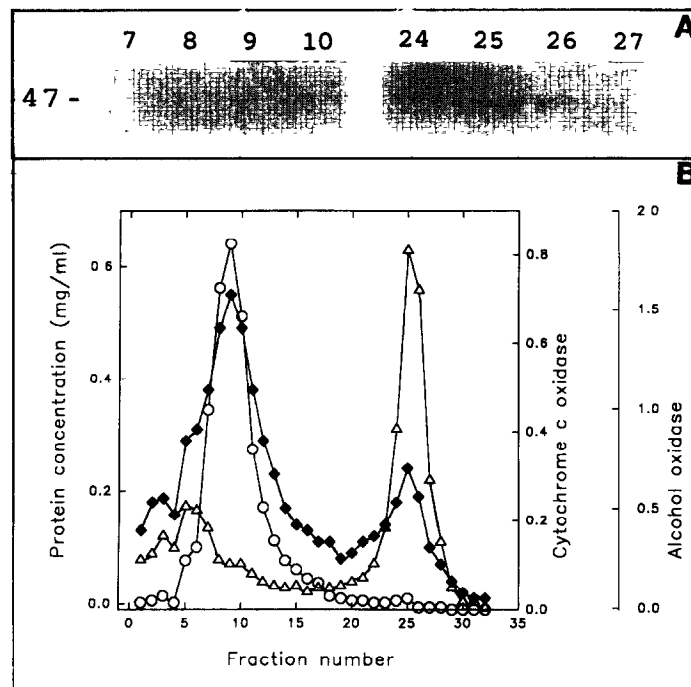
Fig. 4. A. Western blots prepared from the mitochondrial (fractions 7–10) and peroxisomal peak fractions (fractions 24–27) from the sucrose gradient depicted in B. Using monoclonal antibodies against PMP47 of *C. boidinii*, PMP47 protein is solely detected in the peroxisomal peak fractions (10 μg of protein was loaded on each lane). B. Distribution of protein and enzyme activities after sucrose gradient centrifugation of the 30,000 × *g* pellet obtained by differential centrifugation of homogenized protoplasts of transformed WT *H. polymorpha* grown in batch culture on glycerol. (♦) Protein concentration; (○) cytochrome *c* oxidase activity; (△) alcohol oxidase activity.

ous culture on glucose/methanol and grown at restrictive temperature are, as constitutive *per* mutants, characterized by the presence of large cytosolic alcohol oxidase crystalloids [5]. In identically grown cells of the Ts6 transformant, peroxisomes were completely lacking at 43°C (Fig. 3B), indicating that PMP47 could not functionally complement the Ts6 mutation. Differential centrifugation of homogenized protoplasts of such cells revealed that the major fraction of PMP47 was sedimentable and present in the 30,000 × *g* pellet (Fig. 6,

lane A). Subsequent analysis showed that this fraction also contained, beside PMP47, a homologous 68 kDa PMP (PMP68) of *H. polymorpha* (Fig. 6, lane B). Previous experiments revealed that in fully derepressed *per* mutants of *H. polymorpha* different homologous PMP's
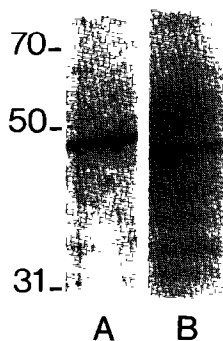

Fig. 5. Western blots using monoclonal antibodies against PMP47, showing the presence of PMP47 in both the peroxisomal (lane A) and mitochondrial peak fraction (lane B) of a sucrose density gradient, prepared from homogenates of transformed WT *H. polymorpha*, grown in a chemostat on glucose/methanol mixture. 20 μg of protein was loaded per lane.
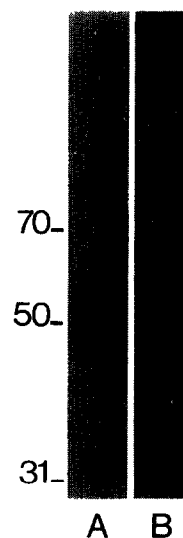

Fig. 6. Western blot, prepared from the 30,000 × *g* pellet fraction, obtained after differential centrifugation of homogenized spheroplasts of transformed Ts6, incubated for 24 h on methanol at 43°C showing, co-localization of PMP47 (lane A) and PMP68 of *H. polymorpha* (lane B) in this fraction. 30 μg of protein was loaded per lane.

(including PMP68) are sedimentable and contained in a small cytosolic proteinaceous aggregate [18]. However, due to the very low yield of these proteins, obtained by the cell fractionation procedure, we were not able to further purify the PMP47-containing fraction from the 30,000 × g pellet by biochemical methods (e.g. sucrose density centrifugation) in order to determine its exact location. Therefore, we have studied the location of PMP47 in transformed Ts6 cells immunocytochemically, using the protein A/gold method. When grown at 30°C, labeling in glycerol-grown cells was almost exclusively on the peroxisomal membrane (Fig. 7A). However, in fully derepressed cells, grown in a chemostat on glucose/methanol at 43°C, specific labeling was observed on cytosolic electron dense aggregates (Fig. 7B), but also on mitochondrial profiles. Labeling of the mitochondria was predominantly on the cristae (Fig. 7C), but infrequently also on electron dense aggregates present in the mitochondrial matrix (Fig. 7C, inset). Under

conditions of low to moderate expression of PMP47 (in batch cultures grown at 43°C on glycerol) labeling was confined to cytosolic aggregates. Double labeling experiments, using antibodies against PMP47 and PMP68 of *H. polymorpha* [15], showed that both proteins were present in the same cytosolic aggregates (Fig. 7B, inset). The above location of PMP47 in transformed Ts6 cells was in line with immunofluorescence observations (Fig. 7D–F). In partly derepressed cells, grown at 30°C on glycerol, peroxisomal membranes showed fluorescence (30°C; Fig. 7D); however, at restrictive growth conditions (43°C) these cells invariably showed one, or infrequently very few, intense fluorescent spots (Fig. 7E). In fully derepressed cells (from glucose/methanol-grown chemostat cultures) grown at restrictive temperatures several spots were observed part of which, on the basis of their elongated morphology, were considered to represent mitochondria (Fig. 7F). Therefore, at enhanced expression rates, also in the absence of peroxisomes part
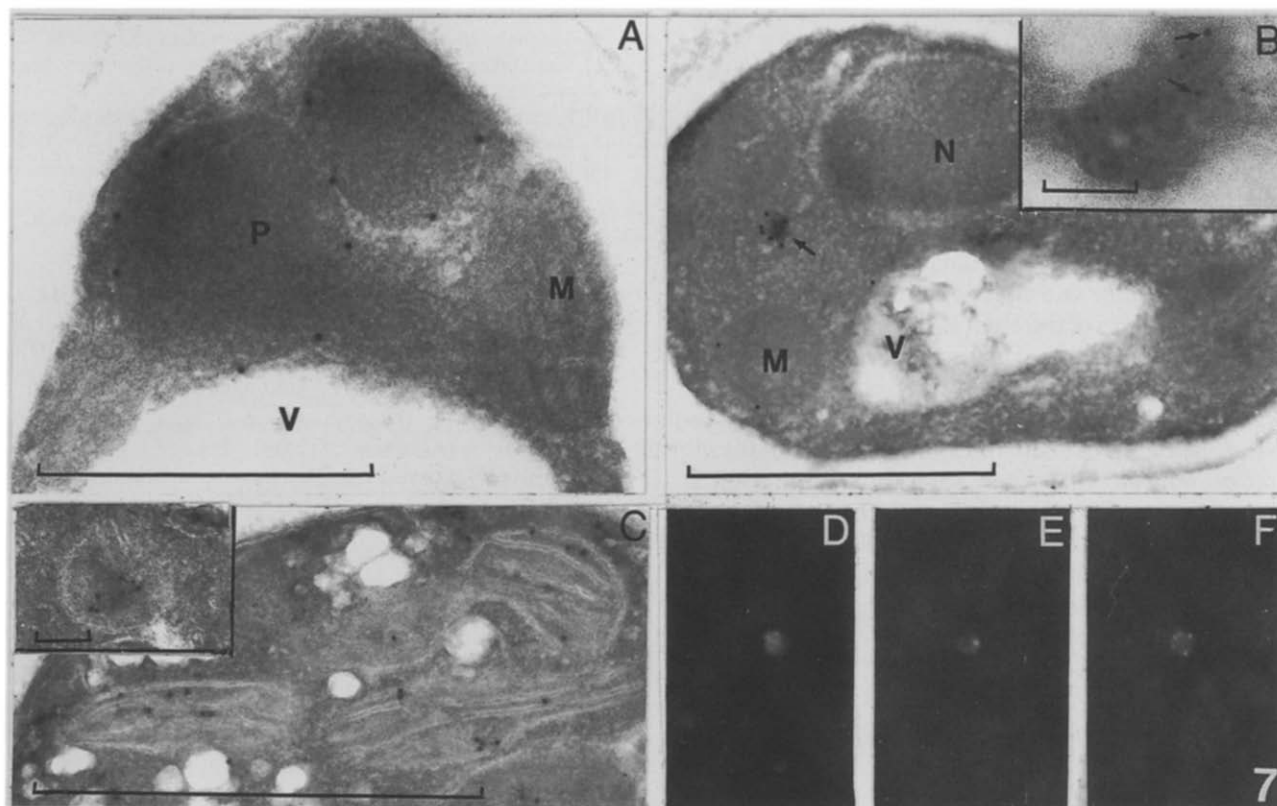

Fig. 7. Immunocytochemical experiments performed on ultrathin sections of glycerol-grown transformed Ts6 using specific antibodies against PMP47 and protein A/gold. At permissive temperatures (30°C) labeling is located on the peroxisomal membrane (A), at restrictive temperatures (43°C) gold particles have accumulated on an electron dense cytoplasmic aggregate (B; arrow); in addition few gold particles are present on mitochondrial membranes. Double labeling experiments (B, inset) showed that PMP47 accumulated in aggregates together with homologous PMP's (8 nm gold: anti PMP68 of *H. polymorpha* [15]; 15 nm gold, anti PMP47, arrows). C. Detail of a fully derepressed Ts6 transformant (grown at 43°C in a chemostat on glucose/methanol) showing labeling on the mitochondria; infrequently labeling was also present on electron dense aggregates in the mitochondrial matrix (C, inset; bar = 0.1 μm; A–C: glutaraldehyde, Lowicryl; uranyl acetate). D–F: immunofluorescence experiments performed on aldehyde-fixed spheroplasts of transformed Ts6, using monoclonal antibodies against PMP47 of *C. boidinii* and FITC. In glycerol-grown cells, grown at 30°C the peroxisomal membranes show fluorescence whereas at restrictive temperature (43°C; E) these cells display a single fluorescent spot. F shows the characteristic fluorescence pattern obtained in fully derepressed Ts6 transformants grown at 43°C in a chemostat on glucose/methanol. The fluorescence in the upper part of the cells is considered to represent mitochondrial staining (magnification Fig. D–F: 1,500×). Electron micrographs are taken of KMnO₄-fixed cells unless otherwise indicated. C, cytosolic alcohol oxidase crystalloid; M, mitochondrion; N, nucleus; P, peroxisome; V, vacuole. The bar represents 1.0 μm unless otherwise stated.

of the PMP47 appeared to be mislocalized into mitochondria rather than being precipitated in additional aggregates.

We have interpreted these results as follows. The initial target of PMP47, after heterologous expression in WT *H. polymorpha*, solely comprises the peroxisomal membrane, indicating that conserved topogenic signals may exist for yeast PMP's [4]. At enhanced expression levels in fully derepressed cells the initial target apparently becomes saturated, resulting in sorting of the additional PMP47 to mitochondria. As expected, an identical fate of PMP47 is observed in permissive Ts6 cells. The physiological experiments revealed that incorporation of a heterologous protein in the peroxisomal membrane of *H. polymorpha* only had a minor effect on growth of cells on methanol and therefore did not severely hamper peroxisome functioning [17].

In restrictive Ts6 cells however, PMP47 initially accumulates in cytosolic protein aggregates in which it is present together with homologous PMP's of *H. polymorpha* [18]. Since in untransformed *per* mutants of *H. polymorpha* PMP's are shown to be located in a single proteinaceous aggregate [18], these results suggest that PMP47 is specifically sorted to the already existing, homologous PMP aggregates. Therefore, also in *per* mutant cells the sorting signals of PMP's may still be functional, providing further evidence for the earlier assumption [18] that the PMP aggregates in these mutants in fact represent peroxisomal remnants. The alternative explanation, namely unspecific aggregation due to a mutual affinity of different PMP's, as is for instance observed for peroxisomal matrix proteins of *H. polymorpha* [9], is less likely since this is expected to result in aggregation of the total amount of PMP's synthesized, irrespective of the expression levels. Our results therefore indicate that upon saturation of the initial target (peroxisome or protein aggregate), additionally expressed PMP47 is targeted to mitochondria, indicating that a cryptic mitochondrial targeting signal might be present on the protein. Based on the derived amino acid sequence [19], the amino terminus of PMP47 is not expected to function as such. However, several regions of basic stretches of amino acids are located within the protein [4] which may be sufficiently amphipathic to serve as a mitochondrial targeting domain. Furthermore, these stretches can bind to acidic phospholipids, providing several 'weak' putative candidates as mitochondrial targeting domains on the protein.

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