A Crucial Role of the Mitochondrial Protein Import Receptor MOM19 for the Biogenesis of Mitochondria

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Abstract. The novel genetic method of "sheltered RIP" (repeat induced point mutation) was used to generate a *Neurospora crassa* mutant in which MOM19, a component of the protein import machinery of the mitochondrial outer membrane, can be depleted. Deficiency in MOM19 resulted in a severe growth defect, but the cells remained viable. The number of mitochondrial profiles was not grossly changed, but mutant mitochondria were highly deficient in cristae membranes, cytochromes, and protein synthesis activity. Protein import into isolated mutant mitochondria was decreased by factors of 6 to 30 for most proteins from all suborganellar compartments. Proteins like the ADP/ATP carrier, MOM19, and cytochrome *c*, whose import into wild-type mitochondria occurs independently of MOM19 became imported normally showing that the reduced import activities are solely caused by a lack of MOM19. Depletion of MOM19 reveals a close functional relationship between MOM19 and MOM22, since loss of MOM19 led to decreased levels of MOM22 and reduced protein import through MOM22. Furthermore, MOM72 does not function as a general backup receptor for MOM19 suggesting that these two proteins have distinct precursor specificities. These findings demonstrate that the import receptor MOM19 fulfills an important role in the biogenesis of mitochondria and that it is essential for the formation of mitochondria competent in respiration and phosphorylation.

THE biogenesis of mitochondria requires the coordinated action of both nuclear and mitochondrial genomes nomes (Grivell, 1989). Proper function of mitochondrial processes depends on accurate import and suborganellar sorting of many preproteins synthesized on cytosolic ribosomes. Protein import into mitochondria is a complex process that requires two separate machineries in the outer and inner membrane each consisting of a large number of components (for reviews see Pfanner and Neupert, 1990; Glick and Schatz, 1991; Maarse et al., 1992; Pfanner et al., 1992; Emtage and Jensen, 1993; Segui-Real et al., 1993*b*). The translocation machinery of the mitochondrial outer membrane is comprised of at least six components organized in a complex (Kiebler et al., 1990; Moczko et al., 1992). MOM19 and MOM72 of *Neurospora crassa* mitochondria have been reported to be involved in the initial step of recognition and binding of preproteins to the mitochondrial surface (Söllner et al., 1989, 1990). MOM22 has recently been shown to function in the passage of preproteins from this receptor binding stage to a site where proteins are fully inserted into the outer membrane (Kiebler et al., 1993). At least part of this site, the so-called "general insertion pore" (GIP)[1] (Pfaller et al., 1988) consists of MOM38 and MOM7/MOM8 (Söllner et al., 1992). So far, most of the functional analysis of preprotein passage across the receptor/GIP complex has been carried out in vitro. For instance, the relative specificities of MOM19 and MOM72 for various precursor proteins have been analyzed in a biochemical approach by using MOM19- and MOM72-specific antibodies. Antibodies against MOM19 inhibited import of the majority of mitochondrial preproteins (Söllner et al., 1989; Moczko et al., 1993), while those against MOM72 appeared to be specific for a subset of proteins like the ADP/ATP carrier (AAC) (Söllner et al., 1990).

In order to understand the role of MOM19 in the initial reaction of protein import, it is important to investigate its function in vivo, e.g., by using mutants defective in MOM19. In the yeast *Saccharomyces cerevisiae*, Mas70p, the counter-

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1. *Abbreviations used in this paper*: AAC, ADP/ATP carrier; fpa, *p*-fluorophenylalanine; GIP, general insertion pore; RIP, repeat induced point mutation.

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part of *N. crassa* MOM72, has been found in vitro to be involved in the transport of a number of preproteins (Hines et al., 1990; Hines and Schatz, 1993). The phenotype of a mas70 disruption mutant, however, was rather inconspicuous showing slower growth only on nonfermentable carbon sources (Riezman et al., 1983) and no drastic effects on protein import in vivo (Hines et al., 1990). Despite the wealth of biochemical information obtained from *N. crassa*, mutants of the protein import machinery have not yet been described, thus precluding in vivo studies on the functional role of individual components. So far, mutants in cloned genes of this organism have been constructed either by introducing the mutant gene by homologous recombination (see, e.g., Nehls et al., 1992) or by altering the gene by the phenomenon of repeat induced point mutations (RIP) previously shown to destroy duplicated target genes by methylation and by GC to AT transitions (Selker, 1990). Unfortunately, these procedures have not allowed the study of genes which might play an important or essential function for the cell. Therefore, a novel genetic strategy was developed to prevent the lethal effects of destroying a potentially essential gene, and to allow the analysis of the effects upon inactivation of that gene. The method referred to as "sheltered RIP" makes use of RIP for destruction of a target gene, but allows the expression and maintenance of the mutated alleles in a heterokaryon in which the normal copy of the gene, present in another nucleus, shelters the cell against potentially lethal effects. We have described the genetic details underlying the technique elsewhere (Harkness et al., 1994). In brief, the duplicated sequence of a target gene present in one nucleus of the mating pair in the ascogenous hyphae will undergo alterations by RIP with a fairly high frequency as the nucleus passes through a genetic cross. The single copy of the corresponding gene in the nucleus of the mating partner, however, remains unaffected by RIP. In a heterokaryotic background the latter copy will provide the functional gene product for the cell, and thus shelter the cells from the effects caused by the RIP alleles. For the study of the consequences of inactivating the target gene, growth of cells containing the unaffected nucleus is inhibited by addition of a drug to which the mutant nucleus carries a resistance marker. Growth in the presence of the drug thus selects for cells harboring the mutant allele, and makes it possible to study the mutant phenotype (for details see Harkness et al., 1994). Sheltered RIP provides a generally applicable procedure for the generation of mutants in any cloned gene of interest in *N. crassa*, since duplicated genes can easily be produced by transformation and integration of that gene into an ectopic site of the genome (Akins and Lambowitz, 1985).

We have applied sheltered RIP to generate a mutant which is deficient in MOM19, when grown under conditions favoring the nucleus affected by RIP. We report here on the severe phenotypic consequences of a lack of functional MOM19 on cell growth and on the ultrastructure, protein composition, and protein import of mitochondria. Our data establish a crucial role of MOM19 for the biogenesis of mitochondria. A detailed investigation of the small amount of protein import still detectable in isolated mutant mitochondria suggests a tight functional cooperation between MOM19 and MOM22. In addition, MOM72 does not serve as a general backup receptor for MOM19 implying that MOM19 is the major entry point for preproteins into mitochondria.

Materials and Methods

Neurospora Strains and Growth Conditions

Strains of *N. crassa* used in this study were: (*a*) host IV (H-IV): LGI, *a*; LGIV, *pyr-1 mom-19$^+$ mtr^R trp-4$^+$*; LGV, *inl inv mei-2*; (*b*) Mate IV (M-IV): LGI, *A*; LGIV, *pyr-1$^+$ mom-19$^+$ mtr^S trp-4*; LGV, *am inl inv mei-2*; (c) TI28.3, derived from H-IV by integrating an ectopic copy of mom-19$^+$ into an unknown chromosome (linkage group, LG); and (*d*) 28.17, a heterokaryon containing one nucleus with chromosome IV derived from M-IV and another nucleus containing chromosome IV derived from TI28.3 in which the two copies of mom-19 have been inactivated by RIP (for genetic details see Harkness et al., 1994).

Cultures were grown at 25°C with vigorous aeration and bright illumination in basal medium containing Vogel's salts including trace elements and biotin (Davis and De Serres, 1970), 1.5% glucose, and 50 mg/ml inositol. As required, media were supplemented with 1 mM uridine (for pyr-1). Liquid medium was inoculated with 1–5 × 10^6 conidia/ml. *p*-Fluorophenylalanine (fpa; Sigma, St. Louis, MO) was used at a concentration of 400 μM. In the absence of fpa all strains were grown for 16–20 h, whereas in the presence of fpa H-IV and TI28.3 were grown for 24 h, and the heterokaryon 28.17 was grown for 32–36 h.

Biochemical Procedures

The following published procedures were used: preparation of antisera and IgG (Söllner et al., 1989); preincubation of mitochondria with IgG (Söllner et al., 1989); immunoblotting and detection by chemiluminescence utilizing the ECL system (Amersham Corp., Arlington Heights, IL) was according to Mayer et al. (1993); in vitro transcription and translation in reticulocyte lysate using [^{35}S]methionine as radioactive label (Söllner et al., 1991); immunoprecipitation by protein A–Sepharose (Zimmermann and Neupert, 1980); protein determination using IgG as a standard was performed with the dye binding assay (Bio Rad Labs, Hercules, CA) or with the BCA reagent (Pierce, Rockford, IL); isolation of mitochondria (Stuart et al., 1990); SDS-PAGE, fluorography (Nicholson et al., 1987), and quantitation by laser scanning densitometry (Pfaller et al., 1988); spectral analysis of cytochromes (Bertrand and Pittenger, 1972).

Protein Import into Isolated Mitochondria

A typical protein import reaction consisted of freshly isolated mitochondria (30 μg protein), 1–5 μl rabbit reticulocyte lysate containing radioactively labeled precursor proteins, 80% import buffer (250 mM sucrose, 3% (wt/vol) fatty acid free BSA, 80 mM KCl, 5 mM $MgCl_2$, and 10 mM MOPS/KOH, pH 7.2) in a total volume of 100 μl. Samples were supplemented with an energy mix (2 mM ATP, 3 mM NADH, 10 mM creatine phosphate, and 100 μg/ml creatine kinase). Import was performed at 10–25°C for 15 min. After chilling on ice, samples were immediately treated with 30–60 μg/ml proteinase K for 15 min on ice. Protease digestion was halted by the addition of 2 mM PMSF from a freshly prepared 200 mM stock solution in ethanol, and samples were diluted with 1 ml SEM buffer (250 mM sucrose, 1 mM EDTA, and 10 mM MOPS/KOH, pH 7.2) containing 1 mM PMSF. In the case of MOM38 import, 50 μg/ml trypsin was used which does not degrade imported MOM38, and protease was halted by addition of a 30-fold excess (wt/wt) of soy bean trypsin inhibitor. Mitochondria were reisolated by centrifugation for 12 min at 10,000 *g* in a JA-18.1 rotor (Beckman Instrs., Inc., Fullerton, CA). Radioactive, imported proteins were subjected to SDS-PAGE and fluorography, and quantitated by laser densitometry.

Electron Microscopy

Hyphae were fixed in 1.5% $KMnO_4$ for 20 min at room temperature followed by intensive washing with H_2O till the suspension was colorless. Protoplasts and isolated mitochondria were fixed in 3% (vol/vol) glutaraldehyde in 0.1 M cacodylate buffer for 30 min on ice and subsequently postfixed in a mixture of 1% (wt/vol) OsO_4 and 1.5% (wt/vol) $K_2Cr_2O_7$. All samples were poststained in 1% (wt/vol) uranyl acetate for 16 h at room temperature, dehydrated in a graded ethanol series, and embedded in Epon 812. Ultrathin sections were cut with a diamond knife and examined in an electron microscope (EM 300; Phillips Technologies, Cheshire, CT).

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Results

Inactivation of the mom-19 Gene by Sheltered RIP

We have applied the method of sheltered RIP to inactivate the mom-19 gene in order to study the functional consequences of a deficiency of MOM19 protein in *Neurospora* cells and mitochondria. An outline for generating mom-19 mutants using this method is given in Fig. 1; a description of the genetic details of the procedure will be published elsewhere (Harkness et al., 1994). Briefly, the *N. crassa* strain H-IV was transformed with genomic mom-19 DNA to generate the TI28.3 isolate containing a single ectopic copy of mom-19 in addition to the endogenous one. TI28.3 was crossed to the mating strain M-IV harboring the appropriate complementing genetic markers for selection of the desired mating products (pyr and trp; Fig. 1). During the cross the duplicated sequences of mom-19 in the TI28.3-derived nucleus were subjected to RIP, whereas the single copy of mom-19 in M-IV remained unaffected. A mutant allele of the mei-2 gene in both H-IV and M-IV caused effective nondisjunction of the chromosomes during meiosis, thereby generating a variety of products, most of which were inviable. The desired products of this sheltered RIP cross were disomic ascospores containing a mutated mom-19^{RIP} allele on one chromosome IV (derived from strain H-IV) and a normal mom-19 allele on the other chromosome IV (derived from strain M-IV). During vegetative growth the disomic spores rapidly and spontaneously converted to heterokaryons thereby separating the two chromosomes IV into different nuclei (Fig. 1). Chromosome IV of the nucleus harboring the functional mom-19 also contained the wild-type version of the mtr gene (mtr^S), which renders cells containing such nuclei sensitive for growth in the presence of fpa. On the other hand, chromosome IV of the nucleus carrying the mom-19^{RIP} allele, contained a mutant version of the mtr gene (mtr^R) conferring resistance to fpa. Thus, growth of the heterokaryon in the presence of fpa inhibited proliferation of cells containing a functional mom-19 copy, and selected for cells harboring the inactivated mom-19^{RIP} gene. This results in an overall reduction of intact MOM19 and allows the study of the phenotypic consequences of MOM19 inactivation. From a number of heterokaryotic isolates displaying a growth defect in the presence of fpa (Harkness et al., 1994) strain 28.17 was chosen for this study on the basis of a complete lack of MOM19 protein (see below). Strain 28.17 also contained the ectopic copy of mom-19^{RIP} on an unidentified chromosome. The DNA alterations caused by RIP resulted in 18 amino acid changes in the endogenous mom-19^{RIP} copy and in at least 12 in the ectopic copy. The latter copy most likely did not yield any functional gene product, as it contained an altered splice junction in the third intron. Details of the alterations have been summarized elsewhere (Harkness et al., 1994).

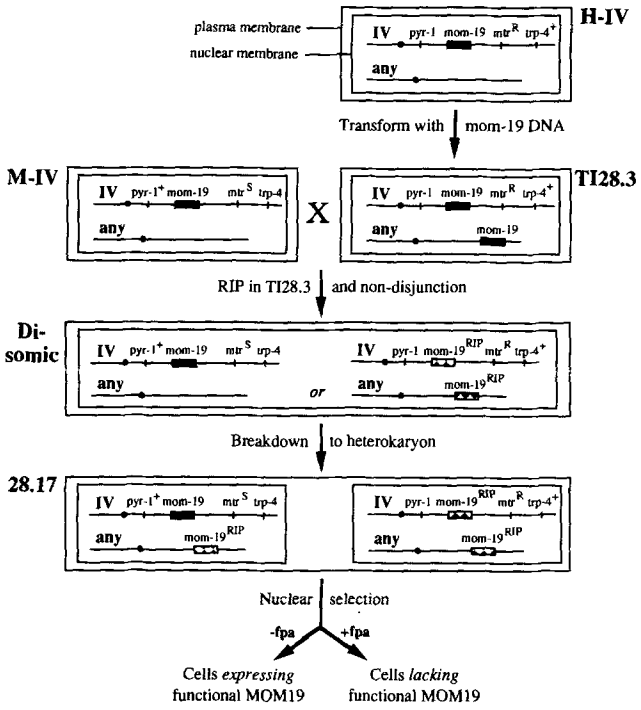

Figure 1. Inactivation of the mom-19 gene by sheltered RIP. The host strain (H-IV) carrying both the endogenous copy of mom-19 and the auxotrophic marker pyr-1 (uridine requirement) on chromosome (linkage group) IV was transformed with genomic mom-19 DNA to produce the strain TI28.3. The transformant thus received a second functional copy of mom-19 (ectopic copy) on an unidentified chromosome. TI28.3 was crossed to the mate strain (M-IV), which contained the trp-4 mutant allele (tryptophan requirement) on chromosome IV. During the sexual cycle the duplicated mom-19 sequences in TI28.3 underwent mutational modifications according to the RIP mechanism (Selker, 1990). Normal chromosome pairing during meiosis was prevented by the inclusion of the mei-2 mutant allele on chromosomes V in both H-IV and M-IV strains. This led to nondisjunction generating a series of aneuploid products including the desired disomic cell harboring both functional mom-19 and mutant mom-19^{RIP} alleles on the two chromosomes IV. Plating on media lacking uridine and tryptophan ensured that only the disomic colonies containing both chromosomes IV were present. The ectopic mom-19^{RIP} copy should segregate with the endogenous mom-19^{RIP} copy 50% of the time. Further culture of the disomic colonies on media lacking uridine and tryptophan allowed rapid and spontaneous breakdown of the disomic nucleus to a heterokaryon containing haploid nuclei with one of the two different chromosomes IV. The strain 28.17 which also contained the ectopic copy of mom-19^{RIP} was isolated. Synthesis of functional MOM19 was inhibited by growth in the presence of the inhibitor *p*-fluorophenylalanine (fpa; mtr as the genetic marker) which selected for cells containing mtr resistant (mtr^R) nuclei. Since cells harboring mtr^S do not grow in the presence of fpa, only defective MOM19^{RIP} protein may be produced, thus allowing the study of the effects caused by inactivation of mom-19.

Depletion of MOM19 Results in Severely Impaired Growth of Neurospora Cells

To examine the effects of MOM19 depletion on the growth of *Neurospora* cells, the heterokaryotic strain 28.17 was grown in liquid media in the presence or absence of fpa to inhibit or allow synthesis of functional MOM19, respectively (Fig. 1). Cell mass was measured after harvesting the cells by filtration. For comparison, growth curves for TI28.3 cells were analyzed in parallel. In the absence of fpa both 28.17 and TI28.3 strains displayed exponential growth at comparable rates (Fig. 2 *A*). In the presence of fpa TI28.3 cells, after an initial lag phase, grew at a similar rate as cells in the absence of fpa. Identical growth characteristics were observed

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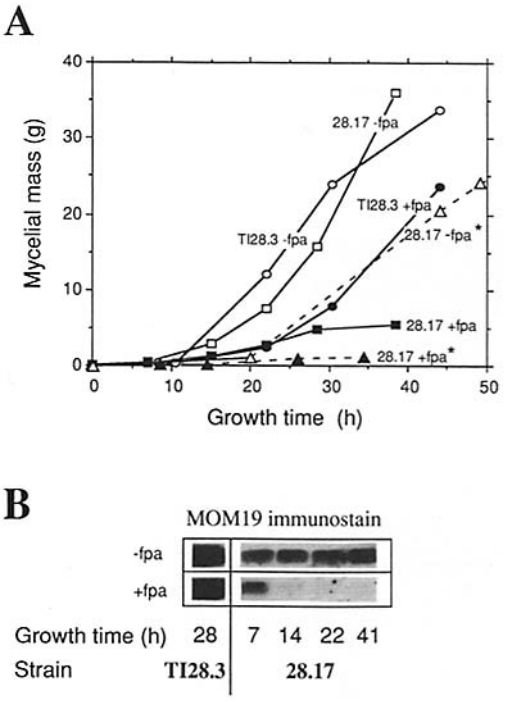

Figure 2. Depletion of functional MOM19 leads to a severe growth defect. (*A*) Cells from the strains TI28.3 and 28.17 were grown at 25°C in the presence or absence of fpa in basal medium as indicated. At various times cells were harvested by filtration, and the wet cell mass was measured. After 38 h mycelia of strain 28.17 grown in the presence of fpa were backdiluted 40-fold into fresh basal medium with (28.17 +fpa*) or without (28.17 −fpa*) fpa, and growth was continued at 25°C for the indicated times before harvesting and weighing of the cells. The mycelial masses are given per liter of cell culture. (*B*) Time course of MOM19 depletion. Cells were grown as described in *A* and harvested at the indicated times by filtration. A cellular protein extract was prepared by grinding with 1 g quartz sand in 1 ml SEM buffer containing 1 mM PMSF and 1% SDS per gram of mycelia. The protein extract was clarified by centrifugation (4,000 *g*, 5 min) and the protein concentration measured by the BCA method (Pierce). 125 μg protein was analyzed for the content of MOM19 protein by SDS-PAGE and immunoblotting.

for H-IV (data not shown). In contrast, 28.17 cells grown in the presence of fpa displayed a drastic reduction in growth rate after ~20 h. When the cells were backdiluted into fresh medium containing fpa, hardly any further increase in mycelial mass was detectable (28.17 +fpa* in Fig. 2 *A*). Upon inoculation of these cells into fresh medium lacking fpa, however, they were able to resume growth at wild-type rates after a lag phase of 20 h (28.17 −fpa* in Fig. 2 *A*). Comparable recovery of growth after exposure to fpa was observed for the fpa-sensitive strain M-IV (data not shown). The results demonstrate that 28.17 cells were severely impaired in growth when fpa was used to inhibit the expression of functional MOM19. The cells, however, remained viable even after prolonged exposure to fpa.

The amount of MOM19 protein in whole cell extracts was measured by immunoblotting. While fpa had no apparent influence on the amount of MOM19 during growth of TI28.3, MOM19 was undetectable by immunostaining in 28.17 cells after growth for 22 h, the time when cells exhibited a marked reduction in growth rate (Fig. 2 *B*). MOM19 was also not detectable by employing an antibody raised against a peptide representing a segment of MOM19 unaffected by RIP (data not shown). This excludes the possibility that the lack of detectable MOM19 was due to alteration of all antigenic sites recognized by the anti-MOM19 antibodies. TI28.3 cells contained threefold higher amounts of MOM19 as compared to wild-type cells (Table I), apparently because of the presence of the additional copy of mom-19. The content of MOM19 in 28.17 cells grown without fpa, on the other hand, was only ~20% of that found in H-IV or M-IV cells (Table I). We have not yet investigated the reason for this decrease in functional MOM19 in 28.17 cells, but the simplest explanation is that the nucleus containing the RIP allele is slightly favored in heterokaryons, possibly due to the nature of the auxotrophic markers used for maintenance of the heterokaryon. The effects described above were not due to alterations introduced into 28.17 cells by RIP outside the coding region of mom-19, since transformation of 28.17 cells with mom-19 cDNA fully relieved the growth defect in homokaryotic isolates, and restored the MOM19 level to that observed in 28.17 cells grown as a heterokaryon without fpa (Harkness et al., 1994). Taken together, these results assign an important role to MOM19 during growth of *Neurospora* cells. This notion is corroborated by independent genetic evidence demonstrating that homokaryotic mom-19^{RIP} mutant cells derived from the heterokaryotic 28.17 strain fail to generate conidia, grow very slowly, and exhibit a "stop-start" phenotype (Bertrand and Pittenger, 1972; Bertrand et al., 1980; Harkness et al., 1994). The availability of a MOM19 mutant now enabled us to address the question of which function of MOM19 might be important for normal growth of *Neurospora* cells.

MOM19-deficient Cells Contain Mitochondria with a Grossly Altered Morphology and Protein Composition

As MOM19 has been reported to represent an important component of the mitochondrial protein import machinery, we first investigated, whether MOM19-deficient cells still contain mitochondria and what the morphological consequences of a loss of MOM19 would be. Cells of strains TI28.3 and 28.17 were grown for various times in the presence and absence of fpa, and examined by electron micros-

Table I. Abundance of Components of the Protein Import Complex of the Mitochondrial Outer Membrane in MOM19-deficient Mitochondria

N. crassa strain	MOM19	MOM22	MOM38	MOM72
H-IV or M-IV	=100	=100	=100	=100
TI28.3 −fpa	322	185	84	106
TI28.3 +fpa	280	128	92	87
28.17 −fpa	24	49	112	96
28.17 +fpa	ND	20	104	62

Mitochondria were isolated from the indicated strains grown in the presence or absence of fpa. Mitochondrial protein (10–100 μg) was analyzed for the relative amounts of the various components of the protein import complex by SDS-PAGE and immunoblotting using the ECL luminescence detection kit. Quantitation was performed by laser densitometry. Data are given relative to the values obtained for wild-type strains H-IV and M-IV. ND, not detectable.

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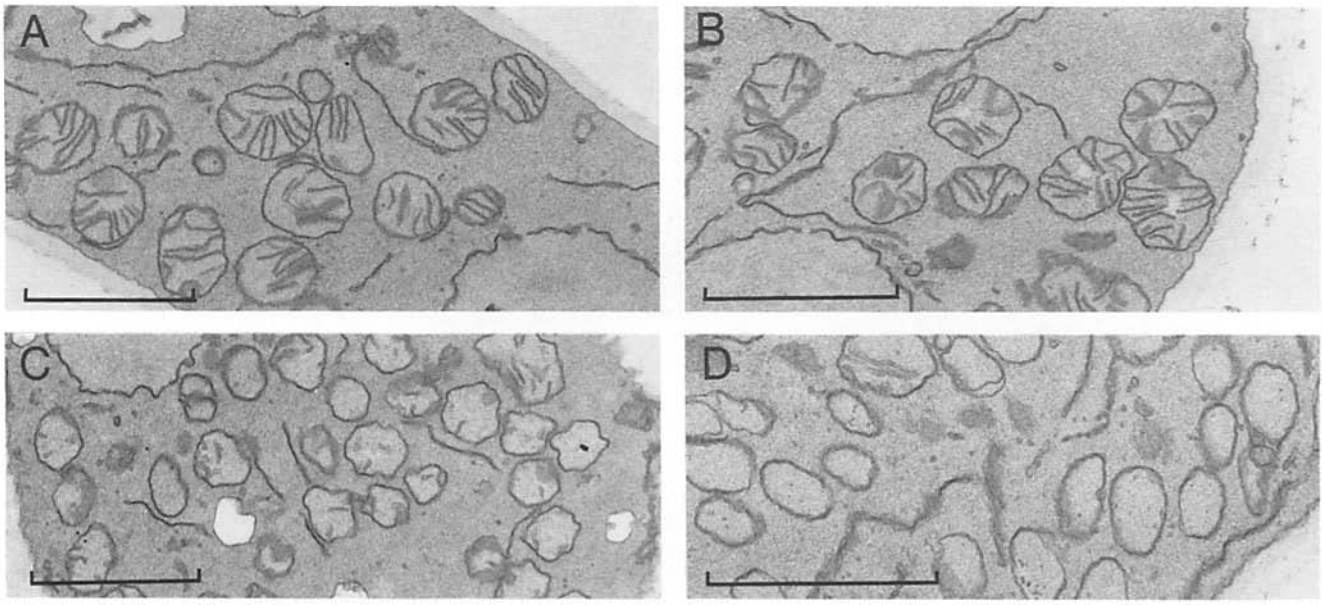

Figure 3. The mitochondrial ultrastructure is grossly changed in MOM19-deficient *N. crassa* cells. 28.17 cells were grown as described in Fig. 2 *A* in the absence of fpa for 15 h (*A*) or in the presence of fpa for 8 (*B*), 16 (*C*), and 32 h (*D*). Cells were examined by electron microscopy after fixation with $KMnO_4$. Bars, 1 μm.

copy. In all cases mitochondrial profiles were clearly visible (Fig. 3). In the presence of fpa 28.17 cells accumulated abnormal mitochondria with a distinct lack of cristae (Fig. 3, *A–D*), while in TI28.3 fpa caused no apparent changes (data not shown). The altered morphology of mitochondria in 28.17 cells grown with fpa appeared concomitantly with the depletion of MOM19 from the cell extracts suggesting that loss of MOM19 function is responsible for the altered morphology (compare Fig. 3, *B–D* with Fig. 2 *B*). Interestingly, the lack of cristae in MOM19-deficient mitochondria resembles the morphology observed for mitochondria in ρ^0- and ρ^--yeast strains (Stevens, 1977, 1981). Despite the substantial reduction in inner membrane content, outer and inner membranes were clearly distinguishable in these mitochondria. The number of mitochondrial profiles per cell and their size did not change significantly upon MOM19 depletion. When mitochondria were isolated from MOM19-deficient cells and visualized by electron microscopy, they appeared as intact organelles despite the complete lack of cristae (data not shown). Furthermore, in glutaraldehyde-fixed samples a lower number in mitochondrial ribosomes was visible in MOM19-deficient cells (data not shown). Taken together, these data demonstrate the importance of MOM19 for the biogenesis and morphology of mitochondria.

To examine the apparent low content in mitochondrial ribosomes in more detail, organellar protein synthesis activity was measured in vivo in the presence of cycloheximide to inactivate cytosolic ribosomes. Hardly any mitochondrial-encoded protein was synthesized in MOM19-deficient cells (from 28.17 grown with fpa) showing a more than 40-fold reduction as compared to wild-type cells or 28.17 cells without fpa (Fig. 4 *A*). The results suggest that MOM19-deficient mitochondria are defective in the expression of mitochondrial-encoded proteins, most of which are components of the oxidative phosphorylation pathway. This observation and the loss of cristae membranes (see above) in MOM19-deficient mitochondria made it likely that the amount of cytochromes was decreased. We therefore recorded absorption difference spectra of mitochondria (Bertrand and Pittenger, 1972) isolated from H-IV and from 28.17 cells grown with and without fpa. Despite the marked reduction of MOM19 in 28.17 cells grown without fpa, no apparent differences to the spectra of mitochondria from wild-type cells were observed (Fig. 4 *B*). On the other hand, mitochondria completely lacking MOM19 (from 28.17 cells grown with fpa) displayed a substantial decrease in cytochromes a/a_3 and *b*, while cytochrome *c* appeared to be virtually unchanged.

The changes in the ultrastructure of mitochondria during MOM19 depletion in 28.17 cells were reflected by a changing mitochondrial protein pattern. While fpa had no apparent effect on the protein composition of wild-type mitochondria, a number of proteins from mitochondria isolated from 28.17 cells strongly increased or decreased in concentration during growth in medium containing fpa (data not shown). To investigate the specific changes of individual mitochondrial proteins, mitochondria were isolated from H-IV, TI28.3, and 28.17 cells grown with or without fpa and analyzed by immunostaining. We first examined the amounts of the other components of the protein import complex in the mitochondrial outer membrane. No significant changes in the steady-state levels were observed for MOM38 and the other surface receptor MOM72 upon depletion of MOM19, not even in 28.17 cells grown in the presence of fpa (Fig. 5 *A* and Table I). In contrast, MOM22 was reduced by 80% in these cells as compared to wild-type cells of strain H-IV. Also in 28.17

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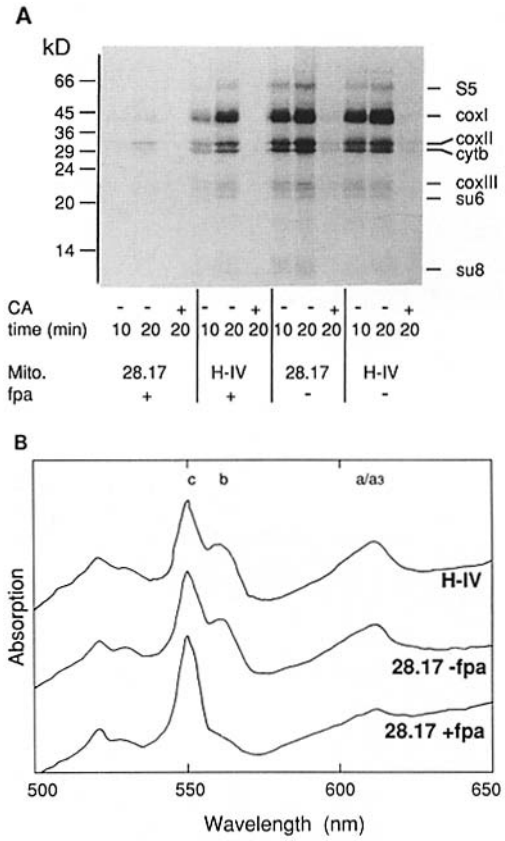

Figure 4. MOM19-deficient mitochondria have a strongly reduced protein synthesis activity and a deficiency in cytochromes. (*A*) Strains H-IV and 28.17 were grown at 25°C in the absence or presence of fpa as described in Fig. 2 *A*. To 100 ml of cell culture 100 μg/ml cycloheximide and, when indicated, 2 mg/ml chloramphenicol (*CA*) were added to block cytosolic and mitochondrial protein synthesis, respectively (Hallermayer et al., 1977). After 3 min the cells were labeled with [^{35}S]methionine (0.5 mCi; 1,100 Ci/mMol) for the indicated times and mitochondria (*Mito.*) were immediately isolated. Radioactively labeled mitochondrial protein (70 μg/sample) was analyzed by SDS-PAGE and fluorography. Quantitation by laser densitometry revealed a more than 40-fold reduction of mitochondrial protein synthesis in mutant (28.17 +fpa) cells as compared to control cells. Some of the mitochondrial protein synthesis products are indicated (S5, ribsosomal protein S5; *coxI–III*, subunits I–III of cytochrome *c* oxidase; *cytb*, cytochrome *b*; *su6* and *su8*, subunits 6 and 8 of F_0-ATPase). Molecular weights of marker proteins are given on the left side of the figure. (*B*) Mitochondria were isolated from strains H-IV and 28.17 grown with and without fpa, and examined by differential absorption spectrophotometry (Bertrand and Pittenger, 1972). The spectra of mitochondria from H-IV grown with or without fpa were identical. The absorption maxima of the cytochromes a/a_3, *b*, and *c* are indicated.

cells grown in the absence of fpa a twofold reduction of MOM22 was seen as a consequence of the decreased amount of MOM19, while in TI28.3, which contains threefold higher amounts of MOM19 as a result of the second copy of mom-19, MOM22 appeared to be increased (Table I). These results suggest that the steady-state levels of MOM19 and MOM22 are adjusted in a coordinated fashion. This might be a consequence of the direct interaction between these two proteins in the receptor complex which is also suggested by independent functional studies (see below).

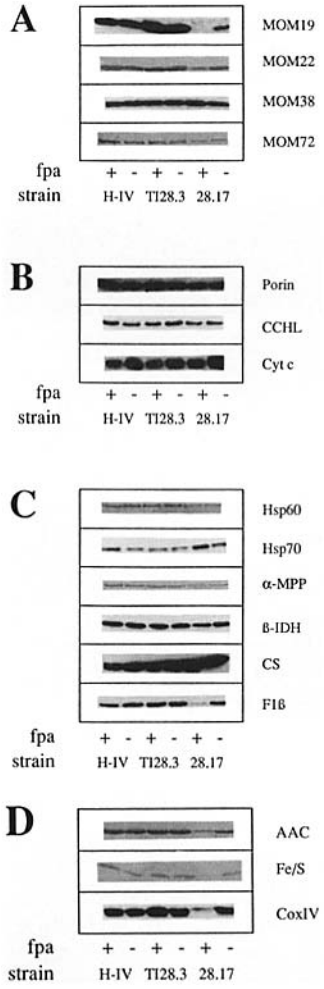

Figure 5. MOM19-deficient mitochondria display a largely altered protein composition. Mitochondria were isolated from strains H-IV, TI28.3, and 28.17 grown in the presence or absence of fpa. 1–100 μg of mitochondrial protein was analyzed by SDS-PAGE and immunoblotting for the amount of the indicated components from (*A*) the protein import complex of the outer membrane, (*B*) the outer membrane and the intermembrane space, (*C*) the matrix space, and (*D*) the inner membrane. *CCHL*, cytochrome *c* heme lyase; *Cyt c*, cytochrome *c*; *Hsp60* and *Hsp70*, heat shock proteins of 60 and 70 kD; α-MPP, α subunit of matrix processing peptidase; *β-IDH*, β subunit of isocitrate dehydrogenase; *CS*, citrate synthase; *F1β*, β subunit of F_1-ATPase; *AAC*, ADP/ATP carrier; *Fe/S*, Rieske iron–sulfur protein; *CoxIV*, subunit IV of cytochrome *c* oxidase.

We next investigated individual components of the outer membrane and the intermembrane space for changes in their steady-state levels as a result of MOM19 inactivation (Fig. 5 *B*). All proteins analyzed from these sub-compartments including cytochrome *c* (see above) were not significantly altered in their levels irrespective of the amount of MOM19 present in the mitochondria. A similar result was observed for proteins from the matrix space, with the exception of Hsp70 which showed a twofold increase, and the β subunit of F_1-ATPase, a protein tightly associated with the inner membrane (Fig. 5 *C*). Components of the inner membrane, on the other hand, were reduced by factors of more than 10 as a result of MOM19 depletion (Fig. 5 *D*). This result is in keeping with the substantial reduction in cristae membranes and with the decrease in cytochromes (see above). From the data in Fig. 5 it becomes obvious that the steady-state levels of specific mitochondrial proteins were altered as a consequence of MOM19 inactivation. These effects are most likely due to a variety of secondary events, e.g., protein import and assembly, reduced expression of the mitochondrial gene products, altered regulation of expression, and different stability of the individual proteins.

MOM19-deficient Mitochondria Are Strongly Impaired in the Import of Most, but Not All Precursor Proteins

To directly study the consequences of a lack of functional

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MOM19 for the transport of individual mitochondrial preproteins, import into isolated mitochondria was measured. Mitochondria from strains H-IV or 28.17 grown in the absence or presence of fpa were incubated with in vitro synthesized preproteins. For a number of preproteins import into MOM19-deficient mitochondria (from 28.17 cells grown with fpa) was substantially reduced in comparison to import into mitochondria derived from wild-type cells H-IV (Fig. 6 *A*). These proteins included components of the outer membrane (porin [Kleene et al., 1987] and MOM38 [Keil et al., 1993]), the intermembrane space (cytochrome *c* heme lyase, CCHL [Lill et al., 1992]), the inner membrane

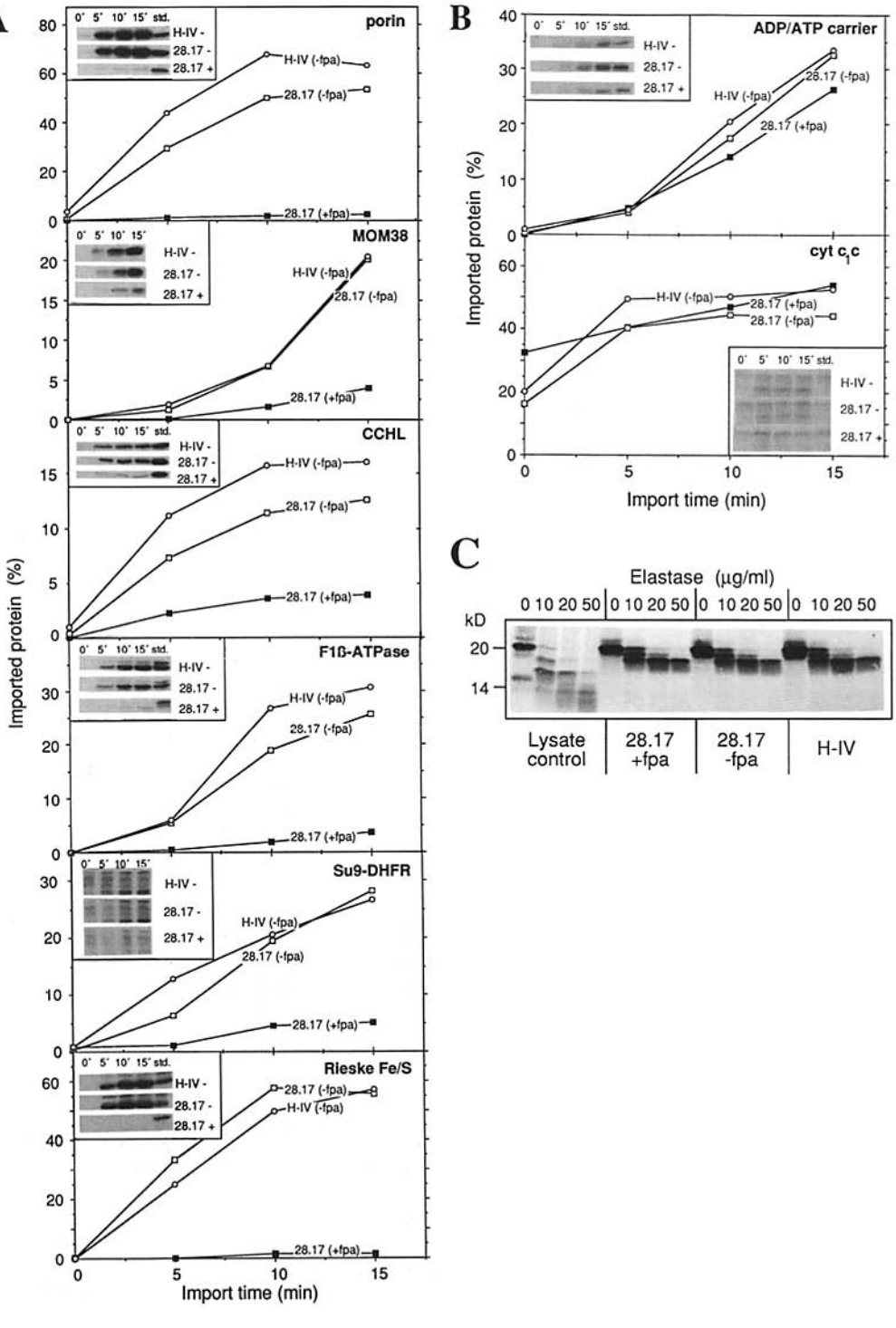

Figure 6. Protein import into MOM19-deficient mitochondria is severely affected for most, but not all preproteins. Radioactively labeled preproteins were incubated in import buffer (supplemented with an energy mix) with 30 µg freshly isolated mitochondria from strains H-IV and 28.17 grown with and without fpa as indicated. To assure that small differences in the rates of import can be detected, import temperatures were kept at 10°C. After the indicated times import was terminated by transfer to 0°C and immediate protease treatment. Mitochondria were reisolated by centrifugation, and the samples were analyzed for imported protein by SDS-PAGE and fluorography. Quantitation of the fluorographs was performed by laser densitometry. Ordinate values are given relative to the amount of radioactively labeled protein associated with mitochondria after 15 min. The standard lane (*std.*) contains 30% of that material. Preproteins that were affected by MOM19 depletion are shown in *A* and those unaffected by MOM19 deficiency are given in *B* and *C*. Su9-DHFR, a fusion protein between subunit 9 of F_0-ATPase and dihydrofolate reductase; cyt c_1c, a fusion protein between the presequence of cytochrome c_1 and cytochrome *c*. Other abbreviations are as in Fig. 5. (*C*) Import of MOM19 was for 10 min at 25°C (Mayer et al., 1993). Samples were treated with the indicated amounts of elastase for 10 min at 25°C followed by addition of 1 ml of SEM buffer containing 1 mM PMSF and an incubation for 5 min at 25°C. Further analysis of the samples was as above. Import was estimated from the characteristic fragmentation pattern of MOM19 which is formed only after correct insertion of MOM19 into the outer membrane (Schneider et al., 1991). In contrast, MOM19 precursor present in reticulocyte lysate becomes degraded at much lower concentrations of protease (Lysate control).

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(Rieske Fe/S protein [Hartl et al., 1986] and Su9-DHFR, a fusion protein between subunit 9 of F_0-ATPase and dihydrofolate reductase [Pfanner and Neupert, 1987]), and a protein from the matrix space (β-subunit of F_1-ATPase [Rassow et al., 1990]). Similar results were obtained for precursors of MOM22 (Keil and Pfanner, 1993), the α-subunit of matrix processing peptidase, α-MPP (Schneider et al., 1989), and pF51-CCHL (Segui-Real et al., 1993*a*), a fusion protein between the presequence of $F_1\beta$-ATPase and CCHL (data not shown). The effects varied for the individual preproteins from a threefold (for CCHL) to a more than 10-fold reduction. The strongly diminished import of matrix and inner membrane components into MOM19-deficient mitochondria was still fully dependent on the presence of a membrane potential (data not shown) required for the import of these proteins across the inner membrane (Schleyer and Neupert, 1985). This shows that the low amounts of protease-protected proteins observed in these experiments still represented authentic protein import. These findings demonstrate the important function of MOM19 for protein import into mitochondria. Surprisingly, import into mitochondria derived from 28.17 cells grown without fpa was virtually unchanged as compared to wild-type mitochondria (Fig. 6 *A*) despite the substantial reduction of MOM19 by 80% in these mitochondria (see Table I). This suggests that MOM19 is not involved in the rate limiting step of the protein import reaction.

Previous findings have shown that a number of mitochondrial proteins become imported into mitochondria independently of MOM19 function including the ADP/ATP carrier (AAC; Söllner et al., 1990), cytochrome *c* (Stuart and Neupert, 1990), and MOM19 (Schneider et al., 1991). Therefore, we expected that import of these proteins should not be affected by the depletion of MOM19. Indeed, AAC was imported with equal efficiencies into mitochondria irrespective of their content in MOM19 protein (Fig. 6 *B*). The fact that AAC needs a membrane potential for import into the inner membrane (Pfanner and Neupert, 1987) supports the conclusion drawn above that the largely diminished import of MOM19-dependent preproteins of the inner membrane and the matrix was not the result of a reduced membrane potential in MOM19-deficient mitochondria. Similar to the import of AAC, no significant differences on import into wild-type and mutant mitochondria were observed for a fusion protein between the presequence of cytochrome c_1 and cytochrome *c* (Fig. 6 *B*). This protein behaves comparable to cytochrome *c* in its import across the outer membrane which occurs independently of the receptor/GIP complex (Stuart et al., 1990). Likewise, no effect of MOM19 depletion was observed on the insertion of MOM19 precursor into the outer membrane (Fig. 6 *C*). The unchanged import of MOM19-independent precursor proteins convincingly demonstrates that MOM19-deficient mitochondria are still fully functional in performing protein import reactions which can occur without the participation of MOM19. For the majority of preproteins, however, MOM19 is needed for efficient transport.

MOM19 Cooperates with MOM22 During Protein Import and Is Not Substituted for by MOM72

Previous biochemical studies have demonstrated that protein import can occur without the function of protease-sensitive components (Pfaller et al., 1989). This so-called "bypass" import reaction occurs at a low efficiency, and preproteins are thought to enter the mitochondria at a later stage of the import reaction, possibly by directly engaging contact with constituents of GIP. We examined MOM19-deficient mitochondria to determine whether the small amount of protein import into these mitochondria occurred via a bypass or a protease-sensitive pathway. Import experiments were performed using mitochondria which had been pretreated with proteinase K (Fig. 7) or trypsin (data not shown). As previously observed import of various precursor proteins into mitochondria derived from a wild-type strain (H-IV) was strongly dependent on the intactness of protease-sensitive components (Pfaller et al., 1988). Similar results were obtained for mitochondria derived from strain 28.17 grown without fpa. Unexpectedly, import into MOM19-deficient mitochondria (from strain 28.17 grown with fpa) also appeared to be strongly susceptible to protease pretreatment suggesting that the import reaction in the absence of functional MOM19 cannot be considered to represent bypass import. This also becomes evident from the observation that import into protease-pretreated wild-type mitochondria (i.e., bypass import) was usually at least twice as high as import into mitochondria lacking MOM19 (Fig. 7, compare lanes with 40 μg/ml proteinase K in H-IV with 0 μg/ml proteinase K in 28.17 +fpa). What might be the explanation for these findings? First, protease sensitivity of import into MOM19-deficient mitochondria indicates the participation

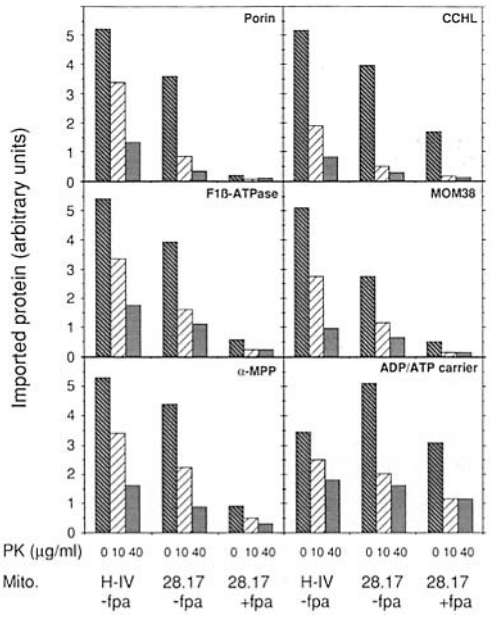

Figure 7. Protein import into MOM19-deficient mitochondria requires protease-sensitive components other than MOM19. Mitochondria (*Mito.*) freshly isolated from the indicated *Neurospora* strains were pretreated with the indicated amounts of proteinase K (*PK*) for 15 min at 0°C. After addition of 1 mM PMSF mitochondria were reisolated by centrifugation (7 min, 6,000 *g*) and resuspended in import buffer. Before import of the indicated preproteins (15 min at 15°C) samples were supplemented with an energy mix. After protease treatment mitochondria were reisolated by centrifugation, and the samples analyzed for imported protein by SDS-PAGE and fluorography. Quantitation of the fluorographs was performed by laser densitometry. Abbreviations are as in Fig. 5.

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of protease-sensitive components other than MOM19. Possible candidates for such components might include (*a*) MOM72 which may act as a backup system for MOM19 (see below), (*b*) MOM22 which has recently been characterized to have a functional role between receptor and GIP stage (Kiebler et al., 1993), (*c*) MOM38, a putative component of GIP (Kiebler et al., 1990), and (*d*) possibly other yet to be identified components of the import machinery. Second, one has to take into consideration that wild-type mitochondria used for bypass experiments still contain the membrane anchor of MOM19. This membrane-embedded protein fragment might still be attached to the protein import complex and thus fulfill an important function in the structural organization of the other subunits in the complex. The complete lack of MOM19, on the other hand, might result in a protein import complex of largely altered composition and stability.

To specifically address the question of whether MOM22 function is still required for protein import after MOM19 depletion, and whether MOM72 can partially replace MOM19, mitochondria were pretreated before import with antibodies against MOM22 or MOM72 and, as a control, against MOM19. In wild-type mitochondria (H-IV) import of MOM38, α-MPP, CCHL, and Su9-DHFR was strongly decreased after blocking of MOM19 and MOM22 (Fig. 8). Comparable results were obtained for the β subunit of F_1-ATPase, Rieske Fe/S protein, and porin (data not shown). Import into mitochondria containing lower levels of MOM19 (28.17 −fpa) was inhibited to a lesser degree. In mitochondria devoid of MOM19 (28.17 +fpa) pretreatment with anti-MOM19 antibodies had no inhibitory effect on the residual low levels of protein import (Figs. 6 and 7). These observations demonstrate that the antibodies specifically interfered with MOM19 function. A similar relief of inhibition upon depletion of MOM19 was seen using antibodies against MOM22 (Fig. 8). Apparently, the lack of MOM19 led to protein import which (*a*) was strongly reduced and (*b*) occurred largely independent of MOM22. Thus, import into MOM19-deficient mitochondria leads to a high degree of bypass of MOM22 function. The remaining weak dependence on MOM22 function shows that MOM22 can still play a role during import, yet at much reduced efficiency. These data suggest a direct functional cooperation of these two proteins during the import reaction.

Using wild-type mitochondria, antibodies against MOM72 displayed an inhibitory effect on the import of MOM38 and α-MPP, whereas the import of CCHL and Su9-DHFR was not affected (Fig. 8). Virtually identical inhibition by anti-MOM72 antibodies was seen using mitochondria with a low content of MOM19 (28.17 −fpa) or mitochondria deficient in MOM19 (28.17 +fpa) showing that MOM72 is used to the same extent in these mitochondria as in wild-type controls. This observation is surprising for proteins like, e.g., MOM38 and α-MPP which are partially inhibited by MOM72 antibodies, yet in the absence of MOM19 display a strong import defect and no higher degree of dependence on MOM72. This suggests that MOM72 cannot functionally replace MOM19 during import of these proteins, at least not at levels present in these mitochondria. The exact role of MOM72 during the import of, e.g., MOM38 remains to be determined. Overlapping specificities of MOM19 and MOM72 have been reported, however, for the import of AAC in that this precursor can use MOM19, when MOM72 is absent (Steger et al., 1990). As expected from earlier observations, import of AAC into wild-type mitochondria was only weakly affected by antibodies against MOM19, but strongly inhibited by anti-MOM-72 antibodies (Fig. 8). In MOM19-deficient mitochondria, however, import of AAC was no longer inhibited by anti-MOM19 antibodies and occurred exclusively and at unchanged efficiency (see Figs. 6 *B* and 7) via MOM72. Thus, AAC can use either MOM72 or MOM19 for efficient import, whereas most other proteins appear to strictly depend on functional MOM19 for high levels of import.

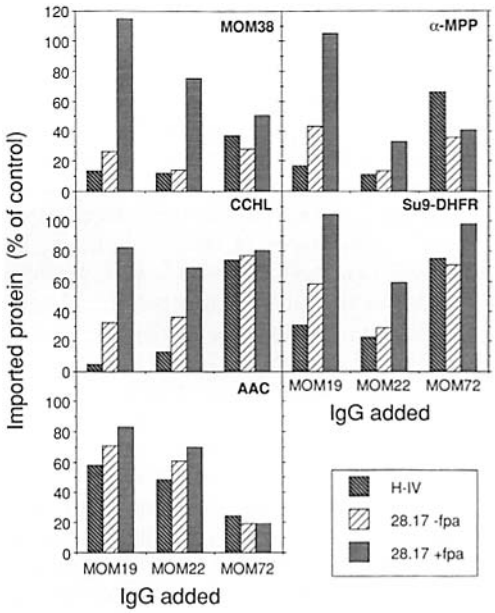

Figure 8. MOM19 cooperates with MOM22 during protein import, but cannot be functionally replaced by MOM72. 20 μg freshly isolated mitochondria were incubated in import buffer for 40 min at 0°C with 70 μg (MOM19 and MOM22) and 130 μg (MOM72) immunoglobulin G (IgG) specific for the indicated proteins. Mitochondria were reisolated by centrifugation and resuspended in import buffer. After addition of an energy mix import of the indicated preproteins was performed for 15 min at 15°C. Following protease treatment mitochondria were reisolated by centrifugation, and the samples analyzed for imported protein by SDS-PAGE and fluorography. Quantitation of the fluorographs was performed by laser densitometry. In order to account for the large differences of import into the various mitochondria (for review see Figs. 6 and 7), data are given relative to the respective control import in the absence of IgGs. Preincubation of the mitochondria with 70 μg IgG isolated from preimmune serum yielded import efficiencies of 93% (±9%). Standard deviation for import into H-IV and 28.17 cells grown without fpa was 9%, whereas it was 18% for the low amounts of import into mitochondria from 28.17 cells grown with fpa.

Discussion

The novel technique of sheltered RIP for inactivating individual genes in *N. crassa* provides a useful tool to analyze the roles of important or essential genes. RIP is highly specific, since transformation of a strain carrying the inactivated gene with a wild-type copy leads to restoration of the original phenotype (Harkness et al., 1994). We took advantage of sheltered RIP to investigate the function of MOM19

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in vivo. Experiments in vitro had suggested a participation of MOM19 in the initial steps of protein import into mitochondria of *N. crassa* (Söllner et al., 1989) and yeast (Moczko et al., 1993). In order to understand the role of MOM19 it was essential to study its action in the intact cell.

Deficiency in MOM19 as a result of inactivation of the mom-19 gene has drastic effects not only on the structure and function of mitochondria, but also on the behavior of whole cells. Growth of cells slows down in conjunction with the falling levels of MOM19 and virtually stops, when MOM19 is fully depleted. This is undoubtedly related to the alterations occurring on both a morphological and a biochemical level. MOM19-deficient mitochondria display a nearly complete loss of cristae membranes and consist only of the outer membrane and the inner boundary membrane. Furthermore, mitochondria lose their capacity for oxidative phosphorylation and for protein synthesis. Clearly, MOM19 performs an essential function in the formation and maintenance of mitochondria which are competent to provide energy for the cell.

The loss or drastic reduction of the ability to import proteins from the cytosol is a prominent characteristic of mitochondria deficient in MOM19. The impaired import competence holds for the majority of mitochondrial proteins (at least of those tested here) and includes proteins from all mitochondrial subcompartments. However, reduced import is not observed with all precursors, as some were found to be imported at the same rate into mutant and wild-type mitochondria. This emphasizes that the changes caused by MOM19 depletion are not unspecific or general, but lead to defined and selective changes in the protein import pathway. Furthermore, this differential effect on various precursor proteins is in excellent agreement with the previously observed differential inhibitory effects of antibodies against the MOM19 and MOM72 components of the mitochondrial receptor complex. Finally, the results strongly argue that MOM19 is the major entry site for precursor proteins into mitochondria. There may be an interesting analogy between MOM19 depletion in mitochondria and the depletion of components of the signal recognition particle in yeast (Hann and Walter, 1991; Hann et al., 1992). Also in this case, e.g., SRP19 is not an essential component, and growth of cells is only severely retarded. There is apparently no absolute requirement of these components for protein translocation, but their participation considerably enhances the rates and/or efficiencies of the process.

Our findings provide detailed insight into the consequences of depletion of MOM19 on the structure and function of mitochondria. These consequences appear to be rather complex. On the one hand, the deficiency in the levels of most inner membrane components is consistent with their strongly reduced import. On the other hand, the steady-state levels of proteins from other compartments in mutant mitochondria and the import rates do not correspond to each other. An interesting example is the AAC. Its precursor is imported into the MOM19-deficient mitochondria with the same apparent efficiency as into wild-type mitochondria. This is consistent with the previously observed preferential use of MOM72 as its import receptor. The observed level of the AAC in MOM19-depleted mitochondria is, however, very low. It seems therefore that some constituents of the mitochondria cannot be assembled because other components limiting for assembly are missing, or because cristae membranes are lacking, and there is no space for integration. It will be interesting to determine whether the precursor of the AAC becomes degraded, and if so, where and how degradation takes place. An opposite behavior was observed with porin. Import of this major component of the outer membrane was drastically reduced in the MOM19-depleted mitochondria, but the steady-state level was nearly the same as in wild-type mitochondria. Apparently, porin existing at the time when depletion of MOM19 starts is not degraded and/or the very low rates of import during the phase of depletion are linked to the slow rate of growth so that normal concentrations are maintained in the outer membrane. Degradation or limitations due to impaired assembly do not appear to have a diminishing effect on porin. A comparable interpretation may explain the unchanged levels of the highly protease-sensitive component MOM72 which has been shown to require MOM19 for its specific association with the outer membrane (Söllner et al., 1990). The almost normal levels of matrix proteins in MOM19-deficient mitochondria may reflect the absence of limitations regarding assembly also of these proteins, since most of them are homooligomers or monomers. The availability of a mutant in which uptake of components is disturbed will be useful for investigating the regulation of assembly and may allow the identification of components critical for the assembly of the mitochondrial membranes.

Mitochondria of cells depleted in MOM19 contain virtually normal levels of MOM72. This and our import experiments using anti-MOM72 antibodies show that MOM72 does not act as a general backup receptor for MOM19. With the ADP/ATP carrier for which MOM72 acts as major receptor, deletion of MOM72 in yeast had only a limited effect on the targeting efficiency. This is because MOM19 can functionally replace MOM72 as a receptor (Steger et al., 1990). It cannot be excluded, however, that MOM72 is competent for low efficiency recognition also of a number of other components and that highly increased levels of MOM72, e.g., after overexpression, could improve the efficiency of targeting, but it is very unlikely that MOM72 can fully replace MOM19.

There appears to be a class of precursor proteins that needs both MOM19 and MOM72 for targeting, representatives being MOM38 (Keil et al., 1993) and MOM22 (Keil and Pfanner, 1993). Antibodies against both MOM19 and MOM72 were found to inhibit import of these precursors. Quite interestingly, in MOM19-depleted mitochondria, import of both precursors was strongly reduced, in full agreement with the data from the in vitro experiments. It is also obvious that MOM72 alone cannot act as a backup system in these cases, even though MOM72 interacts with the preproteins. It is not clear, how the entry of MOM38 is achieved by both receptors acting at the same time. Conceivably, the specificity of targeting could be increased by such a double-check system. In fact, the targeting of MOM38 should be controlled with particular precision, since MOM38 is also believed to control the insertion of MOM19 into the outer membrane (Schneider et al., 1991), and MOM38 may be able to form an essential part of the outer membrane translocation pore (Söllner et al., 1992). Thus, insertion of MOM38 into other cellular membranes could presumably cause deleterious effects due to the mistargeting of mitochondrial proteins such as porin.

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Finally, the results presented here allow new insights into the functional interaction of components of the receptor complex. In particular, a close cooperation of MOM19 with MOM22 is suggested by our findings. It has been observed before, that these two proteins are constituents of the protein import complex of the outer membrane (Kiebler et al., 1990). As described here, the reduction of MOM19 is accompanied by a similar but not as severe decrease in MOM22. This may reflect a requirement for MOM19 to allow stable integration and maintenance of MOM22 in the outer membrane receptor complex similar to what has been observed for subunit IV of the cytochrome *c* oxidase complex (Dowhan et al., 1985). On the other hand, there might also be a regulatory mechanism that ensures a correlated synthesis of both components. A close functional cooperation of MOM19 and MOM22 is strongly suggested by the bypass of residual MOM22 during preprotein import into MOM19-deficient mitochondria. Antibodies against MOM22 were reported to inhibit import of precursors into wild-type mitochondria (Kiebler et al., 1993) but, as reported here, they hardly inhibited import of precursors into MOM19-deficient mitochondria. Obviously, proteins imported via MOM19 also require the function of MOM22, whereas in the absence of MOM19 preproteins enter the outer membrane mainly without the help of MOM22. The molecular basis of this close cooperation between the two proteins remains elusive. The availability of receptor-deficient mutants may now allow the dissection of the initial steps of protein translocation into mitochondria. Moreover, such investigations can take advantage of a recently developed system for the study of protein insertion and translocation into the isolated outer membrane (Mayer et al., 1993), and thereby should allow novel insights into the molecular details of these complex processes.

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