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'Sheltered disruption' of *Neurospora crassa* MOM22, an essential component of the mitochondrial protein import complex

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MOM22 is a component of the protein import complex of the mitochondrial outer membrane of *Neurospora crassa*. Using the newly developed procedure of 'sheltered disruption', we created a heterokaryotic strain harboring two nuclei, one with a null allele of the *mom-22* gene and the other with a wild-type allele. Homokaryons bearing the *mom-22* disruption could not be isolated, suggesting that *mom-22* is an essential gene. The mutant nucleus can be forced to predominate in the heterokaryon through the use of specific nutritional and inhibitor resistance markers. Cultivation of the heterokaryon under conditions favoring the mutant nucleus resulted in selective depletion of MOM22. MOM22-depleted cells did not grow and contained mitochondria with an altered morphology and protein composition. Protein import into isolated, MOM22-depleted mitochondria was abolished for most precursor proteins destined for all subcompartments. In contrast, precursors of MOM19, MOM22 and MOM72 became inserted normally into the outer membrane, defining a novel MOM22-independent import pathway which remained intact in mutant mitochondria. Furthermore, the specific binding of the ADP/ATP carrier to the outer membrane was unaffected, but subsequent transport across the outer membrane did not occur. Our data show that MOM22 is an essential component of *Neurospora* cells specifically required for the biogenesis of mitochondria.

Key words: gene disruption/mitochondria/MOM22/*Neurospora crassa*/protein import

Introduction

Most mitochondrial proteins are encoded by nuclear genes, synthesized as precursors on cytosolic ribosomes, translocated into mitochondria and sorted to the specific mitochondrial subcompartment where they fulfill their function (Hannavy *et al.*, 1993; Kiebler *et al.*, 1993a; Schatz, 1993; Segui-Real *et al.*, 1993). The process of translocating precursor proteins across the membranes requires two separate machineries in the outer and inner mitochondrial membranes. In *Neurospora crassa*, biochemical investigations have led to the identification of a multi-subunit complex which is involved in protein import into and across the outer membrane (Kiebler *et al.*, 1990). Two protease-sensitive components of this complex, MOM19 and MOM72, are thought to be required for the initial recognition and binding of mitochondrial preproteins at the cytosolic side of the mitochondrial outer membrane (Söllner *et al.*, 1989, 1990). From the receptor-bound state, precursors are routed to the general insertion pore (GIP) where they are protected against externally added proteases (Pfaller *et al.*, 1988). GIP is composed of MOM38, MOM7, MOM8, and possibly additional proteins (Söllner *et al.*, 1992). MOM22 represents another protease-sensitive component of the import complex that hitherto has been described only in *N.crassa* (Kiebler *et al.*, 1993b). The N-terminus of the protein is negatively charged and exposed to the cytosol, while the C-terminus extends into the intermembrane space. A function of MOM22 in protein import was deduced from the inhibition of import by prebinding antibodies to MOM22. Based on the observation that blocking of MOM22 does not affect the initial binding of the precursor of the ADP/ATP carrier (AAC) to its specific receptor, MOM72, a role of MOM22 was proposed to be in the transfer of preproteins from receptors to the GIP. However, the function of MOM22 during MOM19-dependent import of preproteins is not presently known.

A more comprehensive understanding of the functional role of MOM22 in the *Neurospora* cell requires the study of mutants lacking the gene. Techniques for the disruption of non-essential genes by targeted replacement have been developed for *N.crassa* (Nehls *et al.*, 1992; Aronson *et al.*, 1994). However, inactivation of essential genes has been achieved only recently by the method of 'sheltered RIP' (repeat induced point mutation; Harkness *et al.*, 1994a). Despite its successful application to the inactivation of MOM19 (Harkness *et al.*, 1994b), the method has certain shortcomings, including the relatively long time required to isolate useful mutants and the fact that the method does not guarantee the creation of null mutants. For the disruption of MOM22, we therefore developed a novel procedure which combines the essentials of the above-mentioned procedures, i.e. targeted gene replacement by homologous recombination and the sheltering effect of the second nucleus in a heterokaryon. The new method, termed 'sheltered disruption', is generally applicable for targeted disruption of any cloned gene in *N.crassa*.

Characterization of null mutants in *mom-22* reveals that the gene is essential for growth. Cells depleted of MOM22 harbor mitochondria with an altered morphology and protein composition. Protein import studies define MOM22 as a key component involved in the translocation of the majority of preproteins into mitochondria. On the other hand, proteins which are integrated into the outer mem-

brane by short trans-membrane segments, like the precursors of MOM19, MOM22 and MOM72, are not affected in their insertion efficiency, indicating that MOM22-independent mechanisms for membrane insertion exist. These data suggest that MOM22 plays an indispensable function for the biogenesis of mitochondria and is specifically required for the transfer of proteins across the outer membrane.

Results

Isolation of mom-22 null mutants by 'sheltered disruption'

The previously described methods for gene disruption in *N.crassa* by homologous recombination (Nehls *et al.*, 1992; Aronson *et al.*, 1994) cannot be applied to essential genes, since the organism would be rendered inviable. We exploited aspects of *Neurospora* biology to overcome this problem. The organism's hyphal system, or mycelium, is divided into compartments that approximate cells. However, these compartments differ from conventional cells in that each may contain several nuclei. Furthermore, large pores allow the passage of cellular components, including nuclei, between these compartments (Fincham and Day, 1963; Davis and De Serres, 1970). *Neurospora* is capable of forming heterokaryons containing two different nuclei within a single hyphal system (Davis and De Serres, 1970). The heterokaryotic state can be forced, if the component nuclei carry different auxotrophic markers, thereby requiring complementation for growth on minimal media. During transformation with a disruption construct, usually only one type of nucleus in heterokaryotic cells undergoes a recombination event leading to disruption of the target gene. At some frequency, this will be a homologous event. We therefore reasoned that a nucleus carrying a lethal disruption could be maintained in a forced heterokaryon, since the essential gene product could still be supplied by the unaffected nucleus. Furthermore, if the two nuclei in the heterokaryon carry recessive genes providing resistance to different inhibitors of growth, the ratio of the nuclei in the mycelium can be shifted by growth in medium containing the appropriate inhibitor. This results in predominance of the nucleus containing the disruption and permits the study of the mutant phenotype. We refer to this procedure as 'sheltered disruption'.

Strain HP1 contains genetic markers selected to achieve the objectives of sheltered disruption (Figure 1) and was used as the parent strain for the disruption of *mom-22*. Spheroplasts of HP1 were transformed with pEH-51, a plasmid containing the *mom-22* gene disrupted by a gene conferring resistance to hygromycin (Figure 2A). Transformants were selected on minimal medium containing hygromycin, and 38 individual isolates were transferred to tubes of the same medium and allowed to conidiate. The isolates were purified through one round of single-colony isolation on the same medium. To identify transformants of the desired type (Figure 1), we analyzed genomic DNA on Southern blots (Figure 2B). Of the 34 strains analyzed, nine showed evidence of targeted replacement in one nucleus of the heterokaryon. However, six of these strains also contained other hybridizing bands, suggesting additional or more complicated integration events in at least one nucleus of the heterokaryon. Three

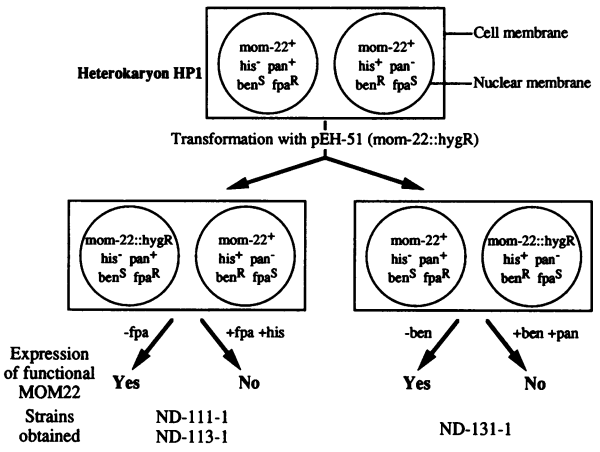

Fig. 1. Sheltered disruption of *N.crassa mom-22*. The starting heterokaryon HP1 contains two nuclei distinguished by nutritional requirements for histidine (his) and pantothenate (pan). These auxotrophic markers allow the heterokaryon to be maintained by growth in minimal medium. In addition, the nuclei contain markers that confer resistance to either fpa or benomyl (ben). The desired event following transformation with plasmid pEH-51 (see Figure 2) is homologous recombination leading to targeted replacement of *mom-22*$^+$ in one of the nuclei by the *mom-22* gene disrupted with a hygromycin resistance gene (hygR). The resulting strains isolated in this study were ND-111-1, ND-113-1 carrying a *mom-22*-disrupted gene in the histidine-requiring nucleus, and ND-131-1 harboring the disruption in the pantothenate-requiring nucleus. The ratio of the two nuclei in these strains can be manipulated by growth in the presence of the appropriate nutrient and inhibitor. This will lead to selective depletion of MOM22.

strains, ND-111-1, ND-113-1 and ND-131-1, showed the patterns predicted for the desired heterokaryotic *mom-22* disruption strain (Figure 2B).

Mom-22 is an essential gene in N.crassa

If MOM22 is important or essential for growth of the organism, conditions forcing the disruptant-containing nucleus to predominate in the heterokaryon should result in a retarded growth rate (see Figure 1). Strains ND-111-1, ND-113-1 and ND-131-1 were tested in race tubes (see Materials and methods) for their ability to grow on medium supplemented with either pantothenate plus benomyl or histidine plus *p*-fluorophenylalanine (fpa). As shown in Figure 3, ND-111-1 and ND-113-1 did not grow on medium containing histidine plus fpa, suggesting that in these strains the disruption was in the histidine-requiring nucleus. ND-131-1 did not grow on medium containing pantothenate plus benomyl, indicating a disruption in the pantothenate-requiring nucleus. These data suggest that MOM22 is essential for growth of *N.crassa* cells.

To further support this conclusion, we attempted to isolate homokaryons of each nucleus from the disruptant strains. Macroconidial spores in *N.crassa* are multinucleate and the segregation of nuclei into spores is random. Therefore, the heterokaryotic strains should give rise to three types of conidia: heterokaryons and each of the homokaryotic types. If *mom-22* is disrupted in a given nucleus of the heterokaryon, and is an essential gene, it should not be possible to isolate the relevant homokaryon from the heterokaryon. Conidia from the three disruptant strains were spread on medium containing both histidine and pantothenate. After 4–5 days incubation at 30°C,

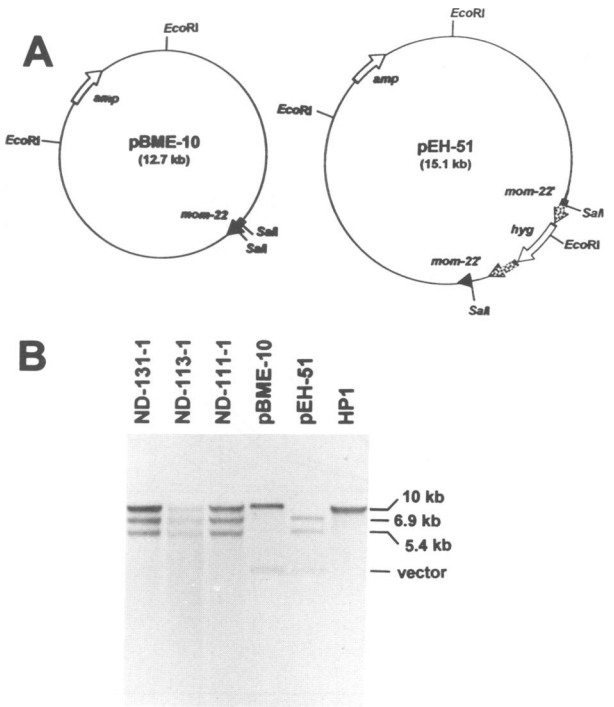

Fig. 2. Southern blot analysis of transformed strains. (**A**) Plasmid maps of pBME-10 and pEH-51 used for transformation. Details for the construction of the plasmids are given in Materials and methods. Relevant restriction sites are indicated outside the circles. The position of genes is shown on the circles. White arrows represent antibiotic-resistant genes, the black bars/arrows define the *mom-22* gene, and the speckled arrows represent promoter and termination sequences from *Aspergillus nidulans* used for the expression of the hygromycin resistance gene (Staben *et al.*, 1989). A small region of pBR322 sequence is present between the end of the termination sequence and the *Sal*I site. (**B**) Genomic DNA was isolated from the indicated *N.crassa* strains, digested with *Eco*RI, electrophoresed on an agarose gel and blotted to a nylon membrane. Plasmids pBME-10 and pEH-51 were included as controls. The blot was probed with DIG-labeled pEH-51. *Eco*RI digestion of the correct heterokaryotic disruptant strain should yield three bands. The wild-type *mom-22* locus should be present on a single fragment that corresponds to the large *Eco*RI fragment (10 kb) cloned in pBME-10. Since there is a single *Eco*RI site in the hygromycin resistance gene, the disrupted version of *mom-22* should give two smaller fragments (6.9 and 5.4 kb) which correspond to those produced from *Eco*RI digestion of pEH-51. The position of the expected bands is indicated. Restriction fragments of the predicted sizes were also observed from cleavage reactions with the enzyme *Xho*I (data not shown).

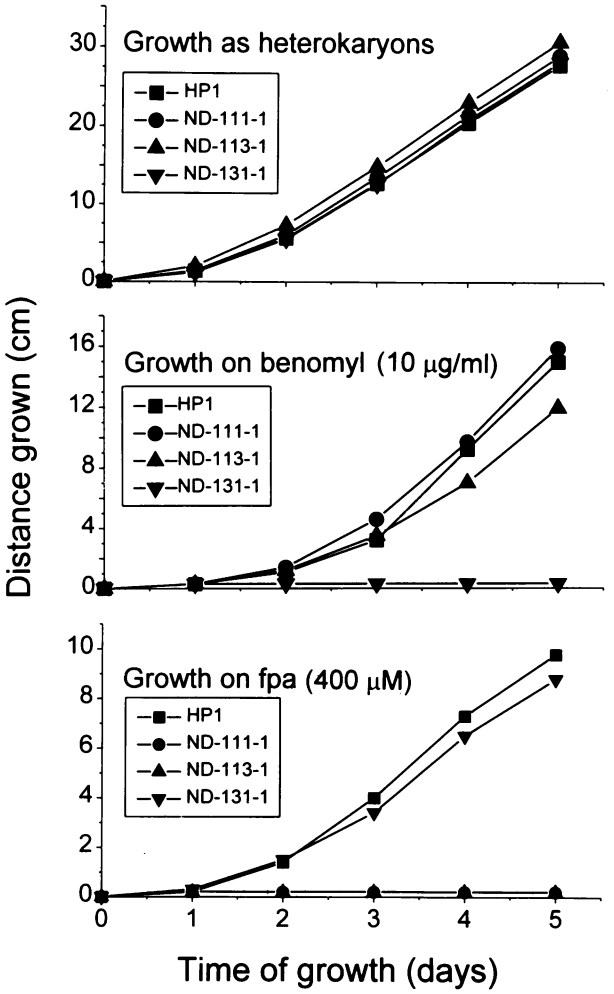

Fig. 3. Growth patterns of *mom-22* disruption strains in race tubes. The indicated strains were inoculated at one end of a 'race tube' (see Materials and methods) and incubated at room temperature. Growth was on minimal media for heterokaryons (top), or on minimal media supplemented with either benomyl plus pantothenate (middle) or fpa plus histidine (bottom).

single colonies representing all sizes present on the plates were transferred to individual culture tubes containing the same medium. These were then tested for nutritional requirements to determine if the isolates were homo- or heterokaryons. As shown in Table I, we were unable to isolate histidine-requiring homokaryotic strains from ND-111-1 and ND-113-1. Likewise, we could not isolate pantothenate-requiring homokaryotic strains from ND-131-1. These results show that *mom-22* is an essential gene. Since all three strains generated seem to fulfill the requirements of *mom-22* sheltered disruptants, we chose only one mutant strain, ND-113-1, for further analysis. Before proceeding to detailed biochemical analysis, we wished to prove that the inability of the strain to grow on medium containing fpa was due solely to the disruption of *mom-22*. This was demonstrated by complementing the mutant phenotype through transformation with the *mom-22* gene. Growth of ND-113-1 on media containing fpa was detectable only when the strain was transformed with *mom-22*-containing genomic DNA or with *mom-22* cDNA (Table II).

Table I. Inability to isolate homokaryons containing the nucleus with the *mom-22* disruption

Strain	Colonies examined	His-requiring homokaryons	Pan-requiring homokaryons	Heterokaryons
ND-111-1	48	0	27	21
ND-113-1	100	0	56	44
ND-131-1	95	48	0	47
HP1	70	8	52	10

Pan, pantothenate; His, histidine.

Characterization of the mom-22 disruption mutant

Upon cultivation of the disruption mutant ND-113-1 in liquid media containing fpa and histidine, hardly any increase in mycelial mass was detectable, whereas in the absence of fpa cells grew like those from wild-type strain

Table II. Rescue of *mom-22* disruption strain ND-113-1 by transformation with *mom-22* DNA

Transforming DNA	Resulting number of colonies
No DNA	0
pBME-10	0
pVoll	0
pAB520	0
pBME-10 + pAB520	250–500
pVoll + pAB520	250–500

Transformation of strain ND-113-1 was with 1 μg of the indicated plasmids. The selective medium contained bleomycin, fpa and histidine. The range in the number of colonies obtained in different trials is shown. pBME-10 contains the genomic copy of *mom-22*, pVoll harbors *mom-22* cDNA. pAB520 confers resistance to bleomycin.

HP1 (Figure 4A). The lag phase observed during growth of heterokaryotic HP1 cells in the presence of fpa is explained by the fact that only conidia containing the fpa-resistant nucleus can grow out into hyphae. A similar growth characteristic was reported for a *mom-19* mutant (Harkness *et al.*, 1994b). When ND-113-1 cells which had been cultivated for 4 days in the presence of fpa were diluted into fresh medium lacking fpa, cells resumed growth, showing that the effects of depleting MOM22 were not lethal (Figure 4A). During cultivation in fpa-containing medium, the content of MOM22 in mutant cell extracts was gradually reduced, until after 2–3 days it reached ~4% of the levels present in either wild-type HP1 cells or in *mom-22* mutant cells grown as a heterokaryon (Figure 4B). Prolonged cultivation of ND-113-1 did not lead to further reduction of MOM22, suggesting that at this residual amount of MOM22 cells cease to duplicate. For further analysis of the mutant phenotype, ND-113-1 cells were used which had been cultivated for 3 days in histidine- and fpa-containing minimal media.

We examined the mitochondrial morphology in MOM22-depleted cells by electron microscopy. Growth in the presence of fpa, i.e. depletion of MOM22, was not accompanied by a loss of mitochondrial profiles (Figure 5). Rather, in comparison with either wild-type cells grown in the presence of fpa (Figure 5A) or with ND-113-1 cells grown in the absence of fpa (not shown), a decrease in the size of the mitochondria and an increase in the number of profiles was evident for ND-113-1 cells cultivated in fpa-containing medium (Figure 5B–D). In addition, a reduction in the number of cristae membranes was apparent. The latter is reminiscent of a number of yeast ρ^0 mutants (Stevens, 1977, 1981) and was similar, but not as pronounced as in *N.crassa* cells depleted of MOM19 (Harkness *et al.*, 1994b).

We next isolated mitochondria from cells grown in the presence of fpa in order to examine their protein composition. In comparison with wild-type mitochondria, the amount of MOM22 was decreased 25-fold (Figure 6A). There was also a 5-fold reduction of MOM19 levels, whereas MOM38 and MOM72 remained virtually unchanged. A correlation between the content of MOM19 and MOM22 was reported in an earlier study of a MOM19-deficient mutant (Harkness *et al.*, 1994b). Thus, the cellular levels of MOM19 and MOM22 are mutually adjusted, indicating a close relationship of these two constituents

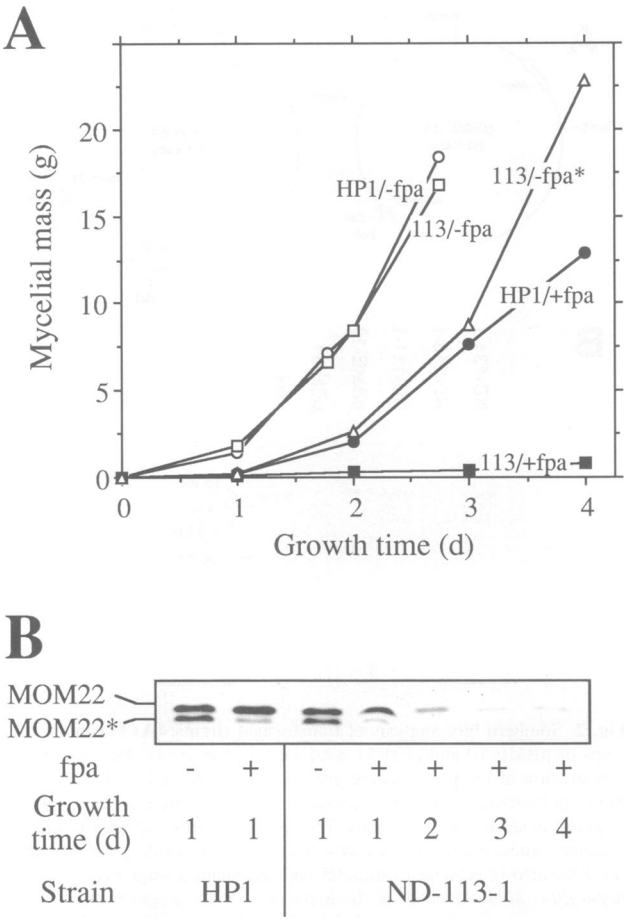

Fig. 4. Depletion of MOM22 strongly impairs growth of *N.crassa* in liquid medium. (**A**) Wild-type strain HP1 and strain ND-113-1 (113) were grown at 25°C in minimal medium in the presence or absence of histidine and fpa as indicated. After various times, samples were withdrawn and the mycelium was harvested by filtration. After 4 days of cultivation in fpa-containing media, cultures were back-diluted 40-fold into medium lacking fpa (113/−fpa*) and incubation continued. The mycelium was dried under a red light, before measuring the dry cellular mass. Mycelial mass is given for 1 l of cell culture. (**B**) Cellular extracts were prepared from these cultures by grinding the mycelium in a mortar in liquid nitrogen for 1 min. Then, 1 ml SEM buffer [250 mM sucrose, 1 mM EDTA and 10 mM MOPS−KOH (pH 7.2)] containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1% SDS was added per gram of mycelium, and the extract was clarified by centrifugation (4000 *g*, 5 min). Total cellular protein (150 μg) of each sample was analysed by SDS−PAGE and immunostaining for MOM22. MOM22* denotes a degradation product of MOM22 generated during the preparation of cell extracts.

of the protein import complex. Another component of the outer membrane, porin, was not significantly changed in MOM22-depleted mitochondria, whereas proteins of the intermembrane space, such as cytochrome *c* and cytochrome *c* heme lyase (CCHL), were reduced ~3-fold (Figure 6B). Slightly decreased levels were observed for various components of the matrix space, with the exception of Hsp70 which was increased (Figure 6C). Similarly, a decrease by ~40% was found for components of the inner membrane, with the exception of cytochrome c_1 which remained normal (Figure 6D). This reduction in inner membrane content is consistent with the decrease in cristae membranes observed in electron micrographs (cf. Figure 5). All these changes in protein levels occur late during

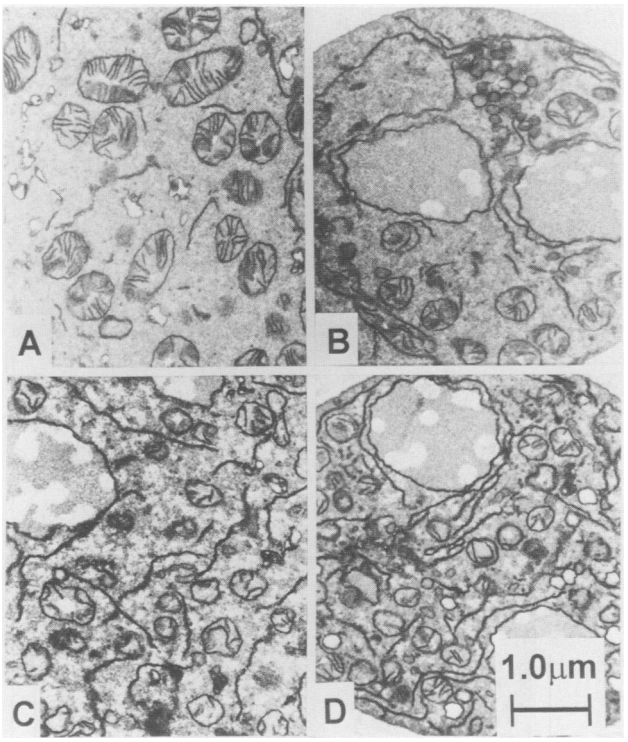

Fig. 5. MOM22-depleted cells contain mitochondria with an altered morphology. Strain HP1 was grown in minimal medium containing fpa and histidine for 18 h (**A**). ND-113-1 cells were grown in the same medium for 18 h (**B**), 42 h (**C**) and 70 h (**D**). Cells were examined by electron microscopy after fixation with $KMnO_4$ (Harkness *et al.*, 1994b).

MOM22 depletion, i.e. when MOM22 has become reduced more than 10-fold (data not shown). It is, therefore, clear that the observed alterations are a result of MOM22 depletion. However, since MOM22-depleted mitochondria are defective in importing proteins from the cytosol (see below), the changes appear to be an indirect consequence of MOM22 depletion, and the altered protein levels might rather reflect the turnover rates of these individual proteins. In summary, the composition of MOM22-depleted mitochondria underwent specific alterations, yet in comparison with wild-type mitochondria the major effects found were on MOM22 and MOM19.

Are the alterations in the protein content of MOM22-depleted mitochondria accompanied by an impairment of the membrane potential, $\Delta\Psi$, or of the F_1F_0-ATPase function? We examined this question by following the $\Delta\Psi$-driven ATP formation. The generation of ATP was determined in a colorimetric assay by the decrease in free phosphate as a result of its incorporation into ADP. The time course of ATP formation in MOM22-depleted mitochondria was similar to that found in wild-type organelles (Figure 7). ATP formation was dependent on the addition of NADH, i.e. required the $\Delta\Psi$ across the inner membrane. Control experiments showed that no ATP was formed upon depletion of $\Delta\Psi$ by uncoupling agents like CCCP or upon inhibition of the F_1F_0-ATPase with oligomycin (R.Lill, unpublished). Thus, mutant mitochondria fully retained their ability to generate a membrane potential which then could be utilized to drive mitochondrial ATP synthesis.

MOM22 is essential for protein import of most, but not all mitochondrial preproteins

MOM22-deficient mitochondria were examined for their ability to import precursor proteins *in vitro*. Preproteins were synthesized in reticulocyte lysate in the presence of [^{35}S]methionine, and incubated with mitochondria isolated from wild-type strain HP1 or from the MOM22 disruption strain ND-113-1, which had both been grown in the presence of fpa. As shown in Figure 8A, import of various precursor proteins destined for all four mitochondrial subcompartments was almost completely abolished in MOM22-depleted mitochondria. Similar results were obtained for fusion proteins carrying the presequences of cytochrome b_2, Rieske iron–sulfur protein or subunit 9 of F_0-ATPase in front of DHFR, subunit IV of cytochrome oxidase or lactalbumin, respectively (data not shown). This suggests that most or all presequence-containing preproteins require MOM22 for import. Quantitation of the efficiency of import into MOM22 mutant mitochondria indicated that import was significantly lower than that reported for 'bypass' import occurring after proteolytic removal of receptors (Pfaller *et al.*, 1989), and lower than the residual import into MOM19-deficient mitochondria (Harkness *et al.*, 1994b). Since proteolytic treatment removes only the N-terminal, cytosolic portion of MOM22 (Kiebler *et al.*, 1993b), these data imply an important function during protein import of the remaining C-terminal part of MOM22 which spans the outer membrane and reaches into the intermembrane space.

A number of preproteins become inserted into or translocated across the mitochondrial outer membrane without the participation of protease-sensitive surface receptors. Examples include the precursors of MOM19 (Schneider *et al.*, 1991) and MOM72 (J.Schlossmann, unpublished). When MOM19 precursor was incubated with MOM22-depleted mutant mitochondria, the protein was imported and assembled into the receptor complex, as evident from the generation of characteristic proteolytic fragments (Figure 8B; Schneider *et al.*, 1991; Mayer *et al.*, 1993). These fragments did not arise from a corresponding protease treatment of the MOM19 precursor in the absence of mitochondria. As compared with wild-type mitochondria, a similar fraction of the bound material became imported (40% in both cases). A similar import competence of MOM22-depleted mitochondria was observed for MOM72 precursor (Figure 8C). Its insertion into the outer membrane was tested by the resistance of the receptor complex-assembled protein against extraction with alkaline buffers.

In contrast to these two preproteins, the precursor of MOM22 has been found to depend on both MOM19 and MOM72 for its insertion (Keil and Pfanner, 1993). To study whether MOM22 is needed for its own biogenesis, membrane insertion was assayed by following characteristic proteolytic fragments generated only from assembled MOM22 (Keil and Pfanner, 1993; Mayer *et al.*, 1993). In comparison with wild-type mitochondria, import into MOM22-depleted organelles was slightly reduced, and a few additional fragments appeared after proteolysis (Figure 8D). These fragments did not arise after trypsin pretreatment of MOM22-depleted mitochondria (data not shown), indicating that they were generated exclusively from imported material. These data demonstrate that MOM22

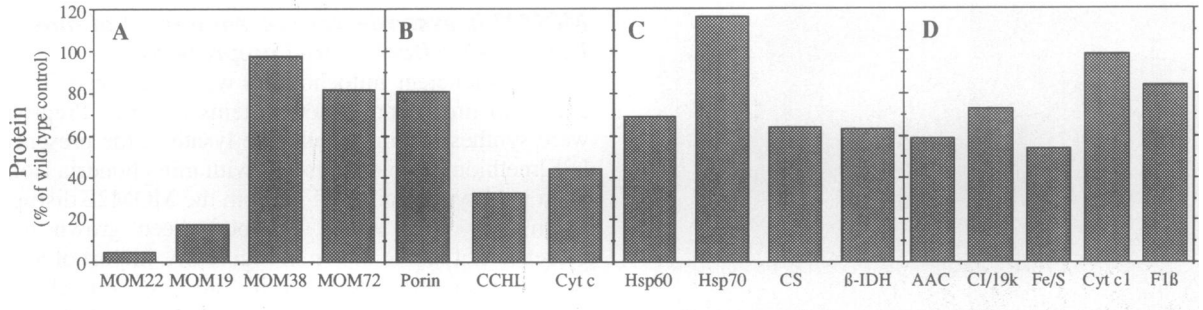

Fig. 6. Protein composition of MOM22-depleted mitochondria. Cells from wild-type strain HP1 and MOM22 disruption strain ND-113-1 were grown in minimal medium containing histidine and fpa. Mitochondria were isolated and mitochondrial protein (150 µg/sample) was analyzed by SDS–PAGE and immunostaining using antibodies specific for the indicated mitochondrial proteins from (**A**) the protein import complex of the outer membrane, (**B**) the outer membrane and intermembrane space, (**C**) the matrix space and (**D**) the inner membrane. Data were collected as the average of at least three independent preparations of mitochondria. The SD varied from 5 to 20% for the individual proteins. CCHL, cytochrome *c* heme lyase; Cyt c, cytochrome *c*; Hsp60 and Hsp70, heat shock proteins of 60 and 70 kDa; CS, citrate synthase; β-IDH, β-subunit of isocitrate synthase; AAC, ADP/ATP carrier; CI/19k, 19 kDa subunit of complex I; Fe/S, Rieske iron–sulfur protein; Cyt c1, cytochrome c_1; F1β, β-subunit of F_0-ATPase.

does not require pre-existing, endogenous MOM22 for its import. Rather, the protein apparently can occupy empty spaces in incompletely assembled receptor complexes. Taken together, our protein import studies demonstrate a severe translocation defect for almost all preproteins. This is due to a specific defect of the general import route. However, preproteins which insert only short segments into the outer membrane became imported normally without a detectable requirement for MOM22.

As shown above, the import of AAC is abolished in MOM22-depleted mitochondria. However, since its receptor, MOM72, was present at unchanged levels in MOM22 mutant mitochondria, we tested whether the binding of AAC to the mitochondrial surface was affected in the mutant. For this purpose, we used outer membrane vesicles (OMV) purified from wild-type or MOM22 mutant strains. OMV are advantageous for such binding studies, since AAC does not become translocated into OMV (Mayer *et al.*, 1993), and non-specific background binding is low in the vesicle system (Mayer *et al.*, 1995). No reduction in AAC binding to MOM22 mutant membranes was observed in comparison with OMV isolated from wild-type strain HP1 (Figure 9). Binding was specific, since it was decreased 5-fold after trypsin pretreatment of the OMV. Thus, it is only the subsequent step of transfer across the membrane that is blocked in the absence of MOM22. In summary, our findings of normal binding of AAC, insertion of MOM22-independent preproteins and unchanged oxidative phosphorylation capacity demonstrate that MOM22-depleted mitochondria are not generally defective. Rather, MOM22-dependent functions are specifically affected in mutant mitochondria.

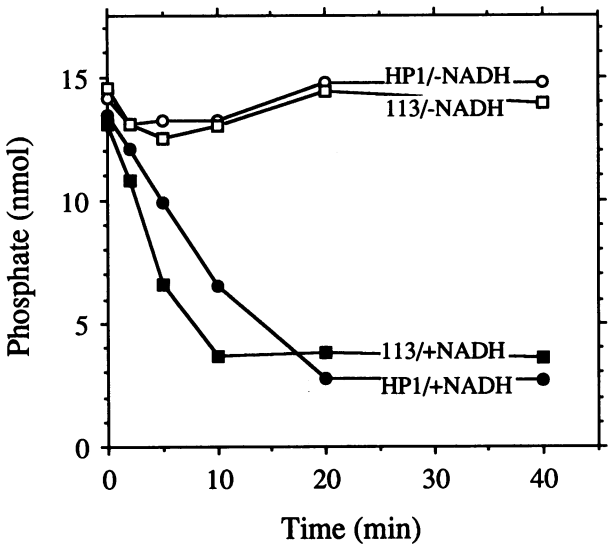

Fig. 7. MOM22-depleted mitochondria retain functional oxidative phosphorylation. Mitochondria (10 µg) freshly isolated from strains HP1 or ND-113-1 (113) which had been grown in the presence of fpa and histidine were incubated in buffer P [20 mM MOPS (pH 7.2), 250 mM sucrose, 0.3 mM potassium phosphate, 5 mM $MgCl_2$, 1 mg/ml fatty acid-free BSA and 1 mM ADP] in the absence or presence of 2 mM NADH at 25°C. The formation of ATP was followed by the decrease in free inorganic phosphate after condensation with ADP. To this end, the reaction was terminated after various times by addition of the malachite green color reagent and citric acid, and residual phosphate was determined at 660 nm as described previously (Lill *et al.*, 1990).

Discussion

For the inactivation of essential genes in *N.crassa*, the method of sheltered disruption developed in this study offers several technical and genetic advantages to the previously reported sheltered RIP procedure (Harkness *et al.*, 1994a). First, sheltered disruption is comparatively rapid, since a genetic cross is not required for the generation of mutants. Second, the screen for potential mutants is less demanding, since it is unnecessary to examine the genomic DNA of potential mutant isolates for evidence of increased methylation or point mutations. Third, the use of sheltered disruption eliminates the possibility of affecting neighboring genes. In sheltered RIP, resulting alterations may extend to sequences in the immediate neighborhood of the target duplication, even when sequences lacking adjacent genes are used (Foss *et al.*, 1991). Finally, sheltered disruption results in a genetically well-defined situation in that it ensures the creation of null mutants. The RIP procedure, on the other hand, yields products containing numerous point mutations in the target gene which may or may not result in the creation of a stop codon or an otherwise totally defective gene product. The availability of sheltered disruption now opens a

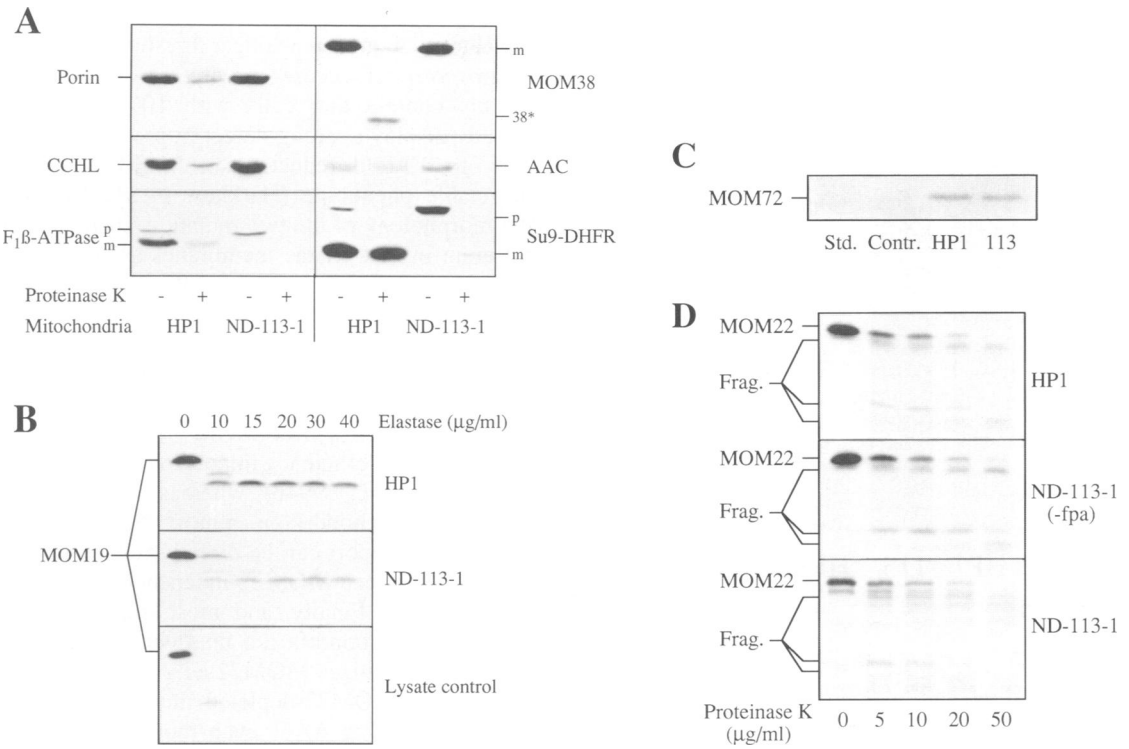

Fig. 8. Import into MOM22-depleted mitochondria is abolished for most, but not all, precursor proteins. For the import studies, 30–50 µg mitochondria were used which had been freshly isolated from strains HP1 and ND-113-1 grown in the presence of histidine and fpa. (**A**) The indicated radiolabeled precursor proteins were incubated with mitochondria in import buffer supplemented with an energy mix (Harkness *et al.*, 1994b). After 10 min at 25°C, samples were placed on ice and split in half. One aliquot was left on ice, while the other one was treated with 100 µg/ml proteinase K for 20 min. Proteolysis was stopped by addition of 1 mM PMSF, and mitochondria from all samples were re-isolated by centrifugation. Radioactive protein was analysed by SDS−PAGE and fluorography of the resulting gels. (**B**) For the import of MOM19, the protocol of Mayer *et al.* (1993) was followed using the indicated concentrations of elastase for protease treatment. As a control for unimported MOM19, a corresponding elastase treatment was performed with MOM19 precursor in the absence of mitochondria (Lysate control). (**C**) Import of MOM72 into mitochondria isolated from strains HP1 or ND-113-1 (113) was for 20 min at 25°C in import buffer containing 0.1% BSA and the energy mix. Mitochondria were re-isolated by centrifugation, and the mitochondrial pellet was resuspended in 200 µl 10 mM HEPES−KOH (pH 7.4). One volume of freshly prepared sodium carbonate (200 mM) was added and samples were incubated for 30 min on ice. Membranes were collected by centrifugation (1 h at 226 000 *g*) and analysed by SDS−PAGE and fluorography. As a control for unassembled MOM72 (Contr.), the precursor was incubated under conditions used for import. Samples were transferred to ice and mitochondria were added. Further analysis was as above. The left lane contains 10% of the MOM72 input as a standard (Std.). (**D**) Import of MOM22 was performed according to Mayer *et al.* (1993). Specific fragments generated from imported MOM22 by digestion with the indicated amounts of proteinase K are assigned (Frag.). As a control, import into mitochondria isolated from ND-113-1 cells grown in the absence of fpa (−fpa) is shown (middle).

variety of genetic techniques to be applied to *N.crassa*. For example, mutated alleles could be introduced into the nucleus harboring the disrupted gene, thus allowing the functional dissection of the gene product. Such an approach should also facilitate the isolation of temperature-sensitive mutants which may be useful for the biochemical analysis of the import process.

Our studies identify MOM22 as an essential constituent of *N.crassa* mitochondria. We provide *in vivo* evidence that MOM22, as part of the protein import complex of the outer membrane, performs an indispensable function during the transport of preproteins across the membrane. MOM22 is the first component of *N.crassa* mitochondria which has been shown to perform an essential function for cell viability. In addition to Isp42p (the yeast homolog of MOM38; Baker *et al.*, 1990), MOM22 is the second essential protein of the outer membrane. Its specific role in protein import is consistent with the fact that, so far, all essential genes encoding mitochondrial proteins are involved in the biogenesis of these organelles, i.e. in transport across the outer and inner membranes, or in protein folding and processing (Baker and Schatz, 1991).

A striking observation is the mutual adaptation of intracellular levels of MOM19 and MOM22. In the present study, we observed that mitochondria depleted of MOM22 were also largely reduced in MOM19 content. Our previous study showed a corresponding increase or decrease in MOM22 as a result of MOM19 overproduction or deficiency, respectively (Harkness *et al.*, 1994b). In addition, a functional cooperation during protein import was noted. The mutual adjustment of cellular concentrations is specific, since it does not affect the other components of the protein import complex. It may therefore reflect a close relationship between these two proteins that could be achieved by many mechanisms. For example, there could be a regulatory connection between the two proteins at the level of transcription or translation. Alternatively, each of these components of the outer membrane import machinery might be required for proper assembly or stability of the other. When considered together with our previous observations (Harkness *et al.*, 1994b), these data strongly suggest a high degree of functional cooperation between the two proteins. It will, therefore, be interesting to determine whether MOM22 and MOM19 directly

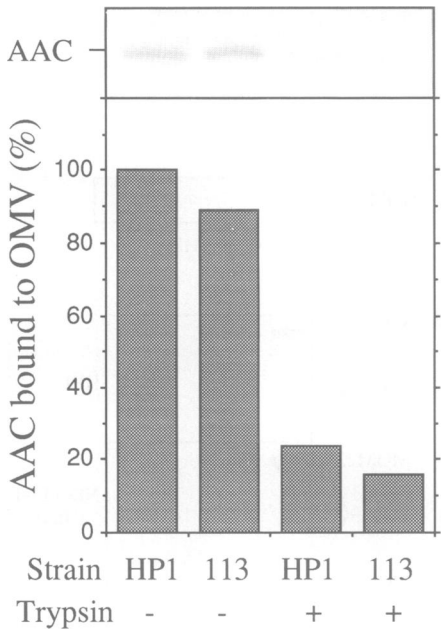

Fig. 9. Binding of AAC to the surface of MOM22-depleted outer membrane vesicles (OMV) is unaffected. Reticulocyte lysate containing the precursor of AAC and, in separate samples, wild-type or MOM22-depleted OMV (5 μg/sample) were treated with apyrase in binding buffer [10 mM MOPS–KOH (pH 7.2), 20 mM KCl, 1.3 mM $MgCl_2$ and 7.5 mg/ml fatty acid-free BSA] as described previously (Mayer *et al.*, 1993). Aliquots of the sample were treated with 25 μg/ml trypsin (+) or trypsin which had been inactivated by 0.5 mg/ml soy bean trypsin inhibitor (−). After 20 min at 0°C, 500 μg/ml soy bean trypsin inhibitor were added to the first aliquot. Binding of AAC to the OMV was allowed for 5 min at 25°C. Samples were placed on ice, diluted with ice-cold buffer D [10 mM MOPS–KOH (pH 7.2), 1 mM EDTA and 20 mM KCl], and membranes were re-isolated by centrifugation (30 min at 125 000 *g*). After aspirating off the supernatant, the tubes were centrifuged for 5 min at 30 000 *g* and the residual supernatant was removed. The pellets were suspended in sample buffer with a pipette, and vesicle-bound AAC was analysed by SDS–PAGE, fluorography and densitometry.

interact with each other and whether a complex of both proteins is involved in forming a binding site which has been shown to recognize mitochondrial targeting sequences at the mitochondrial surface (Mayer *et al.*, 1995).

Depletion of MOM22 in strain ND-113-1 by cultivation in fpa severely decreases the growth rate and results in changes in mitochondrial morphology. In addition, these cells harbor mitochondria which are defective in importing proteins from the cytosol. At first glance, there seems to be a similarity between cells lacking MOM19 (Harkness *et al.*, 1994b) and cells depleted in MOM22. Moreover the cellular levels of these proteins change in a coordinate fashion in the mutants. However, by a number of criteria, the phenotype of MOM22-deficient cells is distinct from that of the previously described MOM19 mutant. First, the *mom-22* gene provides a function that is essential for cell growth. Depletion of *mom-19* results in a 'stop–start' phenotype which is characterized by a growth arrest interrupted by short periods of slow growth (Harkness *et al.*, 1994a). This difference between the phenotypes also appears to be reflected in the observation that mitochondria isolated from liquid cultures of the *mom-19* mutant did not contain detectable MOM19, whereas cultures of the *mom-22* mutant retained some low level of MOM22. This indicates that, after reaching a threshold level of MOM22, *Neurospora* cells cease growing. It should be mentioned in this context that cells with 10% residual MOM22 already display a strong defect in protein import, whereas a 5- to 10-fold reduction in MOM19 resulted in no detectable phenotype (Harkness *et al.*, 1994b). Second, the morphology of the two mutant mitochondria is clearly different in that cristae membranes are practically absent in the MOM19-deficient organelles, whereas in MOM22-depleted mitochondria they were only partially decreased. Despite the reduction in inner membrane content, it is noteworthy that both MOM19- and MOM22-deficient mitochondria are capable of performing oxidative phosphorylation. Third, MOM19-depleted mitochondria maintain a low-efficiency import for most precursors tested (Harkness *et al.*, 1994b), whereas import into MOM22-deficient mitochondria is almost completely abolished. The lack of import can be directly related to a deficiency in MOM22, since MOM22-independent import pathways remain intact. Finally, and most importantly, MOM19-deficient mitochondria are unaffected in their import of AAC which utilizes MOM72 as a receptor for its import. In contrast, MOM22-depleted mitochondria were incapable of importing AAC, even though specific binding to the mitochondrial surface occurred normally. It follows from this comparison that MOM19 and MOM22 play distinct roles in protein import across the mitochondrial outer membrane, and mutants in these two proteins result in specific phenotypes.

Our results fit well with biochemical studies demonstrating that MOM22 is required for the transfer of AAC from the receptor-bound state into and across the translocation pore (Kiebler *et al.*, 1993b). From our data, it is clear that MOM22 also plays an important role during the passage across the membrane of proteins using the MOM19-dependent pathway. Together with earlier studies, our data suggest distinct functions for the cytosolic N-terminal domain of MOM22 and for the C-terminal part. Removal of the cytosolic domain by proteolysis (Pfaller *et al.*, 1989) or blocking with specific antibodies (Kiebler *et al.*, 1993b) still permits protein import, yet at lower efficiency, whereas removal of the whole protein abolishes protein import completely.

In contrast to the majority of preproteins, including those with cleavable N-terminal presequences, some preproteins are unaffected in their import into MOM22 mutant mitochondria. These examples define a novel, MOM22-independent pathway of protein insertion into the outer membrane. Interestingly, these proteins are components of the receptor complex itself and have to transfer only short segments into and across the outer membrane. The major part of these proteins remains exposed to the cytosol. The exceptional mechanism of MOM22-independent membrane insertion may resemble the integration of a class of membrane proteins with C-terminal anchors (for a review, see Kutay *et al.*, 1993). Also in these cases, only a few amino acids have to be transferred across the membrane. Clearly, detailed biochemical investigations are required to substantiate these mechanistic aspects. The possibility of generating specific mutants in the MOM22 disruption strain and the availability of an isolated vesicle

system (Mayer *et al.*, 1993, 1995) should now facilitate such functional studies.

Materials and methods

Strains, media and growth conditions

The heterokaryotic strain HP1 was composed of strains 76-26 (*his-3 mtrR a*) and 71-18 (*pan-2 BmlR a*). The *mtrR* allele confers resistance to fpa, while the *BmlR* allele confers resistance to benomyl. The component strains were a generous gift from R.L.Metzenberg, University of Wisconsin. Strains containing alterations in the *mom-22* gene are described in the text. *Neurospora crassa* cultures were grown and maintained as described previously (Davis and De Serres, 1970; Harkness *et al.*, 1994b). Strain HP1 was maintained as a heterokaryon by growth on minimal medium, which forces complementation of the component strains due to the different nutritional requirements. For the depletion of MOM22, strain ND-113-1 was grown for 3 days in minimal medium containing 0.2 mg/ml histidine and 400 μM fpa. Cells or isolated mitochondria from strain HP1 grown in liquid medium for 24 h in the presence of fpa and histidine served as the wild-type control.

As a simple measure of growth, mycelial elongation was determined in 'race tubes' (Davis and De Serres, 1970). These are long, hollow glass tubes, bent at a 45° angle at each end and maintained in a horizontal position. Agar containing the appropriate growth medium was added to cover the bottom of the tube. Conidia were inoculated at one end of the tube and the extent of growth of the mycelium was recorded each day. Since *N.crassa* only grows at the tips of the hyphae, extension of the mycelium along the surface of the agar occurs in a linear fashion with wild-type cells.

Plasmids

To increase the likelihood of targeted replacements, we wished to isolate large restriction fragments containing *mom-22*. The previously cloned *mom-22* cDNA (Kiebler *et al.*, 1993b), purified from plasmid pVoll, was used to probe a cosmid library of *N.crassa* genomic DNA (Gessert *et al.*, 1994). Several cosmids were isolated and a 10 kb *Eco*RI fragment was identified that contained the *mom-22* locus near its center. This fragment was cloned into plasmid pBM-15, to yield plasmid pBME-10 (cf. Figure 2A). Plasmid pBM-15 is a modified Bluescript vector from which a small fragment extending from the *Eco*RV cleavage site to the *Hin*cII cleavage site has been removed. The plasmid lacks the *Sal*I site which, among others, is normally present in Bluescript, but the reading frame for β-galactosidase remains intact. To generate the *mom-22* disruption, the small *Sal*I fragment in the *mom-22* coding region of pBME-10 was replaced with a 2.4 kb *Sal*I restriction fragment from plasmid pCSN43 (Staben *et al.*, 1989) that contains the bacterial *hph* gene. The latter confers resistance to hygromycin B. The resulting plasmid was named pEH-51 (Figure 2A).

Transformation of N.crassa

Routine transformation of *N.crassa* spheroplasts was performed as described previously (Schweizer *et al.*, 1981) with the modifications of Akins and Lambowitz (1985). To demonstrate rescue of the *mom-22* disrupted strains, a co-transformation system was used. Mutant spheroplasts, generated by growing conidia for a short time in minimal medium, were co-transformed with a plasmid containing *mom-22*$^+$ DNA and a plasmid that provides resistance to bleomycin, pAB520 (Austin *et al.*, 1990). Following transformation with 1 μg of each plasmid DNA, the spheroplasts were plated in medium containing 400 μM fpa, 0.2 mg/ml histidine, 2.5 μg/ml bleomycin and 0.5 mg/ml caffeine, which is selective for both bleomycin resistance and rescue of the *mom-22* mutant phenotype. Colonies appeared after ~3 days incubation at 30°C. Use of this co-transformation system completely eliminated a background that was eventually observed when only fpa in the presence of histidine was used for the selection of transformants. At least in part, this background may arise from spontaneous mutation of the mtrS allele, which encodes a permease allowing the transport of amino acids or their analogs into the cell (Stadler and Kariya, 1969; DeBusk and DeBusk, 1980), to a non-functional version of the gene that results in fpa resistance.

Biochemical procedures

The following published procedures were used: Southern hybridization using DIG-labeled probes (Boehringer Mannheim product information); electron microscopy of mitochondria in hyphal sections (Harkness *et al.*, 1994b); *N.crassa* DNA isolation (Schechtman, 1986); raising antisera and purification of immunoglobulin G (IgG; Söllner *et al.*, 1989); transcription and translation reactions using [^{35}S]methionine (ICN Radiochemicals) as a radioactive label (Söllner *et al.*, 1991); SDS–PAGE and fluorography of the resulting gels (Nicholson *et al.*, 1987); blotting of proteins onto nitrocellulose and immunostaining of blotted proteins using the ECL chemiluminescence detection system (Amersham; Mayer *et al.*, 1993); quantitation of the resulting films and those from fluorography of radioactive proteins on an Image Master densitometer (Pharmacia; Mayer *et al.*, 1993); isolation of mitochondria (Harkness *et al.*, 1994b); purification of outer membrane vesicles (Mayer *et al.*, 1993); and protein import into isolated mitochondria (Harkness *et al.*, 1994b) in import buffer [10 mM MOPS–KOH (pH 7.2), 250 mM sucrose, 80 mM KCl, 5 mM $MgCl_2$ and 30 mg/ml fatty acid-free bovine serum albumin (BSA)] supplemented with an energy mix (2 mM ATP, 3 mM NADH, 10 mM creatine phosphate and 100 μg/ml creatine kinase). Protein concentrations were determined by the Coomassie dye binding assay (Biorad) or by the BCA reagent (Pierce) using BSA as a standard.

Acknowledgements

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