

Cyclosporin A-binding Protein (Cyclophilin) of *Neurospora crassa**

ONE GENE CODES FOR BOTH THE CYTOSOLIC AND MITOCHONDRIAL FORMS

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Cyclophilin (cyclosporin A-binding protein) has a dual localization in the mitochondria and in the cytosol of *Neurospora crassa*. The two forms are encoded by a single gene which is transcribed into mRNAs having different lengths and 5' termini (~1 and 0.8 kilobases). The shorter mRNA specifies the cytosolic protein consisting of 179 amino acids. The longer mRNA is translated into a precursor polypeptide with an amino-terminal extension of 44 amino acids which is cleaved in two steps upon entry into the mitochondrial matrix. *Neurospora* cyclophilin shows about 60% sequence homology to human and bovine cyclophilins.

EXPERIMENTAL PROCEDURES

Strains and Plasmids

N. crassa wild-type strain 74A was grown as described (Stuart *et al.*, 1987). *Escherichia coli* strains used were 5K (Hubacek and Glover, 1970) transformed with the cDNA library which has been cloned into the *Pst*I site of pBR322 (Kleene *et al.*, 1987), and DH1 (Low, 1968) as host for pDS5 (Stüber *et al.*, 1984) and pUC19 (Viera and Messing, 1982; Yanisch-Perron *et al.*, 1985)-derived plasmids.

Isolation of Cyclophilin from Mitochondria

Unless otherwise indicated, all procedures were performed at 4 °C or on ice.

Step 1: Preparation of a Mitochondrial Fraction—Fresh *N. crassa* hyphae (1500 g, wet weight) were macerated and suspended in 4 liters of buffer containing 250 mM sucrose, 10 mM MOPS¹/KOH (pH 7.2), 2 mM EDTA, 1 mM phenylmethyl sulfonyl fluoride (added from a fresh 200 mM stock in ethanol), and 1 mM dithiothreitol (DTT). The suspension was homogenized in a Waring blender for 15 s at low speed and then for 15 s at high speed. The cells were passed through a grind mill (Sebald *et al.*, 1979), and the resulting homogenate was spun for 10 min at 3,100 × g (3,250 rpm; Sorvall H-6000A rotor). The supernatant was collected, and mitochondria were then sedimented by centrifugation for 30 min at 17,700 × g (10,000 rpm; Beckman JA-10 rotor). The resulting pellet was taken as the mitochondrial fraction.

Step 2: Solubilization of Mitochondria with *n*-Octylglucoside—The entire mitochondrial pellet was resuspended to a final volume of 100 ml, in the buffer described in step 1, and solid *n*-octylglucoside (Boehringer Mannheim) was added to a final concentration of 1.8% (w/v). The suspension was stirred for 30 min and then dispersed by homogenization (10 strokes, Dounce S-type). Nonsolubilized material was removed by centrifugation for 60 min at 257,000 × g (Beckman Ti-70 rotor). The clear supernatants were collected, avoiding lipids floating on the surface, and dialyzed extensively against 10 mM Tris/HCl (pH 8.5), 1 mM DTT.

Step 3: DE52 Ion Exchange Chromatography—The dialyzed extract (50 ml containing 500–700 mg of protein) was passed through a DE52 column (Whatman; 2.5 × 32 cm, 1 ml/min) which had been equilibrated in 10 mM Tris/HCl (pH 8.5), 1 mM DTT. The column run was monitored with a flow-through photometer at 280 and 405 nm. The activity appeared in a pass-through fraction which also contained cytochrome *c*, as detected by absorption at 405 nm.

Step 4: Sephadex G-50 Gel Filtration—This step was included only when the fractions collected from the preceding step contained significant high molecular weight contaminants. The active pool from the DE52 column (100 ml containing 10–15 mg of protein) was lyophilized and then resuspended in 5 ml of 0.5 M NaCl, 1 mM DTT. The sample was applied to a Sephadex G-50 column (Pharmacia LKB Biotechnology, Inc., 4.5 × 90 cm, 1 ml/min) that had been equilibrated in 0.5 M NaCl, 1 mM DTT. Two peaks were eluted; the first of which absorbed at 280 nm, and a second peak which absorbed at both 280 and 405 nm. The latter peak was active and was collected.

Step 5: Phenyl-Sepharose Chromatography—The active fractions from the gel filtration column were pooled (80–90 ml containing 5–

Cyclosporin A, a cyclic undecapeptide produced by *Tolypocladium inflatum*, was originally isolated as an antifungal compound (see Borel, 1986; Stähelin, 1986). It has since become an immunosuppressive agent widely used in organ transplantation. In addition, its potential use in the treatment of autoimmune diseases is under clinical investigation (for review see Beveridge, 1986). The immunosuppressive action of cyclosporin A seems to result at least in part from a specific and complete inhibition of lymphokine mRNA production (Elliott *et al.*, 1984; Krönke *et al.*, 1984). On the other hand, hepatocellular toxicity, nephrotoxicity and disturbances of the central nervous system have also been reported (Myers *et al.*, 1984; Atkinson *et al.*, 1984). A cyclosporin A-binding protein has been identified and isolated from the cytosol of bovine thymus and human spleen cells (Handschoemacher *et al.*, 1984; Harding *et al.*, 1986). This protein (cyclophilin) binds cyclosporins with high affinity and was postulated (Handschoemacher *et al.*, 1984; Harding *et al.*, 1986) to mediate the effects of cyclosporin inside the cell. Here we report that cyclophilin is present in both the cytosol and the mitochondria of *Neurospora crassa*, an organism which is sensitive to the antifungal action of cyclosporin A (Dreyfuss *et al.*, 1976). *N. crassa* cyclophilin shows 60% sequence homology to human cyclophilin (Haendler *et al.*, 1987). One gene codes for both the cytosolic and mitochondrial forms. Mitochondrial cyclophilin is translated in the cytosol as a precursor having an amino-terminal presequence of 44 amino acids which is cleaved in two steps upon entry into the mitochondrial matrix.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) J03963.

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¹ The abbreviations used are: MOPS, 3-(*N*-morpholino)-propanesulfonic acid; DTT, dithiothreitol; SDS, sodium dodecyl sulfate.

10 mg of protein), lyophilized, and then resuspended in 10 ml of H₂O (approximate NaCl concentration was therefore 4–5 M). The sample was applied to a column of phenyl-Sepharose (Pharmacia LKB Biotechnology, Inc., 2.5 × 25 cm, 1 ml/min) that had been equilibrated with 0.5 M NaCl, 10 mM MOPS/KOH (pH 7.2), 1 mM DTT. Cytochrome *c*, as detected by its absorption at 405 nm, was eluted with the void fraction, whereas the cyclosporin binding activity was retained on the column. After washing the column with 200 ml of the above buffer, activity was eluted with 50 mM NaCl, 10 mM MOPS/KOH (pH 7.2), 1.8% (w/v) octylglucoside, 1 mM DTT. The active fractions were pooled and dialyzed extensively against 5 mM MOPS/KOH (pH 7.2), 0.5 mM DTT. (Note: a significant proportion of the activity was also removed from the column in the wash buffer.) The sample was lyophilized and resuspended in a minimum volume (~1 ml) of H₂O. Aliquots were stored at -20°C. The final preparation (containing 1 mg of protein) was homogeneous, as judged by SDS-polyacrylamide gel electrophoresis, and was comprised of a single band having an apparent molecular mass of 20,000 Da.

Isolation of Poly(A)⁺ RNA, Northern Blot Analysis, and cDNA Cloning

Total RNA and poly(A)⁺ RNA were isolated from *N. crassa* hyphae as described (Kleene *et al.*, 1987; Stuart *et al.*, 1987). For Northern blot analysis, 10 μg of poly(A)⁺ RNA was resolved on a 1.3% agarose gel containing formaldehyde (Maniatis *et al.*, 1982) and transferred overnight to nylon membranes (Biohyne A; Pall). cDNA inserts were nick-translated (Rigby *et al.*, 1977) and used as labeled probes in buffer containing 50% formamide at 42°C. A cyclophilin cDNA insert (A29/26) was identified from a *N. crassa* library (Kleene *et al.*, 1987) by hybridization selection of mRNA (Parnes *et al.*, 1981; Viebrock *et al.*, 1982; Stuart *et al.*, 1987). Insert A29/26 was used to identify full length cDNA inserts by means of colony hybridization (Grunstein and Hogness, 1975).

Sequencing of Cyclophilin cDNAs

Restriction fragments of the cDNAs were cloned into pUC19 and sequenced on both strands using universal and reverse primers. Parts of the cDNAs were also sequenced using cyclophilin-specific DNA primers. Sequencing analysis was carried out with denatured plasmids (Chen and Seeburg, 1985) according to the dideoxy termination method (Sanger *et al.*, 1977) using ³⁵S-labeled dATP (Amersham Corp., 600 Ci/mmol) (Biggin *et al.*, 1983) and Klenow fragment (Boehringer Mannheim) or a modified T7 DNA polymerase (Sequenase; United States Biochemical Corp.) (Tabor and Richardson, 1987).

Southern Blot Analysis

Southern blot analysis of genomic DNA was carried out as described (Southern, 1975; Maniatis *et al.*, 1982). Restriction digests were resolved on a 0.8% agarose gel, transferred overnight to nylon membranes, and immobilized with UV light. Full length cDNA inserts, or a *SacI/SalI* restriction fragment thereof, were used as labeled probes at 65°C in 2 × SSC, 0.1% SDS, 10 × Denhardt's solution (Denhardt, 1966), 100 μg/ml denatured salmon sperm DNA. Final washing was done with 0.1 × SSC at 65°C.

Amino Acid Sequence Determination of Cyclophilin

The amino acid sequence of cyclophilin isolated from mitochondria was determined by solid-phase Edman degradation. In one degradation run, the entire protein was coupled to *p*-phenylenediisothiocyanate-activated porous glass beads. Subsequent Edman degradation cycles revealed the lack of an amino-terminal methionine. For further protein sequence determination, the protein was first fragmented by CNBr cleavage. The resulting fragment peptides were size-fractionated on a Ultropac TSK-G 2000 high performance liquid chromatography column (2.15 × 60 cm, LKB). The partially purified fractions were treated with absolute trifluoroacetic acid for transformation of the carboxyl-terminal homoserine residues to the corresponding lactones. The activated peptides were immobilized on aminopropyl glass beads via amide linkages. Identification of the phenylthiohydantoin was performed by high performance liquid chromatography on a Shandon ODS column (3 μm) with a gradient of a methanol/2-propanol mixture (1:1; v/v) (Wachter and Werhan, 1979).

Fractionation of Cells and Mitochondrial Subfractionation

For cell fractionation, hyphae of *N. crassa*, grown in the presence of [³⁵S]sulfate (Teintze *et al.*, 1982), were broken by grinding with

quartz sand in a Percoll medium containing 30% (v/v) Percoll (Pharmacia LKB Biotechnology, Inc.), 180 mM KCl, 10 mM MOPS (pH 7.2). (Disruption of isolated mitochondria resuspended in this medium by mild sonication (Hartl *et al.*, 1986) resulted in a complete release of cytochrome *c* (an intermembrane space marker), isocitrate dehydrogenase subunit α (a matrix marker), and cyclophilin.) After pelleting the sand (2 × 5 min at 1,200 × *g* in a Beckman JA-20 rotor), the resulting homogenate was centrifuged for 15 min at 165,000 × *g* in a Beckman Ti-50 rotor. Mitochondria were collected as a sharp band near the bottom of the tube, diluted with Percoll medium, and centrifuged again for 15 min at 165,000 × *g*. The post-mitochondrial supernatant was diluted 1:3 with 180 mM KCl, 10 mM MOPS (pH 7.2) and separated into a vesicle fraction and a cytosolic fraction by centrifugation for 1 h at 165,000 × *g*. Fractionation of mitochondria by digitonin treatment was performed essentially as described (Hartl *et al.*, 1987).

Protein Import into Isolated Mitochondria

Isolation of *N. crassa* mitochondria, import of precursor proteins into isolated mitochondria, and immunoprecipitation were performed as described (Pfanner and Neupert, 1985, 1987b; Schleyer and Neupert, 1985; Hartl *et al.*, 1986; Nicholson *et al.*, 1987).

Miscellaneous

DNA manipulations and *in vitro* expression of full length cDNA inserts were as described (Maniatis *et al.*, 1982; Kleene *et al.*, 1987; Stuart *et al.*, 1987; Pfanner *et al.*, 1987). Binding of [³H]cyclosporin A to cyclophilin-containing fractions was measured as described by Handschumacher *et al.* (1984) and Koletsky *et al.* (1986). Protein was determined as described (Bradford, 1976).

RESULTS

Isolation of Cyclophilin from *N. crassa* Cytosol and Mitochondria—Cyclophilin has been reported to be a cytosolic component in eukaryotic cells. We purified cyclophilin from a *N. crassa* cytosol fraction beginning with the procedure described for bovine and human cells (Harding *et al.*, 1986) but continuing after ultrafiltration with the procedure described below (see also "Experimental Procedures"). The isolated protein has an apparent molecular weight of 20,000 Da and binds cyclosporin A with high affinity ($K_d \sim 2 \times 10^{-7}$ M) (data not shown).

We also found that mitochondrial extracts bound cyclosporin A. In order to purify the putative binding protein from mitochondria, the mitochondrial extract was subjected to DEAE-cellulose chromatography and to hydrophobic interaction chromatography on phenyl-Sepharose (see "Experimental Procedures"). The final preparation had a 78-fold higher specific binding capacity as compared to the total mitochondrial extract (Table I). Analysis by SDS gel electrophoresis showed a single polypeptide band with an apparent molecular weight of 20,000 Da (Fig. 1) that was indistinguishable from the cytosolic cyclophilin. The dissociation constant of the purified mitochondrial protein for cyclosporin A was also 2×10^{-7} M. Thus, the affinities of *Neurospora* cytosolic and mitochondrial cyclophilins are very similar to those found with cyclophilins of higher eukaryotic cells (Handschumacher *et al.*, 1984; Harding *et al.*, 1986). The binding capacity was

TABLE I
Purification of cyclophilin from isolated mitochondria

| Fraction | Total protein | Specific binding of cyclophilin | Purification | Yield |
|---------------------------------|---------------|---------------------------------|--------------|-------|
| | mg | μg/mg | -fold | % |
| Mitochondrial extract | 580 | 0.26 | 1 | 100 |
| DEAE-cellulose chromatography | 11 | 3.4 | 13 | 24 |
| Phenyl-Sepharose chromatography | 1 | 20 | 78 | 14 |

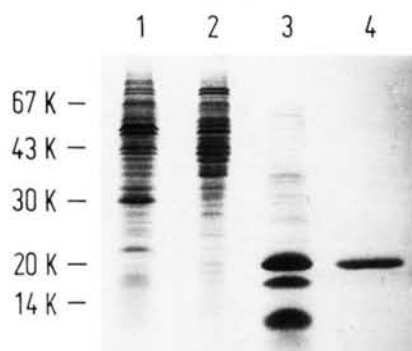


FIG. 1. Gel electrophoretic analysis of purification steps of mitochondrial cyclophilin. The various protein fractions of the purification procedure were subjected to SDS-polyacrylamide gel electrophoresis and protein bands were stained with Coomassie Brilliant Blue. Lane 1, mitochondria (30 μ g); lane 2, octyl-glucoside extract of mitochondria (30 μ g); lane 3, pass-through fraction of DEAE-cellulose chromatography (30 μ g); lane 4, eluate of phenyl-Sepharose (5 μ g).

TABLE II

Subcellular distribution of cyclophilin in *N. crassa*

A total homogenate of 35 S-labeled *Neurospora* hyphae was prepared and separated by Percoll gradient centrifugation into a mitochondrial pellet and a post-mitochondrial supernatant. From the latter, a cytosolic fraction was prepared by high speed centrifugation. Protein was determined in the various fractions. In addition to cyclophilin, ADP/ATP carrier (inner mitochondrial membrane) and cytochrome *c* (intermembrane space) were immunoprecipitated as marker proteins. Immunoprecipitates were separated by SDS gel electrophoresis and analyzed by fluorography and densitometry. Values are given in percent of content in the total homogenate and as relative specific content (RSC).

| Cellular fraction | Protein % | ADP/ATP carrier | | Cytochrome <i>c</i> | | Cyclophilin | |
|-------------------------------|--------------|-----------------|------|---------------------|------|-------------|-----|
| | | % | RSC | % | RSC | % | RSC |
| Total homogenate | 100 | 100 | 1 | 100 | 1 | 100 | 1 |
| Postmitochondrial supernatant | 53.8 | 24.1 | 0.45 | 26.2 | 0.49 | 70.3 | 1.3 |
| Cytosolic fraction | 27.9 | 4.4 | 0.16 | 5.4 | 0.19 | 72.8 | 2.6 |
| Mitochondria | 13.4 | 71.7 | 5.4 | 70.3 | 5.2 | 12.5 | 0.9 |

~40% of that reported for other cyclophilins, probably because the purification procedure led to partial denaturation.

Dual Localization of Cyclophilin in the Mitochondrial Matrix and in the Cytosol—In order to verify the presence of cyclophilin in both the cytosol and mitochondria, a subcellular localization study was performed with *N. crassa* cells. Cells were fractionated into a mitochondrial preparation and a post-mitochondrial supernatant. The mitochondria were further purified by Percoll-gradient centrifugation, and the post-mitochondrial supernatant was separated by centrifugation into a vesicle fraction and a soluble fraction (Table II). As markers, the mitochondrial ADP/ATP carrier (inner membrane) and cytochrome *c* (intermembrane space) contents were determined. The distribution of cyclophilin was measured with antibodies raised against the protein isolated from mitochondria. This antiserum recognized both the cytosolic and mitochondrial cyclophilins with equal efficiency (not shown). Of total cyclophilin present in the cell homogenate, 12.5% was found in the mitochondrial fraction. After correcting for the loss of mitochondria into the postmitochondrial fraction, using the mitochondrial markers, 17% of total cellular cyclophilin could be assigned to mitochondria. The rest was recovered in the cytosol fraction.

In order to determine the submitochondrial localization of

cyclophilin, isolated mitochondria were subfractionated using the digitonin procedure (Hartl *et al.*, 1987). By exposing mitochondria to increasing concentrations of digitonin, the intermembrane space and the matrix space were opened in a successive manner. Cytochrome *c* was determined as a marker for the intermembrane space, and the α -subunit of isocitrate dehydrogenase as a marker for the matrix compartment. Cyclophilin was released from the mitochondria and appeared in the soluble fraction in parallel to α -isocitrate dehydrogenase. This occurred at distinctly higher concentrations of digitonin than those required for the release of cytochrome *c* (Fig. 2). Cyclophilin could also be easily released from mitochondria by sonicating isolated mitochondria in detergent-free 0.1 M KCl-containing buffer (not shown). This established the location of mitochondrial cyclophilin as a soluble component in the matrix.

cDNA Cloning Reveals Two Different mRNAs for Cyclophilin—To analyze how cyclophilin is directed to both the cytosol and the mitochondrial compartment we analyzed the mRNA for cyclophilin. Antibodies against purified mitochondrial cyclophilin were used to screen an ordered *N. crassa* cDNA library (Viebrock *et al.*, 1982; Stuart *et al.*, 1987; Kleene *et al.*, 1987) by means of hybrid-selected translation. A short cDNA clone (A29/26) was used to identify full length clones by means of colony hybridization. About 20,000 clones were screened, and 26 turned out to be positive. One of the clones (cp1-1) had a cDNA insert of about 1,000 base pairs. Two had inserts of about 800 base pairs (cp6 and cp12) and the others were of shorter lengths. Insert cp1-1 was used as a labeled

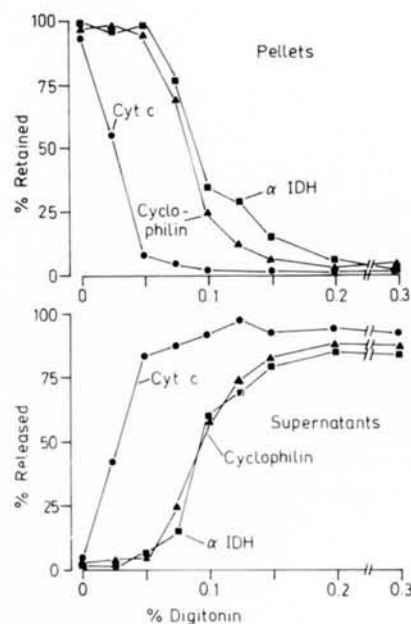


FIG. 2. Release of cyclophilin from mitochondria by treatment with digitonin. Mitochondria were isolated from cells grown in the presence of 35 S sulfate and were adjusted to a protein concentration of 10 mg/ml in 0.25 M sucrose, 1 mM EDTA, 200 mM KCl, 10 mM MOPS, pH 7.2 (SEMK). Digitonin was added to concentrations of 0–0.3% (w/v), as indicated, and the samples were incubated for 1 min at 0 °C in a final volume of 30 μ l and at a protein concentration of 5 mg/ml. Fractions were diluted 5-fold with SEMK and fractionated into pellets (upper panel) and supernatants (lower panel) by centrifugation for 20 min at 48,000 \times *g*. Cyclophilin as well as the marker proteins cytochrome *c* (Cyt *c*) and isocitrate dehydrogenase subunit α (α IDH) were immunoprecipitated (see "Experimental Procedures"). The precipitates were separated by SDS gel electrophoresis and analyzed by fluorography and densitometry. Values are expressed as percentage of total contents measured in corresponding amounts of mitochondria prior to digitonin fractionation.

probe in a Northern blot analysis (Maniatis *et al.*, 1982) of *N. crassa* poly(A)⁺ RNA. Two distinct cyclophilin-specific mRNA species of about 1 and 0.8 kb were seen (Fig. 3, lanes 3–5). cDNA insert cp1-1 (Fig. 3, lanes 6 and 7) comigrated with the 1-kb mRNA species which comprised ~5–10% of the hybridizing mRNA. In contrast, inserts cp6 (Fig. 3, lanes 1 and 2) and cp12 (not shown) comigrated with the 0.8-kb mRNA (~90–95% of hybridizing mRNA). The same hybridization pattern was observed when cDNA inserts cp6 or cp12 were used as labeled probes (not shown).

We concluded that two distinct mRNA species specific for cyclophilin are present in *N. crassa* and that we have cloned full length cDNAs for both forms.

Nucleotide Sequence of Cyclophilin cDNAs and Resulting Amino Acid Sequences for Both Forms of Cyclophilin—The complete nucleotide sequences of cDNA inserts cp1-1, cp6, and cp12 were determined by supercoil sequencing of subcloned restriction fragments (Fig. 4A) and by using cyclophilin-specific DNA primers. cDNA inserts cp6 and cp12 contained an open reading frame potentially coding for 180 amino acids, resulting in a protein of 19,525 Da (Fig. 4B). The cDNA insert cp1-1 was a 5'-extended form of the other two cDNAs. The nucleotide sequence coding for the 180 amino acid sequence is identical to that of cp12 and cp6. The cp1-1 insert, however, contains an upstream initiation codon which extends the open reading frame from 180 to 223 amino acids, thereby potentially coding for a protein of 24,067 Da.

The amino acid sequence of cyclophilin isolated from mitochondria was also determined by solid-phase sequencing. It consists of 179 amino acids which is identical to the 180 amino acid sequence found in the three different cDNAs, the only exception being that the initial methionine is absent.

N. crassa cyclophilin has a high degree of homology (Fig. 5) to human and bovine cyclophilin (Haendler *et al.*, 1987; Handschumacher *et al.*, 1984; Harding *et al.*, 1986). 60% of the amino acid residues are identical. 60% homology is also seen on the level of the nucleotide sequence of the coding regions of *N. crassa* and human cyclophilin mRNA (Haendler *et al.*, 1987) (not shown).

The amino-terminal extension of the 24-kDa form of cyclophilin contains 5 positively charged amino acids (including histidine in position 6), 11 serine residues, 6 threonine residues, and no acidic amino acids (Fig. 4B). Thus, the extra sequence has typical characteristics of a mitochondrial target sequence (see Douglas *et al.*, 1984; Horwich *et al.*, 1986; Hurt and van Loon, 1986; von Heijne, 1986; Zimmermann, 1986; Pfanner and Neupert, 1987a; Nicholson and Neupert, 1988).

One Gene Codes for Both Mitochondrial and Cytosolic Cyclophilin—In order to determine the number of genes coding for both species of cyclophilin mRNAs and thus the two subcellular forms of cyclophilin in *N. crassa*, Southern blot analysis was performed (Southern, 1975). cDNA insert cp1-1 (~1 kb, coding for the 24-kDa mitochondrial precursor protein) and

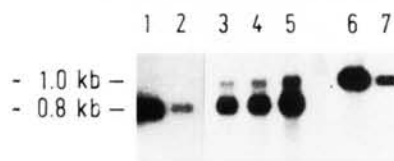


FIG. 3. Northern blot analysis reveals two cyclophilin-specific mRNAs in *N. crassa*. cDNA inserts cp6 (lane 1, 10 ng; lane 2, 1 ng) and cp1-1 (lane 6, 10 ng; lane 7, 1 ng) and three different poly(A)⁺ RNA preparations from *N. crassa* (lanes 3–5, 10 µg each) were subjected to electrophoresis on a denaturing agarose gel containing formaldehyde. The nucleic acids were blotted onto a nylon filter and probed with a nick-translated cp1-1 cDNA insert.

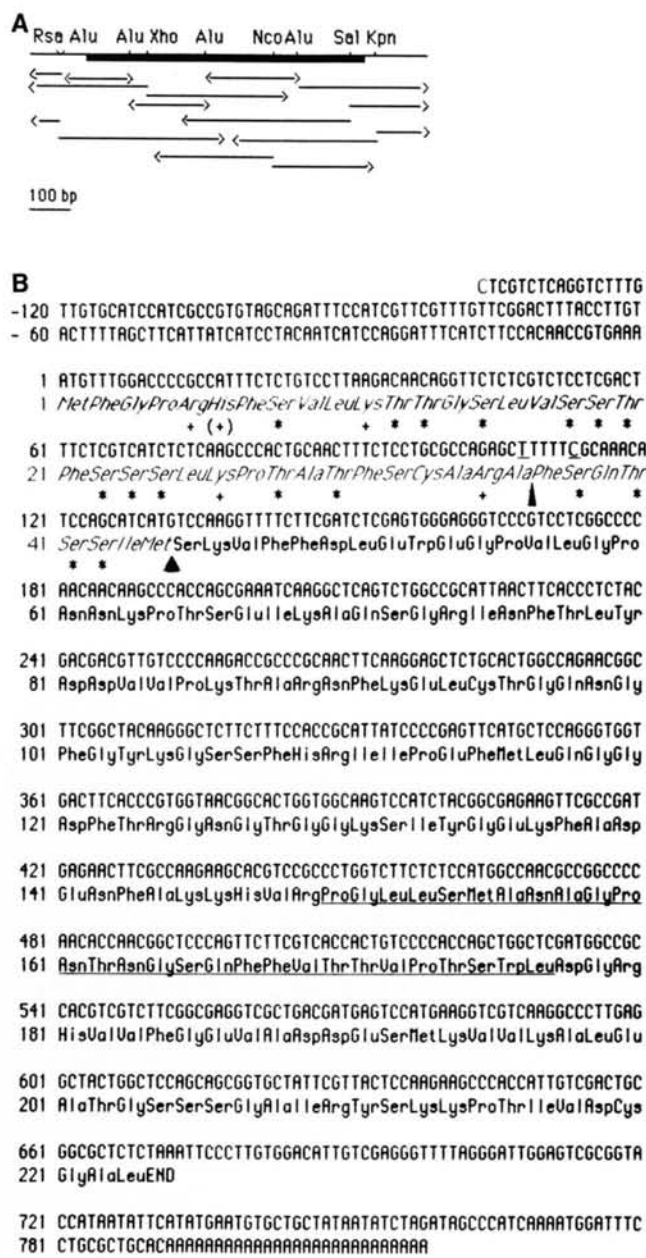


FIG. 4. Restriction map and sequencing strategy for the cDNA insert cp1-1 (A); nucleotide sequence of *N. crassa* cyclophilin cDNA insert cp1-1 and deduced amino acid sequence (B). A, the filled box indicates the coding region for the mitochondrial precursor of cyclophilin. The direction and extent of sequence determinations are shown by horizontal arrows. Each strand was sequenced at least three times. Restriction enzymes used were: *AluI*, *KpnI*, *NcoI*, *RsaI*, *SalI*, and *XhoI*. bp, base pairs. B, the narrow arrowhead points to the putative cleavage site which is the target of the metal-dependent processing peptidase and the beginning of the intermediate-size form. The broad arrowhead indicates the final cleavage site and beginning of the mature protein. The presequence is printed in italics. Hydroxylated amino acids in the presequence are marked by asterisks. Positively charged amino acids in the presequence are also indicated. A long uncharged region in the protein is underlined. The first nucleotides of cDNA insert cp6 (C in position 113) and of cDNA insert cp12 (T in position 108) are also underlined. Small arrows mark the polyadenylation sites of the three inserts.

cDNA insert cp6 (~0.8 kb coding for cytoplasmic cyclophilin) (data not shown) were used as labeled probes. Both probes hybridized with the same restriction fragments of genomic DNA cut with different restriction enzymes (Fig. 6). Each enzyme produced only one major hybridizing band, indicating

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SKUFFDLEWEGPULGPMHKPTSEIKAQSGRIHFTLYDDUUPKTRAMFKELCTGQMGFGYKG 61
*****
UOPTUFFD IAUDEGE P LGRAVSFELFADKUPKTRAMFALSTGEKGFYKG 49
SSFHRI IPEFNLQGGDFTRMGTTGGKSIYGEKFADEMFADKHHVAPGLLSMAHAGPHTNGSQFF 124
*****
SCFHRI IPGFNCGGGDFTRMGTTGGKSIYGEKFADEMFILKHTGPGILSMAHAGPHTNGSQFF 112
UTTUPTSWLDGRAHUUFGEVADDESMAKUKALEATGSSSGAIRYSKKTPIUDCGAL 179
*****
ICTAKTEWLDGKHUUVFGKU KEGMIVAEAMERFSGRNG KTSKKITIAADCGLE 164

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FIG. 5. Comparison of the cDNA derived amino acid sequences of *N. crassa* (upper lines) and human cyclophilin (lower lines). Sequences are given using the single-letter amino acid code beginning with the amino terminus. Gaps are introduced for alignment. Identical amino acids are indicated by asterisks. The sequence of human cyclophilin is taken from Haendler *et al.* (1987).

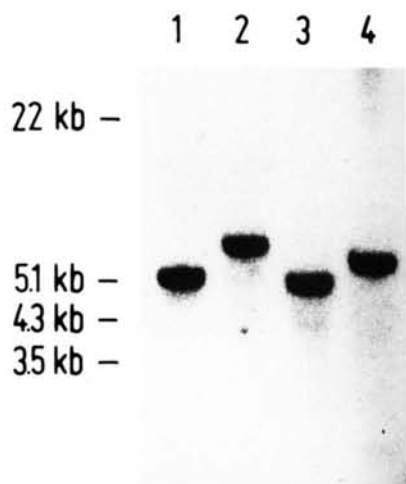


FIG. 6. Southern blot analysis shows the existence of only one gene for cyclophilin. Samples of high molecular weight genomic DNA from *N. crassa* (10 μ g each) were digested with different restriction enzymes, run on an agarose gel, transferred to a nylon filter, and probed with a nick-translated cp1-1 cDNA insert. Lane 1, *Bam*HI; lane 2, *Eco*RI; lane 3, *Hind*III; lane 4, *Pst*I

that there is only one gene for both forms of cyclophilin. The existence of only one cyclophilin gene in *N. crassa* is also supported by the fact that both 24- and 20-kDa specific cDNAs have identical sequences (except the coding region for the presequence) and that all genomic clones sequenced so far have identical sequences.²

In Vitro Expression of the Two Forms of Cyclophilin cDNA—cDNA inserts cp1-1 and cp6 were cloned into the *Pst*I site of the transcription vector pDS5 (Stüber *et al.*, 1984). The inserted DNA was efficiently transcribed under the control of the T5 promoter. The resulting capped RNAs were translated in reticulocyte lysates (Pelham and Jackson, 1976). Insert cp1-1 resulted in the synthesis of two proteins (Fig. 7, lanes 1 and 4) with apparent molecular masses of 24 and 20 kDa. Apparently, due to initiation of protein synthesis at the second methionine (see Fig. 4B) a shorter form, which comigrated with isolated cytosolic and mitochondrial cyclophilin (not shown), was produced in addition to the full sized precursor polypeptide. Both proteins were precipitated by a cyclophilin-specific antiserum (Fig. 7, lane 4). Purified mitochondrial cyclophilin efficiently competed for immunoprecipitation of both proteins to the cyclophilin-specific antibodies (Fig. 7, lane 5). Translation of capped cp1-1 RNA in wheat germ extracts resulted predominantly in the synthesis of the 24-kDa protein (data not shown); initiation in wheat germ

² M. Tropschug and W. Neupert, manuscript in preparation.

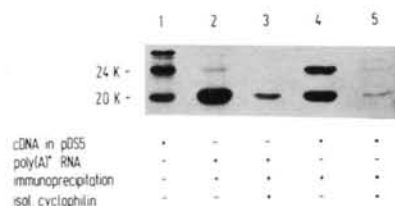


FIG. 7. Cell-free synthesis of cyclophilin. Rabbit reticulocyte lysates were programmed either with RNA derived from transcription of cDNA insert cp1-1 (lanes 1, 4, and 5) or poly(A)⁺ RNA from *N. crassa* (lanes 2 and 3). [³⁵S]Methionine was present to label synthesized protein. A sample of the reticulocyte lysate was applied directly to an SDS-polyacrylamide gel (lane 1); all other samples were immunoprecipitated with a cyclophilin-specific antibody plus protein A-Sepharose (lanes 2–5), and the SDS-dissolved immunoprecipitates were applied to the gel. The samples shown in lanes 3 and 5 received 10 μ g isolated (*isol.*) cyclophilin at the end of the protein synthesis reaction to compete for binding to the antibodies with the radiolabelled proteins.

extracts is known to be more precise (Kozak, 1986).

Transcription and translation of cDNA inserts cp6 or cp12 resulted in the synthesis of only the 20-kDa cyclophilin protein. This protein was also precipitated with the cyclophilin-specific antibody and immunoprecipitation was competed by isolated mitochondrial cyclophilin (data not shown). In order to determine whether both forms of cyclophilin are also translated from total *N. crassa* mRNA, *in vitro* translation of *N. crassa* poly(A)⁺ RNA in reticulocyte lysate and subsequent immunoprecipitation with cyclophilin-specific antiserum were performed. Both the 24- and 20-kDa forms were translated and immunoprecipitated (Fig. 7, lane 2). Precipitation was also competed for by isolated cyclophilin (Fig. 7, lane 3).

These data show that we have indeed cloned full length cDNAs coding for the 24- and 20-kDa forms of cyclophilin (also, see Fig. 3).

Mitochondrial Cyclophilin Is Synthesized as a 24-kDa Precursor Which Is Cleaved in Two Steps Upon Import into Mitochondria—The similarity of the aminoterminal extension in the 24-kDa polypeptide translated from the cp1-1 insert with mitochondrial presequences prompted us to determine whether this is the precursor form of cyclophilin which is imported into mitochondria. This was examined both *in vitro* and *in vivo*.

In a first approach, isolated *N. crassa* mitochondria and reticulocyte lysate containing ³⁵S-labeled 24-kDa polypeptide were incubated at 25 °C in the presence (Fig. 8, lane 1) or absence (Fig. 8, lane 2) of a mitochondrial membrane potential. Mitochondria were reisolated and treated with proteinase K. Only in the presence of a membrane potential was mature-size cyclophilin (20 kDa) found with mitochondria (Fig. 8, lane 1). When the matrix-located processing activity was partially inhibited by the metal chelator *o*-phenanthroline (Zwizinski and Neupert, 1983; Schmidt *et al.*, 1984; Hartl *et al.*, 1986), a 21-kDa product, but no mature cyclophilin, was generated in the presence of a membrane potential (Fig. 8, lanes 3 and 4). In the absence of a membrane potential or in the absence of mitochondria, the 21-kDa form was not generated (data not shown). Since the formation of the 21-kDa polypeptide and of mature-size cyclophilin was dependent on the mitochondrial membrane potential, it reflects protein transport into mitochondria. When the processing peptidase was reactivated by the addition of manganese ions (Zwizinski and Neupert, 1983; Hartl *et al.*, 1986), mature cyclophilin was generated from the 24- and 21-kDa polypeptides (Fig. 8, lanes 7, 8, 11, and 12). We conclude that the 24-kDa polypeptide is imported into mitochondria in a membrane-potential depend-

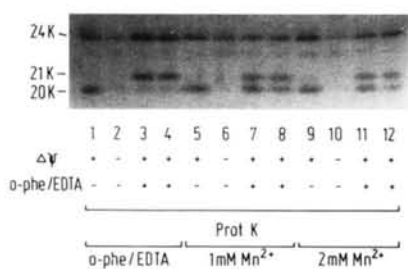


FIG. 8. Import of cyclophilin precursor polypeptide into mitochondria *in vitro*. Isolated *N. crassa* mitochondria were incubated with reticulocyte lysate containing ^{35}S -labeled cyclophilin precursor (24 kDa) and buffer containing bovine serum albumin as described (Pfanner and Neupert, 1985; Hartl *et al.*, 1986). Reactions 1, 3, and 4 contained 2 mM NADH, 8 mM potassium ascorbate, and 0.2 mM *N,N,N',N'*-tetramethylphenylenediamine. Reaction 2 contained 8 μM antimycin A and 20 μM oligomycin. Reaction 3 contained 150 μM *o*-phenanthroline (*o-phe*) and 5 mM EDTA. Reaction 4 contained 250 μM *o*-phenanthroline and 8 mM EDTA. After incubation for 30 min at 25 °C, mitochondria were reisolated, resuspended in bovine serum albumin-containing buffer including antimycin A, oligomycin, *o*-phenanthroline, and EDTA as described above, transferred to new tubes, and treated with proteinase K (50 $\mu\text{g}/\text{ml}$) (*Prot K*) as described (Pfanner and Neupert, 1987b). Reactions were divided into three portions each (reaction 1 into portions 1, 5, and 9; reaction 2 into 2, 6, and 10; reaction 3 into 3, 7, and 11; reaction 4 into 4, 8, 12). Mitochondria were reisolated and resuspended in bovine serum albumin buffer containing *o*-phenanthroline and EDTA (portions 1–4), 1 mM MnCl_2 (portions 5–8), or 2 mM MnCl_2 (portions 9–12). After incubation for 30 min at 25 °C, mitochondria were reisolated. Immunoprecipitation with an antiserum prepared against the isolated mitochondrial cyclophilin was performed as described (Schleyer and Neupert, 1985). Samples were resolved by SDS-polyacrylamide gel electrophoresis. A fluorograph of the dried gel is shown. $\Delta\Psi$, mitochondrial membrane potential. 21 kDa, intermediate-size form of cyclophilin; 20 kDa, mature-size cyclophilin.

ent manner and processed via a 21-kDa intermediate to mature cyclophilin.

The site of the first cleavage of the 24-kDa precursor is not known. A simple consensus sequence for the cleavage sites of the processing peptidase does not exist; however, comparison with a large number of known cleavage sites (see Nicholson and Neupert, 1988) suggests that the first cleavage step is after Ala-36, (see Fig. 4B) since an Arg residue is found in position -2 . Cleavage at this site would also correspond well with the estimated molecular mass differences between the 24-, 21-, and 20-kDa species. The final cleavage most probably occurs after methionine 44 since the isolated mitochondrial cyclophilin starts with Ser-45 of the precursor.

In contrast to this, mature cyclophilin (20 kDa), synthesized by transcription/translation from cDNA inserts cp6 or cp12, was not imported into isolated mitochondria (data not shown) substantiating its extramitochondrial location.

In a second approach, *N. crassa* hyphae were labeled during growth with [^{35}S]methionine for different periods of time followed by a chase with unlabeled methionine. Samples were withdrawn at different time points, precipitated with trichloroacetic acid, lysed in SDS, and cyclophilin was immunoprecipitated (Fig. 9). Both the 24-kDa precursor and mature-size cyclophilin (20 kDa) were found to be synthesized (Fig. 9, lanes 1–4). Labeled 24-kDa polypeptide accumulated during the pulse (lanes 3 and 4) and disappeared during the chase (lanes 5 and 6). This disappearance could be blocked by poisoning the mitochondria with the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (lanes 7 and 8). Mature-size (cytoplasmic) cyclophilin, on the other hand, was labeled without a typical lag phase indicating that it is a direct translation product. In summary, the pulse-chase labeling of

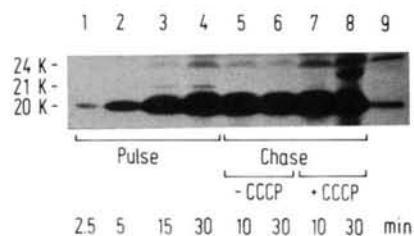


FIG. 9. Pulse-chase labeling of cyclophilin in whole cells. *N. crassa* cells were grown for 14 h to a density of 3 g/l at 25 °C. Cells were harvested by filtration and adjusted to a density of 50 mg of cells/15 ml, cooled to 8 °C, and 3 mCi of [^3H]leucine was added. After 2.5, 5, 15, and 30 min, aliquots containing 5 mg of cells were withdrawn and precipitated with ice-cold trichloroacetic acid (final concentration 0.4 M) (lanes 1–4). To the remaining part of the culture, a chase of 1 mM unlabeled leucine and 50 $\mu\text{g}/\text{ml}$ cycloheximide (final concentrations) were added. The sample then was halved. One portion received 12 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (final concentration) (lanes 7 and 8), the other portion served as a control (lanes 5 and 6). After 1 min, the temperature was raised to 25 °C. At 10 and 30 min after addition of the chase, aliquots of 50 mg of cells were precipitated with trichloroacetic acid (lanes 5–8). All samples were neutralized after treatment with trichloroacetic acid and then immunoprecipitated with a cyclophilin-specific antibody plus protein A-Sepharose. The immunoprecipitates were subjected to polyacrylamide gel electrophoresis. The relevant portion of a fluorograph of the dried gel is shown. As markers, mitochondrial precursor form of cyclophilin (24 kDa) and mature cyclophilin (20 kDa) were precipitated from a reticulocyte lysate which was programmed with mRNA derived from *in vitro* transcription of cDNA insert cp1-1 (see Fig. 7, lane 4) cloned into pDS5 (lane 9).

24-kDa protein shows the typical kinetic behavior of a mitochondrial precursor protein (Zimmermann and Neupert, 1980; Zimmermann *et al.*, 1981).

DISCUSSION

Cyclophilin of *N. crassa* represents one of the rather rare cases in which a protein is present in two different subcellular compartments at the same time. In higher organisms, cyclophilin has been identified as a cytosolic protein, but a possible additional location in mitochondria has not been proven or disproven (Handschumacher *et al.*, 1984; Koletsky *et al.*, 1986). A dual location has been described for proteins such as invertase and amylase (Carlson and Botstein, 1982; Young *et al.*, 1981) which can both occur in the cytosol of the cell and can be secreted by the cell. More recently, several proteins in yeast cells have been described which are at the same time present in the cytosol and in the mitochondrial matrix. Examples include fumarase (Wu and Tzagoloff, 1987), aminoacyl-tRNA synthetases (Gabiuss *et al.*, 1983; Natsoulis *et al.*, 1986; Chatton *et al.*, 1988), tRNA-modifying enzymes (Hopper *et al.*, 1982; Najarian *et al.*, 1987) and α -isopropylmalate synthase (Beltzer *et al.*, 1988). Here we report on the first case of such a situation in a cell other than yeast.

The mechanism by which cells manage to locate a given protein in two different compartments appears to follow the same motif in all the various cases known (for review, see Surguchov, 1987). As with cyclophilin, there is a single gene which is differentially transcribed. One of the two transcripts contains at the 5'-end the information for a "signal" or "target" sequence to direct the precursor out of the cytosol. The shorter transcript lacks the initiation AUG codon at the beginning of the amino-terminal extra sequence and a downstream AUG codon can serve for initiation of translation. Processing of the precursor in the target organelle then leads to the production of the same final product which is made from the second initiation codon, but in another location.

In the case of *N. crassa* cyclophilin, the processing reaction

of the precursor form is of particular complexity. Proteolytic cleavage occurs in two steps. The putative first cleavage site appears to be similar to a number of sites in other *N. crassa* and yeast mitochondrial precursor proteins (for review, see Nicholson and Neupert, 1988). The intermediate formed by this cleavage contains an extension of eight amino acids preceding the amino terminus of the mature protein. Most interestingly, this situation is very similar to that of the two-step processing of the Rieske-Fe/S protein in *N. crassa* and yeast (Hartl *et al.*, 1986). In this case also, an intermediate containing an eight-amino acid extension of the mature form is produced. Furthermore, the overall amino acid composition of this octapeptide is very similar between cyclophilin and the Rieske protein. Although other cases of two-step processing of matrix proteins have been described, nothing is known about the role and the enzymology of this process. The purified metal-dependent processing peptidase (Hawliczek *et al.*, 1988) can only perform the first cleavage of the cyclophilin precursor; on the other hand there are indications that the second cleavage step is also inhibited by metal chelators (see Fig. 8).

Whereas in several cases examined to date the dual localization of the respective enzyme appears to be advantageous on functional grounds, we do not have information as to why the cell places cyclophilin to mitochondria and into the cytosol at the same time. This is related to the problem that the function of the cyclosporin-binding protein is unknown. The fungal toxin cyclosporin may displace a natural ligand of so far unknown nature from cyclophilin. In a preliminary study³ we found that in a number of cyclosporin A-resistant mutants of *N. crassa* both the cytosolic and the mitochondrial forms of cyclophilin are altered. This strongly suggests that cyclosporin sensitivity, at least in *N. crassa*, is mediated by cyclophilin. Cyclophilin may thus also be a mediator of the immunosuppressive and cytotoxic effects of cyclosporin A in higher eukaryotes, although this has to be proven. The successful cloning of the *N. crassa* cyclophilin gene and mRNAs, and establishment of the dual localization of cyclophilin may help to answer this question.

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