Sensitivity to cyclosporin A is mediated by cyclophilin in *Neurospora crassa* and *Saccharomyces cerevisiae*  

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**Cyclosporin A**, a cyclic fungal undecapeptide produced by *Toiyophyllum inflatum*, is a potent immunosuppressive drug originally isolated as an antifungal antibiotic.³⁴ Cyclophilin A (CSA) is widely used in humans to prevent rejection of transplanted organs such as kidney, heart, bone marrow and liver.¹ The biochemical basis of CSA action is not known: its primary cellular target has been suggested to be calmodulin,² the prolactin receptor,⁵ or cyclophilin, a CSA-binding protein originally isolated from the cytosol of bovine thymocytes.⁶⁻⁷⁹⁰ Cyclophilin has been shown to be a highly conserved protein present in all eukaryotic cells tested¹¹⁻¹⁵ and to be identical¹⁶⁻¹⁷ to peptidyl-prolyl cis-trans isomerase,¹⁸⁻¹⁹ a novel type of enzyme²⁰ that accelerates the slow refolding phase of certain proteins in vitro.²¹⁻²² We demonstrate that in the lower eukaryotes *N. crassa* and *S. cerevisiae*, cyclophilin mediates the cytotoxic CSA effect. In CSA-resistant mutants of both organisms, the cyclophilin protein is either lost completely or, if present, has lost its ability to bind CSA.

We selected for CSA-resistant mutants in the St Lawrence 74A wild-type strain of *N. crassa* by applying the lowest CSA concentration (1 μg ml⁻¹) found to prevent growth. Forty-one ultraviolet-induced mutant strains were isolated and four of those, belonging to a high-resistance class (B9; B12; B27; B32; all growing on 10 μg ml⁻¹ CSA), were crossed to a double-mutant strain pan-2 arg-12 to combine the CSA-resistance with 'forcing markers' for heterokaryon formation. We performed the same procedure with a spontaneous mutant (B60) which grew on 5 μg ml⁻¹ CSA. In all five crosses, CSA-resistant and CSA-sensitive segregants occurred in a 1:1 ratio, indicating the involvement of a single gene in each case. Segregants that carried each mutation in wild-type background were selected for biochemical analysis (Fig. 1); segregants with mutations in combination with either pan-2 or arg-12 markers and mating type A were selected for dominance and complementation tests. We found all five CSA-resistant mutations to be recessive in mycelia that were heterokaryotic for either one of them and the CSA-sensitive wild-type allele. Mycelia heterokaryotic for pairs of CSA-resistant mutations displayed the CSA-resistant phenotype, that is, they were unable to complement each other. Apparently the five mutations are allelic, indicating that a single gene, which we name *csr-I*, is responsible for CSA-resistance. Because we noticed a weak linkage (~35%) between CSA-resistant mutations
and mating type a/A alleles (located on N. crassa linkage group I), mutant B12 was crossed to markers on chromosome I, that is nit-2 (left arm) and his-2 (right arm). We observed linkage with his-2 with 19.5% recombination. The linkage with the mating type locus, and his-2 allows us to position csr-1 distal from his-2 on the right arm of chromosome I.

All mutants grew normally at 25 °C. We also tested them for growth at 29 °C and 37 °C, but no temperature-sensitivity was observed.

In wild-type strains of N. crassa cyclophilin is located in both cytosol and mitochondria. Both forms are encoded by a single nuclear gene, which is transcribed into two messenger RNAs with different 5'-ends. The shorter mRNA codes for the cytosolic form of relative molecular mass 20,000 (M. 20K), whereas the longer mRNA codes for a 24K precursor protein whose N-terminal prescence is cleaved, in two steps, on import into mitochondria, leading to the mature 20K cyclophilin protein in the matrix.

To analyse whether the mutations leading to CsA-resistance affected the levels of cyclophilin, we performed western blot assays with cyclophilin-specific antibodies, as well as CsA-binding assays (Fig. 1). Both cytosolic and mitochondrial fractions of wild-type strain and the CsA-resistant segregants in wild-type background (to ensure that only the csr-1 gene was affected), were analysed. Cyclophilin was present in both the cytosolic and mitochondrial fractions of the wild-type strain 74A and the spontaneous mutant B60. None of the other mutants, however, contained immunodetectable cyclophilin in both the cytosol or the mitochondria (Fig. 1a).

In all the mutants we tested (including B60), no significant CsA-binding activity was detectable in the cytosolic fractions (Fig. 1b), in contrast to wild-type cytosol where normal binding was observed. In mitochondrial extracts of all mutant strains (prepared by lysing isolated mitochondria in 1.8% N-octylglucoside and centrifugation), specific CsA-binding activity was reduced to 10–30% of that seen with wild-type mitochondrial extracts. The residual binding activity present in the mutant mitochondria may be due to another CsA-binding component, different from cyclophilin.

Taken together, CsA-resistant mutants of N. crassa that are affected in the gene csr-1 (which is probably the gene coding for cyclophilin) had either completely lost cyclophilin in both cytosol and mitochondria, or, if the protein was still present (as in mutant B60), it had lost its CsA-binding ability. These data indicate that either cytosolic or mitochondrial cyclophilin, or both, is the mediator of CsA action in N. crassa.

We performed similar experiments in the yeast S. cerevisiae, which was previously reported to be insensitive to CsA (ref. 1). We found that CsA-sensitivity seems to be coupled to a Pette phenotype, and is most probably due to alterations in the cell wall which lead to increased permeability to the hydrophobic drug. Forty-eight different laboratory strains of S. cerevisiae were tested for CsA-sensitivity; only two, both with the Pette phenotype, were found to be sensitive to 100 μg ml⁻¹ CsA. CsA-sensitive mutants could easily be selected from CsA-resistant wild-type strains by screening for spontaneous Pette phenotypes.

Starting with the CsA-sensitive ρ0 strain IL 993/5c (ref. 25) (wild type (wt); Fig. 2), eight independent, spontaneous CsA-resistant mutants were isolated (strains 1–8; Fig. 2). Growth tests on nonfermentable carbon sources such as glycerol confirmed that they did not represent ρ0 revertants. All CsA-resistant yeast mutants showed no significant CsA-binding activity in the cytosolic fractions (Fig. 2b). In addition, two of these mutants (mutants 7 and 8; Fig. 2a) contained no immunodetectable cyclophilin. Yeast cyclophilin has an apparent relative molecular mass of 17K, and is recognized by antibodies raised against either N. crassa cyclophilin or isolated yeast cyclophilin. We have cloned and sequenced both a full-length complementary DNA and the gene coding for the cytosolic form of S. cerevisiae cyclophilin (K. Dietmeier and M. T., manuscript in preparation).

Our results demonstrate that cyclophilin is a cellular target of CsA. Both in N. crassa and in yeast the cytotoxic action of CsA is mediated by cyclophilin. In the absence of cyclophilin (or in the presence of a mutated cyclophilin that has lost its CsA-binding activity), CsA does not have any toxic effect.

These findings raise several intriguing questions concerning both the physiological role of cyclophilin and the mechanism.

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**Fig. 1.** Western blot analysis of cyclophilin (20K) (a) and analysis of CsA-binding (b) of mitochondrial and cytoplasmic fractions of N. crassa wild-type and CsA-resistant mutants.

**Methods.** a N. crassa 74A wild-type (wt) and CsA-resistant mutants (B9, B12, B27, B32, B60) were fractionated into cytosol (C) and mitochondria (M) (refs 14, 26). Protein (100 μg) from each fraction was electrophoresed on a 15% SDS-polyacrylamide gel, transferred to nitrocellulose paper, and immunoreacted with polyclonal antibodies raised against N. crassa cyclophilin. Bound antibodies were visualized using peroxidase coupled to antibodies against rabbit IgG. b Cytosolic fractions of the different strains were assayed for CsA binding. With all mutants tested, specific binding of [3H]CsA was less than 5–10% of that of wild-type cytosol. Isolated mitochondria were lysed in 1.8% N-octylglucoside, centrifuged, and the detergent extracts tested for the ability to bind [3H]CsA in a LH-20 assay. In the mitochondrial extracts of the different mutants, specific binding of [3H]CsA was significantly reduced (10–30% of specific binding of wild-type mitochondrial extracts).

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**Fig. 2.** Western blot analysis of cyclophilin (17K) (a) and analysis of CsA-binding (b) of cytosolic fractions of S. cerevisiae wild-type and spontaneous CsA-resistant mutants.

**Methods.** a Wild-type (wt) strain IL 993/5c (ref. 24) was grown at 30 °C in YPD medium, 1% ethanol, 0.1% Tween 80; eight independent spontaneous mutants (strains 1–8) were grown under identical conditions, except that the medium contained 50 μg ml⁻¹ CsA. Zymolyase-spheroplasts were lysed and cytosolic fractions prepared and analysed as in Fig. 1. b Cytosolic fractions of the different strains were assayed for CsA-binding. With all mutants tested (strains 1–8), specific binding of [3H]CsA was less than 5–10% of that of wild-type cytosol.
of CaS action. Cyclophilin is a highly conserved protein in all eukaryotes analysed so far,12-14. How can N. crassa and yeast cells survive without showing any detectable phenotype in the absence of cyclophilin?

Recent work16,17 has demonstrated the identity of cyclophilin to peptidyl-prolyl cis-trans isomerase (PPIase)18,19. Both isolated N. crassa and yeast wild-type cyclophilins were found to possess PPIase-activity similar to the human and bovine enzymes (S. Mayer, F. X. Schmid, D. W. Nicholson and M. T., manuscript in preparation). Furthermore, PPIase-activity measured in cytosolic and mitochondrial fractions of N. crassa mutants that lack cyclophilin, indicate the presence of additional PPIase activity in both the cytosol and mitochondria. In a Southern blot analysis of N. crassa DNA, using low-stringency conditions, we have found two bands that cross-hyridize with a cyclophilin probe, and which correspond to the additional PPIase-activities. The presence of these additional PPIase-activities could explain why a complete loss of cyclophilin does not lead to cell death.

Why does CaS stop growth of wild-type cells but not of cyclophilin-deficient mutant cells? We propose that binding of CaS to cyclophilin leads to the formation of a complex which then interacts with another, unidentified cellular component. This interaction must be lethal to the cell. Other explanations are possible, although less likely. For example, cyclophilin A could be modified by cyclophilin and the derivative could be lethal to the cell.

It is tempting to speculate that cyclophilin may be involved in the unfolding/rolding of newly synthesized proteins, in particular of those which have to traverse the various cellular membranes20-29. In this respect it may be relevant that a cyclophilin-related protein, namely the product of the ninaA gene of Drosophila melanogaster, has been suggested to be involved in the folding of rhodopsin in vivo30,31.

**Note added in proof:** We have learned that T. Chang, B. Weisblum and R. L. Metzenberg (personal communication) have also identified a N. crassa gene for cyclophilin A resistance on linkage group I, left of cyh-1 (1%).

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**Style self-incompatibility gene products of Nicotiana alata are ribonucleases**

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**SELF-INCOMPATIBILITY in flowering plants is often controlled by a single nuclear gene (the S-gene) having several alleles.** This gene prevents fertilization by self-pollen or by pollen bearing either of the two S-alleles expressed in the style. Sequence analysis shows that three alleles of the S gene of Nicotiana alata encode style glycoproteins22 with regions of defined homology. Two of the homologous regions also show precise homology with ribonucleases T1 (ref. 4) and Rh (ref. 5). We report here that glycoproteins corresponding to the S1, S2, S3, and S4 alleles isolated from style extracts of N. alata25 are ribonucleases. These style S-gene-encoded glycoproteins account for most of the ribonuclease activity recovered from style extracts. The ribonuclease specific activity of style extracts of the self-incompatible species N. alata is 100-1,000-fold higher than that of the related self-compatible species N. tabacum. These observations implicate ribonuclease activity in the mechanism of gametophytic self-incompatibility.

Cloned complementary DNAs corresponding to the S1, S2, and S4-allelic glycoproteins have recently been isolated23,24. The primary amino-acid sequences of three S-glycoproteins show some regions that are perfectly conserved and other regions that are less homologous25. Sequence analysis reveals significant homology between the S-glycoproteins and the extracellular ribonucleases (RNases) T1 of Aspergillus oryzae14 and Rh of Rhizopus niveus15 (Fig. 1). The two longest regions of homology, residues 67-72 and 138-142 (Fig. 1) include His 67 and His 139 which are implicated in RNase T1 catalysis8. It is significant that these homologous regions are also conserved among the three S-glycoproteins. In total, of the 122 amino acids perfectly conserved among the three S-glycoproteins, 30 are aligned with identical amino acids in the fungal RNases and another 22 are aligned with closely related amino acids. The positions 5 out of 10 cysteine residues are conserved between the S-glycoproteins and the RNases. Two of these cysteine residues, 86 and 143, in the vicinity of the proposed RNase active site, form a disulfide bond in RNase T1 (ref. 4). These sequence similarities indicate a critical structural relationship between the active-site domain of the two secreted fungal RNases and the homologous region of the S-glycoproteins. This homology prompted us to investigate the RNase activity of the S-glycoproteins.

The S-glycoproteins can be purified from style extracts by a procedure involving ammonium-sulfate fractionation and cation-exchange chromatography26. The S-glycoproteins isolated by this method behave as a single species on SDS-PAGE or reverse-phase HPLC; N-terminal sequencing confirms their identity with the proteins predicted from the cloned cDNAs27. The association of particular S-glycoproteins with the corresponding S allele is further supported by data which show that, in every case examined, the elution profile of proteins resolved by cation-exchange chromatography correctly predicts the genotype of the source material. Also, these isolated S-glycoproteins are active as inhibitors of In vitro pollen tube