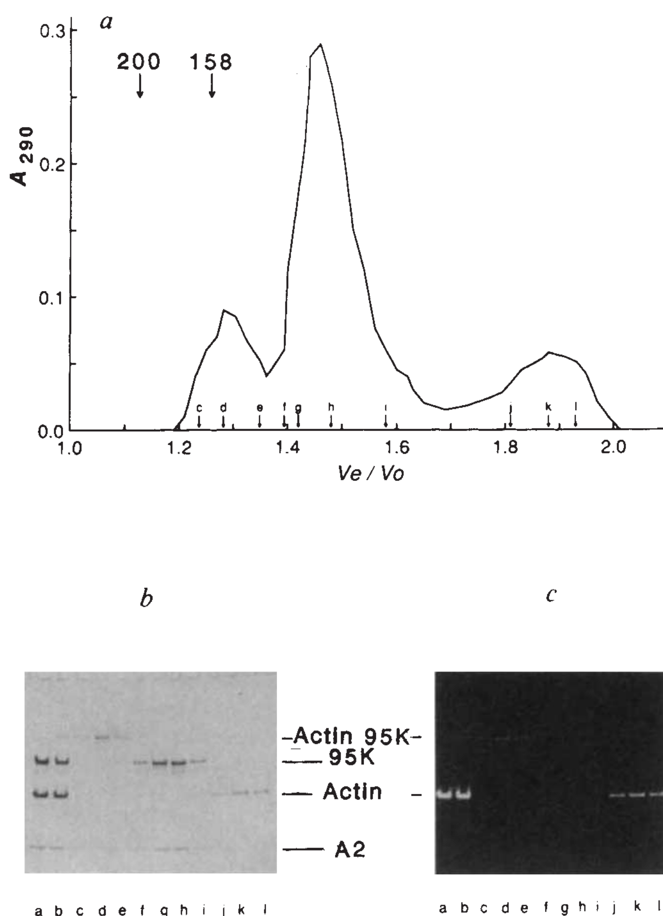

FIG. 3 Purification of the covalent G-actin–S1(A2) complex. *a*, Elution profile of EDC-crosslinked G-actin–S1(A2) complex from a Sephadex G-150 column. Abbreviations: V_e, elution volume; V_0, void volume. Aliquots of fractions c–l were loaded on SDS-PAGE and stained with Coomassie blue (*b*) or viewed in the ultraviolet light (*c*). Lanes a and b, composition of 1,5-IAEDANS-labelled G-actin–S1(A2) mixture before and after EDC-catalysed crosslinking reaction, respectively.
METHODS. 1,5-IAEDANS-labelled G-actin (40 μM) and S1(A2) (50 μM) were mixed in G buffer at 4 °C and a solution of EDC was added to a final concentration of 10 mM. During the 50 min of the crosslinking reaction, the mixture was centrifuged at 160,000*g*, 4 °C. The supernatant was then collected, the reaction was stopped with a 100-fold molar excess of DTE, and the sample was loaded on a G-150 column (2.5 × 140 cm) equilibrated with G buffer at 4 °C. M_r standards ($M_r \times 10^{-3}$): aldolase, 158; α-amylase, 200.

nificance is not clear, because in the presence of 0.5 mM $MgCl_2$, the G-actin-S1(A2) complexes spontaneously form filaments (data not shown). Nonetheless, both the noncovalent and covalent complexes described here are undoubtedly useful for further functional and structural studies, as they provide potential samples for the crystallization and the resolution of the three-dimensional structure of the native G-actin–myosin head complex. □

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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 *Tolypocladium inflatum*, is a potent immunosuppressive drug originally isolated as an antifungal antibiotic[1,2]. Cyclosporin A (CsA) is widely used in humans to prevent rejection of transplanted organs such as kidney, heart, bone marrow and liver[3]. The biochemical basis of CsA action is not known: its primary cellular target has been suggested to be calmodulin[4,5], the prolactin receptor[6,7] or cyclophilin, a CsA-binding protein originally isolated from the cytosol of bovine thymocytes[8–10]. Cyclophilin has been shown to be a highly conserved protein present in all eukaryotic cells tested[11–15] and to be identical[16,17] to peptidyl–prolyl *cis-trans* isomerase[18,19], a novel type of enzyme[20] 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-1*, is responsible for CsA-resistance. Because we noticed a weak linkage (~33%) between CsA-resistant mutations

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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[14]. 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_r 20K), whereas the longer mRNA codes for a 24K precursor protein whose N-terminal presequence is cleaved, in two steps, on import into mitochondria, leading to the mature 20K cyclophilin protein in the matrix[14].

To analyse whether the mutations leading to CsA-resistance affected the levels of cylophilin, we performed western blot assays[23] with cyclophilin-specific antibodies[14], as well as CsA-binding assays[8,9,11] (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 either the cytosol or the mitochondria (Fig. 1*a*).

In all the mutants we tested (including B60), no significant CsA-binding activity was detectable in the cytosolic fractions (Fig. 1*b*), in contrast to wild-type cytosol where normal binding was observed[14]. In mitochondrial extracts of all mutant strains (prepared by lysing isolated mitochondria in 1.8% *N*-octylglucoside and centrifugation[14]), specific CsA-binding activity was reduced to 10–30% of that seen with wild-type mitochondrial extracts[14]. 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 Petite phenotype, and is most probably due to alterations in the cell wall[24] 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 Petite 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 Petite phenotypes.

Starting with the CsA-sensitive ρ^0 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 ρ^+ revertants. All CsA-resistant yeast mutants showed no significant CsA-binding activity in the cytosolic fractions (Fig. 2*b*). In addition, two of these mutants (mutants 7 and 8; Fig. 2*a*) 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[14] 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

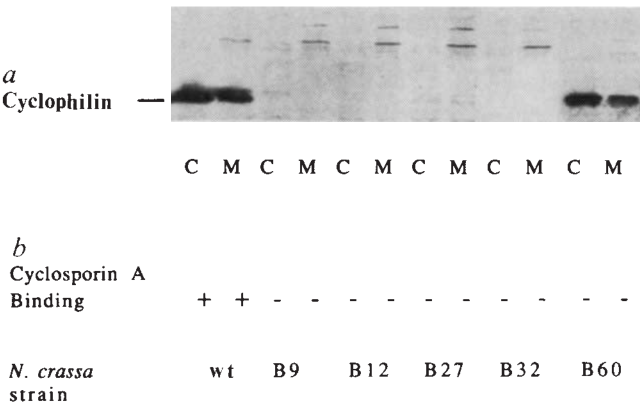

FIG. 1 Western blot analysis of cyclophilin (20K) (*a*) and analysis of CsA-binding (*b*) of cytosolic and mitochondrial fractions of *N. crassa* wild-type and CsA-resistant mutants.
METHODS. *a*, *N. crassa* 74A wild-type (wt) and CsA-resistant mutants (B9, B12, B27, B32, B60) were fractionated into 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[23], and immunodecorated with polyclonal antibodies raised against *N. crassa* cyclophilin[14]. Bound antibodies were visualized using peroxidase coupled to antibodies against rabbit IgG. *b*, Cytosolic fractions of the different strains were assayed for CsA binding[8,9,11,14]. With all mutants tested, specific binding of [^{3}H]CsA was less than 5–10% of that of wild-type cytosol[14]. Isolated mitochondria were lysed in 1.8% *N*-octylglucoside, centrifuged[14] and the detergent extracts tested for the ability to bind [^{3}H]CsA in a LH-20 assay[8,9,11,14]. In the mitochondrial extracts of the different mutants, specific binding of [^{3}H]CsA was significantly reduced (10–30% of specific binding of wild-type mitochondrial extracts[14]).

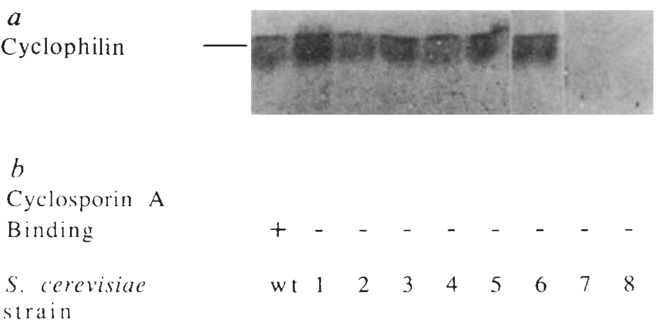

FIG. 2 Western blot analysis of cyclophilin (17K) (*a*) and analysis of CsA-binding (*b*) of cytosolic fractions of *S. cerevisiae* wild-type and spontaneous CsA-resistant mutants.
METHODS. *a*, Wild-type (wt) strain IL 993/5c (ref. 24) was grown at 30 °C in YPD medium[32], 1% ethanol, 0.1% Tween 80; 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[32] and analysed as in Fig. 1. *b*, Cytosolic fractions of the different strains were assayed for CsA-binding[8,9,11,14]. With all mutants tested (strains 1–8), specific binding of [^{3}H]CsA was less than 5–10% of that of wild-type cytosol[14].

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of CsA action. Cyclophilin is a highly conserved protein in all eukaryotes analysed so far[9,12-14]. How can *N. crassa* and yeast cells survive without showing any detectable phenotype in the absence of cyclophilin?

Recent work[16,17] 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-activities 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-hydridize with a cyclophilin probe, and which may 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 CsA stop growth of wild-type cells but not of cyclophilin-deficient mutant cells? We propose that binding of CsA 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, cyclosporin A could be modified by cyclophilin and the derivative could be lethal to the cells.

It is tempting to speculate that cyclophilin may be involved in the unfolding/refolding of newly synthesized proteins, in particular of those which have to traverse the various cellular membranes[26-29]. 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 vivo*[30,31].

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 cyclosporin 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[1]. 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 glycoproteins[2,3] with regions of defined homology. Two of the homologous regions also show precise homology with ribonucleases T_2 (ref. 4) and Rh (ref. 5). We report here that glycoproteins corresponding to the S_1, S_2, S_3, S_6 and S_7 alleles isolated from style extracts of *N. alata*[6] 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 S_2-, S_3- and S_6-allelic glycoproteins[6] have recently been isolated[2,3]. The primary amino-acid sequences of three *S*-glycoproteins show some regions that are perfectly conserved and other regions that are less homologous[3]. Sequence analysis reveals significant homology between the *S*-glycoproteins and the extracellular ribonucleases (RNases) T_2 of *Aspergillus oryzae*[4] and Rh of *Rhizopus niveus*[5] (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 T_2 catalysis[4]. 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 of 5 out of 10 cysteine residues are conserved between the *S*-glycoproteins and the RNases. Two of these cysteine residues, 86 and 143, located in the vicinity of the proposed RNase active site, form a disulphide bond in RNase T_2 (ref. 4). These sequence similarities indicate a close 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-sulphate fractionation and cation-exchange chromatography[6]. 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 cDNAs[6]. 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

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