Mutations That Perturb Cyclophilin A Ligand Binding Pocket Confer Cyclosporin A Resistance in Saccharomyces cerevisiae

(Received for publication, April 6, 1995, and in revised form, May 31, 1995)

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In complex with the peptidyl-prolyl isomerase cyclophilin A, the immunosuppressive antifungal drug cyclosporin A (CsA) inhibits a Ca\(^{2+}\)/calmodulin-dependent protein phosphatase, calcineurin, which regulates signal transduction. We isolated and characterized cyclophilin A mutations that confer CsA resistance in a Saccharomyces cerevisiae strain whose growth is CsA-sensitive. Three mutations (G70S, H90Y, and G102A) alter single amino acids conserved between yeast and human cyclophilin A, which structural analyses implicate in CsA binding to human cyclophilin A. By Western analysis, all three mutant proteins are expressed in yeast. In vitro, two purified mutant cyclophilins (G70S, G102A) retain prolyl isomerase activity and have moderately reduced affinity for CsA and calcineurin but, when bound to CsA, do bind and inhibit calcineurin phosphatase activity. In contrast, the purified H90Y mutant cyclophilin is dramatically decreased in prolyl isomerase activity, CsA affinity, and calcineurin binding and inhibition. These studies identify conserved cyclophilin A residues that participate in CsA binding and catalysis.

The natural product cyclosporin A (CsA) is a potent anti-fungal and immunosuppressive compound via its ability to inhibit signal transduction (for review, see Refs. 1–3). CsA is a hydrophobic cyclic peptide that binds to a family of intracellular proteins, the cyclophilins, which are abundant, ubiquitous, highly conserved, and found in multiple forms in different intracellular compartments. In addition, cyclophilins are enzymes that catalyze protein folding by cis-trans isomerization of peptidyl-prolyl bonds. Cyclophilin A, an 18-kDa cytoplasmic protein, mediates CsA actions in yeast (4–6). The immunosuppressive effects of CsA are also presumed to be mediated by cyclophilin A (7). The target of the cyclophilin A-CsA complex is calcineurin (8, 9), a calcium/calmodulin-dependent serine-threonine-specific protein phosphatase highly conserved from yeast to man (10–12). Calcineurin is also the target of the macrolide FK506 bound to a different prolyl isomerase, FKBP12 (13). Calcineurin regulates nuclear import of the nuclear factor of activated T-cells transcription factor during T-cell activation (14–16), participates in recovery of yeast cells from pheromone-induced cell cycle arrest (4, 10, 12), and is essential in CsA-sensitive yeast strains (5, 17).

A wealth of structural information is available for cyclophilins, both free and in complex with CsA or substrate. The x-ray crystal structures have been solved for cyclophilin A alone (18, 19) and in complex with CsA (20, 21), a tetrapeptide model substrate or a dipeptide (Ala-Pro) (22–24), and a CsA analog (25). Similarly, NMR structures are available for the cyclophilin A-bound conformation of CsA (26), the human cyclophilin A-CsA complex (22, 27), and for wild-type and an F112W mutant of Escherichia coli periplasmic cyclophilin (28, 29). Finally, the x-ray structures of cyclophilin B-CsA and cyclophilin C-CsA have been solved (30, 31). These studies reveal that CsA and substrate bind a common hydrophobic pocket and provide a foundation for mutagenic and biochemical studies to analyze cyclophilin functions in vivo.

Previous studies employing genetic and biochemical analyses established the roles of specific cyclophilin residues in CsA binding, active site function, calcineurin inhibition, and in vivo function. E. coli periplasmic cyclophilin is distantly related to human cyclophilin A, sharing only 34% sequence identity and having prolyl isomerase activity relatively resistant to CsA (32, 33). In E. coli cyclophilin, a tryptophan residue invariant in other cyclophilins is replaced by phenylalanine. The CsA sensitivity of an F112W mutant E. coli protein was increased 75-fold, whereas substitution of Trp\(^{121}\) by phenylalanine in human cyclophilin A decreased CsA affinity 17-fold (34). In the x-ray and NMR structures of cyclophilin A, Trp\(^{121}\) lies in the middle of the Thr\(^{116}\)–Gly\(^{130}\) loop in the CsA binding pocket, near CsA residue 11 and hydrogen-bonded with CsA residue 9 (21, 22). A potential role for cysteine residues in catalysis by cyclophilin A was excluded by isolating active mutants lacking each of four different cysteines (32). Based on the x-ray crystal structure of human cyclophilin A, active site residues (Arg\(^{155}\), Phe\(^{60}\), Phe\(^{113}\), and His\(^{126}\)) were identified by site-directed mutagenesis (35). Interestingly, cyclophilin mutants lacking peptidyl-prolyl isomerase activity retained the ability to bind CsA and inhibit calcineurin (35). Residues surrounding the cyclophilin A ligand pocket (Arg\(^{69}\), Lys\(^{125}\), and Arg\(^{148}\)) that participate in interactions between cyclophilin A-CsA and calcineurin were identified by site-directed mutagenesis (36). Finally, mutations in the Drosophila NinaA cyclophilin homolog (37, 38) were found amongst mutations that disrupt targeting of rhodopsin and distort the fly eye (39). This analysis revealed NinaA regions critical for biological function, including residues within and surrounding the ligand binding pocket and hydrophobic membrane anchor (39–42).

Here we report the isolation of cyclophilin A mutations that confer CsA-resistance in a CsA-sensitive Saccharomyces cerevisiae yeast strain. These mutations identify three residues, Gly\(^{70}\), His\(^{90}\), and Gly\(^{102}\), which participate in CsA binding to.
yeast cyclophilin A and are identical in human cyclophilin A (Gly72, His92, and Gly104). In the structure of human cyclophilin A, the residues all lie within the CSA binding pocket, based on molecular modeling, substitutions at these residues should perturb the cyclophilin A ligand binding pocket. In addition, modeling suggests that the H92Y mutation would most profoundly perturb the ligand binding pocket, and, in vitro, the H90Y cyclophilin A mutant protein has the most severe defect in CSA binding and prolyl isomerase activity. The isolation of spontaneous cyclophilin A mutations that confer CSA resistance in yeast has provided further insight into cyclophilin A structure and corroborates and extends our view based on structural analyses.

**RESULTS AND DISCUSSION**

Isolation of CSA-resistant Yeast Mutants—Previous studies identified an unusual strain of S. cerevisiae, IL993/5c, whose growth is inhibited by CSA (46) and FK506 and in which mutations in cyclophilin A or FKBP12 confer resistance to CSA or to FK506, respectively (5). Our findings established that this strain is CSA-FK506-sensitive because calcineurin is essential to FK506, respectively (5). Preparation of yeast protein extracts from a cyclophilin A-deficient strain (Mn250-2C), cyclophilin affinity chromatography, and Western blot with affinity purified antisera directed against the yeast calcineurin A catalytic subunit CMP1 were as described previously (50).

Calcineurin Phosphatase Assay—Calcineurin phosphatase activity was assayed as described previously (45, 53) with a synthetic peptide from the RII subunit of cAMP-dependent protein kinase (DLLVP1GFRDVRVSAAE) phosphorylated with cAMP-dependent protein kinase. Reactions contained 2 μM phosphopeptide, 40 nM bovine calcineurin (Sigma), 80 nM bovine calmodulin (Sigma), and, where indicated, wild-type or mutant His6-cyclophilin A and CSA in a final reaction of 50 μl. Protein concentrations were determined by comparison of Coomassie Blue-stained SDS-PAGE gel bands to standards of known concentration. Release of 32P from phosphatase activity was quantitated by ion-exchange chromatography and scintillation counting.

**MATERIALS AND METHODS**

Isolation of CSA-resistant yeast mutants—Mutations were isolated from a kanamycin-resistant diploid strain IL993/5c that was transformed with a centromeric low copy number plasmid CPR1 (MATa ura3-52 leu2::hisG a o ura3 tol1-1, and TOC11 o ura3). Mutations conferring CSA-FK506 sensitivity were isolated by streaking to YPD medium containing 100 μg/ml CSA but not to FK506, respectively. Preparation of yeast protein extracts from a cyclophilin A-deficient strain (Mn250-2C), cyclophilin affinity chromatography, and Western blot with affinity purified antisera directed against the yeast calcineurin A catalytic subunit CMP1 were as described previously (50).

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Cyclosporin-resistant Cyclophilin A Mutants

**TABLE I**

| Isolate | CsA | FK | D or Rec | CPR1* | Western (%) | Sequence |
|---------|-----|----|---------|-------|-------------|----------|
| WT      | S   | S  |         |       | 100         | WT       |
| Δcpr1   | R   | S  | Rec     |       | 0           | G705 (GTT to AGT) |
| 7       | R   | S  | Rec     |       | 100         | G102A (GTT to GCT) |
| 46      | R   | S  | Rec     |       | 100         | H90Y (CAC to TAC) |
| 141     | R   | S  | Rec     |       | 25          | A10P (GCT to CCT) |
| 47      | R   | S  | Rec     |       | 100         | ND       |
| 48      | R   | S  | Rec     |       | 0           | ND       |
| 50      | R   | S  | Rec     |       | 0           | H90D (CAC to CGC + Δ) |
| 183     | R   | S  | Rec     |       | 0           | ND       |

CsA-resistant mutant strains, introduction of the cloned CPR1 gene restored CsA sensitivity, whereas introduction of the control plasmid did not. Thus, the mutations that confer CsA resistance are all alleles of the cyclophilin A-encoding gene CPR1 (Table I).

To determine the molecular nature of the cyclophilin A mutations that confer CsA resistance, the CPR1 gene was retrieved from wild-type and mutant genomic DNA by PCR with oligonucleotides flanking the CPR1 gene coding region. DNA sequence analysis of the resulting PCR products revealed that four of the mutations (7, 46, 141, and 47) are single-nucleotide changes that result in amino acid substitutions, G70S, G102A, H90Y, and A10P (Table I). In one additional mutation (183), a single-nucleotide substitution replaces His90 with arginine, but a single-base deletion in the adjacent codon renders the gene out of frame (H90RΔ). In two cases (48 and 50), no changes were identified in the coding portion of the cyclophilin A gene, even though these are clearly cyclophilin A mutations based on failure to complement a cyclophilin A mutation and complementation by the cloned cyclophilin A gene. Because only the coding region of the gene was amplified and sequenced, these two mutants may harbor promoter mutations. In accordance with this interpretation, cells bearing these mutations do not express any cyclophilin A protein (Table I and described below).

As a first step to determine how these cyclophilin A mutations confer CsA resistance, extracts from wild-type and mutant strains were subjected to Western analysis with antisera specific for yeast cyclophilin A. As shown in Fig. 1, this antisera detects wild-type cyclophilin A, an 18-kDa protein, expressed in the wild-type CsA-sensitive strain (Fig. 1, lane 1), and also in a mutant resistant to both CsA and to FK 506 (Fig. 1, lane 2) and a CsA-resistant calcineurin mutant (Fig. 1, lane 3), which both express wild-type cyclophilin A. This protein is not present in extract from a cyclophilin A deletion strain (Δcpr1, Fig. 1, lane 4). In extracts from the mutant strains, three express wild-type levels of cyclophilin A (G70S, H90Y, and A10P) (Fig. 1, lanes 5, 6, and 7), one expresses about one-fourth the wild-type level (H90Y) (Fig. 1, lane 10), and the remaining three do not express cyclophilin A (Fig. 1, lanes 8, 9, and 11). Thus, cyclophilin A mutations that confer CsA resistance can result from a complete lack of protein expression or expression of normal levels of a mutant protein.

**Fig. 1. Expression of cyclophilin A protein in CsA-resistant mutants.** Protein extracts from equal amounts of cells prepared from CsA-resistant strains were fractionated by 15% SDS-PAGE and analyzed by Western blot with a rabbit polyclonal antisera against yeast cyclophilin A. The figure shows results for the CsA-sensitive cyclophilin A wild-type strain TB24 (WT, lane 1), a mutant strain resistant to CsA and to FK 506 (TOC1-1) that expresses wild-type cyclophilin A (WT, lane 2), a CsA-resistant calcineurin A mutant strain (TOC6-1) that expresses wild-type cyclophilin A (WT, lane 3), a cpr1 deletion strain (TB26) lacking cyclophilin A (Δcpr1, lane 4), and CsA-resistant mutant isolates number 7 (G70S, lane 5), 46 (G102A, lane 6), 47 (A10P, lane 7), 48 (ND, lane 8), 50 (ND, lane 9), 141 (H90Y, lane 10), and 183 (H90Δ, lane 11). The arrow indicates the migration position of cyclophilin A. ND indicates cyclophilin A recessive mutants for which the site of mutation lies outside the coding region, presumably in the promoter.

CsA-resistant Cyclophilin A Mutants Have Reduced CsA Affinity—To assist with purification, wild-type cyclophilin A and three cyclophilin A mutant proteins that bear substitutions at residues conserved in human cyclophilin A and which are stably expressed in yeast (G70S, H90Y, and G102A) were fused to a stretch of six histidines at their amino termini, over-expressed in bacteria, and purified by Ni\(^2+\) affinity chromatography to near homogeneity (see "Materials and Methods").

To assess their ability to bind CsA, the purified cyclophilin A proteins were subjected to LH-20 ligand binding assays (51). As shown in Fig. 3A, wild-type yeast cyclophilin A and the G70S and G102A mutant cyclophilins bound CsA, whereas the affinity of the H90Y mutant protein for CsA was dramatically decreased. When LH-20 assays were performed with 5-fold lower amounts of cyclophilin A proteins, CsA binding to the G102A mutant cyclophilin A was reduced in comparison with...
wild-type cyclophilin A (Fig. 3B). Thus, the apparent affinity for CsA by the LH-20 assay was wild-type, G70S > G102A > H90Y.

Peptidyl-Prolyl Isomerase Activity of Cyclophilin A Mutant Enzymes—Because both CsA and substrates associate with the same binding pocket on cyclophilin, as a second measure of CsA affinity we assayed cis-trans peptidyl-prolyl isomerase activity of the purified wild-type and G70S, H90Y, and G102A mutant cyclophilin A proteins. As shown in Fig. 4, the G70S and G102A cyclophilin A mutant proteins had readily detectable prolyl isomerase activity that was comparable with that of wild-type cyclophilin A. This suggests that the G70S and G102A mutations do not dramatically alter the cyclophilin A active site. In contrast, the H90Y mutant protein did not exhibit prolyl isomerase activity (Fig. 4), indicating that this mutation may perturb the cyclophilin A active site such that neither CsA (Fig. 3) nor substrate binds with high affinity.

We next assessed the ability of CsA to inhibit prolyl isomerase activity of the wild-type, G70S, and G102A cyclophilin A enzymes. Prolyl isomerase assays were conducted following preincubation of 20 ng of the different enzymes with CsA at a range of concentrations from 0 to 1000 nM, and the initial reaction velocities were calculated and used to determine the CsA concentration resulting in half-maximal reaction velocity (see "Materials and Methods"). By this analysis, the $K_i$ for inhibition of wild-type cyclophilin A by CsA was approximately 10 nM, whereas for the G70S and G102A, the $K_i$ was increased approximately 10-fold, to 100 nM. Thus, the G70S and G102A mutations result in a moderate reduction in cyclophilin A affinity for CsA. Because the CsA minimum inhibitory concentration in strain TB24 is approximately 25 μg/ml and these mutations confer resistance to 100 μg/ml CsA or 4-fold higher than the MIC, a small reduction in CsA affinity of cyclophilin A should suffice to render the cell drug-resistant. This appears to be the case for the G70S and G102A mutations.

Binding and Inhibition of Calcinurin by Mutant Cyclophilin A-CsA Complexes—To determine if these mutations alter the ability of cyclophilin A to interact with calcineurin, purified wild-type cyclophilin A and the G70S, G102A, and H90Y mutant proteins were coupled to Affi-Gel 10 to produce four different affinity chromatography matrices (see "Materials and Methods"). Equal amounts of cell extract from a cyclophilin A-deficient strain (MH250–2C, prp1::LEU2 mutant) were incubated with each affinity matrix and washed, and bound material was eluted, fractionated by SDS-PAGE, and transferred to nitrocellulose. The yeast calcineurin A catalytic subunit CMP1 was then detected by Western analysis with affinity-purified anti-CMP1 antisera against the calcineurin A catalytic subunit CMP1, whose migration position is indicated by an arrow. Numbers to the left indicate size and migration position of molecular weight standards.
mutant proteins required higher levels of cyclophilin, which is consistent with their decreased affinity for CsA. In addition, these mutations might also perturb nearby residues, such as Arg67 (Arg69 in human cyclophilin A), involved in calcineurin binding. In contrast, the H90Y cyclophilin A mutant protein only weakly inhibited calcineurin activity, which is consistent with its profound CsA binding defect. These observations underscore that, whereas the G70S and G102A mutations subtly alter cyclophilin A properties, the H90Y mutation more dramatically alters the cyclophilin A ligand binding pocket.

Molecular Modeling Based on Human Cyclophilin A—The structures of human cyclophilin A, alone and in complex with either CsA or a peptide substrate, have been determined by x-ray crystallography and NMR (18, 20–24, 27). Residues that comprise the ligand binding pocket of human cyclophilin A (His54, Arg55, Gln63, Asn71, Thr73, Asn102, and Trp121) are conserved in yeast cyclophilin A (His52, Arg53, Gln61, Asn69, Thr71, Asn100, and Trp119). We therefore employed the human cyclophilin A structures as a guide to understand the mutations that render yeast cyclophilin A CsA-resistant. In Fig. 7, these mutations are indicated at the corresponding positions in the x-ray crystal structure of human cyclophilin A (22). The CsA and substrate binding pocket is indicated by the filled Cα–Cα bonds.

All four of the mutations alter single amino acids that lie within the CsA binding site on cyclophilin A. Three mutations alter Gly70, His90, and Gly102 in yeast cyclophilin A, which correspond to Gly72, His92, and Gly104 in human cyclophilin A. By molecular modeling,2 a G72S substitution is predicted to perturb the conformation of the 70s loop of cyclophilin A in which Asn71 and Thr73 bind water molecules that pack against CsA. A G104A substitution would probably alter the conformation of adjacent residues Ala101, Asn102, Ala103, which form part of the hydrophobic pocket (Ala101, Ala103) or hydrogen bond the amino nitrogen of CsA residue Abu2 (Asn102). The G72S and G104A substitutions could also alter the conformation of residues required for cyclophilin A-CsA binding to calcineurin, given our finding that the yeast G70S and G102A cyclophilin A-CsA complexes bound to calcineurin 3-fold less well than wild-type (Figs. 5 and 6). Finally, a H92Y substitution would change the conformation of His126 and possibly also Trp121, which, respectively, hydrogen bond with the carbonyl oxygens of CsA residues MeVal11 and MeLeu9. At present, it is not clear, based on molecular modeling, how an alteration of Val12 (corresponding to yeast A10P) would alter the CsA-binding pocket.

CONCLUSION

Our studies identified yeast cyclophilin A mutant proteins with single-amino acid substitutions at residues conserved between yeast and human cyclophilin A. These mutant proteins were cloned, overexpressed, purified, and characterized in vitro. These analyses reveal that the G70S and G102A cyclophilin A mutant proteins have moderately reduced CsA binding affinity, whereas the H90Y mutant has a more profoundly perturbed ligand binding pocket and fails to bind CsA or catalyze prolyl isomerization. Based on the structure of human cyclophilin A, molecular modeling predicts that these mutations would perturb the ligand binding pocket. None of these residues were identified in earlier studies employing site-directed mutagenesis (32, 34–36), and thus this random collection of mutations has revealed additional residues that partic-

2 Jörg Kallen, personal communication.
ipate in cyclophilin A-ligand interactions. One mutant of the Drosophila NinaA cyclophilin homolog, G96E, has a substitution at the residue corresponding to Gly67 in yeast cyclophilin A (39). Thus, two different studies converged to implicate this residue in cyclophilin interactions with CsA and a presumed in vivo substrate. Further studies in yeast should allow a more precise description of cyclophilin A interactions with substrates, ligands, and calcineurin.

Acknowledgments—We thank N. Rao Movva and Sandoz for materials, Tamara Breuder and Scott Muir for technical assistance, and Jörg Kallen for molecular modeling analyses and discussions.

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