All cyclophilins and FK506 binding proteins are, individually and collectively, dispensable for viability in *Saccharomyces cerevisiae*

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ABSTRACT The cyclophilins and FK506 binding proteins (FKBPs) bind to cyclosporin A, FK506, and rapamycin and mediate their immunosuppressive and toxic effects, but the physiological functions of these proteins are largely unknown. Cyclophilins and FKBPs are ubiquitous and highly conserved enzymes that catalyze peptidyl-prolyl isomerization, a rate-limiting step during *in vitro* protein folding. We have addressed their functions by a genetic approach in the yeast *Saccharomyces cerevisiae*. Five cyclophilins and three FKBPs previously were identified in yeast. We identified four additional enzymes: Cpr6 and Cpr7, which are homologs of mammalian cyclophilin 40 that have also recently been independently isolated by others, Cpr8, a homolog of the secretory pathway cyclophilin Cpr4, and Fpr4, a homolog of the nucleolar FKBP, Fpr3. None of the eight cyclophilins or four FKBPs were essential. Surprisingly, yeast mutants lacking all 12 immunophilins were viable, and the phenotype of the dodecuplet mutant resulted from simple addition of the subtle phenotypes of each individual mutation. We conclude that cyclophilins and FKBPs do not play an essential general role in protein folding and find little evidence of functional overlap between the different enzymes. We propose that each cyclophilin and FKBP instead regulates a restricted number of unique partner proteins that remain to be identified.

Cyclophilin A and FKBP12 originally were isolated as cyclosporin A (CsA) and FK506 binding proteins (FKBPs) and later shown to inhibit the calcium/calmodulin-dependent serine/threonine phosphatase, calcineurin, as protein-drug complexes (reviewed in refs. 1–3). In addition to cyclophilin A and FKBP12, cells express multiple additional cyclophilins and FKBPs (Table 1). The cyclophilins and FKBPs were also independently isolated based on their ability to catalyze cis-trans isomerization of peptidyl-prolyl bonds (4–6), a reaction that can be rate limiting during protein folding *in vitro*. Because prolyl isomerases catalyze peptidyl-prolyl isomerization, are highly conserved from bacteria and yeast to man, and are found in multiple intracellular compartments, it has been suggested that they might play a critical general role in protein folding.

One well characterized cyclophilin is the *Drosophila* ninaA protein (7, 8). NinaA is expressed in the eye, localized within the endoplasmic reticulum (ER), and required for the proper maturation and localization of rhodopsin. In ninaA mutant flies, rhodopsin accumulates in the ER, resulting in visual defects (9, 10). Rhodopsin and ninaA form a stable, stoichiometric complex, and flies lacking a single copy of ninaA exhibit defects (haploinsufficiency), suggesting that ninaA may serve as a chaperone for rhodopsin.

The mammalian cyclophilin-40 and FKBP59 proteins are found in unactivated Hsp90-steroid receptor complexes (11, 12) and may function as chaperones in the assembly of these complexes (13, 14). It previously has been shown that steroid

receptors can be heterologously expressed in yeast, and the receptor complex is functionally conserved (15, 16).

In mammals, FKBP12 is a subunit of both the ryanodine and the IP₃ receptor Ca²⁺ channels (17–19) and is required for proper channel gating (20), possibly by targeting calcineurin to the channel (21). In addition, FKBP12 is necessary for proper function of the multi-drug resistance pump (22). These studies suggest FKBP12 might play a general role in regulating the activity of large membrane proteins.

Five cyclophilins and three FKBPs previously were identified in yeast, each with various subcellular localizations. Cpr1 is the yeast cyclophilin A homolog, the primary target of the immunosuppressant CsA. Strains lacking cyclophilin A are thus resistant to CsA (23–25). Cpr2 is a cyclophilin localized to the secretory pathway (26, 27). Yeast express a mitochondrial cyclophilin, Cpr3, that is required for mitochondrial function at elevated temperature and acceleration of protein refolding after mitochondrial import (28–31). Three immunophilins reside in the ER: Cpr4, Cpr5, and Fpr2. Cpr4 is a cyclophilin with a putative transmembrane domain and has been reported to be essential (32, 33), whereas Cpr5 contains an HDEL retention sequence and has been localized by immunofluorescence to the ER lumen (34). Yeast express one FKBP in the ER, Fpr2, that is induced by stress conditions (35, 36). The FKBP12 homolog in yeast is encoded by the *FPR1* gene. Yeast strains lacking FKBP12 are FK506- and rapamycin-resistant and have a slow growth phenotype (37–39). In addition, *fpr1* mutant strains are resistant to high levels of LiCl and recover faster from α -pheromone induced arrest compared with a wild-type strain (40). Fpr3 is an FKBP localized to the nucleolus and originally was identified as a nuclear localization sequence binding protein (41), a nuclear envelope protein (42), or as an FK520-binding protein (43).

To address the cellular functions of cyclophilins and FKBPs, we have taken a genetic approach in the yeast *Saccharomyces cerevisiae*. We identified three immunophilins: Cpr6 and Cpr7, both cyclophilin-40 homologs; Cpr8, a secretory pathway cyclophilin; and Fpr4, an Fpr3 (Npi46) homolog. Fpr4, like Fpr3, is localized to the nucleus, and both Fpr3 and Fpr4 interact with the ribosomal protein S24. By gene disruption, we find that none of the 12 yeast immunophilins are necessary for viability, although null mutant strains lacking FKBP12 or the cyclophilin 40 homolog Cpr7 do exhibit slow growth (refs. 37 and 44; this study). In addition, we find that transcription of four of the five immunophilins localized in the secretory pathway (*CPR2*, *CPR4*, *CPR5*, and *FPR2*) is regulated by conditions of cellular stress. To test if the individual mutants were viable because of functional overlap between different prolyl isomerases, we constructed yeast strains lacking multiple immunophilins, including a dodecuplet mutant lacking all 12 cyclophilins and FKBPs. Remarkably, these multiple mutant strains are all viable. We conclude that the immunophilins do not play a general essential role in protein folding but rather may perform specific

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Abbreviations: FKBP, FK506 binding protein; CsA, cyclosporin A; ER, endoplasmic reticulum.

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Table 1. The yeast cyclophilins and FKBP

Gene (synonym)	Molecular mass	Mammalian homolog	Localization*	Characteristics/mutant phenotypes	References
Cyclophilins					
<i>CPR1</i> (<i>CYP1</i> , <i>CPH1</i>)	17 kDa	CypA	Cytoplasm	•Primary receptor for CsA •Induced by heat shock	(23–25, 27)
<i>CPR2</i> (<i>CYP2</i>)	20 kDa	CypB	<i>Secretory pathway</i>	•Induced by heat shock and tunicamycin	(26, 27); this study
<i>CPR3</i> (<i>CYP3</i>)	20 kDa	CypD	Mitochondria	• <i>Δcpr3</i> has growth defect on lactate at 37° •Accelerates Su9-DHFR refolding	(28–31)
<i>CPR4</i> (<i>SCC3</i>)	33 kDa	CypC	<i>Secretory pathway</i>	•Induced by heat shock and tunicamycin	(32, 33); this study
<i>CPR5</i>	23 kDa		ER (HDEL)	•Induced by tunicamycin	(34); this study
<i>CPR6</i>	45 kDa	Cyp40	<i>Cytoplasm</i>	•Interacts with Hsp82; also interacts with Rbd3	(44, 52, 53); this study
<i>CPR7</i>	45 kDa	Cyp40	<i>Cytoplasm</i>	•Interacts with Hsp82; also interacts with Rbd3	(44, 52, 53); this study
<i>CPR8</i>	35 kDa	CypC	<i>Secretory pathway</i>	• <i>CPR4</i> homolog	This study
FKBPs					
<i>FPR1</i> (<i>FKB1</i>)	12 kDa	FKBP12	Cytoplasm	•Primary receptor for FK506 and rapamycin • <i>Δfpr1</i> exhibits slow growth	(37–39)
<i>FPR2</i> (<i>FKB2</i>)	12.5 kDa	FKBP13	<i>Secretory pathway</i>	•Induced by heat shock and tunicamycin	(35, 36)
<i>FPR3</i> (<i>NP146</i>)	70 kDa	FKBP25	Nucleolus	•Phosphorylated •Ptp1 substrate	(41–43)
<i>FPR4</i>	60 kDa	FKBP25	Nucleus	• <i>FPR3</i> homolog	This study

*Experimentally established localizations in roman text; localizations inferred by protein sequence or homology in italics.

functions through interactions with unique sets of restricted partner proteins that remain to be identified.

MATERIALS AND METHODS

Media and Strains. Media were prepared as described (45). Strains were as described in Table 2.

Transformations and One-Step Gene Disruptions. Yeast transformation and one-step gene disruption were as described (46). The *Δcpr5::LEU2*, *Δfpr2::URA3*, and *Δfpr3::URA3* gene disruptions were as described (34, 41, 47).

Δcpr4::URA3 Allele. The *CPR4* gene was PCR-amplified from yeast genomic DNA with primers: 5'-GATCGGATCCG-GTTTCAGCTAAACGACACGAGA-3' (*Bam*HI site, bold) and 5'-GATCCTGCAGGGGCAATGCACTAGAGGCTGT-C-3' (*Pst*I site, bold), cleaved with *Bam*HI and *Pst*I, and ligated into the *Bam*HI and *Pst*I sites of pUC18. This plasmid was cleaved with *Sty*I (removes 180 bp), filled in with Klenow, and ligated with a 1.1-kb *Hind*III blunted fragment bearing *URA3*, yielding plasmid pKDd1. The *Δcpr4::URA3* disruption fragment was released with *Bam*HI/*Pst*I digestion.

Table 2. Yeast strains

Strain	Genotype	Reference
JK93da	<i>MATa his4 HMLa leu2-3, 112 rme1 trp1 ura3-52</i>	(37)
MB11	<i>MATα ade2-101 his3-Δ200 ura3-52 trp1Δ1 lys2-801 can1</i>	(28)
MH272-3c	<i>his3 trp1-1</i> (JK93da)	(28)
ED86-1	<i>Δcpr1::LEU2 Δcpr2::TRP1 cpr3::HIS3 fpr1::ADE2</i> (MH272-3c)	(28)
MH250-2c	<i>Δcpr1::LEU2</i> (JK93da)	(40)
ED19	<i>Δcpr2::TRP1</i> (MB11)	(28)
MB11-3	<i>cpr3::HIS3</i> (MB11)	(28)
KDY9	<i>Δcpr4::URA3</i> (JK93da)	This study
KDY19	<i>Δcpr5::LEU2</i> (JK93da)	This study
KDY46	<i>Δcpr6::G418</i> (JK93da)	This study
KDY65	<i>Δcpr7::G418</i> (JK93da)	This study
SMY98-1	<i>Δcpr8::G418</i> (JK93da)	This study
JHY2-1c	<i>fpr1::ADE2</i> (JK93da)	(40)
KDY61	<i>Δfpr2::URA3</i> (JK93da)	This study
KDY86.6a	<i>Δfpr3::URA3</i> (JK93da)	This study
KDY54	<i>Δfpr4::G418</i> (JK93da)	This study
KDY84	<i>Δfpr3::URA3 Δfpr4::G418</i> (JK93da)	This study
KDY81.18c	<i>MATα fpr1::ADE2 Δfpr2::URA3 Δfpr3::URA3 Δfpr4::G418</i> (JK93da)	This study
KDY97.1a	<i>Δcpr1::LEU2 Δcpr2::TRP1 cpr3::HIS3 Δcpr4::URA3</i>	This study
	<i>Δcpr5::LEU2 Δcpr6::G418 Δcpr7::G418 Δcpr8::G418</i> (JK93da)	
SMY101-1	<i>Δcpr2::TRP1 Δcpr4::URA3 Δcpr5::LEU2 Δcpr8::G418</i>	This study
	<i>Δfpr2::URA3</i> (JK93da)	
KDY75.3b	<i>Δcpr1::LEU2 Δcpr6::G418 Δcpr7::G418 fpr1::ADE2</i> (JK93da)	This study
KDY47	<i>Δcpr1::LEU2 Δcpr2::TRP1 cpr3::HIS3 Δcpr4::URA3 Δcpr5::LEU2</i>	This study
	<i>Δcpr6::G418 fpr1::ADE2 Δfpr2::URA3 Δfpr3::URA3</i> (JK93da)	
KDY98.4a	<i>Δcpr1::LEU2 Δcpr2::TRP1 cpr3::HIS3 Δcpr4::URA3 Δcpr5::LEU2</i>	This study
	<i>Δcpr6::G418 Δcpr7::G418 Δcpr8::MET15 fpr1::ADE2 Δfpr2::URA3</i>	
	<i>Δfpr3::URA3 Δfpr4::G418 met15</i> (JK93da)	
SMY136-1	<i>MATα</i> (KDY98.4a)	This study

Δ cpr6::G418 Allele. The *CPR6* gene was PCR-amplified from yeast genomic DNA with primers: 5'-GCCCGGATC-CCCCACTGCATAAATGGACATCGG-3' (*Bam*HI site, bold) and 5'-GCCCGTCGACCCCTTTATAGAACATA-ACTG-3' (*Sal*II site, bold), cleaved with *Bam*HI and *Sal*II, and ligated into plasmid pRS305 (pKDw2) (48). Plasmid pKDw2 was digested with *Hind*III, removing 665 bp of the *CPR6* ORF, blunted with Klenow, and ligated to a *Sma*I and *Eco*RV fragment containing the G418 resistance gene (49). The Δ cpr6::G418 disruption fragment was released with *Bam*HI and *Sal*II.

Δ cpr7::G418, Δ cpr8::G418, and Δ fpr4::G418 Alleles. The *CPR7*, *CPR8*, and *FPR4* genes were disrupted by PCR as described (47, 49). For Δ cpr7::G418, primers were: 5'-CGGCAG-CAACAACCTACATCCAACGCGATGATTCAAGATC-CCCCCCCCGGTTAATTAAGGCGC-3' and 5'-CGTTATCC-ATCTCCAATAAATACATGATACATGATAGCATC-ATCG CGGCGCATAGGCCAC-3' (homology to *CPR7*, roman text; homology to G418 resistance gene, bold).

For Δ cpr8::G418, primers were: 5'-AAAGATTATTA-CAATTTGTATACAT TGGTTCCCAATGAAGCAGCT-GAAGCTTCGTACGC-3' and 5'-TACAAAATGAAT-GAATATTAAGCGGATGGATCGAAACATTGCATA-GGCCACTAGTGGATCTG-3' (homology to *CPR8*, roman text; homology to G418 resistance gene, bold).

For Δ fpr4::G418, primers were: 5'-GGCGGCCATCGAC-CCTGAGCCCTT TGACGATGACAAAAGCCCAGCT-GAAGCTTCGTACGC-3' and 5'-GGAGACCAATTTAAC-ATCAAATGTCAATTCAGAGTTAGCAGGAATACC-GGGCATAGGCCACTAGTGGATCTG-3' (homology to *FPR4*, roman text; homology to G418 resistance gene, bold).

Construction of Multiple Mutants. Strains lacking multiple immunophilins were constructed first by one-step gene disruptions that used the dominant G418 marker in addition to the traditional *URA3*, *TRP1*, *ADE2*, *LEU2*, and *HIS3* yeast markers, and then crossing strains lacking multiple immunophilins. For example, the following strategy was used to engineer the dodecuplet mutant strain. First, an undecuplet mutant strain was generated by the following cross:

MAT α Δ cpr1::LEU2 Δ cpr2::TRP1 *cpr3::HIS3* Δ cpr4::URA3 Δ cpr5::LEU2 Δ cpr6::G418 Δ cpr7::G418 Δ fpr2::URA3 Δ fpr3::URA3 X *MAT α* Δ cpr1::LEU2 Δ cpr2::TRP1 *cpr3::HIS3* Δ cpr4::URA3 Δ cpr5::LEU2 *fpr1::ADE2* Δ fpr2::URA3 Δ fpr4::G418 in which only the Δ cpr6::G418, Δ cpr7::G418, *fpr1::ADE2*, Δ fpr3::URA3, and Δ fpr4::G418 mutations were segregating. From tetrads with meiotic segregation of two G418- and rapamycin-resistant: two G418- and rapamycin-sensitive spores, the G418-rapamycin-resistant meiotic segregants were analyzed phenotypically and by PCR. The 2:2 segregation of the G418 marker indicates that the Δ cpr6::G418, Δ cpr7::G418, and Δ fpr4::G418 alleles cosegregated during meiosis; rapamycin resistance was used to score the *fpr1::ADE2* allele. The Δ fpr3::URA3 allele was identified by PCR. This cross generated an undecuplet mutant strain that lacked *CPR1*, *CPR2*, *CPR3*, *CPR4*, *CPR5*, *CPR6*, *CPR7*, *FPR1*, *FPR2*, *FPR3*, and *FPR4*. Next, the selectable/counter-selectable marker *MET15* was used to disrupt *CPR8* in the undecuplet strain (50). First, a *met15* mutant derived from the undecuplet strain was selected on media containing 1 μ M methylmercury. The resulting *met15* mutant failed to grow on synthetic media lacking methionine, formed dark colonies on media with 0.1% lead nitrate, and failed to complement a known *met15* mutation (50). The *CPR8* gene then was disrupted by using a Δ cpr8::MET15 PCR product as described (47). Primers were: 5'-AAAGATTATTACAAATTTGTATACATGTTCCCAA-TGAAGACCTTGTCCAATTGAACACGC-3' and 5'-TACAAA-ATGAATGAATATTAAGCGGATGGATCGAAACATTT-TGTTTACCATATTTTCTTC-3' (homology to *CPR8*, roman text; homology to *MET15* gene, bold). Finally, we confirmed that all 12 genes had been disrupted by PCR (see Fig. 3), phenotype, and in some cases Northern analysis (see Fig. 4). Other multiple immunophilin mutant strains were constructed similarly.

Northern Analysis. Heat shock. A wild-type yeast strain (JK93da) was grown at 24°C to mid-log phase, heat-shocked at 37°C, and samples removed at 0, 2, 5, 10, and 30 min.

Tunicamycin treatment. Wild type (JK93da) and a strain lacking *cpr1* through *cpr6* and *fpr1* through *fpr3* (KDY47) were grown to mid-log phase. The cultures then were divided and treated with either 10 μ g/ml tunicamycin (dissolved in ethanol) or ethanol alone. For the Northern blot of the *FPR4* gene, the strains treated with tunicamycin were wild type (JK93da) and Δ fpr4::G418 (KDY54). RNA was isolated as described (70). Probes spanning the ORF for each gene were amplified by PCR and radiolabeled with [³²P]dCTP by using the random primer DNA labeling kit (Boehringer Mannheim). Northern blot analysis was as described (51); levels of induction were normalized to actin message and quantified by PhosphorImager.

RESULTS

Identification of All Members of the Yeast Immunophilin Family. We and others previously have identified five cyclophilins (*CPR1*, *CPR2*, *CPR3*, *CPR4*, and *CPR5*) and three FKBP (*FPR1*, *FPR2*, and *FPR3*) in yeast (Table 1). Mutant strains lacking the individual immunophilins found to date exhibit few mild phenotypes. As the sequence of the yeast genome was being completed, we identified three additional cyclophilin genes, *CPR6*, *CPR7*, and *CPR8*, and one FKBP gene, *FPR4*. *CPR6* and *CPR7* encode predicted proteins of 372 amino acids and 394 amino acids ($\approx$ 40 kDa), respectively, share 41% identity, are closely related to mammalian cyclophilin 40, and also have been recently identified by others (44, 52, 53). The yeast Cpr6 protein was expressed as a hexahistidine-tagged protein in bacteria, purified, and found to exhibit CsA-inhibitable peptidyl-prolyl isomerase activity in an *in vitro* chymotrypsin-coupled peptide assay (data not shown). *CPR8* encodes a protein with a predicted molecular mass of about 35 kDa and shares 33% identity with Cpr4, a secretory pathway immunophilin (Fig. 1A) (32, 33).

We also identified an additional FKBP gene, *FPR4*, that encodes a protein of 60 kDa that shares 52% identity with the yeast Fpr3 protein, a 70-kDa nucleolar protein (Fig. 1B). By indirect immunofluorescence we found that an hemagglutinin epitope-tagged form of Fpr4 was localized to the nucleus (data not shown). The 70-kDa Fpr3 protein originally was identified and purified by FK520 affinity chromatography (43). In these studies, a 60-kDa FK520-binding protein was identified but thought to be a degradation product of Fpr3 based on peptide sequence. Reexamination of these data reveals the peptide differs at one position from Fpr3 and is in fact derived from the 60-kDa Fpr4 protein (R. Movva, personal communication). Thus, Fpr4, like Fpr3, binds FK520. Finally, these studies revealed that the ribosomal protein S24 copurified on the FK520 affinity column. We find that both Fpr3 and Fpr4 physically associate with the yeast ribosomal protein S24 in the two-hybrid assay, and these complexes are not disrupted by addition of FK506 (data not shown), indicating that S24 binding to Fpr3 and Fpr4 does not occur at the FKBP active site. In summary, eight distinct cyclophilin genes (*CPR1*-*CPR8*) and four FKBP genes (*FPR1*-*FPR4*), for a total of 12 immunophilins, are encoded by the yeast genome (Table 1). Most if not all of these enzymes are highly conserved and have mammalian homologs (Table 1).

No Cyclophilin or FKBP Is Essential in Yeast. To address the function of these proteins, we disrupted each of the 12 individual immunophilin genes. This analysis revealed that none of the immunophilins are essential for viability (Fig. 2A). Mutations in two of the 12 immunophilins, *cpr7* and *fpr1*, did confer a slow growth phenotype when compared with the isogenic wild-type strain (Fig. 2A; see also refs. 37 and 44). In contrast to a previous report (33), the *CPR4* gene was not essential in our strain background. Each immunophilin mutant strain grew normally at various temperatures (16°, 24°, 30°, and 37°C), under stress conditions (in the presence of 10 μ g/ml tunicamycin or 8% ethanol) and on different carbon sources (glucose, glycerol, galactose, and sucrose), and none were defective in mating or sporulation (in the

A.

CPR8	M--KSFPLLYLVAFMFSCITLPLPVDNKRASSDSDLKKKYAPDPPIITHNVNIGIVFTDPESSEE	64
CPR4	MWLKSLLLCLYSLVL--CQVHAA-PSSGKQITSKDVLQKKYEPSPATHRGITIEYFDPVSKSM	63
CPR1	-----MSQVYFDEADGQ	13
CPR8	A--GRLLITIDLYGTMVFKTVMTCQYVDSVKDLASR-----HSY--SPERDFDKILPNGAIEGS	120
CPR4	KEADL--TFELYGTVPVKTNFAMLAHGVKAVIEGKDPNDIHTYSYRK--INKVYPNKYIQGGV	126
CPR1	PIGRVV--FKLYNDIVFKTAENFRALCTGEGK-----FGYAGSP--FHRVIFDFMLQGGD	63
CPR8	SVSSSSIEETEMLAAPKL--PEENHSLIHDRPGRVSMIK--DDKGLKFIIETSETPLEGE---SVVF	179
CPR4	VAPDVGFPTV---YGFKPDENFYLKHDRPERLAMAYFGPDSNTSEFIITTKADGNEELDCKSVVF	189
CPR1	-FTAGNGTGGKSIYGGKFPDENFKKHDRPGLLSMANAGPNTNGSQFFITTVPCPW--LDGKHVVF	127
CPR8	GQVTAGLKDLMKLANVKTDENGKPEQPIITIGYISSQEHRIQHAKEAHEKYLQRLQDYQNGDLEKG	245
CPR4	GQITSGLDQMDAIQYTETDEYVKPQHELRLFLYFVLEILKISNILDHAAYTEKVEKFRNGDVSVG	255
CPR1	GEVVDGYDIVKVESLGSPSGATKARIVVAKSGEL	162
CPR8	ITLKNLYQGSQRKLEDAKYNQLHHPLPKIMLGISVLLFYVLAKYRKRFNRSSKIVSIRE	309
CPR4	STLENIFRNDKAYTFLTTSTGTAYDLNHPISRALMCLTVLGLCFIAYKGMHEKPHTVSLRHK	319

B.

FPR3	MSDLLPLATYSLNVEPYTPVPAIDVTMPITVRITMAALNPEAIDENKPSLRIKRNPDFEDD	64
FPR4	MSDMLPLATYSLNVEPYSTPALNFKIPVTIRITMAAIDPEPDDDKPSTLRIKRNPELTG	64
FPR3	DFLGGDFDEDEIDEESSSEEEBKTQKKKKSKGKA--ESESDEDEDDDEDFQESVLLTSL	126
FPR4	EYYQDNNDGLEDESESEQADVPKRSVSKKGAVEQSESEDESEDENEIDDEFECVLLTSL	128
FPR3	PEAQYQQSLDLTITPEEEVQFIVTGSYAISSGNYVKKHFPDTPMGVEGEDEDEDADIYDSEYD	290
FPR4	PKQYQQALDITIAPEEDVQFVVTGSYITISLTGNYVKKHFPDNTSSDSDEDEEYYSDESSNGEE	292
FPR3	LTPDEDEIGDDMDLDEEEVEVRIEEVQEEDEEDNDGEEQEEEEEEQKEEVKPEPKKSKK	354
FPR4	EEEEEEDEELSSGDDLDLVDASDIESRLDELVKKDEKKNNKDKSKKHEEDEESAKPA	356
FPR3	EKKRKHKEEKEEKAKVKVKVEFKKDLLEGPTKPKSKQEQDKHPEKSKVLEGGIVIEDRTIGD	418
FPR4	EKKQTTKKDKAEKVKDSEESK-----KPTKLEGGIIIEDRVTKG	400
FPR1	MSEVTEGNVKIDRISPGDG	19
FPR3	PQA--KRGARVGMRYIGLKNKGKVDKNTS--GKFFAFKLGRGEVIGKWDIGVAGMSVGGERRII	479
FPR4	PHA--KKGTRVGMRYVGLKNKGKVDKNTK--GKPFVFKLGGGEVIGKWDIGVAGMAYGGERRIV	462
FPR1	ATFPKTDGLVTIHYTGTLENGQKFDSSVDRGSPFCNIGVQVIGKWDVGIPKLSVGKARLTI	83
FPR3	PAPYAYGKQALPG--IPANSELTFDVKLVSMKN	511
FPR4	PAPYAYGKQALPG--IPANSELTFDVKLVSMK	492
FPR1	PGPYAYGRGFGPLIPFNSTLVDPVDELLKVN	114

FIG. 1. Conservation among novel cyclophilins and FKBP. (A) Alignment of Cpr8, Cpr4, and Cpr1. (B) Alignment of Fpr3, Fpr4, and Fpr1. Identical residues, bold.

homozygous mutant diploid strains). In addition, all of the immunophilin mutants recovered from pheromone-induced cell cycle arrest and were not sensitive to Ca^{2+} ions (100 mM) or cations (200 mM LiCl, 4 mM ZnCl_2) (data not shown).

Mutants Lacking Multiple Immunophilins Are Viable. The lack of phenotypes exhibited by the individual cyclophilin and FKBP mutants could be explained by functional redundancy shared between the immunophilins. For example, all of the immunophilins found in the secretory pathway could share a

common function. If this explanation was the case, all five of the secretory pathway immunophilins (CPR2, CPR4, CPR5, CPR8, and FPR2) might have to be mutated to reveal a mutant phenotype. To investigate this possibility, we generated several different combinatorial immunophilin mutants by targeted gene disruption and genetic crosses between isogenic strains. Strains were engineered that lack: (i) all of the cyclophilins (Δcpr1 through Δcpr8), (ii) all of the FKBP (Δfpr1 through Δfpr4), (iii) all of the immunophilins in the cytoplasm (Δcpr1 , Δcpr6 , Δcpr7 , and Δfpr1), (iv) all of the immunophilins localized to the secretory pathway (Δcpr2 , Δcpr4 , Δcpr5 , Δcpr8 , and Δfpr2), and (v) both of the nuclear FKBP (Δfpr3 and Δfpr4). None of these combinatorial mutant strains exhibited any novel mutant phenotypes or exacerbation or mitigation of the single mutant phenotypes (Fig. 2B and data not shown).

A Dodecuplet Mutant Lacking All Cyclophilins and FKBP Is Viable. To unequivocally exclude that functional redundancy accounts for the viability of these mutants, the genes encoding all 12 of the immunophilins were disrupted in a single strain by a series of one-step gene disruptions and genetic crosses between isogenic strains lacking multiple immunophilins. All gene disruptions were confirmed by PCR analysis, when possible by phenotype, and in some cases also by Northern blot (see *Materials and Methods*, Figs. 3 and 4). Remarkably, the resulting dodecuplet mutant is viable (Fig. 2B). The dodecuplet mutant does exhibit a growth defect, but this defect is attributable to the slow growth caused by the Δcpr7 mutation alone (compare Fig. 2A and B). The additional phenotypes exhibited by the dodecuplet mutant are simply the phenotypes of each individual immunophilin mutation: CsA-, FK506-, and rapamycin-resistance (cpr1 and fpr1), no growth on lactate at 37°C (cpr3), and slow growth (cpr7).

The dodecuplet mutant strain exhibited no defect in mating, or arrest and recovery from α -factor mating pheromone or glycogen accumulation (data not shown). In addition, this mutant strain sporulated normally as a homozygous diploid, and isolated spores had no defect in germination. Thus, the dodecuplet mutant strain suffers no defects under standard growth conditions. We next tested if the dodecuplet mutant exhibits any phenotypes under stress conditions. The mutant strain did not exhibit increased sensitivity to the toxic amino acid analogs canavanine (an arginine

FIG. 2. The immunophilins are not required for viability. A series of yeast strains isogenic with the wild-type strain JK93d but lacking one or more of the 12 immunophilin genes were grown on yeast extract/peptone/dextrose medium for 48 hr at 30°C . (A) Strains lacking individual cyclophilins (Δcpr1 through Δcpr8) or FKBP (Δfpr1 through Δfpr4). (B) Strains lacking all of the cyclophilins (ΔCYPs : Δcpr1 Δcpr2 Δcpr3 Δcpr4 Δcpr5 Δcpr6 Δcpr7 Δcpr8); all the FKBP (ΔFKBPs : Δfpr1 Δfpr2 Δfpr3 Δfpr4); all cytoplasmic immunophilins ($\Delta\text{cytoplasmics}$: Δcpr1 Δcpr6 Δcpr7 Δfpr1); all of the immunophilins localized to the secretory pathway (ΔERs : Δcpr2 Δcpr4 Δcpr5 Δcpr8 Δfpr2); the two nucleolar FKBP ($\Delta\text{nucleolars}$: Δfpr3 Δfpr4); or all 12 cyclophilins and FKBP (Δ12 : Δcpr1 Δcpr2 Δcpr3 Δcpr4 Δcpr5 Δcpr6 Δcpr7 Δcpr8 Δfpr1 Δfpr2 Δfpr3 Δfpr4); WT, isogenic wild-type strain JK93da.

FIG. 3. Disruption of all 12 immunophilin genes in the dodecuplet mutant yeast strain. Genomic DNA was isolated from the isogenic wild type (JK93da) and the dodecuplet mutant strain (SMY136-1) and analyzed by PCR with primers flanking the immunophilin gene indicated above each lane. (A) PCR analysis of the cyclophilins. (B) PCR analysis of the FKBP.

analog, 0.6 $\mu\text{g}/\text{ml}$) or L-azetidine-2-carboxylic acid (a proline analog, 20 $\mu\text{g}/\text{ml}$) and grew normally at 16°, 24°, 30°, or 37°C and on several carbon sources (glucose, glycerol, galactose, and sucrose). The dodecuplet strain was tested for increased sensitivity to extreme heat shock and also induced thermotolerance. Although the dodecuplet did exhibit modest increased sensitivity to extreme heat shock (48°C), this effect is largely attributable to the $\Delta\text{cpr}7$ mutation alone (K.D. and J.H., unpublished results). In addition, the dodecuplet mutant strain was not sensitive to 100 mM CaCl_2 , 4 mM ZnCl_2 , 200 mM LiCl , or nitrogen starvation. Last, the dodecuplet strain did not exhibit any novel auxotrophic phenotypes (data not shown). Because the dodecuplet mutant is viable and suffers no dramatic phenotypes under standard or stressed growth conditions, we conclude that the immunophilins are not essential for life and either do not have overlapping functions, or share overlapping but nonessential functions.

Transcription of Several Immunophilins Is Regulated by Stress Conditions. It previously has been reported that the *CPR1* and *CPR2* genes are modestly induced by heat shock, whereas expression of *FPR2* (the ER FKBP) is induced by both heat shock and tunicamycin, a glycosylation inhibitor that

results in the accumulation of misfolded proteins in the ER (27, 36). We therefore determined if other immunophilins are regulated in a similar fashion. A wild-type yeast strain (JK93da) was grown to mid-log phase at 24°C and then heat-shocked at 37°C for 0, 2, 5, 10, or 30 min. RNA was purified and analyzed with probes specific to the genes encoding immunophilins localized to the secretory pathway. In accord with previous studies (27), we found that the *CPR2* gene was induced ≈ 1.5 -fold by heat shock. In addition, transcription of the *CPR4* gene was induced 2-fold, whereas expression of the *CPR5* and the *CPR8* genes were not regulated by heat shock (Fig. 4 and data not shown).

To analyze gene regulation in response to unfolded proteins in the ER, a wild-type and an immunophilin mutant strain were grown to mid-log phase and then treated with 10 $\mu\text{g}/\text{ml}$ tunicamycin for 3 hr; RNA from these strains was subjected to Northern analysis. All of the immunophilins localized to the secretory pathway, with the exception of *CPR8*, were induced by tunicamycin, though to varying extents (*CPR2*, 1.9-fold; *CPR4*, 2.6-fold; *CPR5*, 2.2-fold; Fig. 4). *FPR4*, the nuclear FKBP, served as a control for these experiments and was not induced appreciably by either heat shock or tunicamycin (Fig. 4).

Because the *CPR2*, *CPR4*, *CPR5*, and *FPR2* genes are regulated by stress conditions, we tested if mutations in these genes genetically interact with other genes known to be involved in similar processes. None of the genes encoding the secretory pathway immunophilins ($\Delta\text{cpr}2 \Delta\text{cpr}4 \Delta\text{cpr}5 \Delta\text{cpr}8 \Delta\text{fpr}2$) were synthetically lethal with a mutation in the *CNE1* gene, which encodes the ER chaperone calnexin (54), with a mutation in the *IRE1* gene, which encodes an ER transmembrane kinase that transduces the unfolded protein response from the ER to the nucleus (55), or with three temperature-sensitive *KAR2* alleles (*kar2-113*, *kar2-159*, *kar2-203*); *KAR2* encodes the yeast BIP homolog (data not shown) (56–58). In addition, strains lacking the secretory pathway immunophilins *CPR2*, *CPR4*, *CPR5*, *CPR8*, and *FPR2*, either alone or in combination, were not hypersensitive to the glycosylation inhibitors tunicamycin (10 $\mu\text{g}/\text{ml}$), castanospermine (1 mM), or 1-deoxynojirimycin (2 mM) (data not shown) (59).

Finally, the $\Delta\text{cpr}2$, $\Delta\text{cpr}4$, $\Delta\text{cpr}5$, $\Delta\text{cpr}8$, and $\Delta\text{fpr}2$ single mutant strains and the $\Delta\text{cpr}2 \Delta\text{cpr}4 \Delta\text{cpr}5 \Delta\text{cpr}8 \Delta\text{fpr}2$ multiple mutant strain had no overt defects in secretion of invertase (as assayed by growth on yeast extract/peptone-sucrose media), barrier protease, or mating pheromone (data not shown).

DISCUSSION

Cyclophilins and FKBP originally were discovered as the intracellular binding targets of immunosuppressive drugs. Simultaneously, these proteins were found to be enzymes that catalyze a rate-limiting step in protein folding: peptidyl-prolyl isomerization. Cyclophilins and FKBP are ubiquitous and highly conserved, suggesting an important conserved cellular function.

We addressed the functions of these enzymes by a genetic approach in yeast. Our findings and previous studies identify a total of 12 cyclophilin and FKBP genes in the yeast genome. The two genes encoding the yeast cyclophilin 40 homologs, *CPR6* and *CPR7*, also have been independently identified recently (44, 52, 53). We deleted the genes encoding each of the 12 yeast immunophilins and find that none are essential. In addition, the genes encoding all of the cyclophilins, all of the FKBP, and all of the immunophilins localized to a common intracellular compartment were deleted. Each of these multiple mutant strains was also viable. Finally, we engineered a strain lacking all 12 of the yeast immunophilins and found that the resulting dodecuplet mutant strain was viable. The phenotype of mutant strains lacking all cyclophilins and FKBP was the simple addition of the few subtle single mutant phenotypes, revealing little or no functional overlap between different enzymes. Because the immunophilins are not essential, either individually or in combination, we conclude that they do not function as critical, general protein folding enzymes in

FIG. 4. Transcription of several immunophilins is induced by heat shock and unfolded proteins. Wild type (WT) and mutant (Δ , which is $\Delta\text{cpr}1$ through $\Delta\text{cpr}6$, and $\Delta\text{fpr}1$ through $\Delta\text{fpr}3$) immunophilin strains were analyzed by Northern blot with probes specific to the gene indicated on the left. Fold induction was determined by normalizing the amounts of immunophilin RNA to actin RNA by PhosphorImaging. The same membrane was used for all blots except for lanes 1–4 of the *FPR4* blot. Lanes 1 and 3, untreated (–). Lanes 2 and 4, treated with 10 $\mu\text{g}/\text{ml}$ tunicamycin (+) for 3 hr. Lanes 5–9, a wild-type strain (JK93da) was grown at 24°C, heat-shocked at 37°C, and RNA samples were analyzed after 0, 2, 5, 10, and 30 min growth at 37°C.

the cell. Instead, each individual immunophilin may interact with a limited number of specific target proteins. Some of these interactions may occur under conditions of cellular stress, and our findings and previous studies of others (27, 36), reveal that transcription of several immunophilins is induced by heat shock or misfolded proteins.

A third family of prolyl isomerases distinct from cyclophilins and FKBP, the parvulins, recently has been discovered (60). The *S. cerevisiae ESS1* gene encodes the only parvulin homolog in yeast, and, interestingly, *ESS1* is essential (61, 62). The viability of the yeast mutant lacking all cyclophilin and FKBP prolyl isomerases, however, is not readily explained by functional overlap with *Ess1* because it is not clear how a single parvulin could mitigate the loss of multiple prolyl isomerases in several different intracellular compartments.

Previous studies support the hypothesis that individual immunophilins have specific functions mediated by interactions with unique target proteins. For instance, *Drosophila ninaA* is eye-specific and forms a very stable, specific complex with rhodopsin (63). Another immunophilin-binding target that has been identified is the HIV-1 capsid protein. Cyclophilin A and the HIV capsid protein form a complex, and cyclophilin A is packaged into HIV virions (64). CsA disrupts the cyclophilin A-capsid protein complex, and HIV-1 virions assembled in the presence of CsA are defective at an early step after infection (65). CsA-resistant HIV-1 isolates have mutations in amino acids within the identified cyclophilin A binding site, and alterations in the cyclophilin A active site prevent capsid protein binding (66, 67). Mammalian cyclophilin 40 is found in complexes with HSP70 and HSP90 and has been shown to directly interact with HSP90. These large complexes are important for the function of several signal transduction pathways, including steroid receptor and v-src signaling (11, 44, 68, 69). Finally, mammalian FKBP12 plays a role in the function of the ryanodine receptor, the IP₃ receptor, and the multidrug resistance pump (17–20, 22). All of these examples argue against a general housekeeping role for the immunophilins and suggest these proteins play more specialized roles involving unique targets.

Why are the immunophilins so highly conserved if they do not play an essential role in protein folding? One explanation is that the immunophilins have conserved binding partners. Although all of the cyclophilins and FKBP are highly conserved from yeast to man, the immunophilin binding targets identified thus far, however, are mammalian proteins that do not have homologs in yeast. One approach to address the conservation and function of the immunophilins will be to identify the physiological targets of the yeast immunophilins by two-hybrid assays or synthetic lethal screens to establish if immunophilin functions have been conserved from yeast to man. Such studies are currently underway.

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