A Novel FK506- and Rapamycin-binding Protein (*FPR3* Gene Product) in the Yeast *Saccharomyces cerevisiae* Is a Proline Rotamase Localized to the Nucleolus

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Abstract. The gene (*FPR3*) encoding a novel type of peptidylprolyl-*cis-trans*-isomerase (PPIase) was isolated during a search for previously unidentified nuclear proteins in *Saccharomyces cerevisiae.* PPIases are thought to act in conjunction with protein chaperones because they accelerate the rate of conformational interconversions around proline residues in polypeptides. The *FPR3* gene product (Fpr3) is 413 amino acids long. The 111 COOH-terminal residues of Fpr3 share greater than 40% amino acid identity with a particular class of PPIases, termed FK506-binding proteins (FKBPs) because they are the intracellular receptors for two immunosuppressive compounds, rapamycin and FK506. When expressed in and purified from *Escherichia coli*, both full-length Fpr3 and its isolated COOH-terminal domain exhibit readily detectable PPIase activity. Both *fpr3*Δ null mutants and cells expressing *FPR3* from its own promoter on a multicopy plasmid have no discernible growth phenotype and do not display any alteration in sensitivity to the growth-inhibitory effects of either FK506 or rapamycin. In *S. cerevisiae*, the gene for a 112-residue cytosolic FKBP (*FPR1*) and the gene for a 135-residue ER-associated FKBP (*FPR2*) have been described before. Even *fpr1 fpr2 fpr3* triple mutants are viable. However, in cells carrying an *fpr1*Δ mutation (which confers resistance to rapamycin), overexpression from the *GAL1* promoter of the C-terminal domain of Fpr3, but not full-length Fpr3, restored sensitivity to rapamycin. Conversely, overproduction from the *GAL1* promoter of full-length Fpr3, but not its COOH-terminal domain, is growth inhibitory in both normal cells and *fpr1*Δ mutants. In *fpr1*Δ cells, the toxic effect of Fpr3 overproduction can be reversed by rapamycin. Overproduction of the NH_2-terminal domain of Fpr3 is also growth inhibitory in normal cells and *fpr1*Δ mutants, but this toxicity is not ameliorated in *fpr1*Δ cells by rapamycin. The NH_2-terminal domain of Fpr3 contains long stretches of acidic residues alternating with blocks of basic residues, a structure that resembles sequences found in nucleolar proteins, including *S. cerevisiae NSR1* and mammalian nucleolin. Indirect immunofluorescence with polyclonal antibodies raised against either the NH_2- or the COOH-terminal segments of Fpr3 expressed in *E. coli* demonstrated that Fpr3 is located exclusively in the nucleolus.

ACQUISITION of the functional state of a polypeptide frequently requires the participation of other proteins which assist in and accelerate the process of protein folding (Gething and Sambrook, 1992; Hartl et al., 1994). Proline rotamases, more formally peptidylprolyl-*cis,trans*-isomerases (PPIases),[1] are thought to participate in protein folding and protein assembly events in vivo (Parsell and Lindquist, 1993) because in vitro these enzymes catalyze interconversion of the *cis* and *trans* isomers of the bond between the carboxyl group of the preceding amino acid and the imino nitrogen of proline in peptide and protein substrates (Kofron et al., 1991; Stein, 1991). It was discovered several years ago that there are two separate types of PPIases. This realization was made possible by investigations into the molecular mode of action of antibiotics that act as immunosuppressants in humans. These drugs effectively block the specific signal transduction pathways required for activation and proliferation of T lymphocytes (for review see Schreiber et al., 1993).

The first PPIase isolated (Fischer et al., 1984) was shown to be the intracellular receptor for an immunosuppressant, cyclosporin A (CsA); and hence, a member of this class of PPIase is called a cyclophilin (Fischer et al., 1989; Taka-

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1. *Abbreviations used in this paper*: CsA, cyclosporin A; FKBP, FK506-binding protein; Gal, 2% galactose supplemented with 0.2% sucrose; Glc, 2% glucose; GST, *Schistosoma japonicum* glutathione S-transferase; HSE, heat shock response element; Hsp, heat shock protein; PPIases, peptidylprolyl-*cis*, *trans*-isomerases.

hashi et al., 1989). It was subsequently shown that an unrelated protein, dubbed an FK506-binding protein (FKBP) because it serves as the intracellular receptor for two other immunosuppressive compounds, FK506 and rapamycin, is also a PPIase (Harding et al., 1989; Siekierka et al., 1989). All three of the immunosuppressive agents contain within their structure a pipecolyl bond that mimics the X-Pro bonds in proteins. The binding of these drugs to their respective binding protiens, collectively termed "immunophilins," potently inhibits their PPIase activity. Furthermore, there is no cross-inhibition; FK506 and rapamycin only interact with FKBPs, and CsA only interacts with cyclophilins. Despite their common enzymic function as PPIases, the cyclophilins and the FKBPs do not possess any significant sequence homology and, thus, represent two distinct, but highly conserved, superfamilies. Members of both families are found in organisms as diverse as bacteria (Hacker and Fischer, 1993), fungi (Tropschug et al., 1988), plants (Luan et al., 1994), insects (Shieh et al., 1989), and mammals (Schreiber, 1991).

CsA, FK506, and rapamycin were initially discovered because they are cytotoxic to many types of microorganisms, including the yeast *Saccharomyces cerevisiae* (Tropschug et al., 1989; Heitman et al., 1991*b*; Koltin et al., 1991). In *S. cerevisiae*, three cyclophilin genes have been identified. The *CYP1* product, an abundant cytosolic protein, is the cellular receptor that mediates CsA toxicity (Tropschug et al., 1989; Koser et al., 1991). The *CYP2* product is a cyclophilin that contains a putative signal sequence and is localized in the ER (Koser et al., 1991). The *CPR3* product encodes a cyclophilin with a mitochondrial targeting sequence and is localized in that organelle (Davis et al., 1992). Two genes encoding FKBPs have been described in *S. cerevisiae*. *FPR1* (*RBP1*, *FKB1*) encodes a 12-kD cytosolic protein, termed FKBP-12, which serves as the sole receptor for rapamycin and is also a major site for FK506 binding (Heitman et al., 1991*a*,*b*; Koltin et al., 1991; Wiederrecht et al., 1991). *FKB2* (*FPR2*) encodes an ER-associated FKBP, designated FKBP-13, which is apparently not involved in mediating either rapamycin or FK506 toxicity (Nielsen et al., 1992).

Several lines of evidence indicate that inhibition of the PPIase activity of the cyclophilins or the FKBPs is not the mechanism by which the drugs exert their immunosuppressive and growth-inhibitory effects. For example, a close structural analogue of FK506, called 506BD, is able to bind in vitro to FKBP-12 (the major cytosolic FK506- and rapamycin-binding protein in human T cells) with the same affinity as FK506 and is able to inhibit its rotamase activity with equivalent efficacy, yet 506BD does not act as an immunosuppressant (Bierer et al., 1990*b*). In yeast, cells carrying a null mutation in the *FPR1* gene grow normally yet are completely resistant to the cytotoxic effects of rapamycin, indicating that loss of Fpr1 activity is not deleterious and that, in the absence of FKBP-12, rapamycin cannot exert its inhibitory action (Heitman et al., 1991*a*,*b*; Koltin et al., 1991; Wiederrecht et al., 1991). These findings suggested that drug binding in vivo generates a novel entity, the immunophilin–drug complex, which causes its effects by interacting with another cellular target(s).

Indeed, recent biochemical studies have demonstrated that both cyclophilin–CsA complex and FKBP12–FK506 complex bind to and inhibit calcineurin, a ubiquitous Ca^{2+}/calmodulin-activated phosphoprotein phosphatase (Liu et al., 1991; Clipstone and Crabtree, 1992; O'Keefe et al., 1992). In T cells, calcineurin action is necessary for activation of the transcription factor, NF-AT, which is required for expression of the genes for interleukin-2 and interleukin-2 receptor (Jain et al., 1993). In yeast, FKBP12-FK506 and cyclophilin–CsA also inhibit calcineurin (Foor et al., 1992). Although calcineurin is not required for the vegetative growth of yeast cells, the function of this enzyme is necessary for the maintenance of viability and the resumption of cell division after cells have been arrested by the action of mating pheromone (Cyert et al., 1991; Cyert and Thorner, 1992). In contrast, the FKBP–rapamycin complex interdicts a different target. In T cells, rapamycin interferes with the signal generated from the occupied interleukin-2 receptor (Bierer et al., 1990*a*; Dumont et al., 1990) and results in an inhibition of the phosphorylation and activation of the 70-kD ribosomal protein S6 kinase (Kuo et al., 1992; Price et al., 1992). In yeast, cellular targets of FKBP12-rapamycin appear to be the *TOR1* and *TOR2* gene products, a set of putative phosphotidylinositol kinases (Flanagan et al., 1993; Kunz et al., 1993; Helliwell et al., 1994), which may regulate, directly or indirectly, activation of p70 S6 kinase. Recent results indicate that animal cells also contain a homologue of Tor2 that is capable of associating with the FKBP–rapamycin complex (Brown et al., 1994; Sabatini et al., 1994).

The normal functions of the PPIases are poorly understood and identification of their physiologically relevant substrates is minimal. There is some support for the view that certain PPIases may assist in the folding (or unfolding) of individual proteins. In *D. melanogaster*, a mutation in the *ninaA* gene, which is expressed specifically in the retina and encodes a cyclophilin anchored in the ER membrane, causes misfolded forms of rhodopsins (but not other proteins) to accumulate in the ER lumen, suggesting that the *ninaA* product is required for correct folding of the rhodopsins. Other evidence indicates that PPIases may play a role in facilitating protein–protein interactions required for the assembly (or disassembly) of multi-protein complexes. For example, before hormone administration, the glucocorticoid receptor resides in the cytosol in a large 9S complex containing two heat shock proteins, Hsp90 and Hsp70, and a previously unidentified 59-kD protein (p59) (Sanchez et al., 1990). Upon binding of steroid, the receptor-containing complex dissociates, and the receptor can now dimerize and translocate to the nucleus in a state competent to bind to its specific target DNA sequence (Pratt, 1987). The p59 component of the inactive receptor complex has recently been characterized and is a new type of FKBP apparently containing two catalytic domains (Ku Tai et al., 1992). Complete understanding of the functions of the PPIases in eukaryotic cells will require elucidation of the number of such enzymes present, their localization within the cell, and the nature of their authentic substrates in vivo.

In an immunological screen to identify previously uncharacterized nuclear proteins, we isolated the gene for an FKBP of unique structure. Here we describe the isolation, structural features, enzymic properties, and subcellular localization of this novel member of the FKBP family. We also

examined the ability of this protein to interact with immunosuppressant drugs and the phenotypic consequences of eliminating or overproducing this new FKBP in the cell.

Materials and Methods

Yeast Strains, Culture Conditions, and Genetic Techniques

The yeast strains used in this work, and their construction, are described in Table I. Unless otherwise specified, cells were grown at 30°C. Rich medium (YPD) and synthetic defined medium (SD), lacking one or a combination of nutrients as required for maintenance of plasmids, were prepared according to Sherman et al. (1986). SD medium contained as the carbon source either 2% glucose (Glc) or 2% galactose supplemented with 0.2% sucrose (Gal). Standard methods were used for genetic crosses, sporulation, and tetrad analysis (Sherman et al., 1986). DNA-mediated transformation was carried out using the lithium acetate method (Ito, 1983), as modified by Schiestl and Geitz (1989). Because of their larger size, a polyploid commercial strain of baker's yeast (Fleischmann's) was used in some of the immunofluorescence experiments.

Preparation of Anti-nuclear Protein Antibodies

Nuclei were prepared from 4 l of strain BJ2168, essentially as described by Aris and Blobel (1991). After exhaustive digestion with DNAse I and RNAse A, the remaining nuclear envelope fraction and other particulate material (~3 mg total protein) were collected by centrifugation for 20 min at 23,500 *g* in a Sorvall HB-4 rotor (Aris and Blobel, 1989), solubilized directly in 1.5 ml of SDS-PAGE sample buffer (Laemmli, 1970), subjected to preparative SDS-PAGE, and visualized by staining with Coomassie blue dye. Prominent polypeptides in this preparation were isolated by preparative SDS-PAGE, recovered from gels using an Elutrap™ elution chamber (Schleicher & Schuell, Keene, NH), and used to immunize adult female New Zealand white rabbits, following standard procedures (Harlow and Lane, 1988). Antibodies directed against a major band that had an apparent molecular mass of ~65 kD were affinity-purified by minor modifications of the method of Smith and Fisher (1984). In brief, samples of the electroeluted 65-kD protein were resolved by SDS-PAGE and electrophoretically transferred to a nitrocellulose filter (Towbin et al., 1979); the strip containing the immobilized 65-kD band then was used as an immunoadsorbant to select specific antibodies from a polyclonal antiserum (no. SB126) that had been raised against the same antigen.

Isolation of the FPR3 Gene and DNA Sequence Analysis

A yeast genomic DNA library (gift of R. Hamataki and A. Sugino, National Institute of Environmental Health Sciences, Research Triangle Park, NC) constructed in the bacteriophage expression vector, λgt11, was screened with the anti-65-kD protein antibodies immunoselected from antiserum no. SB126, using standard procedures (Snyder et al., 1987). One candidate phage (L11B7) was identified by its immunoreactivity and was isolated to homogeneity using four successive rounds of plaque purification and rescreening. Phage DNA was prepared by standard methods (Sambrook et al., 1989), and the insert DNA was released by digestion with EcoRI and subcloned into the pBluescript II KS+ vector (Stratagene Inc., La Jolla, CA). Nucleotide sequence analysis was performed by the dideoxy chain termination method (Sanger et al., 1977) using the universal primers for pBluescript II KS+ and, where necessary, custom-synthesized oligonucleotide primers and Sequenase™ enzyme (US Biochem. Corp., Cleveland, OH), under conditions recommended by the supplier. To confirm the sequence of *FPR3* and to obtain additional flanking sequence, the insert DNA from phage L11B7 was radiolabeled using the random primer method (Feinberg and Vogelstein, 1984) and used to screen a plasmid library of yeast genomic DNA in the vector pSB32 (gift of F. Spencer and P. Heiter, Johns Hopkins University School of Medicine, Baltimore, MD), which yielded plasmid pBYNG1. The entire *FPR3* coding region was contained within a 2.2-kb HindIII fragment of the genomic DNA insert in pBYNG1. This fragment was ligated into pUC19, to generate plasmid pNH2.2, and the DNA sequence of 1711 bp of continuous sequence from the 2.2-kb insert in pNH2.2 was determined on both strands, using the methods described immediately above.

Physical and Genetic Mapping of the FPR3 Locus

To determine the chromosomal location of *FPR3*, the 2.2-kb HindIII fragment from pNH2.2 was radiolabeled by the random primer method (Feinberg and Vogelstein, 1984) and used for hybridization, first, to a blot (gift of Gary Anderson, University of California, Berkeley, CA) on which intact yeast chromosomes had been resolved by electrophoresis in a clamped homogenous electric field (Vollrath et al., 1988) and, second, to filters containing an ordered set of yeast DNA segments (gift of Linda Riles and Maynard Olson, Washington University School of Medicine, St. Louis, MO)

Table I. S. cerevisiae Strains

Strain	Genotype	Source
YPH499	*MAT***a** *ade2-101^{oc} his3-Δ200 leu2-Δ1 lys2-801^{am} trp1-Δ1 ura3-52*	Sikorski and Hieter, 1989
YPH500	*MATα* (otherwise isogenic to YPH499)	Sikorski and Hieter, 1989
YPH501	*MAT***a**/*MATα* (otherwise isogenic to YPH499)	Sikorski and Hieter, 1989
BJ2168	*MAT***a** *gal2 leu2 prb1-1122 pep4-3 prc1-407 trp1 ura3-52*	Jones, 1991
YB100	YPH499 *fpr3-Δ1::URA3*	This Work
YB103	YPH501 *fpr3-Δ1::URA3/FRP3*	This Work
YB104	YPH501 *fpr3-Δ1::URA3/fpr3-Δ1::URA3*	This Work
YB105	YPH500 *fpr3-2::HIS3*	This Work
YB107*	YPH499 *fpr1-2::ADE2*	This Work
YB112	YPH499 *fpr1-2::ADE2 fpr3-2::HIS3*	This Work
YB168	YPH501 *fpr1-2::ADE2/FPR1 fpr2-1::URA3/FPR2*	This Work
YB169A‡	YPH499 *fpr2-1:URA3*	This Work
YB171A	YPH499 *fpr1-2::ADE2 fpr2-1::URA3 fpr3-2::HIS3*	This Work
YB58	YPH500 *hmg1::LYS2*	This Work

* The *fpr1-2::ADE2* insertion mutation was excised from plasmid pYJH25A as described previously (Heitman et al., 1991*b*) and used for DNA-mediated transformation of YPH499 (and YPH501), selecting for Ade$^+$ transformants. Correct gene transplacement of the *FPR1* locus was confirmed by restriction enzyme digestion and Southern hybridization analysis.

‡ The *fpr2-1::URA3* mutation was excised from plasmid pFPR2D1 on a 2.0-kb BamHI-EcoRI fragment (see Materials and Methods) and used for DNA-mediated transformation of YPH499 (and YPH501), selecting for Ura$^+$ transformants. Correct gene transplacement of the *FPR2* locus was confirmed by restriction enzyme digestion and Southern hybridization analysis.

representative of the entire yeast genome (Link and Olson, 1991). To confirm the map location of *FPR3* determined by physical methods, a genetic cross was performed between a strain (YB100) carrying the *fpr3-Δ1::URA3* allele (see below) and a strain (YB58) carrying a marker (*hmg1::LYS2*) known to reside on the left arm of chromosome XIII.

Construction of fpr3 Null Mutations

The *fpr3-Δ1::URA3* deletion mutation was constructed as follows. A 1.2-kb *URA3*-containing HindIII fragment from plasmid pJJ242 (Jones and Prakash, 1990) was converted to blunt ends by incubation with the Klenow fragment of *E. coli* DNA polymerase I and dNTPs, and then ligated into the SmaI site of pBluescript II KS+, to give plasmid pBS*URA3*. The 2.2-kb genomic HindIII fragment containing *FPR3* was transferred into the HindIII site of pUC-RI (a derivative of pUC19 in which the EcoRI site has been destroyed; B.M. Benton, unpublished results), to yield plasmid pNH2.2B. The ClaI site that is most upstream of the *FPR3* coding sequence in the 2.2-kb HindIII fragment in pNH2.2B (see Fig. 1) was destroyed by partial digestion with ClaI followed by incubation with dNTPs and the Klenow fragment and religation, to generate plasmid pNH2.2C. Plasmid pNH2.2C was digested, first with ClaI, and the overhangs so generated were converted to blunt ends, and then with EcoRI, which removed the segment encoding residues 44-278 of the *FPR3* coding sequence. A 1.2-kb *URA3*-containing SmaI-EcoRI fragment excised from plasmid pBS*URA3* was inserted into the blunt[ClaI]-EcoRI sites of pNH2.2C, yielding plasmid pFPR3D1. The linear 2.8-kb HindIII fragment of pFPR3D1 containing the *fpr3-Δ1::URA3* mutation was excised by digestion with HindIII, purified by agarose gel electrophoresis, and introduced into YPH501 by DNA-mediated transformation, selecting for Ura^+ transformants. Gene replacement (Rothstein, 1983) of the *FPR3* locus on one homologue of chromosome XIII was confirmed by restriction enzyme digestion and Southern hybridization analysis (Southern, 1975) of genomic DNA isolated from the Ura^+ transformants (Hoffman and Winston, 1987).

To construct the *fpr3-2::HIS3* insertion mutation, a 3.3-kb BglII fragment containing the *FPR3* coding sequence was excised from the genomic insert in pBYNG1 and ligated into the BamHI site of pBluescript II KS+ (Stratagene Inc.), to create plasmid pNB3.3. pNB3.3 was digested with NdeI, which cleaves at codon 386 of the *FPR3* coding sequence, converted to blunt ends, and ligated to a 2.1-kb PvuII fragment that contains the *HIS3* gene, which was isolated from plasmid pJJ217 (Jones and Prakash, 1990), to create plasmid pFPR3D2. A linear 5.8-kb PvuII fragment from pFPR3D2 containing the *fpr3-2::HIS3* mutation was gel-purified and used for transformation of YPH501, selecting for His^+ transformants. Correct gene transplacement was confirmed by restriction digestion and Southern analysis, as before.

Construction of an fpr2 Null Mutation

The *FPR2* gene was cloned, based on its published sequence (Nielsen et al., 1992), by amplification via the polymerase chain reaction (PCR) using *S. cerevisiae* genomic DNA as the template and a 5′-primer, 5′-*CGGGATCC*ATTATCTCACCAGTTACCCG-3′ (corresponding to nucleotides 107 to 126), and a 3′-primer, 5′-*CGGAATTC*A*TATATTGTAGTTAGAGCCC-3′* (corresponding to nucleotides 847 to 866), which included BamHI (5′-primer) and EcoRI (3′-primer) restriction sites (italicized). Reactions in 50 μl contained 50 ng template DNA, 20 pmol each primer, buffer, dNTPs, and AmpliTaq™ polymerase (Perkin-Elmer-Cetus Instrs., Norwalk, CT), as recommended by the manufacturer, and were subjected to 30 cycles of the following program: 95°C for 30 s, 42°C for 30 s, and 72°C for 30 s. A product of the expected size (760 bp) was obtained, purified by electrophoresis on a 1% agarose gel, reamplified, and digested with BamHI and EcoRI. The resulting fragment was gel-purified and ligated between the BamHI and EcoRI sites in pBluescript II KS+, to create plasmid pBS*FPR2*. The *fpr2-1::URA3* insertion mutation was constructed by digesting pBS*FPR2* with PacI, which cleaves at codon 38 of the *FPR2* coding sequence, converting the overhangs so generated to blunt ends, and ligating with a 1.3-kb SmaI-PvuII fragment containing the *URA3* gene that had been excised from plasmid pJJ244 (Jones and Prakash, 1990), to generate plasmid pFPR2D1.

Plasmids

The 2.2-kb genomic HindIII fragment containing *FPR3* was excised from pBYNG1 and inserted into HindIII-digested YEp351 (Hill et al., 1986), a *LEU2*-containing 2 μm DNA-based multicopy vector, yielding YEp*FPR3*.

As one approach for immunodetection, the *FPR3* coding sequence was tagged near its NH_2-terminal end by the in-frame insertion of a double-stranded synthetic oligonucleotide encoding EEOKLISEEDLD, which contains an antigenic determinant from the c-Myc oncoprotein (underlined) that is recognized by a highly specific monoclonal antibody, 9E10 (Evan et al., 1985). For this purpose, a sense oligonucleotide (5′-CGAAGAACA-AAAATTGATTTCCGAAGAAGATTTGGA-3′) was annealed to an antisense oligonucleotide (5′-CGTCCAAATCTTCTTCGGAAATCAATTTTT-GTTCTT-3′) and then ligated just after codon 43 (at the ClaI site) in the *FPR3* coding sequence (see Fig. 1) in plasmid pNH2.2C. The resulting plasmids were screened by DNA sequence analysis for those containing a single c-*myc* epitope in the correct orientation, and one such plasmid was designated pUC*FPR3*myc. The 2.2-kb HindIII fragment containing *FPR3*myc was excised from pUC*FPR3*myc and inserted into HindIII-digested pRS315, a *LEU2*-containing *CEN*-based low-copy-number vector (Sikorski and Hieter, 1989), to generate pYB141, and also inserted into YEp351, to produce the multi-copy plasmid pYB1010.

To place *FPR3* expression under the control of a regulatable promoter, a new *LEU2*-containing multi-copy vector was constructed as follows. The 850-bp EcoRI-BamHI fragment containing the bidirectional *GAL1-GAL10* promoter (Johnston and Davis, 1984) was inserted between the EcoRI and BamHI sites of YEp351, to create plasmid YEp351GAL. A similar approach was used previously to construct a similar, but *URA3*-containing, multi-copy vector, YEp352GAL (Benton et al., 1990). To facilitate manipulation of the *FPR3* and *FPR3*myc coding sequences, a BamHI site was introduced just upstream (position −18) of the presumptive initiator methionine codon (where +1 is the first base of the ATG codon) by site-directed mutagenesis as follows. A 900-bp blunt[AflIII]-EcoRI fragment containing the 5′-region of *FPR3*, and an otherwise identical fragment containing the 5′-region of *FPR3*myc, were excised from plasmids pNH2.2 and pUC-*FPR3*myc, respectively, inserted into HincII-EcoRI-digested M13mp19 RF DNA, to create mp197125 and mp1971myc, respectively. Single-stranded DNA was prepared using M13KO7 helper phage, according to standard procedures (Sambrook et al., 1989). A mutagenic oligonucleotide (5′-GGGCTGGTTGGATCCTTGCTTTC), which contains a C-to-G transition at position −17, was annealed to the single-stranded mp197125 and mp1971myc DNA and extended using Klenow fragment and a commercial reagent kit (Version 2.1, Amersham Corp., Arlington Heights, IL) for oligonucleotide-directed in vitro mutagenesis, under conditions recommended by the manufacturer. Candidate mutants were screened by digestion with BamHI and confirmed by direct nucleotide sequence analysis. The 900-bp BamHI-EcoRI fragments so generated were inserted into the *E. coli* expression plasmid pGEX2T (Pharmacia LKB Nuclear, Gaithersburg, MD) that had been digested with BamHI and EcoRI, to produce plasmids pGXFPR3A and pGXFPR3mA. To reconstruct the full-length reading frames of *FPR3* and *FPR3*myc, a 700-bp EcoRI fragment containing the COOH-terminal coding segment of *FPR3* was excised from plasmid pNB3.3 and inserted into the EcoRI sites of plasmids pGXFPR3A and pGXFPR3mA, and then screened for the correct orientation by restriction mapping. Two plasmids that met this criterion were designated pGXFPR3B and pGXFPR3mB, rspectively. To place the *FPR3* and *FPR3*myc coding sequences under control of the *GAL1* promoter, the 1440 BamHI-HindIII fragments were excised from pGXFPR3B and pGXFPR3mB and ligated with YEp351GAL that had been digested with BamHI and HindIII, thereby yielding pYB123 (*GAL1-FPR3*) and pYB124 (*GAL1-FPR3myc*).

Construction of NH_2- and COOH-Terminal Truncations of FPR3

To truncate the NH_2-terminus and express just the COOH-terminal domain of *FPR3*, a BamHI site was introduced and a Lys-to-Met mutation was generated at codon 302 of the *FPR3* coding sequence (see Fig. 1) in a single step by PCR amplification, performed as described above, using plasmid pNH2.2 as the template and a 5′-primer, 5′-CC*GGATCC*CATAATGAGT-AAGGTTTTGGAAGGC-3′ (corresponding to nucleotides 1182–1202), and a 3′-primer, 5′-GGCATGC*AAGCTT*ATAAAGAGC-3′ (corresponding to nucleotides 1629–1650), which included BamHI (5′-primer) and HindIII (3′-primer) restriction sites (italicized). The resulting 470-bp product was digested with BamHI and HindIII, purified by electrophoresis in a 1.5% agarose gel, and ligated between the BamHI and HindIII sites in pBluescript II KS+, yielding plasmid pEB22, whose nucleotide sequence was confirmed by direct DNA sequence analysis. To place expression of the COOH-terminal domain of *FPR3* under *GAL1* promoter control, the insert from pEB22 was inserted into BamHI and HindIII-digested YEp351GAL, to create plasmid pYB120 (*GAL1-FPR3C*).

To truncate the COOH terminus and express just the NH_2-terminal do-

main of *FPR3*, the 1406-bp fragment containing *FPR3*myc was released by digestion of YEp351GAL*FPR3*myc with BamHI and HindIII and inserted into pUC19-RI, yielding pJHZ4. pJHZ4 was then digested with EcoRI and HindIII, which excises a fragment that removes all of the sequences beyond codon 229. To reattach sequences corresponding to codons 230–301 (which includes those residues immediately upstream of, but not including, the COOH-terminal catalytic domain) followed by multiple in-frame stop codons and a HindIII site, PCR was carried out, essentially as described above, using YEp351GAL*FPR3*myc as the template and a 5′-primer (5′-GGAGAAGAAGAACAAGAAG-3′), corresponding to nucleotides 685–703, and a 3′-primer (5′-*GCGCG*AAGCTTCACTATTATGGTTTATG-CTTATCTTGTTCC-3′, corresponding to nucleotides 882–903 and containing three in-frame termination codons (underlined), a HindIII site (bold-face), and a GC-clamp (italic) to enhance subsequent digestion by HindIII. The resulting 243-bp product was digested with EcoRI and HindIII and ligated into pJHZ4 that had been digested with EcoRI and HindIII and treated with shrimp alkaline phosphatase. Individual *E. coli* transformants were subjected to DNA sequence analysis and one such isolate that was found to contain no extraneous mutations (other than those purposefully introduced by the PCR strategy) was designated pJHZ2. To place expression of the NH_2-terminal domain of *FPR3* under *GAL1* promoter control, the insert from pJHZ2 was excised with BamHI and HindIII and inserted into BamHI- and HindIII-digested YEp351GAL, to create plasmid pJHZ3, now designated pYB126 (*GAL1-FPR3N*myc).

Bacterial Expression and Purification of Fpr3

To express full-length Fpr3 in *E. coli*, the *FPR3* coding sequence was fused in-frame to the COOH terminus of *Schistosoma japonicum* glutathione S-transferase (GST) in the vector, pGEX-2T (Pharmacia, LKB Nuclear), as an intermediate step (pGXFPR3B) in producing pYB123 (*GAL1-FPR3*) (see above). The resulting plasmid, pGXFPR3, directs synthesis of a GST-Fpr3 chimera in which the Met at the NH_2 terminus of Fpr3 is separated from the COOH terminus of GST by a thrombin cleavage site and a four amino acid (VQPN) spacer. Cleavage of the fusion protein with thrombin releases Fpr3 with a six-residue NH_2-terminal extension (H_2N-GSVQPN). A 2-l culture of *E. coli* strain BL21 carrying plasmid pGXFPR3 was grown at 37°C in 2× LB medium (2% Bacto-tryptone, 1% Bacto-yeast extract, 1% NaCl, 10 mM Tris–Cl pH 7.5, 1 mM $MgSO_4$) supplemented with 100 μg/ml of ampicillin to an A_{600nm} = 0.5. Protein expression was induced by addition of IPTG to a final concentration of 0.5 mM and the culture was incubated for another 2 h. Cells were harvested by centrifugation for 5 min at 4100 *g* in a Sorvall GSA rotor at 4°C, and all subsequent steps were performed at 4°C unless otherwise stated. The cell pellet was washed once by resuspension and recentrifugation in 100 mM KCl, 0.5 mM PMSF, 50 mM K-phosphate (pH 7.5) and resuspended in three times (wt/vol) the amount of the same buffer. Cells were broken by sonic disruption and the lysate was clarified by centrifugation for 30 min at 20,000 *g* in a Sorvall SS-34 rotor. This crude extract was mixed batchwise with 1 ml of preswollen glutathione-Sepharose beads (Pharmacia LKB Nuclear) and incubated for 1 h on a rotary mixer. The beads were collected by brief centrifugation, washed by resuspension and recentrifugation in 10 vol of lysis buffer, and poured into a column, which was then washed exhaustively with 20 vol of 400 mM NaCl, 50 mM K-phosphate (pH 7.5). Adsorbed GST-Fpr3 was eluted with 5 mM reduced glutathione in 100 mM NaCl, 50 mM K-phosphate (pH 7.5) containing 30% glycerol. Fractions (0.2 ml) were collected, subjected to SDS-PAGE, and visualized by both Coomassie blue staining and immunoblotting (see below). Peak fractions were pooled and concentrated to 9 mg/ml by centrifugation in a Centricon-30 chamber (Amicon, Beverly, MA). To release Fpr3 from the fusion, reactions containing 1 mg/ml GST-Fpr3 and 10 μg/ml thrombin (Sigma Chem. Co., St. Louis, MO) in TBS (140 mM NaCl, 20 mM Tris–Cl, pH 7.5) supplemented with 2 mM $CaCl_2$ were performed at 25°C for 30 min. After digestion, the mixture was chilled on ice and the GST fragment was removed by adsorption to glutathione-Sepharose beads, essentially as described above. The beads were removed by centrifugation and the supernatant fraction was stored at 4°C before analysis. Protein concentration was determined by a dye-binding method (Bradford, 1976) using a commercial reagent kit (Bio Rad Labs, Hercules, CA).

To express the COOH-terminal domain of Fpr3 ("Fpr3C") in *E. coli*, the COOH-terminal 134 codons of *FPR3* were fused in-frame to the NH_2-terminal 13 amino acids of T4 lysozyme by inserting a 530-bp blunt-[EcoRI]-HindIII fragment of *FPR3* between the SmaI and HindIII sites in the vector, pEXP1 (Raymond et al., 1990), to create plasmid pT4FPR3C. Protein expression in *E. coli* strain BL21 and preparation of the crude extract were as described above, except the lysis buffer was 5 mM 2-mercaptoethanol, 0.5 mM PMSF, 20 mM Tris–Cl (pH 8.0). The crude extract was adjusted to a final concentration of 2% protamine sulfate and the resulting nucleic acid precipitate was removed by centrifugation. The resulting supernatant fraction was then adjusted with $(NH_4)_2SO_4$ to 40% of saturation, which resulted in nearly quantitative precipitation of Fpr3C. This pellet of precipitated protein was redissolved in lysis buffer containing 5% glycerol, dialyzed overnight against the same buffer, and subjected to chromatography on a Mono-Q column (Pharmacia LKB Nuclear) using an automated FPLC apparatus (Waters Instrs. Inc., Rochester, MN). Fpr3C was found in the flow-through fraction, whereas most of the contaminating proteins were adsorbed. The flow-through fraction was dialyzed against 50 mM NaCl, 5% glycerol, 0.5 mM PMSF, 20 mM Hepes (pH 7.8), and subjected to FPLC on a Mono-S column (Pharmacia LKB Nuclear), which was eluted with a linear gradient from 50 to 600 mM NaCl in the same buffer. Peak fractions were pooled, dialyzed against the same buffer, adjusted to a final concentration of 50% glycerol, and stored at −20°C before analysis.

To express the NH_2-terminal domain of Fpr3 ("Fpr3N") in *E. coli*, the NH_2-terminal 279 codons of Fpr3 were fused in-frame to the COOH terminus of GST in the vector, pGEX-2T (Pharmacia LKB Nuclear), as an intermediate step (pGXFPR3A) in producing pYB123 (*GAL1-FPR3*) (see above). Expression and purification of the GST-Fpr3N fusion protein from *E. coli* strain BL21 carrying pGXFPR3A was performed exactly as described above for full-length Fpr3.

Assay of Peptidylprolyl-cis,trans-Isomerase Activity

Proline rotamase activity was measured as the rate of enhancement of the cleavage of a Pro-containing chromogenic peptide substrate by chymotrypsin using a spectrophotometer (Fischer et al., 1989). Peptides containing Pro exist as an equilibrium mixture of the *cis* and *trans* isomers about the X-Pro bond. Chymotrypsin will only cleave on the C-side of an X-Pro bond when it is in the *trans* configuration. Therefore, conversion of the *cis* to *trans* isomer catalyzed by a PPIase enhances the rate of substrate hydrolysis by chymotrypsin. Measurement of the activity of two different preparations of Fpr3C was kindly performed by Felicia Etzkorn in the laboratory of Christopher Walsh (Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA). Reaction mixtures (1 ml), assembled on ice, contained 70 μM of either *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide or *N*-succinyl-Ala-Leu-Pro-Phe-*p*-nitroanilide, 100 mM Tris–Cl (pH 7.5), and varying amounts of purified Fpr3 or Fpr3C (0.2–1.2 μM). Reaction mixtures were equilibrated to 10°C and initiated by addition of α-chymotrypsin (100 μg/ml). The change in absorbance at 400 nm (or 390 nm) was monitored at 0.1–1.0-s intervals over the course of about 2 min using a Hewlett-Packard 8452A (or a Shimadzu UV160A) spectrophotometer. When the effects of FK506 and rapamycin were tested, Fpr3 was incubated on ice with a 10-fold molar excess of the drug (prepared as a stock in methanol) for 10 min before assembling reaction mixtures. An equal volume of methanol alone had no detectable effect on the PPIase activity of Fpr3.

Preparation of Anti-Fpr3 Antibodies

Antisera to recombinant Fpr3 were raised in adult female New Zealand white rabbits using standard procedures (Harlow and Lane, 1988) as follows. Serum no. 1138 was raised against purified Fpr3C (Mono-Q fraction). Serum no. 1139 was raised against the purified GST-Fpr3N fusion protein. The anti-Fpr3 immunoglobulins from both antiserum no. 1138 and antiserum no. 1139 were enriched by affinity purification of these antisera on columns consisting of GST-Fpr3 that had been immobilized on CNBr-activated Sepharose beads (Pharmacia LKB Nuclear). After washing these columns extensively with 1 M NaCl, the specific antibodies were eluted with 50 mM glycine (pH 2.4) and the resulting eluate was immediately neutralized by the addition of one-tenth volume of 0.5 M Na_2HPO_4. Anti-yeast fibrillarin antibodies (mAb A66) were the gift of Dr. John Aris (Dept. of Anatomy and Cell Biology, University of Florida, Gainesville, FL).

Gel Electrophoresis and Immunoblotting

Yeast cells were grown to mid-exponential phase (A_{600nm} = 0.75-1) in rich medium (YPD), harvested by brief centrifugation, washed once in water, and resuspended in 0.3 ml ice-cold lysis buffer (150 mM NaCl, 50 mM Tris–Cl, pH 8.0, 1 mM PMSF, 1 mM EDTA, 1% Triton X-100, 0.5% SDS) at 40–50 A_{600nm} units per ml. The cells in the suspension were lysed by six 30-s periods of vigorous vortex mixing (separated by 1-min periods of cool-

ing on ice) with 0.3 g of prechilled glass beads (0.45–0.6 mm diameter). The lysates were adjusted to 2% SDS, boiled for 3 min, cooled to room temperature, and clarified by centrifugation at 10,000 *g* for 10 min to remove the beads and cell debris. Protein in the clarified extracts was precipitated with trichloroacetic acid (10% final concentration), resolubilized in SDS-PAGE and sample buffer, and subjected to SDS-PAGE (Laemmli, 1970). Proteins were visualized by staining with Coomassie blue (Weber and Osborn, 1969) or silver (Oakley et al., 1980).

Transfer of proteins resolved by SDS-PAGE to nitrocellulose (BA85, 0.45 mm: Schleicher & Schuell) was performed using procedures described in detail elsewhere (Towbin et al., 1979). Blots were blocked and incubated with the primary antibodies using standard procedures (Harlow and Lane, 1988) and immune complexes were detected using secondary anti-IgG antibodies coupled to horseradish peroxidase and a chemiluminescence detection system (ECL™, Amersham Corp.), according to the manufacturer's instructions. *E. coli* cell extracts and purified fractions containing Fpr3 or any of its derivatives were solubilized and analyzed by SDS-PAGE and immunoblotting using procedures essentially identical to those used for yeast extracts.

Immunofluorescence

Staining of yeast cells by indirect immunofluorescence was performed essentially as described by Rose and Fink (1987), with the following modifications. Cell walls were removed by incubation with 20 μg/ml Zymolyase-100T (Seikagaku America Inc., Rockville, MD) at 30°C for 30–45 min. The primary antibodies (either affinity-purified IgGs from antisera no. SB126, no. 1138, and no. 1139, all diluted to 1 μg/ml, or ascites fluid containing anti-*c*-Myc mAb 9E10, diluted 1:200) were incubated with the fixed cells at room temperature for 1–2 h. Secondary antibodies (1:200 diluted, affinity-purified, FITC-, Texas Red- or rhodamine-labeled, goat anti-rabbit IgG antibodies or goat anti-mouse IgG antibodies; Jackson ImmunoResearch Labs., Inc., West Grove, PA) were applied for 1 h. Cells were examined with a Nikon Optiphot epifluorescence microscope using a 100× objective and photographed with Kodak Ektachrome Elite 400 color slide film.

Measurement of Sensitivity of Rapamycin Action

A bioassay was used to determine the relative sensitivity of cells to the growth inhibition caused by rapamycin. Samples (200 μl) of cell suspensions (exponential cultures at ~1 × 10^7 cells per ml) of the strains to be tested were used to inoculate 3 ml of molten soft (0.7%) agar containing SD medium and were immediately poured uniformly over the surface of an SD plate containing nutrients appropriate for the maintenance of plasmids, if present. After solidification of the top agar, sterile cellulose filter disks (0.6 cm diameter; Difco Laboratories, Detroit, MI), each containing a different known concentration of rapamycin that was spotted in an identical volume of ethanol containing 10% Tween-20, were placed aseptically on the surface of the agar. The plates were incubated at 30°C for ~48 h until the lawns were clearly visible. As in other agar diffusion assays of drug sensitivity, the radius of the halo of growth inhibition in the lawn surrounding the disk is proportional to the logarithm of the initial concentration of the drug applied to the disk (Cooper, 1963). Alternatively, various amounts of the drugs were added directly to vigorously stirred molten (60°C) agar from a concentrated stock in ethanol containing 10% Tween-20 just before pouring into Petri plates. Rapamycin was the generous gift of Suren Sehgal (Wyeth-Ayerst Research Laboratories, Princeton, NJ). FK506 was the generous gift of Ihor Bekersky (Fujisawa USA, Inc., Melrose Park, IL).

Results

Isolation and Characterization of the FPR3 Gene

As one means toward identifying previously unknown proteins that may be involved in events that regulate the architecture and metabolism of the yeast cell nucleus, we raised polyclonal antisera directed against several polypeptides that were abundantly represented in preparations of *S. cerevisiae* nuclei which we believed to be highly enriched in the nuclear envelope fraction. The methods we used for subcellular fractionation have been reported to yield a subnuclear fraction that is supposed to be devoid of chromatin and its associated proteins (Aris and Blobel, 1988, 1989, 1991). Prominent polypeptides in these preparations, as detected by SDS-PAGE and Coomassie blue staining, were excised from preparative gels and injected into rabbits. The same protein species immobilized by transfer to nitrocellulose filters were used to immuno-select specific antibodies from the polyclonal sera. The affinity-purified antibodies then were used to stain fixed and permeabilized yeast cells by indirect immunofluorescence. Antibodies obtained from an antiserum that was raised against a relatively abundant nuclear protein with an $M_{r_{app}}$ of 65 kD decorated a polypeptide of identical mobility on immunoblots of fractionated nuclear extracts and displayed exclusively nuclear staining when examined by indirect immunofluorescence.

The affinity-purified antibodies directed against the 65-kD species were used to screen a yeast genomic DNA library in the λgt11 expression vector. The insert from the single immunoreactive phage so obtained was subcloned, and DNA sequence analysis revealed a open reading frame of 1239 bp (Fig. 1 *A*). Using the phage insert as a probe, a genomic fraction covering this same region was recovered from an independent yeast genomic DNA library in a plasmid vector. DNA sequence analysis of the insert in the plasmid confirmed the sequence of the open reading frame and extended the sequence in the regions immediately flanking the open reading frame (Fig. 1 *A*). The open reading frame predicts a protein of 413 amino acids, with a calculated molecular weight of 46,800. The NH_2-terminal two-thirds of the predicted primary sequence contains an unusually large number of charged residues (Fig. 1 *A*), arranged as long tracts of acidic residues separated by shorter stretches of basic residues, which is known to cause an anomalously slow mobility upon SDS-PAGE (for example see Fuller et al., 1989) and presumably accounts for its higher apparent molecular mass (~65 kD) when determined by this method. The sequence contains at least one match to the consensus bipartite nuclear localization signal, -KK-$(X)_{10}$-KAK-KVKK- (residues 258–276) (Robbins et al., 1991). Comparison of the open reading frame to all available protein sequences in the GenBank database using the BLASTP algorithm (Altschul et al., 1990) revealed that the COOH-terminal 111 residues of the open reading frame (Fig. 1 *A*, *underlined*) share over 40% identity with various members of the FKBP family from yeast, *Neurospora crassa*, and humans (Fig. 2). Hence, we designated the gene encoding this protein *FPR3* (for FK506-binding proline rotamase number 3) because it was the third yeast FKBP identified (Kunz and Hall, 1993). The *FPR3* gene product, Fpr3, was also termed yFKBP-47, in keeping with a suggested nomenclature for FKBPs (Jin et al., 1991).

To determine if *FPR3* is an expressed gene, DNA probes internal to the coding region were hybridized to preparations of poly(A)$^+$ RNA isolated from *MAT***a** and *MAT*α haploids and *MAT***a**/*MAT*α diploids. A transcript of about 1,400 nucleotides, which is of sufficient length to contain the entire predicted open reading frame, was readily detectable in all three cell types (data not shown). The location of the *FPR3* gene on the physical and genetic maps of the *S. cerevisiae* genome was then determined (see Materials and Methods). DNA probes internal to the coding region hybridized to the band representing chromosome XIII on blots of intact chro-

A

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- 279  CACTAAATCTGGTTGCATCTACATCTATATAGTCAAAACAGGCAAGAACAGAGTTATGATACATGCTTCATGTTCCAT
- 201  TGTTAGAATCCACTAGTATTAGCTCGCTGTTCAATTTTCAGCCCTCTACGGACTGTCGTCATTCCTAAAGGACGGCAA
- 123  AATTTAAAAGTGAAGGTAAAATTTTTAAATTCGAAAAAAGAGCCTACTAACACGTTTCTATATAATACATAATTGTGT
-  45  GAAAGTTCATACATAATTGAAAGCAAGCATCCAACCAGCCCAAT

+   1  ATGTCTGATTTGTTACCACTAGCTACCTACAGTTTGAATGTTGAACCTTATACCCCGGTTCCAGCAATCGACGTCACG
       M  S  D  L  L  P  L  A  T  Y  S  L  N  V  E  P  Y  T  P  V  P  A  I  D  V  T    26

+  79  ATGCCCATCACCGTTCGTATTACTATGGCTGCTTTGAACCCGGAAGCCATCGATGAAGAGAACAAACCATCGACTTTA
       M  P  I  T  V  R  I  T  M  A  A  L  N  P  E  A  I  D  E  E  N  K  P  S  T  L    52

+ 157  AGAATTATCAAAAGAAACCCGGACTTTGAAGATGATGATTTTTTAGGTGGTGATTTTGATGAAGACGAAATAGACGAA
       R  I  I  K  R  N  P  D  F  E  D  D  D  F  L  G  G  D  F  D  E  D  E  I  D  E    78

+ 235  GAATCCTCTGAGGAAGAAGAAGAGGAAAAAAACCCAAAAGAAAAAGAAGAGTAAAGGCAAGAAGGCTGAAAGTGAAAGT
       E  S  S  E  E  E  E  E  E  K  T  Q  K  K  K  K  S  K  G  K  K  A  E  S  E  S   104

+ 313  GAGGATGATGAAGAAGACGATGACGAGGACGATGAGTTCCAAGAATCCGTCCCTTTGACTTTATCTCCGGAAGCCCAA
       E  D  D  E  E  D  D  D  E  D  D  E  F  Q  E  S  V  L  L  T  L  S  P  E  A  Q   130

+ 391  TACCAACAATCTTTGGACTTGACCATTACTCCAGAAGAAGAAGTCCAATTCAATGTCACTGGTTCTTACGCTATCTCC
       Y  Q  Q  S  L  D  L  T  I  T  P  E  E  E  V  Q  F  I  V  T  G  S  Y  A  I  S   156

+ 469  TTGAGCGGTAACTATGTTAAGCATCCATTTGATACTCCAATGGGAGTCGAAGGTGAAGACGAAGATGAAGACGCTGAC
       L  S  G  N  Y  V  K  H  P  F  D  T  P  M  G  V  E  G  E  D  E  D  E  D  A  D   182

+ 547  ATCTATGACAGTGAAGACTACGACTTGACCCCAGATGAGGATGAAATTATTGGCGACGACATGGACGACTTGGATGAC
       I  Y  D  S  E  D  Y  D  L  T  P  D  E  D  E  I  I  G  D  D  M  D  D  L  D  D   208

+ 625  GAAGAGGAAGAAGAAGTTCGTATTGAAGAAGTCCAAGAAGAAGATGAAGAAGATAATGATGGAGAAGAAGAACAAGAA
       E  E  E  E  E  V  R  I  E  E  V  Q  E  E  D  E  E  D  N  D  G  E  E  E  Q  E   234

+ 703  GAAGAAGAAGAAGAAGAAGAAGAACAAAAAGAAGAAGTTAAGCCAGAACCTAAGAAAAGCAAAAAGGAAAAAAAGAGA
       E  E  E  E  E  E  E  E  Q  K  E  E  V  K  P  E  P  K  K  S  K  K  E  K  K  R   260

+ 781  AAGCACGAAGAGAAAGAAGAAGAAAAGAAAGCTAAAAAAGTAAAGAAGGTCGAATTCAAGAAGGACTTAGAGGAGGGT
       K  H  E  E  K  E  E  E  K  K  A  K  K  V  K  K  V  E  F  K  K  D  L  E  E  G   286

+ 859  CCAACAAAACCCAAAAGCAAAAAGGAACAAGATAAGCATAAACCAAAGAGTAAGGTTTTGGAAGGCGGCATAGTAATC
       P  T  K  P  K  S  K  K  E  Q  D  K  H  K  P  K  S  K  V  L  E  G  G  I  V  I   312

+ 937  GAAGACCGTACTATCGGTGATGGCCCACAGGCTAAGAGAGGTGCCAGAGTAGGCATGAGGTACATTGGTAAGTTCAAG
       E  D  R  T  I  G  D  G  P  Q  A  K  R  G  A  R  V  G  M  R  Y  I  G  K  L  K   338

+1015  AACGGTAAAGTTTTCGACAAGAACACCAGCGGTAAACCATTTGCATTCAAACTTGGCCGTGGTGAAGTTATVAAAGGC
       N  G  K  V  F  D  K  N  T  S  G  K  P  F  A  F  K  L  G  R  G  E  V  I  K  G   364

+1093  TGGGACATTGGTGTTGCCGGTATGTCTGTTGGTGGCGAACGTAGAATCATCATTCCAGCACCATATGCCTACGGGAAG
       W  D  I  G  V  A  G  M  S  V  G  G  E  R  I  I  I  P  A  P  Y  A  Y  G  K   390

+1171  CAAGCTCTGCCAGGTATTCCTGCCAATTCCGAACTGACATTCGACGTTAAATTGGTTTCTATGAAAAACTAGTACACG
       Q  A  L  P  G  I  P  A  N  S  E  L  T  F  D  V  K  L  V  S  M  K  N  .        413

+1249  CGCTCGTACGGATAGATGTTTATATATATTATTCTTTTTACATAATATTGTAACCTTCTGTATGTAAATCCGAAAACA
+1327  TTCTATTTCTTTTTAGTATCTATTGCTCTTTATAAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCCTCGGGTACC
+1405  GAGCTCGAATTAATTCACTGGCCGTCGTT
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B

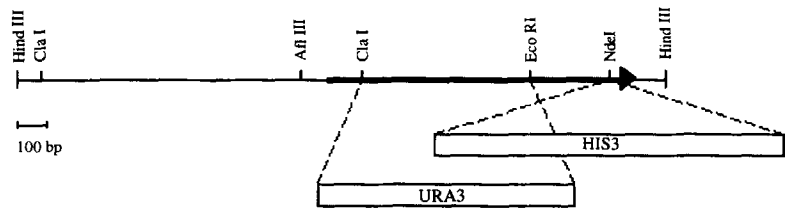

Figure 1. Sequence of the *FPR3* gene and structure of *fpr3* null mutations. (*A*) Nucleotide sequence and deduced amino acid sequence of the *FPR3* gene. Numbers to the left represent bases in the nucleotide sequence (where +1 is the A of the presumptive ATG initiator codon). Numbers to the right represent amino acids in the predicted primary sequence (given in the single letter code). Arrowhead indicates the position (between codons 43 and 44) where a *c-myc* epitope tag was inserted to create the *FRP3*myc allele (see Materials and Methods). The COOH-terminal region homologous to FKBPs is underlined (see also Fig. 2). The GenBank accession number for this sequence is L34569. (*B*) Restriction endonuclease cleavage site map of the 2.2-kb genomic HindIII fragment that contains the *FRP3* locus. In the *fpr3-Δ1::URA3* allele, codons 44–279 were deleted and replaced by the *URA3* gene. In the *fpr3-2::HIS3* allele, the coding sequence was disrupted by insertion of the *HIS3* gene immediately after codon 386.

mosomes separated by clamped homogeneous field electrophoresis (data not shown) and hybridized specifically to a lambda phage carrying a particular subfragment of the far left arm of chromosome XIII, just centromere-distal to the *HMG1* gene (data not shown). To confirm this conclusion, a genetic cross was performed between a *MAT***a** strain (YB100) carrying a *URA3* marker inserted into the *FPR3* gene and a *MATα* strain (YB58) carrying a *LYS2* marker inserted into the *HMG1* gene. Tetrad analysis (PD:NPD:T:: 29:0:0) demonstrated that the *FPR3* locus is indeed very tightly linked to the *HMG1* locus.

FPR3 Gene Product Has Peptidylprolyl-cis, trans-Isomerase Activity In Vitro

Although the COOH-terminal portion of Fpr3 shares a high

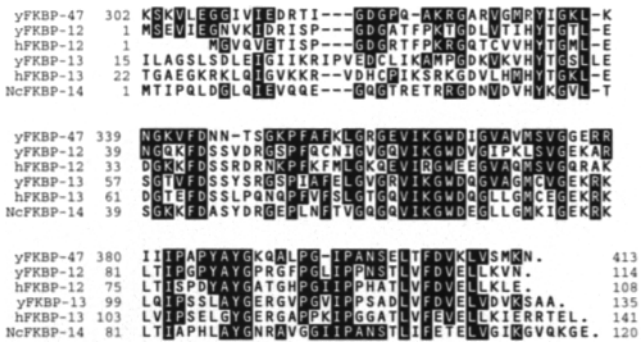

Figure 2. Homology of the COOH-terminus of Fpr3 to known members of the FKBP family. The COOH-terminal 111 amino acids (residues 302–413) of Fpr3 (yFKBP-47) are compared with the predicted primary sequences of yeast FKBP-12 (yFKBP-12) (Heitman et al., 1991*b*; Koltin et al., 1991; Wiederrecht et al., 1991), human FKBP-12 (hFKBP-12) (Standaert et al., 1990), yeast FKBP-13 (yFKBP-13) (Nielsen et al., 1992), human FKBP-13 (hFKBP-13) (Jin et al., 1991), and *Neurospora crassa* FKBP-14 (NcFKBP-14) (Tropschug et al., 1990). White-on-black letters indicate amino acid identities. Dashes indicate single-residue gaps introduced to maximize the alignment.

degree of amino acid sequence identity with known FKBPs and each of the residues known to line the substrate-binding pocket in human FKBP-12 (Tyr27, Phe37, Trp60, Tyr 83 and Phe100; Fig. 2) (Becker et al., 1993; Rotonda et al., 1993) are completely conserved in Fpr3, it was nonetheless important to demonstrate that, like other FKBPs, Fpr3 is indeed a functional PPIase. For this purpose, a construct was made (see Materials and Methods) that permitted expression of the COOH-terminal 134 residues, which includes the presumptive catalytic domain, in *E. coli*. This soluble protein, Fpr3C, was purified to apparent homogeneity (Fig. 3 *A*). In addition, full-length Fpr3p was expressed in *E. coli* as a fusion protein to *S. japonicum* glutathione S-transferase, yielding a GST-Fpr3 chimera of $M_{r_{app}}$ ~95kD. The soluble GST-Fpr3 fusion protein was purified to near homogeneity by adsorption and elution from glutathione-Sepharose beads (Fig. 3 *B*). Fpr3 (with a short 6-amino acid NH_2-terminal extension) was released by digestion of the GST-Fpr3 fusion with thrombin, and the bulk of the free GST and uncleaved GST-Fpr3 were removed by their readsorption to glutathione-Sepharose (Fig. 3 *B*). The isolated catalytic domain, Fpr3C, possessed readily detectable PPIase activity, as mea-

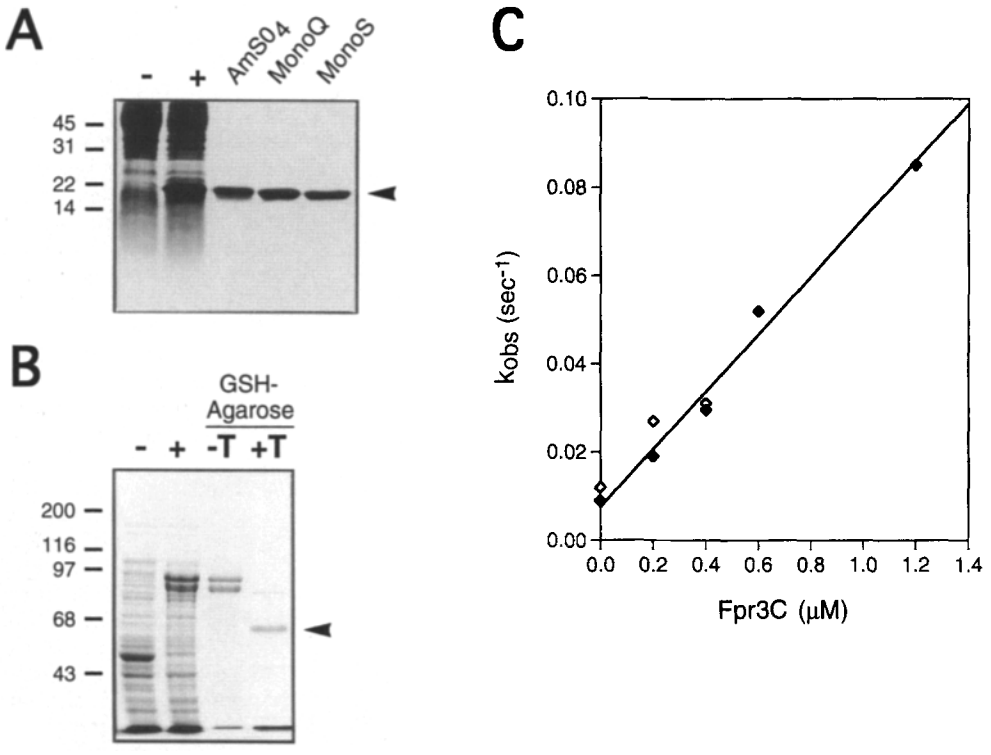

Figure 3. Purification and in vitro PPIase activity of bacterially expressed Fpr3. (*A*) Purification of the Fpr3C domain. *E. coli* (2×10^{11} cells) carrying plasmid pT4FPR3C were grown, induced, harvested and lysed, and Fpr3C was purified from the resulting extracts, as described in detail in Materials and Methods. Samples (~10 μg total protein each) from various stages of the purification were resolved by SDS-PAGE in a 15% gel and stained with Coomassie blue dye. Clarified lysates of uninduced (−) and IPTG-induced (+) cultures; material precipitated from the extract of IPTG-induced cells by addition of $(NH_4)_2SO_4$ to 40% of saturation (*AmSO4*); flow-through fraction following chromatography of the redissolved $(NH_4)_2SO_4$ precipitate on Mono-Q (*MonoQ*); pooled peak fractions of the eluate following chromatography of the Mono-Q pass-through on Mono-S (*MonoS*). Arrowhead indicates purified Fpr3C. (*B*) Purification of full-length Fpr3. *E. coli* carrying pGEXFPR3 were grown, induced, harvested and lysed, and the GST-Fpr3 fusion protein was purified and cleaved, as described in detail in Materials and Methods. Samples (~5 μg total protein each) from various stages of the purification were resolved by SDS-PAGE in an 8% gel and stained with Coomassie blue dye. Clarified lysates of uninduced (−) and IPTG-induced (+) cultures; glutathione eluate of the material bound upon incubation of the extract of IPTG-induced cells with glutathione-agarose before treatment with thrombin (−*T*); material that no longer adsorbed to glutathione-agarose after treatment with thrombin (+*T*). Arrowhead indicates purified Fpr3. Molecular masses ($\times 10^{-3}$) of markers were: 14 kD, egg white lysozyme; 22 kD, soybean trypsin inhibitor; 31 kD, carbonic anhydrase; 43/45 kD, ovalbumin; 68 kD, bovine serum albumin; 97 kD, phosphorylase b; 116 kD, *E. coli* β-galactosidase; and 200 kD, myosin heavy chain. (*C*) PPIase activity of the Mono-Q (*open symbols*) and Mono-S (*closed symbols*) fractions of the preparation of Fpr3C shown in *A* were assayed using 70 μM *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide as the substrate in a coupled assay with chymotrypsin, as described in Materials and Methods. The initial rate of hydrolysis (k_{obs}) was measured in the absence and in the presence of the indicated concentrations of Fpr3C.

sured by enhancement of the rate of chymotrypsin cleavage of a synthetic Pro-containing peptide substrate in a coupled spectrophotometric assay (Fig. 3 *C*). The specific activity of full-length Fpr3 was not significantly different from that of its isolated COOH-terminal domain (data not shown). Similar to other FKBPs from humans (Park et al., 1992) and yeast (Nielsen et al., 1992), both full-length Fpr3 and Fpr3C reproducibly displayed a significantly faster rate (≥twofold) of isomerization of a peptide substrate containing a Leu-Pro bond compared to an otherwise identical substrate containing an Ala-Pro bond (data not shown). Also, as predicted from its homology to other FKBPs, Fpr3 appeared to be both an FK506- and rapamycin-binding protein in vitro because addition of a 10-fold molar excess of either compound to reaction mixtures completely inhibited its PPIase activity (data not shown). This conclusion was further supported by in vivo studies (see below).

Genetic Analysis of FPR3 Function

Two different *fpr3* null mutations were constructed (Fig. 1 *B*). The *fpr3-Δ1::URA3* allele is a deletion (codons 44 to 278), which eliminates a large portion of the open reading frame. The *fpr3-2::HIS3* allele contains a large insertion at codon 386, which disrupts the catalytic domain. Appropriate DNA fragments containing these mutations were used to transplace the *FPR3* locus on one homologue of chromosome XIII in *MATa*/*MATα* diploid recipients. Viable haploid spores containing either of the two null alleles were readily obtained upon sporulation and dissection of the corresponding heterozygous *fpr3/FPR3* diploid transformants (see, for example, Fig. 7 *A*). It has been observed previously that *fpr1* (Heitman et al., 1991*b*) and *fpr2* (Nielsen et al. 1992) null mutants generated in an identical fashion are also viable. Therefore, like the *FPR1* and *FPR2* genes, the *FPR3* gene is dispensible for spore germination and vegetative cell growth.

Haploid *fpr3-Δ1* and *fpr3-2* strains were examined systematically for potential phenotypes that might reveal the physiological function of Fpr3. Compared to otherwise isogenic controls, growth of *fpr3* mutants was unaffected on rich or synthetic defined medium containing glucose, glycerol or acetate. The *fpr3* mutants were no more cold- (14°C) or heat- (36°C) sensitive, or susceptible to heat shock, than the parental cells; also, *fpr3* mutants were mating proficient and displayed no defect in response to (or recovery from) mating pheromone-induced cell cycle arrest. Homozygous *fpr3/fpr3* diploids sporulated and germinated at a frequency indistinguishable from normal diploids. Similarly, normal *FPR3*$^+$ cells also expressing *FPR3* from a 2-μm DNA plasmid, which resulted in an ~10-fold overproduction of Fpr3, as judged by immunoblotting (data not shown), showed no difference from control cells with respect to growth, temperature-sensitivity, mating ability, or sporulation. Finally, both *fpr3* mutants and normal cells carrying *FPR3* on a 2-μm DNA plasmid displayed no morphological aberrations when examined microscopically either by phase contrast or by indirect immunofluorescence when stained with the DNA-specific dye, DAPI, or with anti-actin or anti-tubulin antibodies (data not shown).

One possible explanation for the lack of a discernible phenotype of *fpr3* mutations was the presence of genes in the yeast genome whose products might substitute for *FPR3* function. Low stringency hybridization of *FPR3* DNA probes to total cellular DNA digested with various restriction enzymes did not reveal any evidence for a highly homologous gene in the *S. cerevisiae* genome. However, as mentioned earlier, two other FKBP-encoding genes, *FPR1* (Heitman et al., 1991*a*; Koltin et al., 1991; Wiederrecht et al., 1991) and *FPR2* (Nielsen et al., 1992), were previously identified in *S. cerevisiae*. Therefore, we tested whether either *FPR1* or *FPR2* might be partially redundant in function with *FPR3* by determining if any phenotypes were revealed by combining an *fpr3* mutation with mutations in the other two genes. For this purpose, a set of otherwise isogenic strains carrying *fpr1* and *fpr2* null mutations, either individually or in combination, were constructed and crossed to *fpr3* mutants. Sporulation and dissection of the resulting diploids readily yielded *fpr1 fpr3* and *fpr2 fpr3* double mutants and even *fpr1 fpr2 fpr3* triple mutants at the expected frequencies. Furthermore, like *fpr3* single mutants, strains carrying any of these combinations of mutations, including the *fpr1 fpr2 fpr3* triple mutant, had no detectable phenotypes with respect to growth, temperature-sensitivity and mating ability.

Fpr3 Can Bind Rapamycin and FK506 In Vivo

Standard laboratory strains of *S. cerevisiae*, including YPH-499 and the derivatives we used in this work, are quite sensitive to growth inhibition by rapamycin, but are essentially unaffected by FK506. Rapamycin-Fpr1 complexes appear to be targeted to the PI lipid kinases, Tor1 and Tor2, which are enzymes essential for viability (Kunz et al., 1993; Helliwell et al., 1994), whereas FK506-Fpr1 complexes are targeted to calcineurin (Foor et al., 1992), an enzyme that is not essential for cell growth (Cyert et al., 1991; Cyert and Thorner, 1992) (although FK506 at high concentrations can inhibit the growth of certain amino acid auxotrophs because it can block the transporters for those amino acids [Heitman et al., 1993]). Several groups have demonstrated that cells bearing an *fpr1* null mutation are completely resistant to the growth-inhibitory action of rapamycin (Heitman et al., 1991*ab*; Koltin et al., 1991). Moreover, because FK506 and rapamycin compete for binding to Fpr1, FK506 at moderate concentrations can actually antagonize the toxicity of rapamycin (Heitman et al., 1991*a*).

To determine whether the *FPR3* product was capable of binding either of these drugs and contributing to their growth-inhibitory effect, we first compared the sensitivity of isogenic *FPR3* cells and *fpr3* mutants, in both *FPR1* and *fpr1* backgrounds, to different concentrations of rapamycin (data not shown). The *FPR1 fpr3* cells were just as sensitive to rapamycin as *FPR1 FPR3* cells. Furthermore, *fpr1 fpr3* double mutants were no more resistant to rapamycin than *fpr1 FPR3* cells. Moreover, the resistance of *fpr1* cells and the sensitivity of *FPR1* cells to rapamycin was unaffected when these strains expressed *FPR3* from a 2-μm DNA plasmid.

Because Fpr1 is a cytosolic protein (also referred to as yFKBP-12) and because Fpr3 was initially identified as a nuclear protein, it seems possible that neither drug can affect Fpr3 function because these compounds cannot enter the nucleus or because the downstream targets for the growth-inhibitory action of immunophilin-drug complexes are not present in the nucleus. To address this issue, we constructed

a vector to express just the catalytic domain of Fpr3 because we reasoned that the NH_2-terminal domain of Fpr3 might be responsible for sequestering Fpr3 in the nucleus and/or for targeting Fpr3 to a specific substrate(s) in the nuclear compartment. For this purpose, we placed the coding sequence for the COOH-terminal 112 residues (where the Lys301 codon had been changed to a Met codon) under control of the *GAL1* promoter in a multi-copy vector. As a control, we inserted the entire *FPR3* gene into the same expression vector.

These plasmids were introduced by transformation into otherwise isogenic *FPR1* (rapamycin-sensitive) and *fpr1* (rapamycin-resistant) strains. First, cells carrying each of the two different plasmids were grown on glucose medium to mid-exponential phase, and then shifted to medium containing galactose for several hours to induce expression of the corresponding genes. As demonstrated by immunoblotting of cell lysates fractionated by differential centrifugation or by indirect immunofluorescence with specific anti-Fpr3C antibodies, the Fpr3C domain was expressed as an abundant cytosolic protein (data not shown), whereas full-length Fpr3 was associated exclusively with the nucleus (see below). Expression of neither plasmid detectably affected (either enhanced or reduced) the rapamycin sensitivity of the *FPR1* cells (data not shown). As expected, however, in *fpr1* cells, which are otherwise resistant to rapamycin, expression of Fpr3C in the cytosol (but not full-length Fpr3) restored sensitivity to rapamycin (Fig. 4), although at the level about 10-fold lower than that typically displayed by *FPR1* cells (data not shown). The ability of the Fpr3C domain to functionally substitute for Fpr1 in vivo to mediate rapamycin growth inhibition indicates that the COOH-terminal catalytic domain of Fpr3 is capable of binding rapamycin, confirming the results of the in vitro assays performed on the purified protein. Furthermore, we found that the presence of a large excess of FK506 (which by itself is not an efficacious growth inhibitor, as mentioned above) was able to partially antagonize the toxic effect of rapamycin in the *fpr1* cells expressing Fpr3C (data not shown), presumably by preventing formation of the Fpr3C-rapamycin complex. This observation also supports the conclusion that the COOH-terminal domain of Fpr3 is capable of binding FK506, in agreement with the results of the in vitro assays.

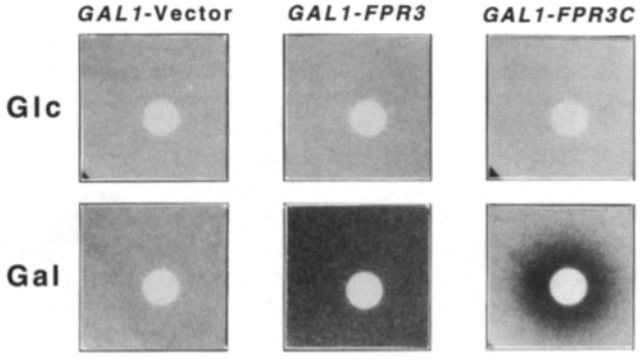

Figure 4. The COOH-terminal domain of Fpr3 can function as a receptor for rapamycin in vivo. Strain YB112 (*MATa fpr1-2::ADE2 fpr3-2::HIS3*) carrying either vector alone, YEp351GAL (*GAL1-Vector*), or the same plasmid expressing either full-length Fpr3, pYB123 (*GAL1-FPR3*), or just the COOH-terminal domain of Fpr3, pYB120 (*GAL1-FPR3C*), were plated on SD-Leu medium containing either glucose (*Glc*) or galactose (*Gal*) and their sensitivity to growth inhibition by rapamycin (10 μg total per disk applied in ethanol containing 10% Tween-20) was tested by the halo bioassay described in Materials and Methods.

Overproduction of Fpr3 or its NH_2-terminal Domain is Deleterious to Growth

Although overproduction of full-length Fpr3 from the *GAL1* promoter did not confer rapamycin sensitivity to *fpr1* cells, unlike the overproduced Fpr3C domain, we noticed that nascent lawns of cells harboring the *GAL1-FPR3* plasmid grew very slowly when plated on galactose-containing medium, but not when plated on glucose-containing medium (Fig. 4). Immunoblotting indicated that the level of Fpr3 achieved in cells expressing *GAL1-FPR3* from the multi-copy plasmid after several hours of growth on galactose medium was at least 5–10-fold higher than the level observed when *FPR3* was expressed from its own promoter on a multi-copy plasmid (data not shown). To confirm that this level of Fpr3 overproduction was deleterious to growth, we compared the ability of otherwise wild-type cells carrying plasmids expressing either *GAL1-FPR3*, *GAL1-FPR3*myc (which contains a 12-residue insert harboring a *c*-Myc epitope introduced in-frame between codons 43 and 44 of the *FPR3* coding sequence), *GAL1-FPR3C*, or the vector alone, to form single colonies on selective medium. When the selective medium contained glucose as the carbon source (which represses expression of the *GAL1* promoter), cells grew equally well, regardless of the plasmid they contained (Fig. 5 *A*). In marked contrast, when the selective medium contained galactose as the carbon source (which induces high-level expression from the *GAL1* promoter), cells expressing full-length Fpr3 (either the normal protein or the epitope-tagged version) grew extremely slowly, whereas growth of the cells expressing Fpr3C, or of the control cells carrying the vector alone, was unaffected (Fig. 5 *A*). This phenotype was observed at all temperatures examined, and was no more pronounced at elevated (36°C) or reduced (14°C) temperatures (data not shown).

To determine if the NH_2-terminal domain contributes to the toxicity caused by Fpr3 overproduction, a construct was made that expresses the epitope-tagged version of the NH_2-terminal 301 codons of the *FPR3* gene under control of the *GAL1* promoter in the same vector. When otherwise wild-type cells harboring either *GAL1-FPR3*, *GAL1-FPR3C*, *GAL1-FPR3N*myc, or vector alone, were grown on selective medium with glucose as the carbon source, the efficiency of plating of 5-fold serial dilutions of all four strains was essentially identical (Fig. 5 *B*). In contrast, when plated on galactose-containing medium, cells expressing both full-length Fpr3 and Fpr3Nmyc plated with at least a 10–100-fold lower efficiency than the cells expressing Fpr3C or the vector only control (Fig. 5 *B*).

Because *fpr1* mutants are resistant to growth inhibition by rapamycin and because overproduction of full-length Fpr3 did not substitute for Fpr1 to mediate rapamycin killing, we were able to employ an *fpr1* strain background as means to determine if the catalytic domain contributes to the toxicity caused by Fpr3 overproduction. Binding of rapamycin to the

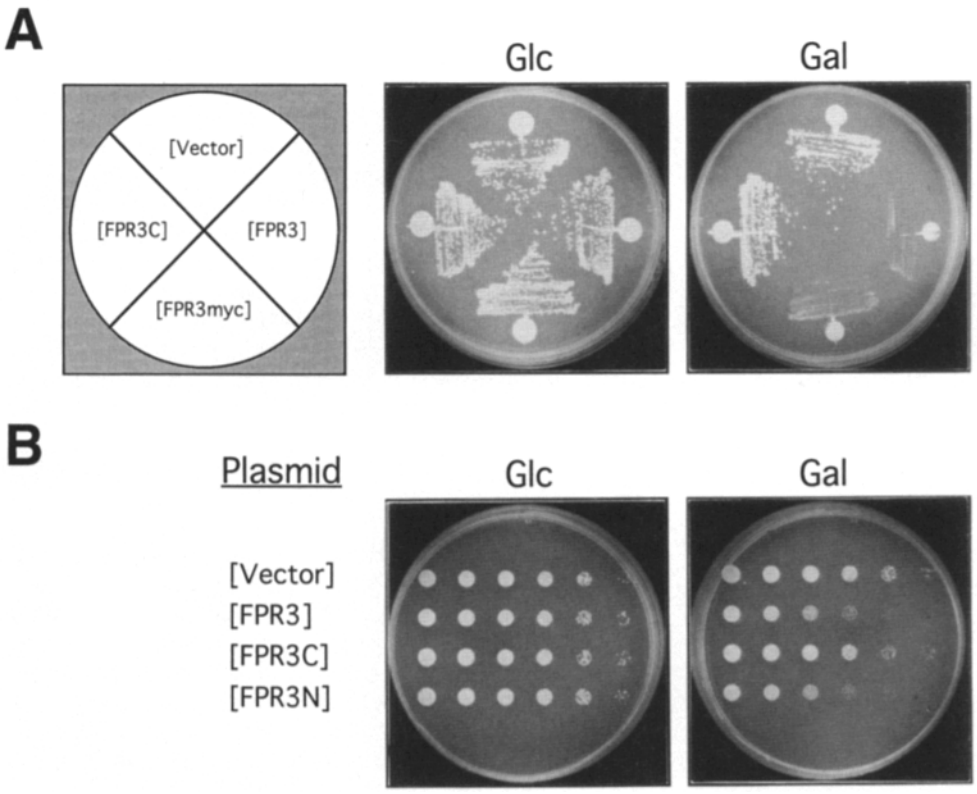

Figure 5. Overexpression of full-length Fpr3 or its NH_2-terminal domain is toxic. (*A*) Samples of cultures ($A_{600\ nm}$ = 0.02) of strain YB112 (*MATa fpr1-2::ADE2 fpr3-2::HIS3*) carrying either vector alone, YEP351GAL [*Vector*), or the same plasmid expressing either full-length Fpr3, pYB123 [*FPR3*], or a derivative of full-length Fpr3 containing a c-Myc epitope inserted in-frame between residues 43 and 43, pYB124 [*FPR3myc*), or the COOH-terminal domain of Fpr3, pYB120 [*FPR3C*), were spotted onto plates containing SD-Leu medium with either glucose (*Glc*) or galactose (*Gal*) as the carbon source and then streaked for single colonies. (*B*) Identical initial concentrations of the same strains as shown in *A*, as well as of the same strain (YB112) carrying the same vector expressing just the NH_2-terminal domain of Fpr3, pYB126 [*FPR3N*), were spotted on SD-Leu plates containing either glucose (*Glc*) or galactose (*Gal*) at serial dilutions ranging from $A_{600\ nm}$ = 0.5 (left-most) to $A_{600\ nm}$ = 0.001 (right-most).

active site of the catalytic domain of Fpr3 would presumably preclude its ability to interact with its normal in vivo substrate(s). When plated on glucose medium, *fpr1* cells carrying either *GAL1-FPR3*, *GAL1-FPR3C*, *GAL1-FPR3N*myc, or the vector alone, grew equally well (Fig. 6). As observed in wild-type cells (data not shown), when the same strains were plated on galactose medium, the *fpr1* cells expressing full-length Fpr3 or Fpr3Nmyc grew much more poorly than cells expressing Fpr3C or the vector only control (Fig. 6). Remarkably, however, when the galactose medium contained

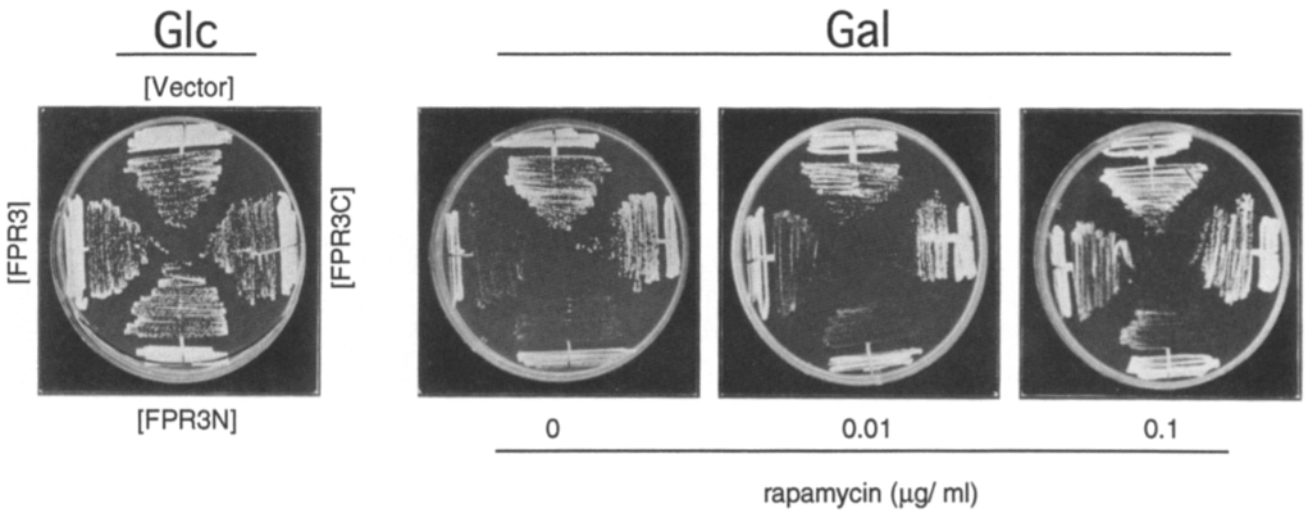

Figure 6. Rapamycin ameliorates the toxicity of Frp3 overproduction. The same strains described in the legend to Fig. 5 were streaked to single colonies on plates containing either glucose (*Glc*) or galactose (*Gal*) as the carbon source in the absence and presence of the indicated concentrations of rapamycin.

even low concentrations of rapamycin, the poor growth of cells expressing Fpr3 was largely reversed, whereas the poor growth of cells expressing Fpr3Nmyc was not spared. (Partial reversal was observed even at concentrations of rapamycin, 0.01–0.1 μg/ml, that are below those necessary to see growth inhibition mediated by the Fpr3C domain). Taken together, these findings indicate that both its COOH-terminal catalytic domain and its NH_2-terminal charged domain contribute significantly to the toxic effect of overproduced Fpr3.

Fpr3 Is Localized to the Nucleolus

The BLASTP search that revealed striking homology of the COOH-terminal 111 residues of Fpr3 to FKBPs also showed that the NH_2-terminal domain possesses nearly as great a degree of homology (36% amino acid sequence identity) over nearly as long a region (98 residues) to nucleolin, a mammalian protein localized in the nucleolus. This similarity is largely confined, however, to long Glu-rich tracts found in both Fpr3 and nucleolin. Although other known nucleolar proteins from both *S. cerevisiae*, like Nsr1 (Lee et al., 1991) and mammals, like Nopp140 (Meier and Blobel, 1992), also possess long tracts of acidic residues punctuated by shorter stretches of basic residues, such strikingly acidic domains are also found in other classes of both nuclear and nonnuclear proteins.

To definitively determine the subcellular compartment in which Fpr3 resides, polyclonal antisera were raised against both the Fpr3N and Fpr3C domains that were expressed in and purified from bacterial cells, and specific antibodies directed against each domain of the protein were further enriched by affinity purification (see Materials and Methods). To demonstrate the specificity of these antibodies, extracts were prepared from cultures derived from a tetrad of *FPR3*$^+$ and *fpr3* null mutant spores (Fig. 7 *B*). As expected, the antibodies specifically recognized the Fpr3 gene product because they detected a species with an $M_{r_{app}}$ of 65 kD that was present in the *FPR3*$^+$ cells, but was absent in the otherwise isogenic *fpr3* null mutants (Fig. 7 *B*). Furthermore, cells carrying multi-copy plasmids expressing *FPR3* greatly overproduced the 65-kD species (data not shown). Similarly, the major 65-kD band normally observed by Coomassie blue staining of the material in our particulate nuclear fractions was missing when such fractions were prepared from an *fpr3* null mutant and more prominent when prepared from cells overexpressing *FPR3* from a plasmid (data not shown).

In our initial experiments, *FPR3* and *fpr3* haploids (and *FPR3/FRP3* and *fpr3/fpr3* diploids) were fixed, permeabilized, stained with the anti-Fpr3 antibodies, and examined by indirect immunofluorescence. We found that a signal could be readily detected in *FPR3*$^+$ cells, but not in *fpr3* mutants, and that Fpr3 was confined to a portion of the nucleus (data not shown). These results provided, first, confirmation of the specificity of the antibodies. Second, Fpr3 could be readily detected even when expressed from its chromosomal locus, consistent with the fact that Fpr3 was a reasonably abundant species in our original nuclear preparations.

To provide greater resolution, however, we also examined the localization of Fpr3 in a commercial brand of baker's yeast, which has a much larger cell size than our standard laboratory strains because it is highly polyploid. Both the anti-Fpr3N antibodies (Fig. 8) and the anti-Fpr3C antibodies (data not shown) specifically stained the nucleolar region of the nucleus in the polyploid cells, which stains very poorly with DAPI. Furthermore, in double labeling studies (Fig. 8), the Fpr3 staining was completely congruent with that of a known and well-characterized yeast nucleolar protein, fibrillarin (*NOP1* gene product) (Aris and Blobel, 1988; Tollervey et al., 1993). Thus, in agreement with the homology of its NH_2-terminal domain to extended portions of other known nucleolar proteins, Fpr3 is likewise localized exclusively in the nucleolus. Furthermore, even when rampantly overproduced from the *GAL1* promoter on a multi-copy vector, Fpr3 protein was present predominantly in the nucleolus, although there was a substantial increase in fluorescence around the entire nuclear periphery that appeared to represent association with the nuclear envelope (data not shown).

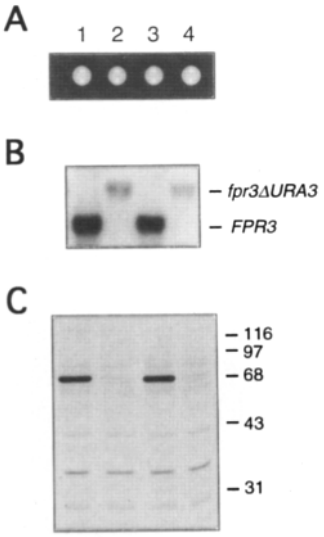

Figure 7. Immunological detection of the *FPR3* gene product. (*A*) Strain YB103 (*MATa/MATα frp3-Δ1::URA3/FPR3*) was sporulated, dissected, and the four spores (lanes *1–4*) of one tetrad were incubated at 30°C on YPD medium for 4 d. (*B*) Genomic DNA isolated from sub-cultures of each of the four corresponding spore clones shown in *A* was digested with HindIII, subjected to electrophoresis in a 1% agarose gel, blotted to a nitrocellulose filter, and hybridized to a radioactively labeled 2.2-kb genomic fragment containing the *FPR3* gene, which revealed the fragments corresponding to the normal *FPR3* locus (lanes *1* and *3*) and the *fpr3-Δ1::URA3* mutation (lanes *2* and *4*). (*C*) Cell extracts were prepared from sub-cultures of each of the four corresponding spore clones shown in *A*, fractionated by SDS-PAGE in a 10% gel, transferred electrophoretically to a nitrocellulose filter, and incubated with affinity-purified antibodies directed against the NH_2-terminus of Fpr3 derived from serum 1139 (prepared as described in Materials and Methods). Molecular weight markers are the same as those described in the legend to Fig. 3.

Discussion

In a search for novel nuclear proteins, we have identified a new gene, *FPR3*, which encodes a previously undescribed type of FKBP and is the third FKBP identified in *S. cerevisiae* to date. Unlike the cytoplasmic Fpr1 (114 residues) and the ER-localized Fpr2 (118 residues, after removal of the signal sequence), which consist only of a folded catalytic and drug-binding domain (Van Duyne et al., 1991), Fpr3 (413 residues) is nearly four times longer by virtue of its striking NH_2-terminal extension. Another unusually large FKBP, p59, has been identified recently in mammalian cells. However, this molecule is comprised of two tandem repeats of the catalytic domain, separated by a spacer sequence (Tai et al., 1993), and is found in both the cytosol and the nucleoplasm

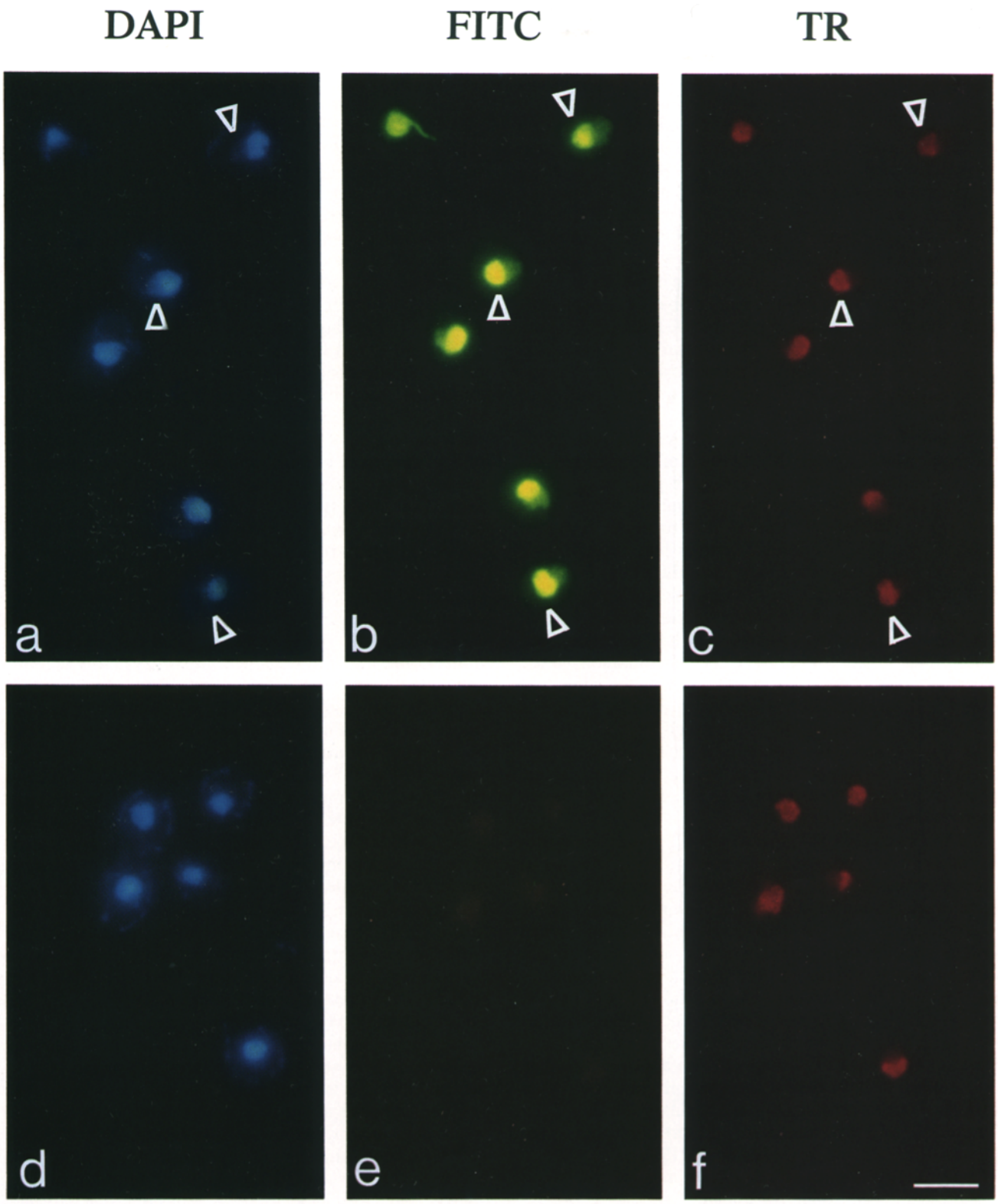

Figure 8. Fpr3 is localized in the nucleolus. Cells of a commercial polyploid strain of baker's yeast (Fleischmann's) were grown in YPD, harvested, fixed, permeabilized, and then incubated simultaneously either with affinity-purified rabbit polyclonal antibodies directed against the NH_2-terminus of Fpr3 (derived from antiserum 1139) and a mouse monoclonal antibody (mAb A66) directed against a known yeast nucleolar marker, fibrillarin (*NOP1* gene product) (*a–c*), or with the IgG fraction from preimmune serum drawn from the rabbit used to prepare anti-Fpr3 antiserum 1139 and the mouse anti-fibrillarin monoclonal antibody (*d–f*). The rabbit antibodies were then stained using FITC-conjugated goat anti–rabbit immunoglobulin (*FITC*) and the mouse antibody was stained using Texas Red-conjugated goat anti–mouse immunoglobulin (*TR*). Both sets of cells were counter-stained with DAPI to reveal the position of the chromosomal DNA within the nucleus (*DAPI*). The nucleolus stains relatively poorly with DAPI because of its high RNA and protein content. Bar, 2 μm.

(Alnemri et al., 1993). Hence, this mammalian FKBP differs in both structure and localization from Fpr3.

Despite its unusual structure, Fpr3 possesses PPIase activity in vitro, and can be inhibited by both rapamycin and FK506. Because the first 60 amino acids of Fpr3 contain eight X-Pro bonds (almost as many X-Pro bonds as there are in the remainder of the 301-residue NH_2-terminal extension), it seemed reasonable that the NH_2-terminus might act as an intramolecular autoinhibitory domain. However, we found that the specific activities of purified full-length Fpr3 and the isolated COOH-terminal catalytic domain, Fpr3C, were virtually indistinguishable within the limits of sensitivity of the assay used. As has been observed for other FKBPs (Park et al., 1992), Fpr3 promoted a somewhat faster rate of isomerization for a substrate containing a Leu-Pro bond compared to an otherwise identical substrate with an Ala–Pro bond. Members of the other family of PPIases, the cyclophilins, display the opposite substrate preference (Harrison and Stein, 1990).

We found that *fpr3* null mutations had no discernible phenotype. We suspected that, because of its small size (12 kD), the normally cytosolic *FPR1* gene product might be able to pass through nuclear pores and might, therefore, be able to substitute for the loss of Fpr3 function. However, even *fpr1 fpr2 fpr3* triple null mutants had no detectable growth defect. If the critical function of FKBPs is related to their PPIase activity, it is possible that the cyclophilins may serve a redundant, or overlapping, function in cells. Indeed, in *S. cerevisiae*, cyclophilin homologues have been described which reside in the cytoplasm (*CYP1* gene product), in the lumen of the ER (*CYP2* gene product), and in the mitochondrion (*CYP3* gene product) (Haendler et al., 1989; Koser et al., 1991; Davis et al., 1992; McLaughlin et al., 1992). Thus, it would not be too surprising if one or more cyclophilins also were localized in the nucleus and/or the nucleolus, which may explain the absence of an obvious phenotype when the cell lacks Fpr3.

If FKBPs and cyclophilins are involved in promoting protein folding and protein assembly events in vivo, it might be anticipated that in the absence of these functions cells might be more sensitive to conditions that place more stress on the cells capacity to carry out such processes, for example when proteins are caused to unfold by a heat shock or if normal folding reactions are slowed down by reducing the temperature. Indeed, it has been found that mutations in either of two cyclophilin genes, *CYP1* or *CYP2*, reduce the survival of yeast cells following a heat shock (Sykes et all, 1993). In contrast, we found that *fpr3* mutants are not more sensitive to heat shock than their otherwise isogenic controls (neither were they more cold sensitive). Similarly, it has been reported that *fpr1* null mutants are not more heat shock sensitive than *FPR1*$^+$ controls (Sykes et al., 1993).

Conversely, in the absence of FKBPs and cyclophilins, it is possible that cells might accumulate higher levels of misfolded proteins and/or aggregated unassembled proteins than wild-type cells, which might act as a signal to induce stress responses. In fact, consistent with a potential role during stress response, transcription of both the *CYP1* and *CYP2* genes is modestly induced (two- to threefold) when yeast cells are subjected to a heat shock; and the promoters of both genes contain sequences that match the consensus heat shock response element (HSE) (Sykes et al., 1993). In contrast, transcription of the *FPR1* gene is not heat shock-responsive (Sykes et al., 1993). Similarly, the 5′-flanking region of the *FPR3* gene lacks any sequence resembling an HSE. As one means to determine if misfolded proteins might be accumulating in cells lacking *fpr3* function even under normal growth conditions, we introduced a reporter plasmid, *SSA1-lacZ*, which contains an HSE and is highly induced upon heat shock (Slater and Craig, 1987). We found that *fpr3* mutants did not display any elevation in the basal level of expression of the *SSA1-lacZ* reporter plasmid, compared to isogenic *FPR3*$^+$ control cells, and did not show any defect in the induced level of expression of this reporter gene (B. Benton, unpublished results). Hence, currently there is no evidence that Fpr3, or any of the other FKBPs yet identified in yeast, play any role during response to stress.

One of the most striking features of Fpr3 is its strict subcellular localization. Using either of two different polyclonal antibodies, each directed against a different domain of the protein, it was found that Fpr3 resides exclusively in the nucleolar region of the nucleus. The original identification of Fpr3 as a major protein in preparations of isolated nuclei, and cell fractionation experiments which have been performed subsequently (B. Benton, unpublished results), are fully consistent with the compartmentation of Fpr3 in the nucleolus. Furthermore, these observations suggest that Fpr3 is very tightly associated with other elements in the nucleolus because little or no Fpr3 appears to leach out from the nuclei during cell lysis and fractionation. Moreover, only 20% or so of the Fpr3 can be extracted into soluble form from isolated nuclei by treatment with high salt (0.5 M NaCl) and none is extracted by the non-ionic detergent (1% Triton X-100). On the other hand, more chaotropic agents are somewhat more efficacious in extracting Fpr3 into the soluble fraction. For example, when isolated nuclei are treated with 0.3 M $(NH_4)_2SO_4$, about half of the Fpr3 is released into the soluble fraction (B. Benton, unpublished results).

We also attempted to localize Fpr3 using a derivative in which a 12-residue c-Myc epitope-containing tag was inserted between amino acids 43 and 44 in the Fpr3 sequence because the cognate anti-c-Myc mAb 9E10 gives little or no background staining of yeast cells (see, for example, Kuchler et al., 1993). However, even when the corresponding gene (*FPR3*myc) was carried on a low-copy-number (*CEN*-based) plasmid and introduced into strain YB104 (*MATa*/*MATα*-*fpr3-Δ1::URA3*/*fpr3-Δ1::URA3*), which lacks any endogenous Fpr3, the c-Myc–tagged Fpr3 was not confined solely to the nucleolus, but was found more diffusely throughout the nucleus. In most cells, the Fpr3myc colocalized with the nuclear DNA, but in some cells it was also observed in small vesicle-like bodies in the cytosol. Furthermore, in cells that were apparently premitotic because they contained a large bud in which the nucleus had migrated from its central location in the mother cell into the bud neck, Fpr3myc was frequently found within a DNA-less projection that extended from the mother cell nucleus into the bud. This kind of structure is thought to define an early stage of nuclear migration (Davis and Fink, 1990), but it is also reminiscent of the "tracks" seen by Meier and Blobel (1992) that appear to shuttle other nucleolar proteins between the nucleolus and the cytoplasm in animal cells. Curiously, when just the NH_2 terminus of the c-Myc–tagged Fpr3 was expressed in yeast cells, this domain of the protein targeted very efficiently and

specifically to the nucleolus, just like authentic Fpr3 (Zang, 1994). Thus, it is not clear why, in the context of full-length Fpr3, the presence of the short epitope sequence appears to perturb its normal localization. Nonetheless, based on at least this case, conclusions about subcellular distribution based on an epitope-tagged protein should be interpreted with caution.

Localization of Fpr3 to the nucleolus suggests possible roles for this PPIase in the folding of ribosomal proteins or rRNA processing enzymes (perhaps during their import into this region of the nucleus), in the assembly of preribosomes, and/or in the export of ribosomes to the cytoplasm. Most of the nucleolar genes believed to be involved in the maturation of rRNA or of ribsomes cause a pronounced growth defect when mutated. Furthermore, mutations in these genes lead to an accumulation of the 35S pre-rRNA precursor, and result in a concomitant diminution in the steady-state level of the 18S and 25S rRNAs (Woolford and Warner, 1991). However, neither *fpr3* null mutants nor normal cells acutely overproduce *FPR3* from the *GAL1* promoter on a multi-copy vector (a condition that is toxic to the cells) showed any marked change in the steady-state level of the 18S and 25S rRNA species compared to control cells, although in *fpr3* mutants there is some modest accumulation of the 35S rRNA precursor (B. Benton, unpublished results). Thus, Fpr3 appears to have a more subtle role in the metabolism of the nucleolus.

Whether Fpr3 has a specific role in the nucleolus as a consequence of its localization to this compartment or because it has specificity for substrates that are restricted to the nucleolus, or both, remains to be determined. There are both genetic and biochemical approaches that can be used to help elucidate the function of Fpr3 and to identify its physiologically relevant substrates. Because overproduction of Fpr3 is toxic to cells, we have been able to select several dosage suppressors from a cDNA library driven by the *GAL1* promoter (Liu et al., 1992), which rescue this growth inhibition when coexpressed with *GAL1-FPR3* in the same cell. One of these suppressors encodes ribosomal protein L34 (B. Benton and J.-H. Zang, unpublished results), for which there are two separate genes in the *S. cerevisiae* genome (Woolford and Warner, 1991). We are currently pursuing other approaches to determine if the interaction between Fpr3 and L34 is physiologically meaningful. Furthermore, like certain other nucleolar proteins, including nucleolin (with which Fpr3 shares significant homology in its NH_2-terminal domain) (Belenguer et al., 1990; Peter et al., 1990), Fpr3 is phosphorylated in vivo and the phosphorylation sites are confined to the NH_2-terminal extension (L. Wilson, B. Benton, J. Thorner, and G. S. Martin, manuscript in preparation). Whether these modifications have any consequence for the activity, regulation, localization or stability of Fpr3 is currently under investigation.

Finally, some of our results may bear on the spatial restriction of the signal transduction components that are the targets for the growth inhibitory effects of rapamycin-FKBP complexes. Since Fpr3 was properly localized to the nucleolus even when greatly overproduced, yet rapamycin was able to reverse the toxic effects of Fpr3 overproduction, rapamycin can presumably enter the nucleus. In contrast, the isolated COOH-terminal domain (Fpr3C) of Fpr3 was a cytosolic protein, was not deleterious to growth when overproduced, yet was able to mediate the growth inhibitory action of rapamycin in cells lacking Fpr1, the major cytosolic FKBP. Similarly, overproduction of a normally ER-localized cyclophilin does not increase the sensitivity of T cells to cyclosporin A-mediated inhibition of calcineurin; but, if its signal sequence is removed, such that the cyclophilin is now overproduced in the cytosol, calcineurin inhibition can be achieved at lower doses of cyclosporin A (Bram et al., 1993). Therefore, our results suggest, first, that the target(s) of the growth inhibitory action of rapamycin-FKBP complexes (presumably Tor1 and Tor2) are only accessible from the cytoplasm. Since rapamycin binds to the active site of FKBPs and inhibits their PPIase activity, the second conclusion from our observations is that overproduced Fpr3 is toxic because it is acting inappropriately on one (or more) of its normal substrates or is interacting with an inappropriate substrate within the nucleolus. Further analysis of the dosage suppressors we have isolated may help resolve this latter issue.

The authors thank Felicia Etzkorn and Christopher Walsh for measuring the proline rotamase activity of our purified Fpr3C protein; Linda Riles, Maynard Olson, and Mark Johnston for providing the collection of lambda phages carrying the entire *S. cerevisiae* genome and for the communication of gene locations on the physical map; Robert Hamataki, Akio Sugino, Forrest Spencer, and Phil Hieter for the gift of yeast genomic DNA libraries; John Aris for supplying mAb no. A66 directed against yeast fibrillarin; J. Michael Bishop for providing hybridoma cell line c-*myc* 9E10; Gary Anderson for providing the yeast chromosome blot; Randy Hampton for the gift of plasmid pJR350; Michael Hall for providing plasmid pYJH25A; Suren Sehgal and Wyeth-Ayerst Research Laboratories, Inc. for the gift of rapamycin; Ihor Bekersky and Fujisawa USA, Inc. for the gift of FK506; Randy Schekman for use of his PhosphorImager; Jim Onuffer, Mallory Haggart, and Michael Moore for the synthesis of oligonucleotides; and Paul A. Fisher for his advice and encouragement.

This work was supported by a postdoctoral fellowship (PF-3566) from the American Cancer Society and by a postdoctoral traineeship CA09041 from the National Cancer Institute (to B. M. Benton), by a Chancellor's Undergraduate Scholarship from the University of California at Berkeley (to J.-H. Zang), and by National Institutes of Health Research Grants GM21841 and GM44173 (to J. Thorner).

Received for publication 16 June 1994 and in revised form 28 July 1994.

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