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Purification of FKBP-70, a novel immunophilin from *Saccharomyces cerevisiae*, and cloning of its structural gene, *FPR3*

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Abstract A novel protein, belonging to the yeast family of FKBPs (FK-binding proteins), FKBP-70, was isolated from *Saccharomyces cerevisiae* by its interaction with the immunosuppressive agent FK-520. Its structural gene, *FPR3*, was cloned and the protein expressed and purified from *Escherichia coli*. This third member of the FKBP family in yeast is homologous to the other FKBPs at its carboxy terminus, showing conserved ligand binding and proline isomerase regions. It is, however, a longer acidic protein with several potential nuclear targeting sequences and a region of homology to nucleolins. Yeast strains deleted for *FPR3*, as well as a triple deletion mutant of this family of genes, *FPR1*, *FPR2* and *FPR3*, are viable under normal conditions of growth, indicating that the *FPR* genes are not essential for life.

Key words: Immunosuppression; FK506; PPIase; Ascomycin; Yeast; CsA

1. Introduction

The immunosuppressive macrolides, FK-506 and its derivative FK-520 (ascomycin) interfere with T-cell activation. These agents promote the association of a cytosolic binding protein FKBP-12 and the type 2B phosphatase calcineurin, inhibiting its activity. Calcineurin is thought to be required for dephosphorylation and nuclear translocation of NF-AT, a positive transcription factor for lymphokine IL-2. The immunosuppressive cyclic peptide cyclosporin A (CsA), extensively used in organ transplantation, also inhibits calcineurin in association with its binding protein, cyclophilin (for general reviews see [1,2]). In addition, several novel proteins that bind to either FK-derivatives or CsA, collectively termed immunophilins, have been identified in many species from prokaryotes to man. They constitute two highly conserved protein families [3]. The peptidyl-prolyl *cis-trans* isomerase (PPIase or rotamase) activity associated with these immunophilins is inhibited by the respective ligands and is thought to play a role in protein folding [4]. Biochemical and genetic studies in yeast have also identified immunophilins and demonstrated that the activity of the immunosuppressive agents in yeast is analogous to their activity in mammalian cells [5–7]. At present, a cytosolic FKBP-12 [8], and an endoplasmic reticulum-associated FKBP-13 [9] have been identified. We have now isolated a novel protein, FKBP-70, a third member of the FKBP family by ascomycin affinity chromatography. We report the partial FKBP-70 protein sequence, the complete primary sequence of the gene, FPR3, and the encoded protein as well as its expression in *E. coli*. We show by gene disruption experiments that the presence of FKBP-70 is not essential for life and in addition that yeast strains simultaneously disrupted for all three *FPR* genes are viable.

2. Materials and methods

2.1. Microbial strains

Diploid (a/α) and haploid (a and α) derivatives of JK93d (*leu2-3,112, his4, trp1, ura3-52, rme1*) and MH287 a/α, JH2-1ba and JH2-1cα (derived from the respective JK93d strains by introduction of the *fpr1::ADE2* disruption) were previously described [10]. JH4-9B is a derivative of JK93d a/α with *cpr1::LEU2* and *fpr1::URA3* disruptions (a gift of J. Heitman). The following strains were constructed for this work: RH211, derived from JK93dα by introducing the *fpr3::LEU2* disruption; RH201α, derived from JK93dα by introducing the *fpr2::URA3* disruption, and RH202α, derived from JH2-1c by introducing the *fpr3::LEU2* disruption. RH205α is the RH201 derivative with all three *FPR* gene disruptions, *fpr1::ADE2, fpr2::URA3* and *fpr3::LEU2*.

2.2. General protocols

All procedures employing *E. coli* (strain JM 109) and other molecular biology protocols were from Sambrook et al. [11]. Yeast protocols were those decribed in [12]. DNA sequencing was performed using the Pharmacia T7 sequencing kit. PCR protocols were according to the instructions included in the GeneAMP kit (Perkin-Elmer).

2.3. Protein purification and sequencing

An affinity resin was prepared by incubating 25 ml Affigel-10 (Bio-Rad) and 30 mg of 32-*O*-(3-amino-propanoyl)-ascomycin in 25 ml of 10 mM $NaBO_3$ pH 8.5, 50% v/v ethanol overnight. The resin was quenched with 1 M ethanolamine-HCl pH 8.5, 50% ethanol, washed using alternating cycles of 5 mM glycine-HCl pH 2.5 or 0.5 M NaCl, 20 mM Tris-HCl pH 8.0, and then stored in 100% ethanol at −20°C until use.

A 5-l yeast culture was harvested in the exponential growth phase and cells were lysed using Zymolyase (Seikagaku, Japan). Homogenization was performed in buffer A (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% w/v Triton X-100, 5 mM β-mercaptoethanol, 1 mM EDTA, and protease inhibitors (0.5 mM Pefabloc, 5 μg/ml soybean trypsin inhibitor, 2 μg/ml antipain, 5 μg/ml leupeptin, 1 mM benzamidine) using a Dounce homogenizer. A precleared cell extract (250 ml) was prepared and loaded onto a 5 ml affinity column, equilibrated in buffer A without the protease inhibitors. The column was washed with 250 ml buffer A containing 0.1% Triton X-100, and then with detergent-free buffer. Elution was with 20 mM glycine-HCl pH 2.5; the fractions were immediately neutralized with Na-phosphate buffer and then analyzed by reducing SDS-PAGE. For protein sequencing, the peak fractions (40 μg FKBP-70) were precipitated using trichloroacetic acid (10% w/v), and run on 12.5% polyacrylamide gels. These were then blotted

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onto PVDF membranes, the proteins detected by Coomassie-blue staining, and the appropriate bands excised [13]. Sequencing was done on an ABI 470A gas phase protein sequencer. N-terminal sequencing was attempted directly from the blotted material using the method developed by Shively et al. [14]. For sequences of internal peptides, the membrane pieces were first incubated with 1 ml 0.5% (w/v) polyvinylpyrolidone for 30 min at room temperature, washed once with 100% methanol and four times with water. Lys-C protease was added at 1/10th (w/w) the estimated amount of blotted protein, and incubated overnight with the membrane in 200 μl 100 mM Tris-HCl pH 8.1 at 37°C. The peptides were solubilized by adding guanidinium-HCl, before the sample volume was reduced to 180 μl using a speed-vac concentrator (final guanidium- HCl concentration was 6 M). The material was then treated with 100 equivalents of dithiothreitol dissolved in 10 μl 6 M guanidium-HCl,0.5 M Tris-HCl pH 8.1 (buffer B) under argon for one hour at 37°C, and furthermore with 300 equivalents of iodoacetamide dissolved in 10 μl buffer B for 30 min at room temperature, again under argon and in the dark. The supernatant was then injected onto reverse phase HPLC (Applied Biosystems 130A system equipped with a Brownlee RP 300 C8 column, 2.1 × 30 mm) and eluted with a gradient of solvents A and B (solvent A: 0.1% (v/v) trifluoroacetic acid (TFA); solvent B: 70% (v/v) acetonitrile, 30% water, 0.08% TFA): 0–10 min 0% B, 11–30 min 0–60% B, 31–40 min 60–100% B. The peaks were collected manually and the material from individual peaks was applied directly to precycled glassfilter disks (exposed to 3 mg Polybrene) for sequencing. PTH amino acid analysis was performed using an on-line ABI 120A PTH analyzer.

Recombinant FKBP-70 was purified from a 1-l culture of *E. coli* W3110C^{I} that was induced for expression of this protein by shifting an exponentially growing culture from 30°C to 42°C and continued incubation for 3 h. Bacterial pellet was lysed in the presence of 1 mg/ml lysozyme, 20% w/v Sucrose and 30 mM Tris-HCl pH 8.0 for 15 min on ice. The suspension was then diluted 10-fold in buffer A and treated subsequently as detailed for the preparation from yeast.

2.4. Generation of PCR probes, library screening and gene isolation

Two degenerate oligonucleotides designed from two internal peptide sequences were used in a PCR with yeast chromosomal DNA as template: a sense oligonucleotide, 5′-TGG GAY ATY GGI GTK GCY GGI ATG TC-3′ (from peptide WDIGVAGMS) and an antisense oligonucleotide 5′-GC WGG RAT ICC WGG HAR RGC TTG-3′ (from peptide QALPGIPA), where Y = C or T, I = Inosine, K = G or T, W = A or T, H = A or C or T, and R = A or G. The resulting 124 bp PCR fragment was subcloned into Bluescript SK^{+} vector (Stratagene) and sequenced using adjacent primers. Based on the internal DNA sequence unique to this fragment, two novel oligonucleotides with the sequence 5′-GTTGGTGGCGAACGTAGAATCA-3′ (bases 2626–2647, Fig. 2) and 5′-GGAATACCTGGCAGAGCTTGCT-3′ (complimentary sequence to bases 2675–2696, Fig. 2) were synthesized and utilized in PCR with the parent vector as template to generate a 70 bp product. A radioactive probe of this fragment obtained by random primer labelling was used to screen a cosmid library of yeast genomic DNA in the *E. coli* vector pWE15 (Stratagene). A positive cosmid clone containing yeast genomic DNA of approximately 35 kb was identified by hybridisation. This was mapped by restriction enzyme analysis and Southern hybridisation to the 70 bp probe. A 4 kb *SalI–NotI* DNA fragment containing the protein coding sequence was subcloned into the pGEM5Zf^{+} vector (Promega), generating plasmid pFC1. A segment of yeast genomic DNA of 2943 bp was sequenced from this plasmid by the dideoxy sequencing method using adjacent vector primers and several internal oligonucleotides (data not shown).

2.5. Construction of E. coli *plasmids for expression*

A sense oligonucleotide with the sequence 5′-GGGCCCGGATCCATGGCTGATTTGTTACCACTA-3′ corresponding to the amino-terminal portion of the open reading frame and an antisense oligonucleotide 5′-GTCGACGGATCCAAGCTTTCTAGACTAGTTTTTCATAGAAACCAA-3′ derived from the carboxy-terminal part were used in a PCR with the plasmid pFC1 as template. The product of nearly 1250 bp containing the coding region (with encompassing *NcoI* and *BamHI* sites specified by the primers) was cloned into the *E. coli* expression vector pL-RCV downstream from the thermoinducible lambda promoter pL. The resulting plasmid, pLFKBP-70 was introduced into the *E. coli* strain W3110C^{I} (carrying a temperature sensitive lambda repressor on its chromosome). Growth at high temperature (42°C) of this bacterial strain resulted in the production of recombinant FKBP-70 (for a description of pL-RCV and subsequent protein induction methods, see Lokker et al. [15]).

2.6. Yeast plasmids and strains deleted for structural genes

A 2.2 kb DNA fragment specifying the complete *FPR3* gene was obtained from plasmid pFC1 by treatment with restriction endonuclease *HindIII* and subcloned into the 2-micron vectors YEPlac112 and YEPlac195 and into the centromeric vectors YCPlac22 and YCPlac111 [16], resulting in plasmids YPH 105, YPH 103, YPH 106 and YPH 76 respectively. A 2.2 kb *XhoI–SalI* fragment from plasmid YEP13 [12] specifying the *LEU2* gene was cloned into the single *AccIII* site within the coding region of the *FPR3* gene in plasmid pFC1, by treatment with Klenow enzyme prior to ligation. Plasmid pF70L, resulting from this ligation contained the *LEU2* gene inserted within the *FPR3* gene in antisense orientation, flanked on its 5′ and 3′ sides by *FPR3* fragments of 1.3 and 1 kb, respectively. The entire region was reisolated from pF70L as a 4.5 kb DNA fragment upon treatment with HindIII and transformed into yeast cells. The *FPR2* gene disruptions contained a 1.12 kb *SmaI–ClaI* fragment from YEP24 [12], specifying the *URA3* gene, cloned into the structural gene between *PacI* and *NaeI* sites (thus replacing amino acid residues 30 to 80 of FKBP-13 ([9], and our unpublished results). Transformants were selected for growth on media lacking leucine. Chromosomal *FPR* gene disruptions were verified by restriction analysis, Southern blotting and hybridization to labeled probes (data not shown). Diploid strains with confirmed disruptions were sporulated and dissected. Segregation of the inserted *LEU2* gene was monitored both by testing growth on media lacking leucine and by Southern blotting of DNA prepared from the haploid progeny. Growth was assessed directly on rich medium, at 14°, 20°, 30°, 37°, and 40°C, and at 30°C in the presence of 1 M NaCl, 1 M sorbitol, 100 μg/ml FK-520 or 50 mM Tris-HCl pH 8.6 buffer to score for altered phenotypes.

2.7. Computer analysis

EMBL, Gen Bank, SWISSPROT, PIR data bases were accessed for analyses of proteins and nucleic acids using UW-GCG software.

3. Results and discussion

3.1. Purification and microsequencing of FKBP-70

Affinity chromatography of yeast extract prepared from strain JH4–9B, lacking FKBP-12, reproducibly yielded three major bands in a Coomassie-stained SDS-polyacrylamide gel (Fig. 1A, lane 4): a 70, 60 and 12 kDa species. The yield of the 70 kDa protein was typically estimated to be in the range of 100 μg with variable quantities of the 60 kDa and 12 kDa proteins. Direct sequencing of both the 70 and 60 kDa species was not possible due to blocked N-termini. The 12 kDa species was identified as the the YS24 gene product, the S22 protein [17] of the 40S small ribosomal subunit, based on its amino-terminal sequence. Copurification of this protein could have resulted from its interaction with either the ascomycin resin (even though this protein shows no homology to any known FKBPs) or with FKBPs bound to the column. Both 70 and 60 kDa proteins were treated with Lys-C endoprotease and several resulting peptides were sequenced. Two peptides A and B of sequence GWDIGVAGMSVGGERRII (362–379, Fig. 2) and QALPGIPANSELTFD (389–405, Fig. 2) were obtained from both the 70 kDa and 60 kDa species. This suggested that the 60 kDa protein may have resulted from limited proteolysis of the 70 kDa FKBP during purification. Incidentally, sequencing of the peak fraction containing peptide A from the 70 kDa species also yielded a second minor sequence, RNPDFEDDDFLGGD (57–70, Fig. 2). Two additional peptide sequences, HPFDTPMGVEGEDEDEDADI (164–183,

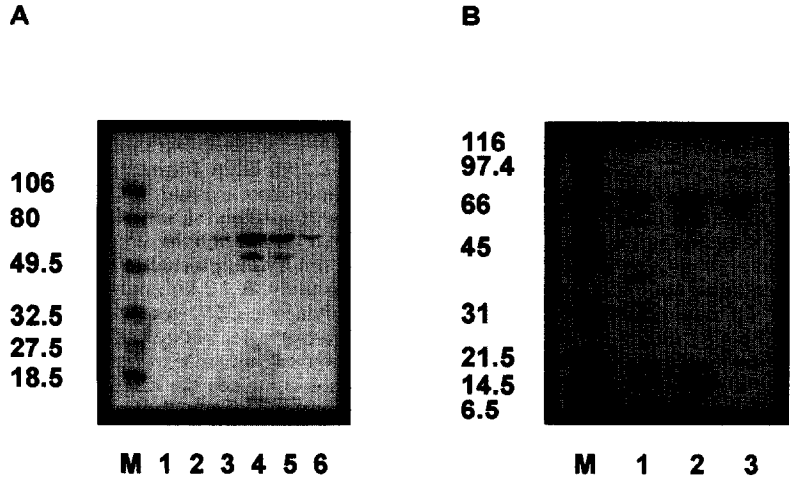


Fig. 1. Purification of FKBP70. (A) Elution of yeast FKBP70 from the Ascomycin affinity resin. Fractions 4 and 5 contain the majority of the material released by low pH. (B) Comparison of FJBP70 isolated from yeast (lanes 1 and 2) and FKBP70 expressed in *E. coli* (lane 3). Sizes of molecular mass standards are indicated on the left.

Fig. 2) and VLEGGIVIEDRTIGDGPQ (303–320, Fig. 2) were obtained from the 70 kDa species.

3.2. *Isolation of the* FPR3 *gene and homology of FKBP-70 with other proteins*

The amino acid sequences of peptides A and B and their connecting sequence as derived from the initial PCR product, revealed high similarity to members of the FKBP family including FKBP-12 and FKBP-13 from yeast (Fig. 3c). Subsequent isolation and sequencing of a yeast DNA fragment from pFC1 identified a reading frame of 1233 bp encoding a novel protein of 411 amino acids (Fig. 2). Detection of a 1.5 kb messenger RNA by Northern analysis (data not shown) and the presence of upstream transcription regulatory sequences were consistent with the length of the reading frame. This structural gene for the novel FKBP was designated *FPR3* (*FK-binding proline rotamase 3*), consistent with the nomenclature of previously identified yeast FKBPs, *FPR1* and *FPR2* [8,10].

Comparison of FKBP-70 with existing sequence databases revealed two separate regions of homology to previously known proteins. The carboxy-terminal quarter of the molecule (321–411) was homologous to the other yeast FKBP proteins (Fig. 3c) as well as the non-yeast members of the FKBP immunophilin family (data not shown). The high degree of conservation included the residues involved in drug binding and constituting the rotamase active site (see legend, Fig. 3c). Recombinant FKBP-70 showed FK-506 sensitive rotamase activity as measured by the method of Kofron *et. al* [32], albeit six fold lower than recombinant human FKBP-12 (Fig. 4).

The region of FKBP-70 extending from amino acids 41 to 266 is extremely rich in acidic residues (99 out of 226). The primary sequence from residues 41 to 300 contains several clusters of negatively charged residues and four positively charged regions. This kind of charge clusters in polypeptides have been implicated in membrane association and nuclear localization [19]. Amino acids 209 to 270 show high homology (32/61 identical) to a region (amino acids 239 to 297) of Chinese hamster nucleolin ([20] and Fig. 3b) and its counterparts in other vertebrates [21]. In this alignment the functional nuclear localization signal (NLS) of nucleolin (KRKKEMTKQKEVPEAKKQK, positions 279–297) is displayed at the carboxy-terminal end [22]. Potential NLSs [23] in FKBP-70 include the homologous sequence KSKKEKKRKHEEKEEEKKAKK, (residues 251–270) as well as other stretches of basic amino acids (in particular KKKKSKGKK, residues 91–99). The acidic domain of nucleolin has been shown to regulate chromosome decondensation [24], and bind to the NLSs of other proteins destined for import into the nucleus [25]. However, the RNA binding domain and Gly/Arg domains of nucleolin, important for its localization and function in the nucleolus [26], have no counterpart in FKBP-70. A mammalian protein with a similar FKBP domain, FKBP-25, that localizes to the nucleolus and associates with casein kinase II and nucleolin was recently described [27]. FKBP-70 may play a role in protein folding in the nucleus, analogous to that of FKBP-12 in the cytosol [28], and of FKBP-13 in the endoplasmic reticulum [29]. The unique aminoterminal portion of the FKBP-70 may be responsible for its localization and target specificity, while the rotamase domain would participate in the protein folding functions.

3.3. *Expression of FKBP-70 in* E. coli

The calculated molecular weight of FKBP-70, based on its amino acid sequence, is 46.5 kDa, and is different from the observed size of 70 kDa. The recombinant FKBP-70, purified from *E. coli* displayed an electrophoretic mobility of approximately 69 kDa (Fig. 1B, lane 3) raising the possibility that, in yeast, FKBP-70 may have additional post translational modifications. Sequencing of the recombinant protein confirmed the initial 30 amino acids residues. We conclude that the cloned gene encodes the complete protein and that its abnormal migration in SDS-PAGE is due to its primary sequence. Its high content of acidic amino acid (theoretical pI 4.2) might be responsible for this aberrant migration, as reported for the clathrin assembly protein AP180 [30].

3.4. *Disruption of the FPR3 gene*

Disruption mutants of *FPR3*, interrupting the coding region at proline 127 in the structural gene, were viable, indicating that FKBP-70 is not essential for vegetative growth. Therefore, all

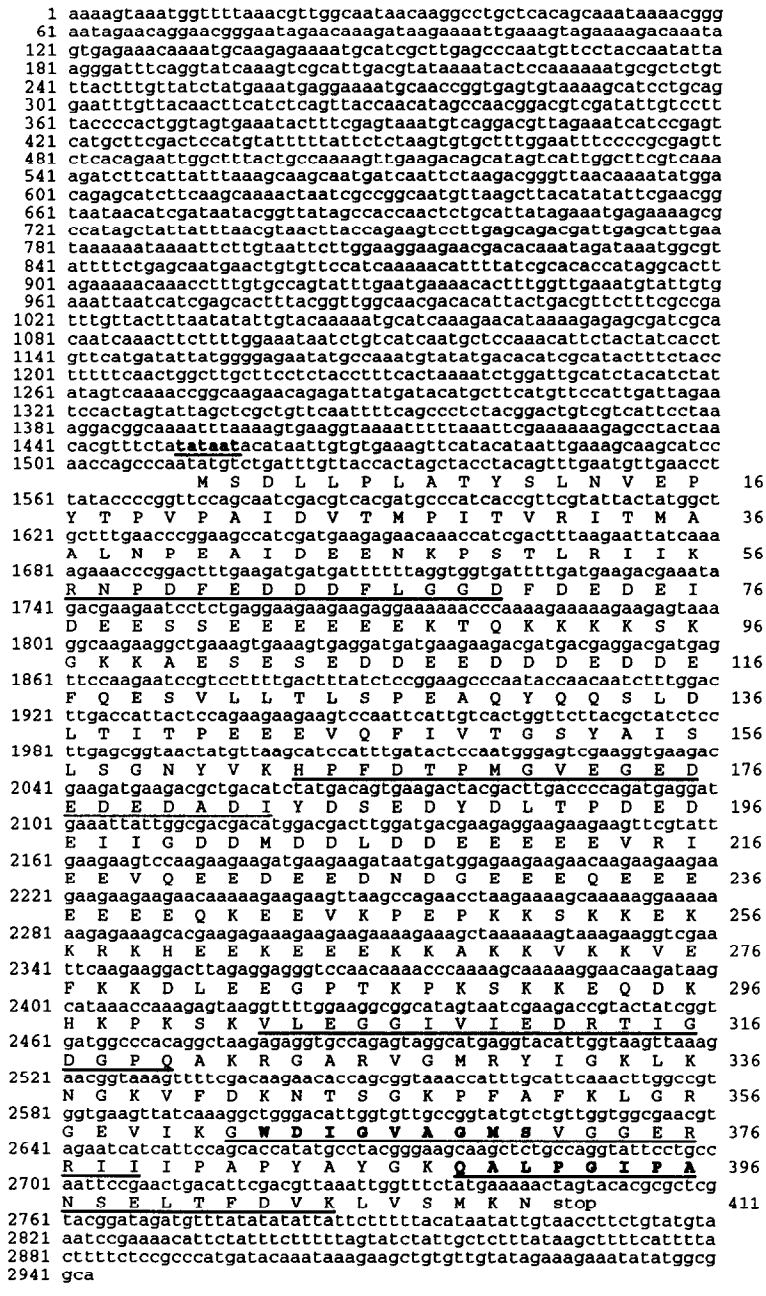
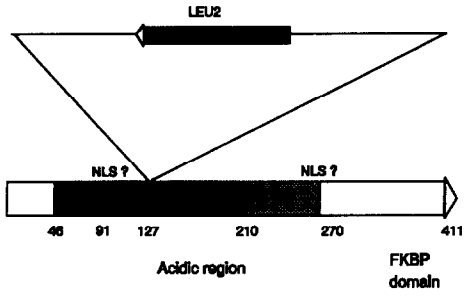


Fig. 2. Nucleotide sequence of the cloned FPR3 gene (numbered on the left) and amino acid sequence of the FKBP70 protein (numbered on the right). A potential promoter element is indicated on the nucleotide sequence (bold, underlined). Partial protein sequence obtained from internal peptides (underlined), as well as peptide sequence used to derive PCR primers (bold) is indicated.

three members of the *FPR* gene family, *FPR1*, *FPR2* and *FPR3*, share this characteristic ([8,9] and this work). We were unable to observe altered growth phenotypes in strains disrupted for *FPR3* under different conditions of temperature, osmolarity, pH of the growth medium or in the presence of ascomycin. The *FPR3* null mutants would survive if FKBP-70 did not encode an essential function or if this function could be provided by other genes. If the latter were the case, mutations in multiple genes would be required for defective growth phenotypes. Southern blot analysis of genomic DNA from haploid strains with an *FPR3* probe indicated that it is present as a single copy gene (data not shown). Other likely candidates for this shared role are the two homologous *FPR1* and *FPR2* rotamases, although specific subcellular compartmentalisation of these proteins may prevent them from compensating for each other. We have examined this possibility by constructing a triple mutant yeast strain, simultaneously disrupted for the three *FPR* genes. As this strain was viable, we conclude that the yeast strains can survive with the reduction in the isomerase activity contributed by these three proteins and the survival of individual *FPR* null mutants cannot be a consequence of one of the other two homologous genes compensating for its function. Neither low nor high copy number yeast plasmids with the cloned *FPR3* gene inhibited the growth of wild type yeast strains on selective

3a.



3b.

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       210        220        230        240        250        260        270
FKBP70  EEEEE-VRIEEVQEEDEEDNDGEEEQEEEEEEEEQKEEVKPEPKKSKKEKKRKHEEKEEEKKAKKVK
        ||:::    || :|::|||:::||::|||||||:.|.|||.| |.|||.. |:.| .|.||.|
NucH    EEDDDD---EEEDEDEEEDEEEEEDEEEEEEEEEEEPVKPAPGKRKKEMT-KQKEVPEAKKQKV
       240          250        260        270        280        290      298
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3c.

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FKBP-12  SEVIEGNVKI-DRISPGDGATFPKTGDLVTIHYTGTL-ENGQKFDSSVDRGSPFQCNIGV
FKBP-13  GSLSDLEIGIIKRIPVEDCLIKAMPGDKVKVHYTGSLLESGTVFDSSYSRGSPIAFELGV
FKBP-70                        AKRGARVGMRYIGKL-KNGKVFDKN-TSGKPFAFKLGR
                               .. *. *  .*.* * ..* .**..  .*.*... .*.

FKBP-12   GQVIKGWDVGIPKLSVGEKARLTIPGPYAYGPRGFPGLIPPNSTLVFDVELLKVN
FKBP-13   GRVIKGWDQGVAGMCVGEKRKLQIPSSLAYGERGVPGVIPPSADLVFDVELVDVKSAA
FKBP-70   GEVIKGWDIGVAGMSVGGERRIIIPAPYAYGKQALPG-IPANSELTFDVKLVSMKN
          * ****** *.....**..... ** ..*** .. ** **... *.***.*. ..
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Fig. 3. (a) Domain organization of the FKBP-70 protein, indicating point of disruption with the LEU2 gene fragment (black). Numbers indicate key aminoacid residues. Acidic domain (dark and light gray), with region of homology to nucleolin (light gray) and immunophilin homology domain (FKBP domain) are highlighted. Arrowheads indicate C-terminus. NLS?, potential nuclear localization signals (hatched). (b) Sequence comparison between FKBP70 and Hamster nucleolin (NucH), showing the region of highest homology using GAPcomputer program (UW-GCG). Residues identical (|), highly conserved (:), and less conserved (·) are indicated. (c) Homology among FKBP proteins of *S. cerevisiae*. The complete amino acid residues of FKBP-12 and FKBP-13, and the amino acid residues 321 to 411 of FKBP-70 were compared: (*) indicates identical residues; (·) indicates common residues in any two proteins. The CLUSTAL multiple sequence alignment program (UW-GCG) was used. The amino acid residues Y331, F341, D342, I360, W363, Y386, I394 and F404 in FKBP-70 are not only conserved in all three yeast FKBP proteins, but the corresponding residues in human FKBP-12 were shown to interact directly with the FK-506 ligand in the crystals [18]. Similarly, the conserved signature sequence for the FKBP family of proteins [3] can be seen in FKBP-70 as **YIGKLKNGKVFDKN** (aa 331–344, bold letters indicating the identical residues).

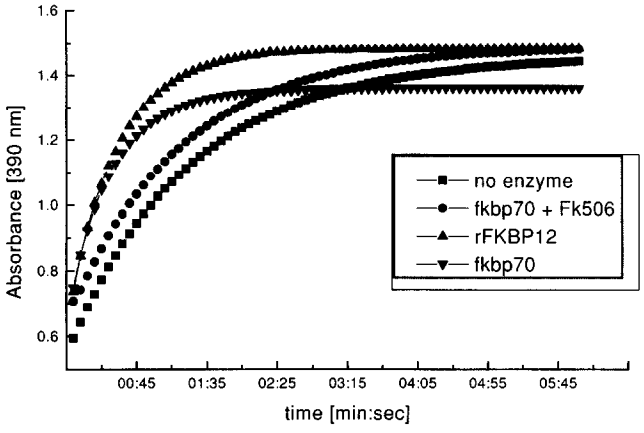

Fig. 4. Peptidyl-prolyl isomerase (PPIase) activity of recombinant FKBP-70 as measured by the method of Kofron et al. [32]. The data is generated by using 100 nM recombinant human FKBP-12 (rFKBP12) and 660 nM recombinant FKBP-70 (fkbp70). FK506 was used at tenfold molar excess to inhibit the enyme reaction.

media, suggesting that a high expression level of FKBP-70 is not toxic to yeast.

In summary, we characterized a novel member of the *FPR* gene family in yeast and showed that its presence is not essential for growth under normal conditions. However, conditional growth phenotypes were observed for other immunophilins in yeast [28,29,31]. A better understanding of the physiological role of FKBP-70 may require screening for its synthetic lethality with mutations in other yeast proteins.

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