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Dominant Missense Mutations in a Novel Yeast Protein Related to Mammalian Phosphatidylinositol 3-Kinase and VPS34 Abrogate Rapamycin Cytotoxicity

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Rapamycin is a macrolide antifungal agent that exhibits potent immunosuppressive properties. In *Saccharomyces cerevisiae*, rapamycin sensitivity is mediated by a specific cytosplasmic receptor which is a homolog of human FKBP12 (hFKBP12). Deletion of the gene for yeast FKBP12 (*RBP1*) results in recessive drug resistance, and expression of hFKBP12 restores rapamycin sensitivity. These data support the idea that FKBP12 and rapamycin form a toxic complex that corrupts the function of other cellular proteins. To identify such proteins, we isolated dominant rapamycin-resistant mutants both in wild-type haploid and diploid cells and in haploid *rbp1::URA3* cells engineered to express hFKBP12. Genetic analysis indicated that the dominant mutations are nonallelic to mutations in *RBP1* and define two genes, designated *DRR1* and *DRR2* (for dominant rapamycin resistance). Mutant copies of *DRR1* and *DRR2* were cloned from genomic YCp50 libraries by their ability to confer drug resistance in wild-type cells. DNA sequence analysis of a mutant *drr1* allele revealed a long open reading frame predicting a novel 2470-amino-acid protein with several motifs suggesting an involvement in intracellular signal transduction, including a leucine zipper near the N terminus, two putative DNA-binding sequences, and a domain that exhibits significant sequence similarity to the 110-kDa catalytic subunit of both yeast (VPS34) and bovine phosphatidylinositol 3-kinases. Genomic disruption of *DRR1* in a mutant haploid strain restored drug sensitivity and demonstrated that the gene encodes a nonessential function. DNA sequence comparison of seven independent *drr1^{dom}* alleles identified single base pair substitutions in the same codon within the phosphatidylinositol 3-kinase domain, resulting in a change of Ser-1972 to Arg or Asn. We conclude either that DRR1 (alone or in combination with DRR2) acts as a target of FKBP12-rapamycin complexes or that a missense mutation in DRR1 allows it to compensate for the function of the normal drug target.

The macrolide drug rapamycin exhibits immunosuppressive as well as antineoplastic and antiproliferative properties (reviewed in reference 52). Despite the structural similarity between rapamycin and FK506, FK506 (as well as the cyclic undecapeptide cyclosporin A [CsA]) abrogates early events in T-cell activation by specifically blocking transcription of interleukin-2 (IL-2) (47, 70; reviewed in references 62 and 64), whereas rapamycin blocks subsequent lymphokine receptor-mediated processes (16, 18).

The blockade of T-cell signal transduction results from the interaction of these agents with specific intracellular receptors (or immunophilins). CsA binds to a class of proteins called cyclophilins (reviewed in reference 73), whereas the primary targets for both rapamycin and FK506 are the FKBPs (for FK506-binding proteins) (28, 67, 69). One FKBP subtype (FKBP12) has been purified from a variety of organisms and, like the cyclophilins, shown to be an enzyme with peptidyl-prolyl *cis-trans* isomerase (PPIase) activity (28, 67). It is well established, however, that although ligand binding specifically inhibits enzymatic activity in vitro, this loss of function is not required for immunosuppression (6, 24, 29, 30, 37, 45, 74). Instead, the pharmacological effects of these drugs are derived from the formation of cyclophilin- or FKBP12-drug complexes which exhibit a gain of function by interacting with other downstream cellular proteins. Thus, the immunophilins act as chaperones for these drugs, delivering them to another site of action in the cell.

Both the cyclophilin-CsA and FKBP12-FK506 complexes bind to a specific protein phosphatase (calcineurin) which is hypothesized to control the activity of IL-2 gene-specific transcriptional activators (12, 24, 45, 55; reviewed in reference 63). In contrast, the downstream cellular targets for the rapamycin-sensitive signaling pathway have not been genetically characterized, although rapamycin has been shown recently to block the phosphorylation and activation of 70-kDa S6 (pp70^{S6K}) and p34^{cdc2} kinases in animal cells (8, 11, 51).

Since rapamycin is a potent antifungal agent, we have used the power of yeast genetics to rapidly dissect the rapamycin-sensitive pathway, with the hope that a parallel pathway exists in mammalian cells. We and others previously identified and characterized the gene encoding a yeast homolog of human FKBP12 (hFKB12) (29, 30, 37, 39, 74). Deletion of this gene (which we call *RBP1*, for rapamycin-binding protein; also known as *FPR1* and *FKB1* [30, 37, 74]) results in a recessive rapamycin-resistant phenotype, and expression of human FKBP12 in an *rbp1* deletion mutant restores rapamycin sensitivity (37).

In this study, we have identified two yeast genes that define proteins that appear to interact with the RBP1-rapamycin complex (as well as with the complex formed between rapamycin and recombinant hFKBP12 expressed in

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TABLE 1. Yeast strains

Strain[a]	Genotype	Source or reference
RS188N	*MAT***a** *trp1-1 ura3-1 his3-11,15 leu2-3,112 ade2-1 can1-100 erg6*	This study
LF07	*MAT***a** *trp1-1 ura3-1 his3-11,15 leu2-3,112 ade2-1 can1-100 erg6 fkh1-1*	This study
RJ06	*MAT***a** *trp1-1 ura3-1 his3-11,15 leu2-3,112 ade2-1 can1-100 erg6 fkh1-1 drr1-29*	This study
SX50-8A	*MAT***a** *trp1 ura3-52 his3Δ*	J. B. Hicks
GL45	*MATα trp1Δ1 ura3-52 leu2-3,112*	38
GL53	*MAT***a** *trp1 ura3-52 his3Δ +* *MATα trp1Δ1 ura3-52 + leu2-3,112*	38
RC11-8D	*MATα trp1 ura3-52 his3Δ leu2-3,112 drr1-1*	This study
RC94	*MAT***a** *trp1 ura3-52 his3Δ + +* *MATα trp1 ura3-52 his3Δ leu2-3,112 drr1-1*	This study
MM3	*MATα trp1Δ1 ura3-52 leu2-3,112 rbp1::URA3*	61
MM9	*MAT***a** *trp1Δ1 ura3-52 leu2-3,112 rbp1::URA3 TY1::LEU2-hFKBP12*	This study
MM9-9	*MAT***a** *trp1Δ1 ura3-52 leu2-3,112 rbp1::URA3 TY1::LEU2-hFKBP12 drr2-1*	This study
F762	*MATα trp1Δ1 ura3-52*	G. R. Fink

[a] Strains SX50-8A and GL45 are the parents of GL53. Strain RC11-8D is a haploid segregant of a rapamycin-resistant isolate of GL53 containing the *drr1-1* allele. Strains SX50-8A and RC11-8D are the parents of RC94.

yeast cells). Molecular genetic analysis of one of these genes reveals that it encodes a novel protein homologous to the catalytic subunit of phosphatidylinositol (PI) 3-kinases which, in mammalian cells, has been shown to interact with activated cell surface receptors through a phosphorylated tyrosine and regulate the formation of a family of phosphoinositides that may function as second messengers in cell signaling.

MATERIALS AND METHODS

Yeast strains and selection and genetic characterization of dominant rapamycin-resistant mutants. The genotypes of all strains relevant to this study are listed in Table 1. Direct selection of rapamycin-resistant mutants of *S. cerevisiae* was carried out as described previously (37), using either diploid strain GL53 (38) (Table 1) or haploid strains MM9 and RS188N. Strain MM9 was constructed as follows. hFKBP12 was previously engineered for functional expression in yeast strains by using the copper-inducible *CUP1* promoter and the *CYC1* transcriptional terminator (plasmid p138-FKBP [37]). A single copy of the *CUP1*-hFKBP12-*CYC1* expression cassette from p138-FKBP was integrated into the yeast genome, using Ty1 homology. For this, the unique *Xho*I site of p138-FKBP was first destroyed by Klenow polymerase and the vector was self-ligated. The *CUP1*-hFKBP12-*CYC1* cassette was then subcloned into a Ty1::*LEU2* construct carried on a derivative of plasmid pTCD5 (kindly provided by E. Jacob, SmithKline-RIT) called pTCD-AF; the expression cassette, carried on a *Bam*HI-*Mlu*I fragment, was subcloned into the unique *Bam*HI and *Mlu*I polylinker sites in pTCD5-AF which lie adjacent to *LEU2* (within Ty1). The resulting plasmid was digested with *Xho*I (which cuts within each of the Ty1 long terminal repeats), and the linear Ty1::*LEU2*-hFKBP12 cassette was used to transform strain MM3 (61) to leucine prototrophy by the lithium acetate method (33). Several Leu^+ prototrophs were analyzed by genomic Southern blotting to determine the copy number of the integrated cassette, using a ^{32}P-labeled *Sal*I-*Xho*I fragment of *LEU2* as a probe and quantifying band intensity relative to the endogenous *leu2-3,112* allele; one transformant (strain MM9) was found to contain a single Ty1::*LEU2*-hFKBP12 insertion. ^{32}P labeling was performed by random priming (Pharmacia oligolabeling kit).

In all cases, rapamycin-resistant mutants were isolated from independent yeast cultures. Cells from single colonies or from individual overnight cultures derived from single colonies were patched on either YEPD or synthetic agar medium, incubated for 2 to 3 days at 30°C, and replica plated to the same medium containing 80 ng of rapamycin per ml. For selection of mutants from strain MM9, expression of hFKBP was induced by growing cells in the presence of 150 μM $CuSO_4$.

Isolation and molecular characterization of genomic clones. Chromosomal DNA was prepared from strains RC11-8D and MM9-9 (Table 1), partially digested with *Sau*3A, size selected on 1% agarose gels (selecting for inserts >10 kb in size), and subcloned into the unique *Bam*HI site of the yeast centromere plasmid YCp50. Each genomic library contained an average insert size of >10 kb. The libraries were introduced individually into diploid strain GL53 by the spheroplast transformation method (4), selecting for Ura^+ transformants. In each case, ~10,000 original transformant colonies were recovered, collected into pools representing ~2,000 colonies, and replated onto both synthetic complete minus uracil and YEPD media, each supplemented with 80 ng of rapamycin per ml. The presence of drug-resistant colonies was scored after several days of incubation at 30°C. In pools which gave drug-resistant colonies, two colonies were picked and tested for cosegregation of the Ura^+ and rapamycin resistance phenotypes during growth on nonselective medium (YEPD); ~25% plasmid loss was observed over 3 days, and in all cases, we observed mitotic instability for both phenotypes. Plasmid DNA was recovered from each clone and reintroduced into strain GL53 to test for drug resistance. From the RC11-8D-derived library (*drr1-1*), three plasmids were recovered; restriction mapping indicated that two of the plasmids were identical (pRC1), whereas the third plasmid was distinct but contained an overlapping insert (pRC5). From the MM9-9 library, two identical plasmids able to confer drug resistance were recovered (47a).

Regions of the genomic inserts in pRC1 and pRC5 were subjected to double-stranded DNA sequence analysis using the method of Sanger et al. (60) and the Sequenase kit (U.S. Biochemical); both universal and synthetic oligodeoxyribonucleotides were used as sequencing primers. Some portions of the DNA sequence were determined from unidirectional deletions of subclones carried in pUC18 which were created by using the Pharmacia double-stranded nested deletion kit.

***DRR1* gene disruption.** Two *drr1::URA3* constructs were made and introduced into the genome in place of the chromosomal allele (see Fig. 2A). In the first (genomic disruption) construct, the *URA3* gene carried on an ~1.1-kb *Bgl*II-*Bam*HI fragment was inserted into the unique *Bam*HI site contained within the *Bgl*II-*Sph*I subfragment of pRC5 which had been subcloned into the *Bam*HI and *Sph*I polylinker sites of pUC18, creating pUC-drr1::URA3-1. The resulting *drr1::URA3* construct was linearized by digestion with *Sph*I and *Kpn*I (polylinker site) and used to transform diploid strain RC94 (4), selecting for Ura$^+$ prototrophs. In the second (genomic deletion-insertion) construct, a large portion of the pRC1 insert carried on a *Bgl*II-to-*Spe*I fragment was first subcloned into the unique *Bam*HI and *Spe*I sites of pBluescript (Stratagene), and the resulting plasmid was digested with *Bam*HI in order to delete ~4.2 kb of internal sequence. The vector DNA fragment was gel purified and ligated to the same ~1.1-kb *Bgl*II-*Bam*HI *URA3* fragment used above; the resulting plasmid (pBS-drr1::URA3-2) was linearized by digestion with *Spe*I and *Kpn*I, and the DNA was again used to transform RC94 as described above. That the genomic disruption and deletion-insertion mutations were targeted to the homologous region in the genome was confirmed by genomic Southern blotting, using published procedures (46).

PCR amplification and sequence analysis of *drr1^{dom}* alleles. The polymerase chain reaction (PCR) was performed according to published procedures (53), with minor modifications (61), using ~5 ng of genomic DNA from strain RC11-8D, and from other strains carrying different *drr1^{dom}* alleles, as a template. Synthetic oligodeoxyribonucleotide primers for PCR were designed to amplify the ~1.07-kb *Hin*dIII-*Bam*HI fragment within *drr1-1* (see Fig. 2A); the sequences of these primers were 5′-GCATGATCTTGGTTTGGATCCGAATA-3′ and 5′-TATCCCAAGCTTGGTTCAAATTATTG-3′. Amplified DNA fragments were used directly as substrates for PCR sequencing (using a Bethesda Research Laboratories double-stranded cycle sequencing kit); all reactions were performed according to the manufacturer's instructions.

Inverse PCR. Attempts to isolate additional genomic clones containing sequences upstream of the 5′ end of the pRC1 insert, using the RC11-8D-derived YCp50 library and 5′-specific DNA probes, were unsuccessful. To obtain upstream genomic sequences, we used an inverse PCR strategy (71). By genomic Southern blot analysis, we identified an ~1.1-kb *Hha*I fragment which overlapped the 5′ end of the pRC1 insert and extended ~700 bp into flanking sequences. Total genomic DNA from strain RC11-8D was digested with *Hha*I, diluted to 3.4 μg/ml, and self-ligated. Approximately 50 ng of DNA from the ligation mix was used as a template for PCR (performed in duplicate), using synthetic divergent primers homologous to the known sequence; the sequences of the primers were 5′-GCCCCTACCCTAGTGTTCG-3′ and 5′-TGGCTTACTGCCTCCACGG-3′. A DNA fragment of the expected size was generated, gel purified, and subcloned into the pCRII TA cloning vector (Invitrogen). DNA sequence analysis of both PCR products indicated that they were identical and contained ~400 bp of sequence overlapping the 5′ end of the pRC1 insert plus ~700 bp of upstream sequence.

Isolation and analysis of RNA. Total RNA was extracted from strain F762 (*MAT*α *trp1*Δ*1 ura3-52*) by the method of Carlson and Botstein (10), and poly(A)$^+$ RNA was selected following passage over oligo(dT)-cellulose (2). Poly(A)$^+$-selected RNA was size fractionated by electrophoresis through 1.5% agarose in the presence of 2.2 M formaldehyde (43), transferred to nitrocellulose, and probed with a ^{32}P-labeled ~2.6-kb *Bgl*II-*Sph*I fragment of the pRC5 insert. Hybridizations were carried out according to previously published procedures (46).

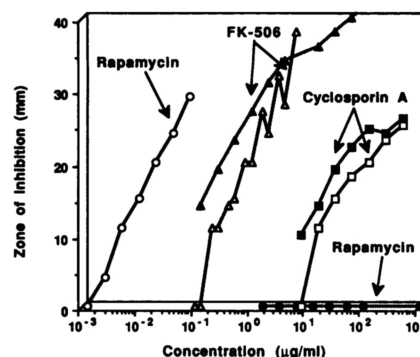

FIG. 1. Effect of a *drr1^{dom}* mutation on rapamycin, FK506, and CsA sensitivity. Cells of a haploid strain carrying a mutation conferring hypersensitivity to CsA and FK506 (LF07 [18a]) and a strain with the same genetic background plus a *drr1^{dom}* allele (*drr1-29*; strain RJ06) were spread on separate YEPD plates and exposed to either CsA, FK506, or rapamycin. The indicated concentrations of each drug were added to wells cut into the agar, and the sizes of the zones of growth inhibition were measured after 2 days of incubation at 30°C (37). Open circles, rapamycin (LF07); closed circles, rapamycin (RJ06); open triangles, FK506 (LF07); closed triangles, FK506 (RJ06); open squares, CsA (LF07); closed squares, CsA (RJ06).

Chromosome mapping. An *S. cerevisiae* cantour-clamped homogeneous electric field (CHEF) gel chromosome blot (purchased from Clonetech Laboratories, Inc.) was probed with a ^{32}P-labeled ~2.6-kb *Bgl*II-*Sph*I fragment of the pRC5 insert. Hybridization and washing conditions (at moderate to low stringency) were as previously reported (46).

Sources of drugs. FK506 and rapamycin were prepared by fermentation and purified at SmithKline Beecham Pharmaceuticals in Brockham Park, United Kingdom. CsA was obtained from Sandoz Pharmaceuticals, East Hanover, N.J.

Nucleotide sequence accession number. The GenBank accession number for the DNA sequence reported here is L19540.

RESULTS

Isolation and genetic analysis of dominant rapamycin-resistant mutants of *S. cerevisiae*. To identify *S. cerevisiae* proteins other than RBP1 (yeast FKBP12) involved in mediating rapamycin sensitivity, we initially selected rapamycin-resistant mutants in haploid cells (strain RS188N) and screened them for those identifying genes other than *RBP1*. Among a total of 277 independently isolated rapamycin-resistant mutants, 258 contained recessive mutations in *RBP1*, whereas the remainder contained unlinked dominant mutations in a single gene (39), which we designate *DRR1* (for dominant rapamycin resistance). We tested whether there was any effect of *drr1^{dom}* mutations on sensitivity to FK506 and CsA. Since wild-type yeast cells are naturally highly resistant to FK506 and CsA, we introduced a *drr1^{dom}* mutation into a genetic background containing a mutation that confers hypersensitivity to these two drugs (*fkh1-1* [18a]). As shown in Fig. 1, the *drr1^{dom}* mutation results in complete rapamycin resistance but has no detectable effect on the response to the

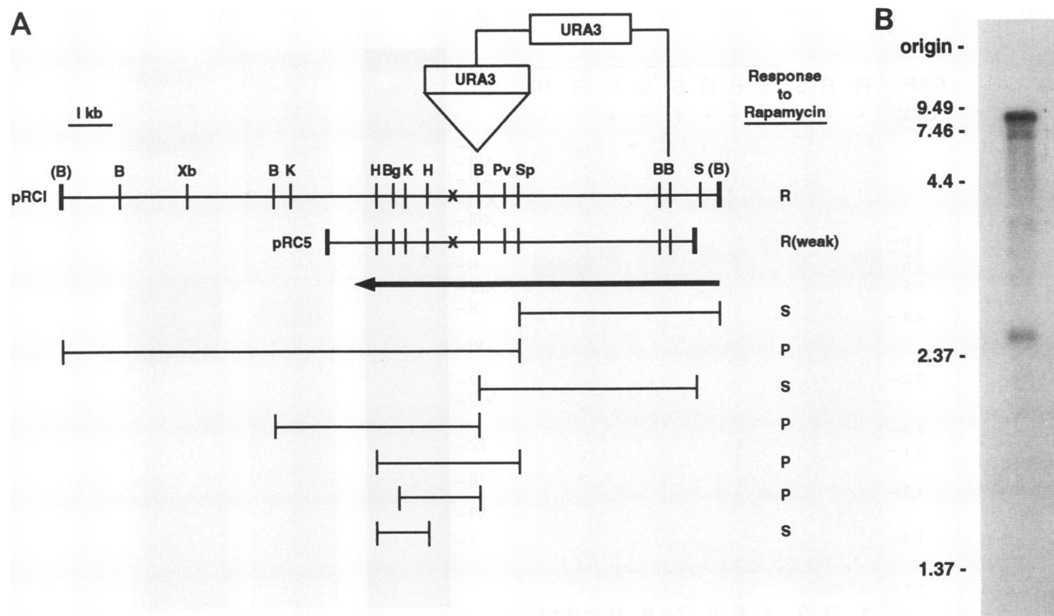

FIG. 2. (A) Restriction map of the pRC1 and pRC5 inserts and representative subclones used to identify the location of the *drr1-1*^{dom} rapamycin-resistant mutation. All constructs were introduced into wild-type strain GL53 (see Table 1) and scored for their effects on rapamycin sensitivity. The response to rapamycin is indicated as R (resistant), S (sensitive), or P (papillae) (see Materials and Methods for a complete description of phenotypes). The position of insertion of the *S. cerevisiae URA3* gene is shown for the genomic disruption and genomic insertion-deletion constructs described in the text. B, *Bam*HI; Xb, *Xba*I; K, *Kpn*I; H, *Hin*dIII; Bg, *Bgl*II; Pv, *Pvu*II; Sp, *Sph*I; S, *Spe*I; (B), *Bam*HI-*Sau*3A. Only relevant *Hin*dIII sites are shown. The approximate positions of *drr1* missense mutations are noted by X's on the pRC1 and pRC5 inserts. The thick arrow corresponds to the transcribed region. (B) Northern blot analysis. Poly(A)$^+$ RNA was prepared from total RNA extracted from *S. cerevisiae* F762 (*MAT*α *trp1*Δ*1 ura3-52*), size fractionated, blotted, and probed with a ^{32}P-labeled ~2.6-kb *Bgl*II-*Sph*I fragment of pRC5 (A) as described in Materials and Methods. Size markers are in kilobases.

other drugs, suggesting that *DRR1* is specific to the rapamycin-responsive pathway.

We next isolated rapamycin-resistant mutants in diploid cells. The rationale for this approach was to generate only dominant drug-resistant mutants that define proteins with an essential function. We reasoned that recessive (loss of function) mutations in *RBP1* would be excluded, whereas downstream proteins in the rapamycin pathway could be defined by missense mutations that alter the interaction with the RBP1-rapamycin complex. Starting with diploid strain GL53 (Table 1), we isolated 52 independent rapamycin-resistant mutants by direct selection on YEPD plates containing 80 ng of rapamycin per ml (see Materials and Methods). Segregation analysis of 45 mutants showed 2:2 segregation for drug resistance and all other genetic markers. However, a total of seven mutants displayed either weak drug resistance or ambiguous segregation patterns upon retesting; these strains were not chosen for further study.

One of the originally isolated *drr1*^{dom} mutants was crossed to rapamycin-resistant haploid segregants from each of the 45 GL53-derived mutants. Segregation analysis showed that 33 contained mutations in *DRR1* (i.e., only rapamycin-resistant haploid segregants were obtained), whereas the remaining 12 contained mutations in a separate gene or genes (i.e., some rapamycin-sensitive segregants were obtained). Further genetic analysis revealed that at least 3 of the remaining 12 mutants define a second gene, which we designate *DRR2* (data not shown).

In a separate study aimed at generating structure-function information on recombinant hFKBP12 expressed in *S. cerevisiae*, we identified seven dominant rapamycin-resistant mutants in a haploid strain engineered to contain a single expression cassette for hFKBP12 in place of *RBP1*. Genetic analysis revealed that four of these strains contained alleles of *drr1*, whereas three contained alleles of *drr2*.

Isolation of *drr1-1* and *drr2-1* genomic clones. To isolate the dominant mutant *drr* genes, separate genomic plasmid libraries were constructed (in YCp50) from individual drug-resistant mutants, i.e., strain RC11-8D (*drr1-1*) and MM9-9 (*drr2-1*) (Table 1). Each library was introduced into the rapamycin-sensitive diploid strain GL53, and Ura$^+$ transformants were screened for those containing plasmids conferring rapamycin resistance (using 80 ng of rapamycin per ml in synthetic complete medium minus uracil) (see Materials and Methods). Two independent clones were isolated for *drr1-1*, whereas one clone was obtained for *drr2-1*. The activity of the transforming plasmids was confirmed by demonstrating cosegregation of rapamycin resistance and the Ura$^+$ phenotype in cells grown in nonselective medium. The plasmids also were found to confer drug resistance when reintroduced into GL53. The *drr1-1* genomic clone was subjected to molecular genetic characterization as described below; the characterization of *drr2-1* will be described elsewhere.

Restriction analysis of the two putative *drr1-1*-containing plasmids (pRC1 and pRC5) revealed the presence of two overlapping inserts of ~8.5 and ~14 kb (Fig. 2A). pRC5 transformants grew less well than pRC1 transformants on rapamycin-containing medium. Genomic Southern blot analysis indicated that the insert in pRC1 is present at a single copy number and represents a contiguous portion of the genome (data not shown). Using the internal *Bgl*II-*Sph*I fragment as a probe, we detected a prominent large (~8-kb) mRNA species by Northern (RNA) blotting (Fig. 2B), with

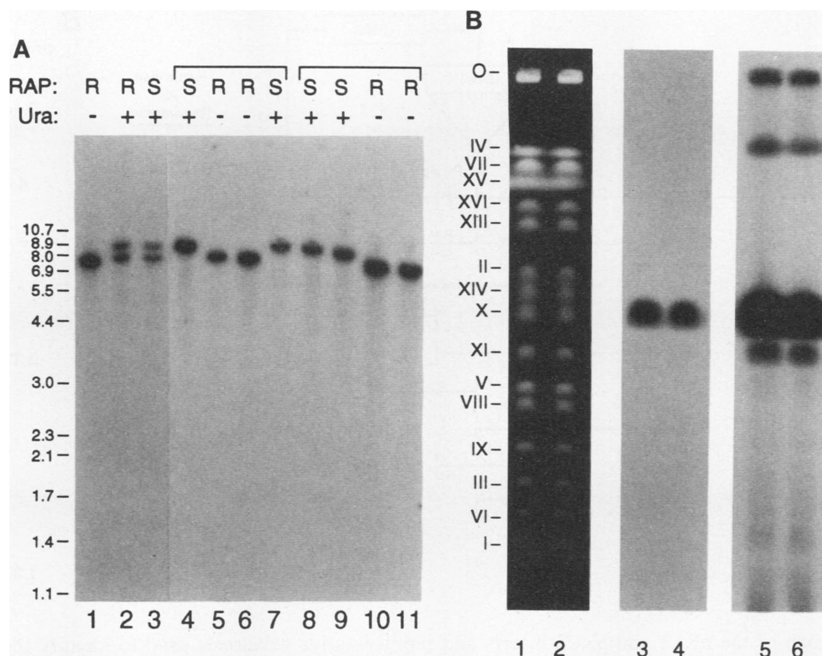

FIG. 3. (A) Genomic Southern blot analysis of *drr1::URA3* gene disruption. Genomic DNA was prepared from untransformed diploid strain RC94 (lane 1), a rapamycin-resistant Ura$^+$ transformant of RC94 (lane 2), a rapamycin-sensitive Ura$^+$ transformant of RC94 (lane 3), and the progeny of two tetrads derived from the rapamycin-resistant Ura$^+$ transformant shown in lane 2 (lanes 4 to 11), digested with *Bgl*II, run on a 0.8% agarose gel, blotted to nitrocellulose, and probed with a ^{32}P-labeled ~2.6-kb *Bgl*II-*Sph*I fragment of the pRC5 insert (Fig. 1A). The rapamycin-resistant (R) or -sensitive (S) phenotype of each strain is shown at the top along with the Ura$^+$ (+) or Ura$^-$ (−) phenotype. Size markers are in kilobases. (B) Chromosome blot analysis of *DRR1*. An ~2.6-kb *Bgl*II-*Sph*I fragment of the pRC5 insert was ^{32}P labeled and used to probe an *S. cerevisiae* CHEF gel chromosome blot (Clonetech Laboratories). Lanes: 1 and 2, the ethidium bromide-stained CHEF gel which corresponds to the blot (lanes 3 to 6); 3 and 4, 1-day exposure; 5 and 6, 7-day exposure. Chromosome identification (roman numerals) was provided by Clonetech.

two additional minor bands of smaller size. Fragments of each insert were subcloned into YCp50 and assayed for the ability to confer drug resistance in strain GL53. Consistent with the size of the mRNA, we were unable to identify a subclone smaller than the ~8.5-kb insert in pRC5 (Fig. 2A and data not shown). However, as shown in Fig. 2A, certain subclones resulted in the appearance of stable rapamycin-resistant papillae against a background of nongrowing cells which could have arisen by gene conversion of the chromosomal wild-type gene from the mutant allele. Further subcloning localized the mutation to an ~1-kb *Hin*dIII-*Bam*HI fragment (Fig. 2A; see below).

Confirmation that the cloned gene corresponds to the *drr1-1* allele was obtained by reverse genetics (59) (see Materials and Methods). Briefly, a genomic disruption allele was constructed by subcloning the *URA3* gene, carried on a 1.1-kb *Bam*HI-*Bgl*II fragment, into the unique *Bam*HI site of the *Bgl*II-*Sph*I insert piece of pRC5 (Fig. 2A) which had been subcloned into a shuttle vector. The linearized *URA3*-disrupted fragment was introduced into a heterozygous *drr1-1*/+ (rapamycin-resistant) diploid (strain RC94), and Ura$^+$ prototrophs were selected and analyzed for drug sensitivity. We obtained equal numbers of rapamycin-resistant and rapamycin-sensitive transformants. Genomic Southern blot analysis confirmed that in both cases, the gene replacement event occurred at a region homologous to the cloned sequence (Fig. 3A). This finding suggests that in the rapamycin-resistant diploid transformant, the gene disruption occurred in the wild-type allele, whereas in the rapamycin-sensitive diploid transformant, the gene replacement occurred in the *drr1-1* allele. Furthermore, the existence of rapamycin-sensitive diploid transformants indicated that the insertion inactivated the dominant mutation. Tetrad analysis of one rapamycin-resistant transformant showed 2:2 segregation for resistance and sensitivity and cosegregation for sensitivity and Ura$^+$ (in 17 four-spored asci) (Fig. 3A). In addition, we constructed and analyzed in vivo a deletion-insertion mutation in which a ~4.2-kb portion of the pRC5 insert (between the distal *Bam*HI sites) (Fig. 2A) was replaced with *URA3*. The gene replacement event was confirmed by genomic Southern blotting, and haploid *drr1::URA3* segregants were again found to be viable and rapamycin sensitive (19 four-spored asci). Thus, *DRR1* is nonessential.

DRR1 was mapped to chromosome X by chromoblot analysis (Fig. 3B). Prolonged exposure of autoradiographs revealed additional homologous sequences on chromosomes IV and XI (Fig. 3B), which may reflect genetic redundancy.

Analysis of the *drr1-1* DNA and deduced protein sequence. We determined the DNA sequence of ~7.5 kb within the insert of pRC5. Analysis of the sequence identified a single long open reading frame (ORF) which extended beyond the 5′ end of the insert. When the nucleotide sequence of the upstream region in pRC1 was determined, it was found to contain an extension of the same ORF. Apparently, the expression of *drr1-1* in these plasmids is being controlled by flanking plasmid sequences, and the truncation in pRC5 may account for the difference in growth phenotype (in response to rapamycin) observed between cells containing it and pRC1 (data not shown). We noted that both the pRC1 and pRC5 ORFs were found to be in frame with a potential ORF (starting with a Met codon) running 3′ to 5′ in the Tet^r gene

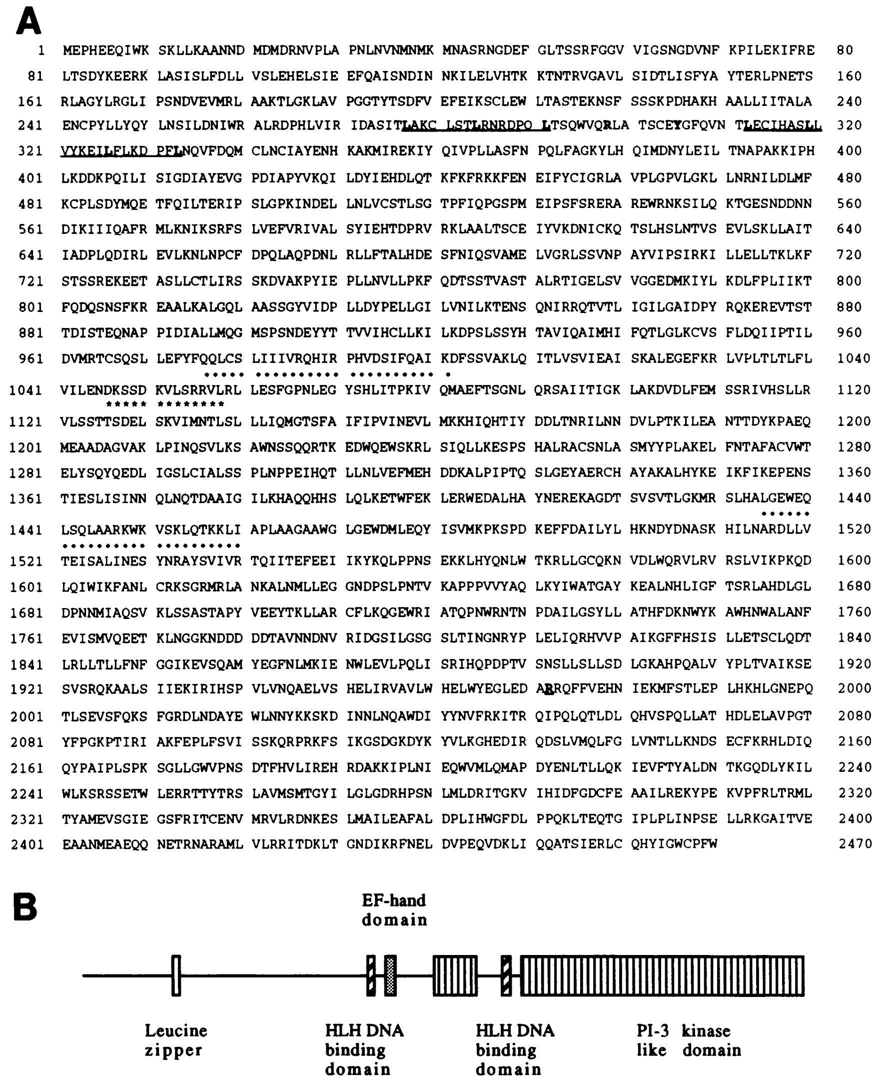

FIG. 4. (A) Predicted amino acid sequence of *drr1-1*. The amino acid sequence shown corresponds to the deduced translation product (starting with the first Met residue) of the long ORF present in the pRC1 and pRC5 inserts as well as the overlapping sequence cloned by inverse PCR (see Materials and Methods). The nucleotide sequence (determined for both strands of DNA) has been deposited in GenBank (accession number L19540). The putative translation product from the pRC1 insert begins at residue 98 (D-98), whereas the product from the pRC5 insert begins at residue 330 (D-330). A putative leucine zipper domain is underlined (solid line). A putative EF-hand motif is indicated by asterisks (starting at residue 1046). Two putative helix-turn-helix DNA-binding motifs are indicated by dotted lines (residues 976 to 1001 and 1435 to 1460). The mutant residue identified in the *drr1-1*^{dom} mutant protein (Arg-1972) is underlined and in boldface. The coordinates for amino acid sequences similar to the 110-kDa catalytic subunit of bovine PI 3-kinase and the yeast VPS34 PI 3-kinase are ~1220 to 1349 and ~1490 to the end (see text and legend to Fig. 5 for details). (B) Locations of functional domains in the putative *drr1-1* translation product. Open box, leucine zipper; diagonally hatched box, helix-turn-helix (HLH) DNA-binding domain; stippled box, EF-hand domain; vertically hatched box, PI 3-kinase-related domain (see text and legend to panel A for details).

of YCp50. We next used inverse PCR to isolate an ~1.1-kb overlapping fragment containing 700 bp of upstream sequence (see Materials and Methods); nucleotide sequence analysis of this fragment extended the ORF to a putative initiation codon. DNA sequence upstream of this site contains multiple stop codons in all reading frames (data not shown).

The entire translation product of 2,470 amino acids predicted by the ORF is shown in Fig. 4. This product is expected to correspond to a protein of 281.2 kDa that is very hydrophilic and does not appear to contain any hydrophobic signal or transmembrane regions. There are several Met residues near the N terminus of the deduced protein, and we have not yet established which one represents the start of translation.

Comparison of mutant DRR1 (drr1-1) with proteins in the latest update of the protein sequence data bases by using FASTA (57) or BLAST (1) revealed significant sequence similarity in the C-terminal half to bovine PI 3-kinase (32) and yeast VPS34 (31), which has also been shown to be a PI 3-kinase (65). The similarity is most evident in the last 970 amino acids of drr1-1, a region which corresponds to the putative catalytic domain of bovine PI 3-kinase (32) (Fig. 5B). However, a further region of weak sequence similarity

A

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         Vps34  ........MS LNN......I TFCVSQDLDV P.LKVKIKSL EGHKPLLKPS Q.....KILN PELMLIGSNV FPSSDLIVSL QVFDKERNRN LTLPIYTPYI   80
   Bovphos3kin  LNREIGFAIG MPV......C EFDMVKDPEV QDFRRNILNV CKEAVDLRDL N.....SPHS RAMYVYPPNV ESSPEL..PK HIYNKLDKGQ IIVVIWVIVS  199
          Drr1  MEAADAGVAK LPINQSVLKS AWNSSQQRTK EDWQEWSKRL SIQLLKESPS HALRACSNLA SMYYPLAKEL FNTAFACVWT ELYSQYQEDL IGSLCIALSS 1300
     consensus  .......... lp........ .f..sqd..v .d....ik.l ......l.ps ......s.l. ...y....nv f.s..l.v.. ..y.k..... i...i....s

         Vps34  PFRNSRTWDY WL........ ....TLPIRI KQLTFSS.HL RIILWEYNG.   116
   Bovphos3kin  PNNDKQKYTL KINHDCVPEQ VIAEAIRKKT RSMLLSSEQL KLCVLEYQGK   249
          Drr1  PLNPPEIHQT LLNLVEFMEH .DDKALPIPT QSLGEYAERC HAYAKALHYK  1349
     consensus  P.n....... .ln.....e. ....alpi.t .sl..sse.l .....ey.gk
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B

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         Vps34  FRYDVIDHCE VVTDNKD... ........QE NLN.KY.... ...FQGEFTR LPWLDEITIS KLRKQRENRT WPQGTFVLNL EFPMLELPVV FIEREIMNTQ  226
   Bovphos3kin  VRTGIYHGGE PLCDNVNTQR VPCSNPRWNE WLNYDI.... ...YIPDLPR AARLC.LSIC SVKGRKGAKE .EHCPLAWG. NINLFDYTDT LVSGKMALNL  445
          Drr1  LHKNDYDNAS KHILNARDLL VTEISALINE SYNRAYSVIV RTQIITEFEE IIKYKQLPPN SEKKLHYQNL WTKRLLGCQK NVDLWQRVLR VRSLVIKPKQ 1599
     consensus  .r...yd..e ...dN..... v.......nE .lN..y.... ....i.ef.r ...l..l.i. s.kk...... w....l.... n..l...... ..s..i...q

         Vps34  .......... .......MNI P....TLKNN PGLSTDLREP NRNDPQIK.. .......... ........IS LGDKYHSTLK FYD....PDQ PNNDPIEEKY  281
   Bovphos3kin  .......... .......WPV PHGLEDLLNP IGVTGS..NP NKETPCLE.. .......... ........LE F.DWFSSVVK FPDMSVIEEH ANWSVSREAG  505
          Drr1  DLQIWIKFAN LCRKSGRMRL ANKALNMLLE GGNDPSLPNT VKAPPPVVYA QLKYIWATGA YKEALNHLIG FTSRLAHDLG LDPNNMIAQS VKLSSASTAP 1699
     consensus  .......... .......m.. p.....lln. .G...sl.np nk..P..... .......... ........i. f.d...s.lk f.d...i... .n.s...ea.

         Vps34  RRLERASKNA N.LDKQVKPD IKKRDYLNKI INYPPGTKL. ..TAHEKGSI WKYRYYLMNN KKALTKLLQS TNLR.....E ESERVEVLEL MDSWAEIDID  372
   Bovphos3kin  FSYSHAGLSN R.LARDNELR ENDKEQLRAI CTRDPLSEI. ..TEQEKDFL WSHRHYCVTI PEILPKLLLS VKWN.....S RDEVAQMYCL VKDWPPIKPE  596
          Drr1  YVEEYTKLLA RCFLKQGEWR IATQPNWRNT NPDAILGSYL LATHFDKNWY KAWHNWALAN FEVISMVQEE TKLNGGKNDD DDDTAVNNDN VRIDGSILGS 1799
     consensus  ...e.a.l.a r.l.kq.e.r i.....lr.i ....pl.... ..T..eK... w..r.y...n .e.l.kll.s tkln...... .de.a....l v..w..I...

         Vps34  DALELLGSTF KNLSVRSYAV NRLK...... .......... .........K .ASDKELELY LLQLVEAVCF ENLSTFSDKS NSEFTIVDAV SSQKLSGDSM  446
   Bovphos3kin  QAMELLDCNY PDPMVRGFAV RCLE...... .......... .........K YLTDDKLSQY LIQLVQVLKY EQY....... .......... ..........  644
          Drr1  GSLTINGNRY PLELIQRHVV PAIKGFFHSI SLLETSCLQD TLRLLTLLFN FGGIKEVSQA MYEGFNLMKI ENWLEVLPQL ISRIHQPDPT VSNSLLSLLS 1899
     consensus  .alellg..y p...vr..aV ..lk...... .......... .........k ...dkelsqy l.qlv...k. En........ .s.....d.. .s..l.....

         Vps34  LLSTSHANQ. ..KLLKSISS ESETSGTESL PIVI...... .......SPL AEFLIRRA.. .......... ......LVNP RLGSFF.... ..YWYLKSES  516
   Bovphos3kin  .......... .......... .......... ..LD...... .......NLL VRFLLKKA.. .......... ......LTNQ RIGHFF.... ..FWHLKSEM  675
          Drr1  DLGKAHPQAL VYPLTVAIKS ESVSRQKAAL SIIEKIRIHS PVLVNQAELV SHELIRVAVL WHELWYEGLE DARRQFFVEH NIEKMFSTLE PLHKHLGNEP 1999
     consensus  .l...h.... ...l...i.s es.......l .i........ ........ll ..fLir.A.. .......... ......lvn. rig.fF.... ...whLksE.

         Vps34  E.......DK PY...LDQIL SSFWSRLDKK SRNILNDQVR L.INVLRECC ETIKRLKDTT AKKMELLVHL LETKVRP..L VKVRPIALPL DPDVLICDVC  593
   Bovphos3kin  H.......NK TVSQRFGLLL ESY.CRACGM YLKHLNRQVE A.MEKLINLT DILKQEKKDE TQKVQMKF.L VEQMRRPDFM DALQGFLSPL NPAHQLGNLR  765
          Drr1  QTLSEVSFQK SFGRDLNDAY EWLNNYKKSK DINNLNQAWD IYYNVFRKIT RQIPQL.... .QTLDLQHVS PQLLATHDLE LAVPGTYFPG KPTIRIAKFE 2094
     consensus  .........K .....l...l es...r...k ..n.LN.qv. ...nvlr..t ..ikqlk... .qk..l...l .e...rpd.. .av.g...Pl .P...i....

         Vps34  PETSKVFKSS LSPLKITFKT T......LNQ PYHLMFKVGD DLRQDQLVVQ IISLMNELLK NENV....DL KLTPYKILAT GPQEGAIEFI PN.DTLASIL  682
   Bovphos3kin  LEECRIMSSA KRPLWLNWEN PDIMSELLFQ NNEIIFKNGD DLRQDMLTLQ IIRIMENIWQ NQGL....DL RMLPYGCLSI GDCVGLIEVV RNSHTIMQIQ  861
          Drr1  PLFS.VISSK QRPRKFSIKG SD......GK DYKYVLKGHE DIRQDSLVMQ LFGLVNTLLK NDSECFKRHL DIQQYPAIPL SPKSGLLGWV PNSDTFHVLI 2187
     consensus  pe.s.v.sS. .rPlk...k. .d.....l.q .y...fK.gd DlRQD.Lv.Q ii.lmn.llk N.......dL ...pY..l.. gp..Glie.v pNsdT...i.

         Vps34  SKYHG..... ..ILGYLKL. .....HYPDE NATLGVQGWV LDN....... .......... .........F VKSCAGYCVI TYILGVGDRH LDNLLVTP.D  742
   Bovphos3kin  CK.GG..... ..LKGALQFN SHTLHQWLKD KNKGEIYDAA IDL....... .......... .........F TRSCAGYCVA TFILGIGDRH NSNIMVKD.D  926
          Drr1  REHRDAKKIP LNIEQWVMLQ MAPDYENLTL LQKIEVFTYA LDNTKGQDLY KILWLKSRSS ETWLERRTTY TRSLAVMSMT GYILGLGDRH PSNLMLDRIT 2287
     consensus  .k..g..... ..i.g.l.l. .......l.. ..k.ev...a lDn....... .......... .........f trScAgycv. tyILG.GDRH .sNlmv...d

         Vps34  GHFFHADFGY ILGQDPKPF. ......PPLM KLPPQIIEAF GGAESS...N YDKFRSYCFV AYSILRRNAG LILNLFELMK TSNIPDIRID PNGAILRVRE  832
   Bovphos3kin  GQLFHIDFGH FLDHKKKKFG YKRERVPFVL TQDFLIVISK GAQECTKTRE FERFQEMCYK AYLAIRQHAN LFINLFSMML GSGMPE..LQ SFDDIAYIRK 1024
          Drr1  GKVIHIDFGD CFEAAILREK Y.PEKVPFRL TRMLTYAMEV SGIEGS.... ...FRITCEN VMRVLRDNKE SLMAILEAFA LDPLIHWGFD LPPQKLTEQT 2379
     consensus  G..fHiDFG. .l....k.f. y..e.vPf.l t....i.... gg.E.s.... ...Fr..C.. ay..lR.na. l..nlfe.m. .s..p....d ....il..r.

         Vps34  RFNLNM.... ........SE EDATVHFQNL INDSVNALLP IVIDH.LHNL AQYWRT.... .......... .......... .......... .  875
   Bovphos3kin  TLALDK.... ........TE QEALEYFMKQ MNDAHHGGWT TKMDWIFHTI KQHALN.... .......... .......... .......... . 1068
          Drr1  GIPLPLINPS ELLRKGAITV EEAANMEAEQ QNETRNARAM LVLRRITDKL TGNDIKRFNE LDVPEQVDKL IQQATSIERL CQHYIGWCPF W 2470
     consensus  ...L...... ........te eeA...f..q .Nd..na... .v.d.i.h.l .q........ .......... .......... .......... .
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FIG. 5. Amino acid sequence alignment between part of the putative *drr1-1* translation product (Drr1), the 110-kDa catalytic subunit of bovine PI 3-kinase (Bovphos3kin) (32), and the *S. cerevisiae* VPS34 PI 3-kinase (Vps34) (31). The optimum alignment obtained by using the PILEUP program (University of Wisconsin Genetics Computer Group [15]) is shown. The consensus sequence is shown on the bottom line, with uppercase letters indicating residues conserved among all three proteins. Panels A and B correspond to two regions of the three proteins showing sequence similarity (see text). The mutant Arg-1972 residue in *drr1-1* is shown in boldface (B).

can be detected between residues 1220 and 1349 of drr1-1 (Fig. 5A). There are several residues conserved between the PI 3-kinases and protein serine/threonine or tyrosine kinases, such as Asp-166, Asn-171, Asp-184, Phe-185, and Gly-186, using the numbering in cyclic AMP (cAMP)-dependent serine/threonine kinase (27, 44), that are proposed to be involved in ATP binding and also found in drr1-1 (at residues 2275, 2280, 2294, 2295, and 2296) (Fig. 5B). On the other hand, other residues which also help to define the active site of the protein kinases, such as xxHRDLKPEN or xxHRD LAARN, found in protein kinases at residues 164 to 171 (cAMP-dependent protein kinase numbering [44]), are replaced by ILGxGDRHxxN in the PI 3-kinases and drr1-1 (residues 2270 to 2280) (Fig. 5B). These and other similarities between drr1-1 and the PI 3-kinases that specifically differ with the protein kinases suggest that drr1-1 is not a protein kinase but instead is likely to act upon substrates similar to those acted on by the PI 3-kinases.

A search for protein motifs within drr1-1 (University of Wisconsin Genetics Computer Group package, version 7) identified other putative functional domains (Fig. 4). These include a possible leucine zipper near the N terminus (residues 312 to 333) with several basic domains nearby (e.g., KAKMIREK starting at residue 351 and KFKFRKK starting at residue 441) (Fig. 4). Interestingly, there is a second shorter leucine zipper repeat further upstream; the two

TABLE 2. Molecular genetic characterization of independently isolated *drr1* alleles

Allele	Parent strain[a]	DNA sequence[b]	Amino acid at residue 1972[c]
Wild type	SX50-8A	---AGC---	Ser
Wild type	GL45	---AGC---	Ser
drr1-1	SX50-8A	---AGG---	Arg
drr1-7	SX50-8A	---AGG---	Arg
drr1-9	GL45	---AGG---	Arg
drr1-18	SX50-8A	---AGG---	Arg
drr1-19	GL45	---AGA---	Arg
drr1-27	SX50-8A	---AAC---	Asn
drr1-49	GL45	---AGG---	Arg

[a] Strain designations were deduced from sequence polymorphisms (silent differences) between the alleles in SX50-8A and GL45, which were the parents of strain GL53 (see Table 1).

[b] The ~1-kb *Hin*dIII-*Bam*HI fragment shown in Fig. 1A was cloned by PCR (in duplicate reactions) from the genomic DNA prepared from each of the strains shown. The location of the mutation in *drr1-1* was identified by comparing the nucleotide sequence of the entire fragment with that from each parent; the nucleotide sequence of ~200 bp flanking this region was determined for the other alleles.

[c] See Fig. 2.

regions are separated by 21 amino acids, an exact multiple of 7. Conceivably, this could be an extension of the first region, with an interruption by an Arg and a Tyr in the repeat.

A single EF-hand-like motif can be found at residues 1046 to 1058, although it may not be able to bind Ca^{2+} since it has a single mismatch at one of the positions which coordinates the Ca^{2+} (i.e., Val-1057 rather than an Asp or Glu).

Finally, two putative helix-turn-helix DNA-binding motifs were found at residues 976 to 1001 and 1435 to 1460 which differed from the consensus by one amino acid. The second motif, which is like the DNA-binding domains of Myb (5) and yeast REB1 (34), is inserted between the two PI 3-kinase-homologous regions identified above. There is no homologous region in either VPS34 or bovine PI 3-kinase.

Characterization of *drr1* mutant alleles. As indicated in Fig. 2A, plasmids containing an ~1-kb *Hin*dIII-*Bam*HI fragment of *drr1-1*, when introduced into wild-type cells, gave a high frequency of rapamycin-resistant papillae. We reasoned that these may have arisen via a gene conversion or double-reciprocal recombination event which introduced the mutant allele into the chromosome. To determine whether this region contains the mutation, we compared the sequence of the entire *Hin*dIII-*Bam*HI fragment with the corresponding regions cloned from strains GL45 and SX50-8A, which were the parents of diploid strain GL53. We identified a single nucleotide change in the mutant allele (AGC to AGG) resulting in a Ser-to-Arg conversion at position 1972 (Table 2). In addition, we observed six silent nucleotide changes between the parental wild-type genes which allowed us to assign the *drr1-1* allele to the SX50-8A-derived locus. We next determined the nucleotide sequence of 200 bp flanking the Ser-1972 codon in eight additional independently selected mutants. Five of the alleles encoded the same Ser-to-Arg conversion; four of these contained the same nucleotide change, whereas one contained a AGC-to-AGA mutation. One allele contained an AGC-to-AAC mutation at this position resulting in a Ser-to-Asn conversion. The remaining two alleles did not show any nucleotide sequence alterations within this region. Again, the presence of silent mutations allowed us to determine the parental origin of each mutant allele (Table 2).

Given that seven of the nine *drr1* alleles tested were found to contain mutations in the same residue and that fragments of the gene spanning this site cause a high incidence of drug-resistant papillae, we conclude that replacement of Ser-1972 with either Arg or Asn results in dominant rapamycin resistance. This Ser residue lies downstream of both *URA3* insertions (Fig. 2A) and within the region of sequence similarity with PI 3-kinases; however, Ser-1972 is present in a short stretch of nonconserved residues (Fig. 5B). Since Ser-1972 matches the qualifications for a protein kinase C phosphorylation site (35), it is possible that the mutations therein change the state of phosphorylation, which could affect protein activity and/or localization.

DISCUSSION

The pharmacological effects of the immunosuppressive agents CsA, FK506, and rapamycin in mammalian cells are a direct consequence of their ability to block signal transduction pathways leading to T-cell activation (62). Since all three drugs possess antifungal activity, one way to rapidly elucidate their mechanisms of action has been to exploit the power of yeast genetics, with the hope that the pathways leading to cytotoxicity in yeast cells may parallel those leading to immunosuppression in animals. This approach has resulted in the discovery that yeast cells (as well as cells of other lower eucaryotes) possess many of the same primary receptors for these drugs, namely, the cyclophilin- and FKBP-related peptidyl-prolyl *cis-trans* isomerases, collectively referred to as immunophilins (13, 14, 19, 23, 26, 30, 37, 38, 40, 41, 48, 54, 56, 72, 74).

It has been firmly established that although CsA, FK506, and rapamycin bind tightly to and inhibit the enzymatic activity of their cognate receptor proteins, peptidyl-prolyl *cis-trans* isomerase inhibition cannot account for the immunosuppressive behavior of these drugs (6, 24, 45). The cumulative genetic and biochemical data indicate that immunosuppressive drugs act through the formation of an immunophilin-drug complex that selectively poisons signal transduction. Both CsA and FK506 block the same step in T-cell activation, namely, a Ca^{2+}-dependent signal transduction step that affects the phosphorylation of NF-AT, a factor required for IL-2 transcription (18, 21, 70). This effect is mediated by the specific inhibition of calcineurin protein phosphatase(s) (12, 24, 45, 55).

In contrast, rapamycin does not inhibit IL-2 synthesis but instead blocks T-cell proliferation dependent upon IL-2 (6, 16). The antiproliferative effects of rapamycin result from the formation of a complex with FKBP12 which appears to inhibit biochemical events required for the progression of IL-2-stimulated T cells from G_1 to S phase (6, 16). Recently, rapamycin has been shown to inhibit the activation of p70 S6 kinase in growth factor-stimulated T cells and fibroblasts (8, 11, 58). Moreover, rapamycin blocks the activation of $p34^{cdc2}$ (51), a cyclin-dependent kinase, which (in both yeast and mammalian cells) is required for the G_1-to-S-phase transition (17, 22, 25). In yeast cells, the $p34^{cdc2}$ homolog CDC28 is required for passage through START, the point in G_1 at which cells commit to a new cell division cycle (22, 50). $p34^{cdc2}$ kinase activity fluctuates during the cell cycle as a result of (i) transient complex formation with specific cyclins and (ii) changes in its own state of phosphorylation. Thus, the FKBP12-rapamycin complex appears to target a step in the pathway leading to the generation of activated $p34^{cdc2}$ kinase, which may implicate either a specific cyclin family member, p70 S6 kinase (which might be involved in the regulatory cascade leading to $p34^{cdc2}$ activation) (51), or an

upstream kinase (or phophatase) that coordinately activates both p70 S6 and p34^{cdc2}.

The fact that cells of *S. cerevisiae* lacking FKBP12 are viable and possess a recessive drug-resistant phenotype supports the model in which an FKBP12-rapamycin complex mediates rapamycin cytotoxicity in yeast strains (29, 37). In this study, we have identified mutations in two genes that encode proteins apparently involved in the rapamycin-sensitive pathway; these genes (*DRR1* and *DRR2*) may be equivalent to the *TOR1* and *TOR2* genes (for target of rapamycin) described by Heitman et al. (29). Molecular genetic analysis of the *drr1-1*^{dom} allele has revealed that the pathway leading to rapamycin cytotoxicity in yeast cells involves a novel protein with sequence similarity to the catalytic subunit of PI 3-kinase. The significance of this finding is not immediately clear. In mammalian cells, the cellular effects of peptide growth factors and hormones are mediated through their interactions with cell surface receptors, which stimulate an intracellular cascade of signaling events (3, 9, 68). Among these events are tyrosine kinase activation and the formation and activation of receptor-enzyme complexes that regulate the production of second messengers in cell signaling (66). One cytosolic enzyme associated with activated tyrosine kinases is PI 3-kinase, an enzyme that catalyzes the formation of a family of phosphorylated phosphoinositides with phosphate at the D-3 position of the inositol ring (9). The role of these phosphoinositides in the signal transduction process is unknown.

DRR1 is more closely related in sequence to the PI 3-kinases than to the protein kinases, suggesting that it may act upon a similar substrate. However, it is not clear whether it has PI 3-kinase activity, since yeast strains lacking VPS34 (65) have no residual PI 3-kinase activity. DRR1 may act on a completely different substrate or phosphorylate a different position in the inositol ring and thus may represent a PI kinase other than PI 3-kinase. Given its size, however, DRR1 is not likely to correspond to the known yeast PI 4-kinases, which have apparent molecular masses of 45 and 125 kDa (7, 20). Another possibility is that wild-type DRR1 kinase activity is inducible, perhaps requiring translocation to a membrane compartment where the specific PI substrate resides in order for activity to be detected.

An analogy here exists with the proto-oncogene *c-abl*, which encodes a protein with a tyrosine kinase and DNA-binding domain (36). During mitosis, the domain which binds DNA is phosphorylated, resulting in loss of DNA binding and movement from the nucleus to the cytoplasm, where it can associate with the cytoskeleton. Association with the cytoskeleton can also be stimulated by mutation or protein fusion, which leads to increased kinase activity and tumorigenicity (49) and decreased nuclear localization. Thus, nuclear localization seems to be associated with inhibition of its kinase activity. The presence of putative DNA-binding domains in DRR1 is consistent with the possibility that it has a nuclear function and that its kinase activity correlates with its subcellular localization.

Genomic disruption of *DRR1* indicated that it encodes a nonessential function. Furthermore, chromosome blot analysis revealed the existence of unlinked related sequences that may encode functional homologs of DRR1; the presence of these related sequences may account for the minor RNA species observed by Northern analysis. This possibility presents the following question: is DRR1 the true target of rapamycin, or rather do mutations in DRR1 allow it to bypass the function of the true target? Our original prediction was that the downstream target of the FKBP12-rapamycin complex would be an essential protein. It is interesting to note that during our mutant search, we obtained dominant drug-resistant mutations in the same two genes (*DRR1* and *DRR2*) with use of both a wild-type genetic background and a cell line in which rapamycin sensitivity is mediated by recombinant hFKBP12; thus, hFKBP12 is capable of chaperoning the drug to the same target that is normally recognized by the yeast FKBP12-rapamycin complex. The fact that yeast cells lacking DRR1 are viable but remain sensitive to rapamycin, however, implies the existence of additional target proteins, some of which may correspond to either DRR2 or proteins encoded by the putative homologs of *DRR1*. Although *DRR1* alone is nonessential, it may be part of a member of a small family of related genes that collectively encode an essential function. We conclude that although DRR1 appears to mediate rapamycin sensitivity, it may not be the sole target of rapamycin.

It is tempting to speculate that wild-type DRR1 possesses a PI kinase activity that is cell cycle regulated and/or regulated by nuclear localization. A reasonable corollary is that specific missense mutations in DRR1 can constitutively activate its kinase activity. Thus, the amino acid changes that we have identified in the rapamycin-resistant DRR1 protein may allow it to compensate for the loss of the proliferative signal inhibited by rapamycin by constitutively activating an alternative signal rather than by preventing its association with the FKBP12-rapamycin complex. The positions of the mutations within the kinase domain, but in a region not shared by the PI 3-kinases, support this idea. Therefore, it is entirely possible that DRR1 is not a component of the rapamycin-sensitive pathway in wild-type yeast cells. Instead, missense mutations in DRR1 at Ser-1972 may alter its normal activity and allow it to substitute for the function of an essential protein which is the true target of rapamycin. If Ser-1972 is, in fact, a site of phosphorylation, the state of phosphorylation may also control the cellular localization of the protein, analogous to the yeast SWI5 transcription factor (50). Alternatively, the mutations may be affecting the interaction of DRR1 with the real drug target in such a way as to overcome the effect of rapamycin. For example, if the activity of DRR1 is downstream of and modulated by the real rapamycin target, mutation at Ser-1972 may eliminate the need for activation for the protein to perform its function. In this model, the direct rapamycin target could be a specific phosphatase that normally dephosphorylates DRR1 at Ser-1972; thus, changes at Ser-1972 could constitutively activate its kinase activity. Obviously, further biochemical studies will be required to elucidate the cellular function of DRR1 and related proteins in order to ascertain their role in signal transduction and in the pathway leading to rapamycin cytotoxicity in *S. cerevisiae.*

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ADDENDUM

After submission of this report, Kunz et al. (41a) described the cloning and sequence analysis of the *TOR2* (target of

rapamycin) gene of *S. cerevisiae* and presented a partial protein sequence (73 amino acids) derived from a related gene, *TOR1*. Like *DRR1*, *TOR2* encodes a large PI 3-kinase-related protein. Unlike *DRR1*, however, *TOR2* is essential. Kunz et al. (41a) have proposed that rapamycin most likely acts by inhibiting the TOR PI kinase activity. From DNA sequence comparison, chromosome mapping, and the phenotype of gene disruptions, it is clear that *DRR1* is identical to *TOR1* (chromosome X) and *DRR2* is identical to *TOR2* (chromosome XI). A Bestfit comparison of the deduced protein sequences of DRR1/TOR1 (2,470 amino acids) and DRR2/TOR2 (2,474 amino acids) revealed that they are 67% identical (80% similar), with a high degree of conservation within the PI 3-kinase domain and a perfect C-terminal alignment. This sequence similarity suggests that these proteins have a related biological function. Furthermore, Ser-1972, which we have identified as the site of dominant drug-resistant mutations in DRR1 (TOR1), is conserved in DRR2 (TOR2), and we have recently found that our mutant *drr2-1^dom^* clone also contains a single base pair mutation altering this identical residue (in this case, Ser-1975 to Arg) (47a). This finding suggests a common mechanism for rapamycin resistance in both mutant proteins. Whether the DRR (TOR) proteins act as selective rapamycin targets or, instead, the mutant proteins acquire a new function that rescues cells from G_1 arrest in the presence of the drug will require further biochemical experiments to assay their physical interaction with the FKBP12-rapamycin complex.

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