Sit4 Phosphatase Is Functionally Linked to the Ubiquitin-Proteasome System

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ABSTRACT

Using a synthetic lethality screen we found that the Sit4 phosphatase is functionally linked to the ubiquitin-proteasome system. Yeast cells harboring *sit4* mutations and an impaired proteasome (due to *pre1-1 pre4-1* mutations) exhibited defective growth on minimal medium. Nearly identical synthetic effects were found when *sit4* mutations were combined with defects of the Rad6/Ubc2- and Cdc34/Ubc3-dependent ubiquitination pathways. Under synthetic lethal conditions, *sit4 pre* or *sit4 ubc* mutants formed strongly enlarged unbudded cells with a DNA content of 1N, indicating a defect in the maintenance of cell integrity during starvation-induced G_1 arrest. Sit4-related synthetic effects could be cured by high osmotic pressure or by the addition of certain amino acids to the growth medium. These results suggest a concerted function of the Sit4 phosphatase and the ubiquitin-proteasome system in osmoregulation and in the sensing of nutrients. Further analysis showed that Sit4 is not a target of proteasome-dependent protein degradation. We could also show that Sit4 does not contribute to regulation of proteasome activity. These data suggest that both Sit4 phosphatase and the proteasome act on a common target protein.

PROTEIN activity within the cell is controlled by three major mechanisms. Chemical modification of amino acid residues modulates the activity of proteins, while regulation of synthesis and selective proteolysis is implicated in controlling the cellular levels of proteins. Proteasomes are highly sophisticated protease complexes, which act as the major device for regulatory protein degradation in the cytoplasm and nucleus of the eukaryotic cell (for reviews see Coux *et al.* 1996; Hilt and Wolf 1996; Voges *et al.* 1999). The 26S proteasomes consist of a proteolytic core module, the 20S proteasome, and two 19S regulatory cap complexes attached to both ends of the 20S complex (Peters 1994). The 20S proteasome is a hollow cylindrically shaped complex, which includes three different proteolytic activities. *pre1-1 pre4-1* double mutants bearing mutations within β-subunits in the center of the 20S proteasome show defective chymotrypsin-like and peptidyl-glutamyl-peptide-hydrolyzing (PGPH) activity and are strongly impaired in proteasome dependent proteolysis (Hilt *et al.* 1993).

Nearly all proteins degraded via the 26S proteasome are marked by polyubiquitin chains. Ubiquitination of proteasomal substrates is performed by a complex enzyme system consisting of E1 (ubiquitin-activating enzymes), E2 (ubiquitin-conjugating enzymes), and, in many cases, E3 enzymes (ubiquitin-ligating enzymes; for reviews see Jentsch 1992; Ciechanover 1994; Hochstrasser 1996; Scheffner *et al.* 1998; Sommer 2000).

The ubiquitin-proteasome system is linked to a variety of different cellular pathways. Proteasomes are implicated in stress response by removal of abnormal proteins generated by heat stress, exposure of cells to amino acid analogs, or certain mutations (Heinemeyer *et al.* 1991, 1993; Hilt *et al.* 1993; Hiller *et al.* 1996; Gerlinger *et al.* 1997; Plemper *et al.* 1997). Proteasome-mediated destruction of defined substrate proteins is an essential regulatory step in many different cellular pathways, such as metabolic adaptation (Murakami *et al.* 1992; Kornitzer *et al.* 1994; Schork *et al.* 1995), cell differentiation (Chen *et al.* 1993; Richter-Ruoff *et al.* 1994), or cell cycle control (for reviews see Deshaies 1995; Hilt and Wolf 1996; King *et al.* 1996; Mann and Hilt 2000).

We performed a search for components that are physically or functionally linked to the proteasome system. We thereby discovered that a mutation residing in the *SIT4* gene causes a synthetic effect when combined with proteasomal mutations. Originally, *SIT4* was isolated in a screen for mutations that allowed expression of the *HIS4* gene in the absence of its native transcription factors Bas1, Bas2, and Gcn4 (Arndt *et al.* 1989). The *SIT4/PPH1* gene codes for a serine/threonine protein phosphatase of the PP2A family (for overview see Stark 1996). Sit4 is implicated in the transcription of various genes and also has a function in control of the G_1 phase of the cell cycle (Sutton *et al.* 1991a,b). A complex of Sit4 and the Tap42 protein, which acts as a regulator of Sit4 activity, is part of the TOR signaling pathway (Di Como and Arndt 1996; Jiang and Broach 1999). The TOR pathway, which can specifically be inhibited by the

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drug rapamycin, controls protein translation in response to nutrient availability (Hall 1996; Thomas and Hall 1997).

In some yeast strains the Sit4 phosphatase provides essential functions. Such vital necessity on *SIT4* depends on the character of the polymorphous *SSD1* gene (***s**uppressor of **s**it4 **d**eletion*; Sutton *et al.* 1991a). Deletion of *SIT4* is lethal in cells harboring an *ssd1-d* allele. In contrast, *sit4*Δ cells containing an *SSD1-v* allele are viable but grow with a significantly reduced rate. Phenotypic effects caused by the *sit4-102* mutation also depend on the *SSD1* allele present in the cell (Sutton *et al.* 1991a). The *SSD1* gene codes for a 1250-amino-acid protein, whose exact function is still unclear. The *SSD1* gene product has multisuppressor characteristics. Besides suppression of the *sit4* deletion, *SSD1-v* alleles also partially cure defects that are caused by hyperactivation of the Ras-cyclase-cAPK pathway (Sutton *et al.* 1991a; Wilson *et al.* 1991), by *ins1* (Wilson *et al.* 1991), by a *bem2* mutation (Kim *et al.* 1994), or by failures of the cell integrity/protein kinase C (PKC1) pathway (Costigan *et al.* 1992; Laporte *et al.* 1996). Ssd1 also functions in cell cycle control (Sutton *et al.* 1991a; Cvrckova and Nasmyth 1993; Stettler *et al.* 1993; Kikuchi *et al.* 1994; Uesono *et al.* 1997).

In this study we show that the Sit4 phosphatase and the proteasome are functionally connected. Because Sit4 does not control proteasomal activity and the proteasome does not degrade Sit4, this linkage seems to be indirect. The concerted action of both systems—which appears to be independent from the role of Sit4 in TOR-signaling—is required for cell maintenance during starvation-induced G_1 arrest.

MATERIALS AND METHODS

Growth conditions: Plasmids were amplified in *Escherichia coli* strains DH5α, GM2163 (New England Biolabs, Beverly, MA), or DB6656. Bacteria were grown at 37° in LB medium, containing 50 μg/ml ampicillin for plasmid selection when necessary. Yeast cells were grown in YPD (1% yeast extract, 2% peptone, 2% glucose) or in synthetic minimal medium, SD (2% glucose, 0.67% Difco yeast nitrogen base without amino acids containing 20 μg/ml uracil, 20 μg/ml histidine, 30 μg/ml leucine as supplements). Ammonia-based synthetic complex dextrose (SC) media contained 120 mg/liter myo-inositol, 12 mg/liter para-aminobenzoic acid, and all amino acids at a concentration of 120 mg/liter with the exception of L-leucine, L-tryptophan, L-lysine, and L-histidine. If required for growth of strains containing auxotrophic mutations, SC medium was supplemented with 220 mg/liter L-leucine, 62.8 mg/liter L-histidine, 180 mg/liter L-lysine, 81.7 mg/liter L-tryptophan, 55.3 mg/liter adenine, or 22.4 mg/liter uracil. Selection for uracil auxotrophs was performed on SD medium containing 50 mg/liter uracil and 0.1% w/v 5-fluoroorotic acid (5-FOA). Yeast cells were sporulated in 1% potassium acetate medium.

General methods: Transformation of *E. coli* cells, restriction reactions, DNA ligations, and other recombinant DNA techniques were performed following standard protocols as described in Sambrook *et al.* (1989) and Ausubel *et al.* (1990). Mating of yeast cells, sporulation, tetrad dissection, plasmid segregation, and plasmid rescue were done as described (Sambrook *et al.* 1989; Ausubel *et al.* 1990; Guthrie and Fink 1991). Yeast transformations were performed by a modified version of the DMSO method (Heinemeyer *et al.* 1991) or by the lithium acetate method (Gietz *et al.* 1992).

Plasmids: Plasmid pTS4 encoding the *PRE1* and *PRE4* genes was constructed by inserting a 1.15-kb *Eco*RI/*Bam*HI fragment carrying *PRE1* into the multicloning site of pDP83 [*URA3 CEN14 ARS1*] followed by the insertion of a 2-kb *Hin*dIII/*Sph*I fragment carrying *PRE4*. Plasmid pTS5 was generated by insertion of a 1.5-kb *Eco*RI fragment containing the *pre1-1* allele into the *Eco*RI site of plasmid pRS313 (Sikorski and Hieter 1989). Plasmid pTS6 was obtained by insertion of a 1.85-kb *Eco*RI/*Sac*I fragment that contained the *pre4-1* sequence into *Eco*RI/*Sac*I sites of plasmid pRS313. Plasmid pTS9 [*PRE1 PRE4 HIS3 CEN6 ARSH4*] was made by consecutive insertion of a 1.9-kb *Eco*RI/*Sac*I *PRE4* fragment and a 1.5-kb *Eco*RI *PRE1* fragment into plasmid pRS313. A 0.6-kb *Xba*I/*Eco*RV fragment containing the *MET3* promoter was excised from plasmid pHAM8 (Mountain and Korch 1991) and inserted into *Xba*I/*Eco*RV-digested pRS313 [*HIS3 ARSH4 CEN6*] (Sikorski and Hieter 1989), yielding plasmid pTS12. To generate plasmid pTS13, which contains *PRE1* under the control of the *MET3* promoter, a 0.6-kb fragment containing the *PRE1* gene was amplified by PCR and inserted into the *Eco*RV site of pTS12. Plasmid pTS14 containing *SIT4* controlled by the *MET3* promoter was obtained by insertion of a 1-kb *SIT4* fragment, which was amplified by PCR using p102/20 as the template. Plasmids pCK1 and pCK2 were obtained by insertion of a 2-kb *Sac*II/*Pvu*II fragment containing *SIT4* into the *Sac*II/*Sma*I sites of plasmid pRS305 [*LEU2*] and pRS315 [*LEU2 ARSH4 CEN6*], respectively (Sikorski and Hieter 1989). To construct plasmid pES1, which contains a truncated version of *SIT4*, plasmid pCK2 was digested with *Bgl*II/*Nru*I, blunted, and religated. To generate pTS100 [Δ*sit4::URA3 LEU2*], the *Hin*dIII site in the backbone of plasmid pCK2 was removed by digestion with *Hin*dIII, filling of the ends by treatment with Klenow enzyme, and religation. Thereafter, almost the complete *SIT4* open reading frame (codons: 14–312 and 8 bp from the 3′-region) was excised by digestion with *Xba*I/*Sph*I. Overhanging ends were blunted by treatment with Klenow enzyme and T4-DNA polymerase and, using a *Hin*dIII linker, a 1.2-kb *Hin*dIII fragment containing the *URA3* gene was inserted, yielding pTS100. The plasmid pTS64 [*SSD1-v LEU2 ARSH4 CEN6*] was constructed by insertion of a 4.7-kb *Pvu*II fragment containing the *SSD1-v* allele into the *Sma*I site of vector pRS315 (Sikorski and Hieter 1989). The plasmids CB239 [*HA-SIT4 LEU2*] and CB243 [*SIT4-HA LEU2*] containing epitope-tagged versions of *SIT4* were a gift of K. Arndt. To isolate the *sit4-51* allele, plasmid pES1 was gapped by *Xba*I/*Sph*I digestion. The linear fragment that contained *SIT4* 5′- and 3′-flanking regions (572-bp *Sph*I/*Pvu*II fragments and 464-bp *Sac*II/*Xba*I fragments) at its ends was used to transform the *sit4-51* strain YTS98/5b. The repaired plasmid pES2 harboring the *sit4-51* mutant sequence was isolated from leucine prototrophic transformants.

Yeast strains: YTS40 cells were made by transforming WCG4a/α cells with a linear 1.98-kb *Sac*II/*Sal*I fragment derived from pTS100 that contained the *sit4::URA3* allele. Correct integration of the *sit4*Δ sequence was verified by Southern analysis. A *sit4*Δ strain that was viable due to the presence of a plasmid-encoded *SSD1-v* allele (YTS43) was isolated from tetrads of YTS40 diploids that had been transformed with pTS64 (*SSD1-v*). WCG4 isogenic *sit4-51* cells were made by one-step gene replacement. YTS43 cells were transformed with a linear 2-kb fragment harboring the *sit4-51* mutant sequence isolated from the *Sac*II/*Pvu*II-digested pES2 plasmid. Cells

that had lost the *URA3* marker gene due to homologous recombination of the *sit4-51* sequence with the chromosomal *sit4*Δ*::URA3* locus were selected on 5-FOA medium. Correct integration of the *sit4-51* mutant gene was verified by Southern analysis. After growth on nonselective medium, cells were screened for clones that had lost the *SSD1-v LEU2* encoding plasmid during mitotic division, yielding the *sit4-51* single-mutant strain YTS100. WCG4 isogenic *sit4-102* cells (strain YSH2) were obtained by a similar strategy. The *sit4 pre1-1 pre4-1* triple-mutant YTS102 was constructed by dissection of tetrad spores from a diploid strain (YTS101) obtained by crossing strains YTS100 (*sit4-51*) and YHI29/14 (*pre1-1 pre4-1*). Both the *sit4-51* single mutation and the *pre1-1 pre4-1* double mutations cause temperature sensitivity (ts; Hilt *et al.* 1993). Therefore, temperature-sensitive segregants derived from a nonparental ditype tetrad showing a 2:2 segregation for the ts phenotype should harbor all three mutations. Such clones were confirmed to contain the *pre1-1* and *pre4-1* mutations by measuring their defects for 20S proteasomal chymotrypsin-like and PGPH activity. The identity of a segregant with these properties, YTS102, as a *sit4-51 pre1-1 pre4-1* triple-mutant strain was further confirmed by backcrossing with wild-type cells (WCG4α). As expected, tetrads derived from such diploids showed 4:0, 3:1, and 2:2 segregation patterns for the ts phenotype. The *sit4-102 pre1-1 pre4-1* mutant strain YSH3 was constructed following a similar strategy using diploid cells obtained by crossing YSH2 (*sit4-102*) with YHI30/14 cells (*pre1-1 pre4-1*). Strains YMHO17 [*sit4*Δ (*HA-SIT4*)] and YMHO18 [*sit4*Δ (*SIT4-HA*)] were derived from tetrad dissection of YTS40 diploids transformed with CB239 and CB243, respectively. *cdc34-1* cells (strain YSH10) and *rad6*Δ cells (strain YSH12) with the genetic background of WCG4 were generated by backcrossing strains W432 and YWO62 four times with WCG4 cells. To generate *sit4-51 ubc1* double mutants, YHI124/12D cells [*ubc1*-Δ (*UBC1, URA3*)] were crossed with YTS100 cells, then sporulated, and dissected. Resulting *sit4-51 ubc1* segregants were then selected for loss of the complementing *UBC1* plasmid by streaking on 5-FOA plates. *ubc10 sit4-51*, *ubc11 sit4-51*, and *ubc13 sit4-51* cells were obtained from a cross of YTS100 cells with Y04763 (*ubc10*Δ*::kanMX4*), Y01636 (*ubc11*Δ*-kanMX4*), and Y04027 (*ubc13*Δ*::kanMX4*) strains (Euroscarf), respectively. The other *sit4 ubc* mutants were derived from crosses of *sit4-51* cells with strains YWO23, W303BP, W303BQ, and 8b. All yeast strains are listed in Table 1.

Genetic mapping of the chromosomal *sli1-1* locus: A 2-kb *Sac*II/*Pvu*II complementing fragment (derived from plasmid pCK1) containing the *SIT4* gene linked to the *LEU2* auxotrophic marker gene was integrated in strain YTS91 at its native chromosomal locus. The resulting strain YTS93 was backcrossed with YTS21α. All tetrads derived from the resulting diploids showed 4:0 segregation of viability on 5-FOA medium, evidencing that the *sli1-1* mutation is allelic to the *SIT4* gene. An identical genetic analysis using integration of the complementing *SSD1*-containing DNA resulted in both viable and nonviable spores on 5-FOA, confirming that *sli1-1* is not allelic with *SSD1*.

Purification of the 20S proteasome and activity assays: 20S proteasomes were partially purified by gel filtration as described in Fischer *et al.* (1994). *In vitro* assays for proteasomal activities were done according to Hilt and Wolf (1999). Pulse-chase experiments for fructose-1,6-bisphosphatase (FBPase) degradation were done as described in Gueckel *et al.* (1998). For cycloheximide chase cells were grown in SC medium to an OD_{578} of 1.5–2. To block protein synthesis, cycloheximide was added to a final concentration of 0.5 mg/ml. At indicated times, cells were harvested and suspended in 1 ml H_2O (OD_{578} = 3) and thereafter lysed by the addition of 150 μl 1.85 M NaOH, 7.5% 2-mercaptoethanol solution (10 min incubation at 0°). Proteins were precipitated by addition of trichloroacetic acid (final concentration 5%). Precipitates were solubilized in 200 mM Tris/HCl pH 6.8, 8 M urea, 5% SDS, 0.1 mM EDTA, and 1.5% dithiothreitol and proteins were separated by electrophoresis on 10% SDS-polyacrylamide gels. After blotting onto nitrocellulose membranes, proteins were visualized using monoclonal mouse-anti-hemagglutinin (HA) antibodies (12CA5; Roche Diagnostics) by enhanced chemiluminescence detection (Amersham Pharmacia Biotech).

RESULTS

Mutations that cause synthetic lethality with an impaired proteasome: To identify genes/proteins whose function is linked to the proteasome system we performed a screen for mutations that cause synthetic lethality when combined with impaired proteasomal proteolysis (*sli* mutations: *s*ynthetic *l*ethality with *i*mpaired proteasome function). The yeast strain used for this screen (YTS21) harbored mutations in two chromosomal 20S proteasome β-type genes (*pre1-1 pre4-1*) and the complementing plasmid pTS4 containing both wild-type genes *PRE1* and *PRE4* and the *URA3* marker gene. As evidenced by activity tests for proteasome-mediated peptide cleavage, YTS21 cells exhibit wild-type activity. *pre1-1 pre4-1* mutant cells are viable (Hilt *et al.* 1993). Thus, on nonselective medium YTS21 cells are able to lose plasmid pTS4 during mitotic division. Cells can thereby switch from wild type to proteasome mutant background and become uracil auxotrophs. YTS21-derived mutants containing a *sli* mutation are expected to grow only when the proteasome is functional and therefore depend on the presence of plasmid pTS4. Thus *sli* mutants cannot lose pTS4 and can therefore be detected by their inability to grow on 5-FOA medium, which is toxic for uracil prototrophs.

YTS21 cells were mutagenized with ethyl methanesulfonate to a residual viability of ~10% and plated on SC (Ura^-) plates. To enable mitotic loss of the plasmid pTS4, the cells were transferred to nonselective medium (YPD). Thereafter a total of 2500 clones were replica plated on 5-FOA containing minimal agar medium and screened for strains that could not grow under these conditions. Potential *sli* mutants were retested by streaking on 5-FOA medium, yielding 15 *sli* mutants. These mutants were backcrossed to their respective wild-type cells (YTS21α) and the diploids were subjected to sporulation and tetrad analysis. By these means, seven mutants were proven to contain single gene mutations as shown for the *sli1-1* (*sit4-51*) mutant YTS91 (Figure 1). Crossing of these *sli* mutants resulted in the identification of at least four complementation groups. To exclude the possibility that 5-FOA-induced lethality of the mutants was based on an undesired chromosomal integration of the *URA3* gene, the *sli* mutants were transformed with plasmid pTS9 containing the *PRE1* and *PRE4* wild-type genes. With the exception of one clone, all pTS9 transformants regained the ability to lose plasmid pTS4, dem-

TABLE 1

Yeast strains used

Strain	Genotype	Source
WCG4a	*MAT***a** *his3-11,15 leu2-3,112 ura3 Can^S Gal$^+$*	Heinemeyer *et al.* (1991)
WCG4α	*MATα his3-11,15 leu2-3,112 ura3 Can^S Gal$^+$*	W. Hilt
WCG4a/α	*MAT***a**/α *his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 ura3/ura3 Can^S/Can^S Gal$^+$/Gal$^+$*	W. Hilt
YHI29/14[a]	*MAT***a** *pre1-1 pre4-1 ura3 leu2-3,112 his3-11,15 Can^S Gal$^+$*	Spore YHI29a/α
YHI29/1[a]	*MATα pre1-1 ura3 leu2-3,112 his3-11,15 Can^S Gal$^+$*	Spore YHI29a/α
YHI29/4[a]	*MATα pre4-1 ura3 leu2-3,112 his3-11,15 Can^S Gal$^+$*	Spore YHI29a/α
YHI30/14[a]	*MATα pre1-1 pre4-1 ura3 leu2-3,112 his3-11,15 Can^S Gal$^+$*	Spore YHI29a/α
YHI124/12D[a]	*MAT***a** *ubc1Δ::HIS3 ura3 his3-11,15 leu2-3,112 trp1Δ::loxp Can^S Gal$^+$ [UBC1 URA3]*	This work
YTS21[a]	*MAT***a** *pre1-1 pre4-1 ura3 leu2-3,112 his3-11,15 Can^S Gal$^+$ [PRE1 PRE4 URA3]*	YHI29/14 transformed with plasmid pTS04
YTS21α[a]	*MATα pre1-1 pre4-1 ura3 leu2-3,112 his3-11,15 Can^S Gal$^+$ [PRE1 PRE4 URA3]*	YHI30/14 transformed with plasmid pTS04
YTS40[a]	*MAT***a** *MATα SIT4/sit4Δ::URA3 ura3/ura3 leu2-3,112/ leu2-3,112 his3-11,15/his3-11,15 Can^S/Can^S Gal$^+$/Gal$^+$*	*sit4* deletion in WCG4a/α
YTS43[a]	*MATα sit4-Δ::URA3 ura3 his3-11,15 leu2-3,112 Can^S Gal$^+$ [SSD1-v LEU2]*	This work
YTS91[a]	*MAT***a** *sli1(sit4-51) pre1-1 pre4-1 ura3 leu2-3,112 his3-11,15 Can^{S}Gal$^+$ [PRE1 PRE4 URA3]*	This work
YTS93[a]	*MAT***a** *MATα SIT4/sit4-51::LEU2::SIT4 pre1-1/pre1-1 pre4-1/pre4-1 ura3/ura3 leu2-3,112/leu2-3,112 his3-11,15/ his3-11,15 Can^S/Can^S Gal$^+$/Gal$^+$ [PRE1 PRE4 URA3]*	YTS91 × YHI30/14
YTS94[a]	*MAT***a** *sit4-51 ura3 leu2-3,112 his3-11,15 Can^S Gal$^+$ [pGAL1::PRE1 LEU2]*	YTS91 transformed with pDP60GAL1.E1
YTS95[a]	*MAT***a** *sit4-51 ura3 leu2-3,112 his3-11,15 Can^{S}Gal$^+$ [pMET3::PRE1 HIS3]*	YTS91 transformed with pTS13
YTS96[a]	*MAT***a** *sit4-51 ura3 leu2-3,112 his3-11,15 Can^{S}Gal$^+$ [pMET3::SIT4 HIS3]*	YTS91 transformed with pTS14
YTS98[a]	*MAT***a** *MATα SIT4/sit4-51(sli1-1) pre1-1/pre1-1 pre4-1/pre4-1 ura3/ura3 leu2-3,112/leu2-3,112 his3-11,15/his3-11,15 Can^S/Can^S Gal$^+$/Gal$^+$ [PRE1 PRE4 URA3]*	YTS21 × YHI91
YTS98/5b[a]	*MATα sit4-51 pre1-1 pre4-1 ura3 leu2-3,112 his3-11,15 Can^S Gal$^+$ [PRE1 PRE4 URA3]*	Spore of YTS98
YTS100[a]	*MATα sit4-51 ura3 leu2-3,112 his3-11,15 Can^{S}Gal$^+$*	This work
YTS101[a]	*MAT***a** *MATα SIT4/sit4-51 PRE1/pre1-1 PRE41/pre4-1 ura3/ura3 leu2-3,112/leu2-3,112 his3-11,15/his3-11,15 Can^S/Can^S Gal$^+$/Gal$^+$*	YTS100α × YHI29/14
YTS102[a]	*MAT***a** *sit4-51 pre1-1 pre4-1 ura3 leu2-3,112 his3-11,15 Can^{S}Gal$^+$*	This work
YMHO17[a]	*MAT***a** *sit4Δ::URA3 [HA-SIT4 LEU2] ura3 leu2-3,112 his3-11,15 Can^S Gal$^+$*	Spore of YTS40 transformed with CB239
YMHO18[a]	*MAT***a** *sit4Δ::URA3 [SIT4-HA LEU2] ura3 leu2-3,112 his3-11,15 Can^S Gal$^+$*	Spore of YTS40 transformed with CB243
YMHO19[a]	*MAT***a** *MATα PRE1/pre1-1 PRE4/pre4-1 SIT4/sit4Δ::URA3 [HA-SIT4 LEU2] ura3/ura3 leu2-3,112/ leu2-3,11 his3-11,15/his3-11,15 Can^S Gal$^+$*	YMHO17 × YHI30/14
YMHO20[a]	*MAT***a** *MATα PRE1/pre1-1 PRE4/pre4-1 SIT4/sit4Δ::URA3 [SIT4-HA LEU2] ura3/ura3 leu2-3,112/ leu2-3,11 his3-11,15/his3-11,15 Can^S Gal$^+$*	YMHO18 × YHI30/14
YMHO21[a]	*MATα sit4Δ::URA3 [HA-SIT4 LEU2] ura3 leu2-3,112 his3-11,15 Can^S Gal$^+$*	Spore of YMHO19
YMHO23[a]	*MATα pre1-1 sit4Δ::URA3 [HA-SIT4 LEU2] ura3 leu2-3,112 his3-11,15 Can^S Gal*	Spore of YMHO19
YMHO26[a]	*MATα pre1-1 pre4-1 sit4Δ::URA3 [HA-SIT4 LEU2] ura3 leu2-3,112 his3-11,15 Can^S Gal*	Spore of YMHO19
YMHO27[a]	*MATα sit4Δ::URA3 [SIT4-HA LEU2] ura3 leu2-3,112 his3-11,15 Can^S Gal*	Spore of YMHO20
YMHO29[a]	*MATα pre1-1 sit4Δ::URA3 [SIT4-HA LEU2] ura3 leu2-3,112 his3-11,15 Can^S Gal*	Spore of YMHO20
YMHO33[a]	*MATα pre1-1 pre4-1 sit4Δ::URA3 [SIT4-HA LEU2] ura3 leu2-3,112 his3-11,15 Can^S Gal*	Spore of YMHO20
W432	*MAT***a** *cdc34-1 ade2-1 leu2-3,112 his3-11 trp1-1 ura3*	W. Seufert
YWO23	*MATα ubc4Δ::HIS3 ubc5Δ::LEU2 lys2-801 leu2-3,112 ura3-52 his3-200 trp1-1*	W. Seufert
YWO62	*MAT***a** *rad6Δ::HIS3 lys2-801 leu2-3,112 ura3-52 his3-200 trp1-1*	W. Seufert

(*continued*)

TABLE 1

(Continued)

Strain	Genotype	Source
W3031BP[c]	*MAT*α *ubc6*Δ*::LEU2 ade2-1 leu2-3,112 his3-11,15 trp1-1 ura3*	Hiller *et al.* (1996)
W3031BQ[c]	*MAT*α *ubc7*Δ*::LEU2 ade2-1 leu2-3,112 his3-11,15 trp1-1 ura3*	Hiller *et al.* (1996)
8b[c]	*MAT***a** *ubc8*Δ*::URA3 ade2-1 leu2-3,112 his3-11,15 trp1-1 ura3*	W. Seufert
YO4763[b]	*MAT***a** *his3*Δ*1 leu2*Δ*0 ura3*Δ*0 met15*Δ*0 ubc10*Δ*::kanMX4*	Euroscarf
YO1636[b]	*MAT***a** *his3*Δ*1 leu2*Δ*0 ura3*Δ*0 met15*Δ*0 ubc11*Δ*::kanMX4*	Euroscarf
YO4027[b]	*MAT***a** *his3*Δ*1 leu2*Δ*0 ura3*Δ*0 met15*Δ*0 ubc13*Δ*::kanMX4*	Euroscarf
YSH2[a]	*MAT***a** *sit4-102 ura3 leu2-3,112 his3-11,15 Can^{S}Gal$^+$*	This work
YSH2α[a]	*MAT*α *sit4-102 ura3 leu2-3,112 his3-11,15 Can^{S}Gal$^+$*	This work
YSH3[a]	*MAT***a** *sit4-102 pre1-1 pre4-1 ura3 leu2-3,112 his3-11,15 Can^{S}Gal$^+$*	This work
YSH4[a]	*MAT***a** *sit4-102 pre1-1 ura3 leu2-3,112 his3-11,15 Can^{S}Gal$^+$*	This work
YSH5[a]	*MAT***a** *sit4-102 pre4-1 ura3 leu2-3,112 his3-11,15 Can^{S}Gal$^+$*	This work
YSH6[a]	*MAT***a** *sit4-51 pre4-1 ura3 leu2-3,112 his3-11,15 Can^{S}Gal$^+$*	This work
YSH7[a]	*MAT***a** *sit4-51 pre1-1 ura3 leu2-3,112 his3-11,15 Can^{S}Gal$^+$*	This work
YSH10[a]	*MAT***a** *cdc34-1 ura3 leu2-3,112 his3-11,15 Can^{S}Gal$^+$*	Four time backcross of W432 × WCGα
YSH11[a]	*MAT***a** *sit4-51 cdc34-1 ura3 leu2-3,112 his3-11,15 Can^{S}Gal$^+$*	YSH10 × YTS100α
YSH12[a]	*MAT***a** *rad6*Δ*::HIS3 ura3 leu2-3,112 his3-11,15 Can^{S}Gal$^+$*	Four time backcross of YWO62 × WCGα
YSH13[a]	*MAT***a** *sit4-51 rad6*Δ*::HIS3 ura3 leu2-3,112 his3-11,15 Can^{S}Gal$^+$*	This work
YSH19[a]	*MAT***a** *sit4-102 cdc34-1 ura3 leu2-3,112 his3-11,15 Can^{S}Gal$^+$*	YSH13 × YSH2α

[a] These strains are isogenic with WCG4.
[b] These strains are isogenic with BY4741; WCG4 and BY4741 are derivatives of strain S288c.
[c] These strains are isogenic with W303.

FIGURE 1.—YTS91 cells contain a single gene mutation that confers synthetic lethality with an impaired proteasome. Growth of yeast cells on SC-5-FOA medium (30°, 3-day incubation). YTS21 [*pre1-1 pre4-1* (*PRE1 PRE4 URA3*)]: strain used for EMS mutagenesis and synthetic lethal screening; YTS91 [*sli1-1* (*sit4-51*) *pre1-1 pre4-1* (*PRE1 PRE4 URA3*)]: mutant strain isolated in the synthetic lethal screen. Tetrad spores derived from a cross of YTS21α × YTS91 show 2:2 segregation of synthetic lethality (shown is a typical tetrad: YTS98/1a/1b/1c/1d).

onstrating that lethality was due to a new mutation in the genetic background of strain YTS21. To confirm that lethality of *sli* mutants was not based on undesired lethal mutations within the *pre1* or *pre4* coding sequences, we tested whether growth of the clones could be restored by complementation with plasmid-derived *pre1-1* or *pre4-1* alleles. After transformation with either pTS1 (*pre1-1 HIS3*) or pTS6 (*pre4-1 HIS3*), all clones remained unable to grow on 5-FOA medium, demonstrating that the *sli* mutations were not allelic with *pre1-1* or *pre4-1*.

***SIT4/PPH1* and *SSD1* complement the synthetic growth defect of *sli1* mutants:** A *sli1-1* mutant strain (YTS98/5b) was used to isolate complementing DNA. Cells were transformed with a YCplac111-derived *Saccharomyces cerevisiae* genomic DNA library. Screening of ~25,000 transformants yielded 50 clones with restored viability on 5-FOA medium. To avoid isolation of library plasmids harboring a complementing *PRE1* gene, plasmids were isolated from transformants that still showed defective proteasomal chymotrypsin-like activity. In this way two plasmids, p102/20 and p102/25, which contained independent genomic inserts, were obtained.

Plasmid p102/20 harbored a 2.8-kb genomic fragment and contained the entire open reading frame of *SIT4/PPH1*, which by subcloning was proven to be responsible for complementation of the synthetic defect of the *sli1-1* mutants (YTS91 cells). *SIT4* codes for a serine/threonine phosphatase involved in gene expression and cell cycle control (ARNDT *et al.* 1989; POSAS *et al.* 1991; SUTTON *et al.* 1991a; FERNANDEZ *et al.* 1992). The second plasmid, p102/25, harbored a genomic insert of ~12 kb in length. Subcloning restricted the complementing DNA to a 4.7-kb *Pvu*II fragment, containing the complete open reading frame of the *SSD1* gene. *SSD1* was known to function as a low-copy suppressor of certain Sit4-related defects (SUTTON *et al.* 1991a).

A genetic analysis (ROSE and BROACH 1991) was performed to uncover the chromosomal location of the mutation conferring the synthetic growth defect to YTS91 cells (for details see MATERIALS AND METHODS). This analysis demonstrated that *sli1-1* resides within the *SIT4* locus and is nonallelic with *SSD1*. Therefore, *SSD1*-mediated complementation of the synthetic growth defect of YTS91 could be assigned to a low-copy suppressor effect. Deletion of *SIT4* in the wild-type strain WCG4 resulted in lethality. This result confirms that YTS91 cells, which are derived from WCG4, harbor an *ssd1-d* allele. The lethality caused by the deletion of *SIT4* in WCG4 was cured after transformation of the cells with the centromeric plasmid pTS64 (*LEU2 CEN6*), which contained the *SSD1* allele isolated from the YCplac111-derived genomic library. Therefore, we conclude that pTS64 contains an *SSD1-v* allele. These data clearly evidence that the synthetic defects of YTS91 cells depend on the presence of an *ssd1-d* allele and are suppressed by low-copy expression of an *SSD1-v* allele.

The *sit4-51* mutation causes temperature sensitivity: The *sit4* mutant allele was rescued from strain YTS91 by "gap repair" (ROTHSTEIN 1991). Sequencing of the *sit4* mutant allele revealed a single point mutation within

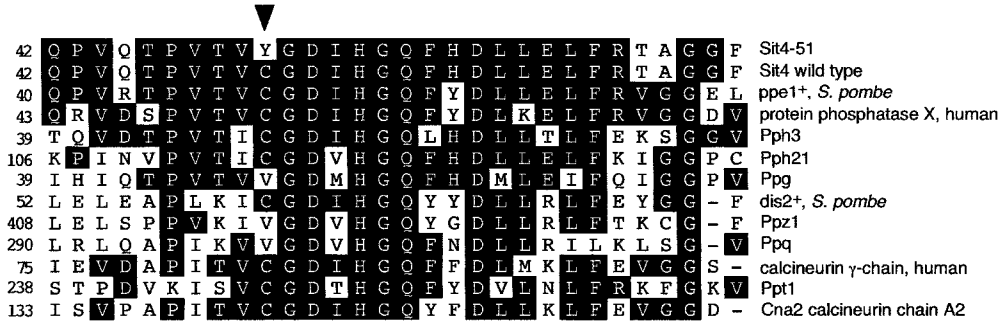

FIGURE 2.—The *sit4-51* mutation is located in a highly conserved region among protein phosphatases, marked by an arrowhead. Cysteine 51 is conserved within many protein phosphatases. Sequences are arranged according to decreasing overall homology of the respective protein with the Sit4 sequence. *Schizosaccharomyces pombe* (ppe1$^+$, dis2$^+$) and human (protein phosphatase X, calcineurin γ-chain) proteins are indicated. The others are *S. cerevisiae* proteins.

TABLE 2

Growth of *sit4*, *pre1-1*, *pre4-1* double, single, and triple mutants on SD and YPD medium

Strain	Genotype	25°	28°	30°	37°
SD medium					
WCG4a	Wild type	+++	+++	+++	++
YHI29/14	*pre1-1 pre4-1*	++	++	+	−
YTS100	*sit4-51*	+++	+++	++	−
YTS102	*sit4-51 pre1-1 pre4-1*	++	+	−	−
YSH2	*sit4-102*	+++	+++	+	−
YSH3	*sit4-102 pre1-1 pre4-1*	++	−	−	−
YHI29/1	*pre1-1*	ND	ND	++	−
YSH7	*sit4-51 pre1-1*	ND	ND	−	−
YSH4	*sit4-102 pre1-1*	ND	ND	−	−
YHI29/4	*pre4-1*	ND	ND	+++	++
YSH6	*sit4-51 pre4-1*	ND	ND	+	−
YSH5	*sit4-102 pre4-1*	ND	ND	−	−
YPD medium					
WCG4a	Wild type			+++	+++
YHI29/4	*pre1-1 pre4-1*			++	−
YTS100	*sit4-51*			+++	+
YTS102	*sit4-51 pre1-1 pre4-1*			++	−
YSH2	*sit4-102*			+++	−
YSH3	*sit4-102 pre1-1 pre4-1*			++	−

Wild-type cells and mutants containing different combinations of *sit4-51*, *sit4-102*, *pre1-1*, and *pre4-1* mutations were streaked on SD and YPD agar medium and incubated for 50 hr at different temperatures as indicated. Cell growth was classified according to size of colonies using arbitrary units: +, ++, +++. −, no visible colonies (synthetic lethal cells); ND, not determined.

the *SIT4* coding region (guanine 152 was mutated to adenine, resulting in replacement of cysteine 51 by tyrosine). This mutation, referred to as *sit4-51* hereafter, resides in a region that is highly conserved among Ser/Thr-phosphatases in yeast and other species (Figure 2). Moreover, this cysteine residue is conserved in many members of the protein phosphatase family (Figure 2). To study the phenotypic effects of the *sit4-51* mutation we constructed a *sit4-51* mutant strain (YTS100) with the genetic background of WCG4a by one-step gene replacement (see MATERIALS AND METHODS). Similar to the previously described *sit4-102* mutation (SUTTON *et al.* 1991a), *sit4-51* caused ts under certain conditions. YTS100 cells show normal growth on YPD at 30°. However, when shifted to 37°, *sit4-51* mutants grow at reduced rates (Table 2). On minimal medium YTS100 cells can form colonies at 30° but do not grow at elevated temperatures (37°; Table 2). The *sit4-51* mutation is recessive and *sit4-51*-induced temperature sensitivity can be fully complemented by a plasmid-encoded *SIT4* wild-type gene. The temperature sensitivity of *sit4-51* mutants could also be complemented by expression of a plasmid-derived *SSD1-v* gene (data not shown), demonstrating that, as previously shown for *sit4-102* (SUTTON *et al.* 1991a), *sit4-51* causes temperature sensitivity only in the *ssd1-d* background.

***pre1-1 pre4-1 sit4-51*-induced lethality depends on limitation of nutrients:** We performed shut-off experiments to determine the terminal phenotype of *sit4-51 pre1-1 pre4-1* cells under synthetic lethal conditions. For this purpose, we constructed *sit4-51 pre1-1 pre4-1* mutant cells kept viable by the presence of plasmids encoding wild-type *SIT4* or *PRE1* under the control of repressible promoters. Surprisingly, no synthetic growth defect was observed when *sit4-51 pre1-1 pre4-1* cells harboring a plasmid encoding the *PRE1* gene under the control of the *GAL1* promoter (strain YTS94) were shifted to repressive conditions (glucose-containing media). By recording the proteasomal chymotrypsin-like activity under repressive conditions we found that YTS94 cells exhibited weak residual activity compared with *pre1-1* cells containing an empty vector. This result indicated that *GAL1*-controlled expression of the plasmid-encoded *PRE1* gene could not completely be repressed and that the presence of low amounts of wild-type Pre1 protein were sufficient to complement the synthetic defect of *sit4-51 pre1-1 pre4-1* mutants. Therefore, promoter shut-off experiments were performed with *sit4-51 pre1-1 pre4-1* cells that contained plasmid-encoded *SIT4* or *PRE1* wild-type genes under the control of the *MET3* promoter (strains YTS95 and YTS96). When the plasmid-encoded *SIT4* or *PRE1* genes were repressed on minimal medium (SD), corresponding to the conditions used during the synthetic lethality screen, YTS95 and YTS96 cells showed the expected lethality. However, YTS95 and YTS96 cells formed colonies on YPD or syn-

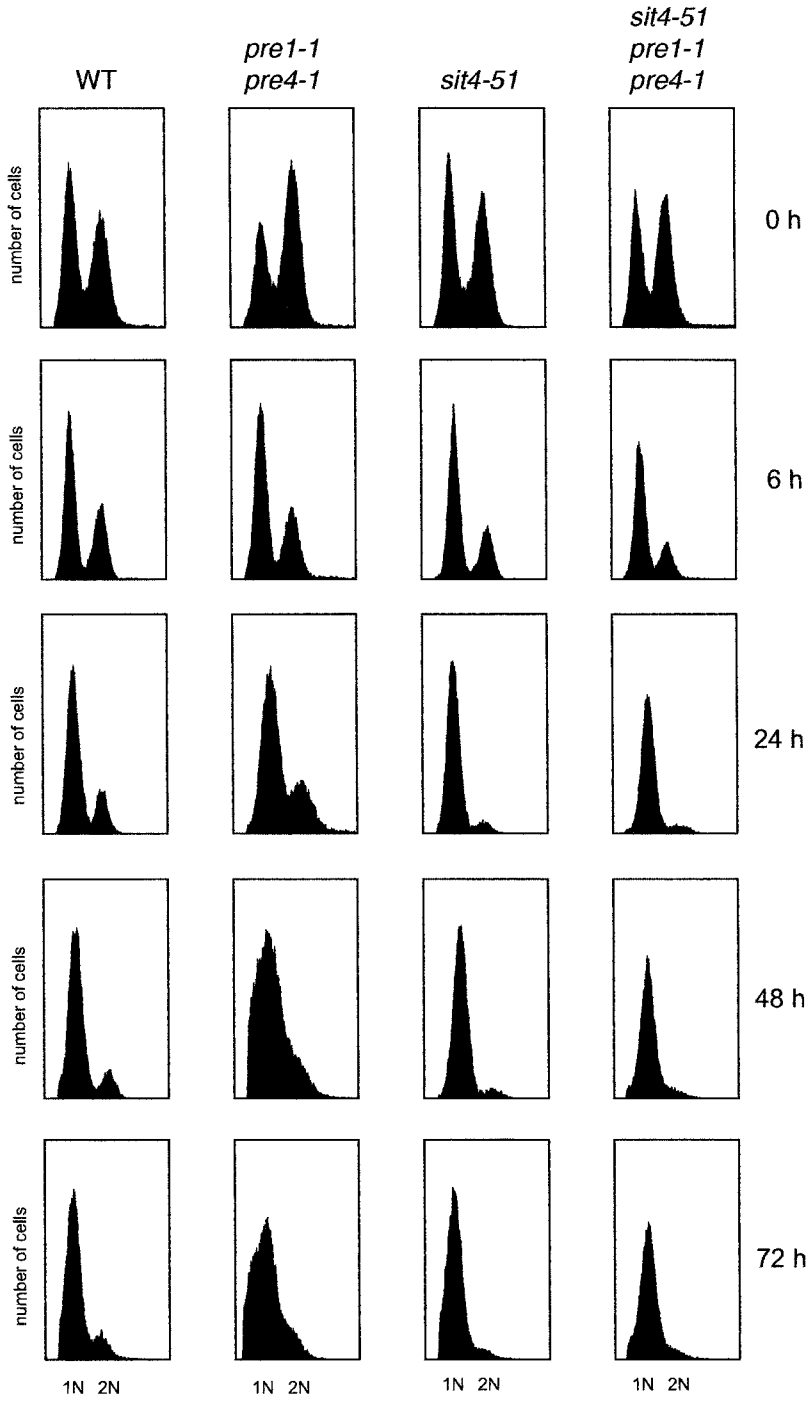

FIGURE 3.—*sit4-51 pre1-1 pre4-1* mutants undergo G_1 arrest when incubated in minimal medium. Wild-type (WCg4a), *pre1-1 pre4-1* double-mutant (YHI29/14), *sit4* single-mutant (YTS100), and *sit4-51 pre1-1 pre4-1* triple-mutant (YTS102) cells were grown in YPD medium to logarithmic phase (30°) and then transferred to liquid SD medium. After different incubation times samples were taken, DNA was stained with propidium iodide, and cells were analyzed for their DNA content by FACS. The vertical axes indicate the cell count, the horizontal axes the DNA content.

thetic complete (SC) media, indicating that viability of *sit4-51 pre1-1 pre4-1* mutants depended on the availability of nutrients. These results suggested that the triple mutants might grow on rich medium. In agreement with this idea we were able to generate haploid *sit4-51 pre1-1 pre4-1* and *sit4-102 pre1-1 pre4-1* triple-mutant strains by crossing the *sit4* single mutants with *pre1-1 pre4-1* double-mutant strains (for details see MATERIALS AND METHODS). As expected, the *sit4-51 pre1-1 pre4-1* (YTS102) and also *sit4-102 pre1-1 pre4-1* (YSH3) cells obtained were able to grow on rich media—YPD and SC medium—but not on SD (Table 2). YTS102 cells did not grow on minimal medium at temperatures >30°. YSH3 cells showed lethality on minimal medium even at 28°, indicating that, compared to *sit4-51*, *sit4-102* leads to a slightly stronger defect of Sit4 function. This finding was further confirmed by the stronger effects observed when the *sit4-102* mutation was combined with the proteasomal single mutations (Table 2). Interestingly, slight synthetic effects occurred even when *sit4* mutations were combined with the *pre4-1* mutation (Table 2). Until now it was thought that this proteasomal mutation, like other mutations that solely

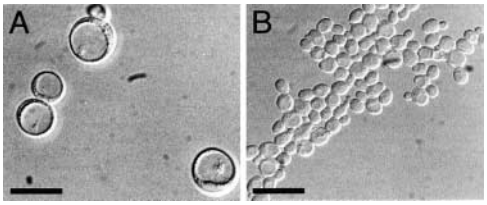

FIGURE 4.—Morphology of synthetic lethal and sorbitol-cured *sit4-51 pre1-1 pre4-1* mutants. *sit4-51 pre1-1 pre4-1* mutants (YTS102) grown on YPD were transferred to SD plates containing (A) no sorbitol (seen are four enlarged unbudded cells, two of which are accidentally positioned near each other) or (B) 0.5 M sorbitol and incubated at 30° for 36 hr. Cells were scraped off the plates, dissolved in liquid SD medium, mounted on slides, and inspected with a microscope (Zeiss axioscop). Bar, 22 μm.

affect the 20S proteasomal PGPH activity, does not cause any detectable defects in proteasome-dependent protein degradation or *in vivo* proteasome function (HILT *et al.* 1993; GUECKEL *et al.* 1998).

The synthetic growth defect of *pre1-1 pre4-1 sit4-51* cells is based on an osmosensitivity during starvation of cells: The terminal phenotype of *sit4-51 pre1-1 pre4-1* cells (YTS102) exposed to synthetic lethal conditions was determined. As shown by fluorescence-activated cell sorter (FACS) analysis, the vast majority of *sit4-51 pre1-1 pre4-1* triple-mutant cells—as found for wild-type, *pre1-1 pre4-1*, and *sit4-51* mutant cells—switched to a state with 1 N DNA content during prolonged incubation in liquid minimal medium. Twenty-four hours after shift to SD medium, most YTS102 cells (>90%) were arrested as single unbudded cells. As previously observed for mutants deficient in proteasomal activity, the asynchronous cultures of *pre1-1 pre4-1* cells cultivated in rich medium harbored a higher amount of 2 N DNA-containing cells (GUECKEL *et al.* 1998). Nevertheless, these cells switched to a 1 N DNA content during incubation in minimal medium. After >48 hr incubation, a shoulder appeared at the 1 N DNA peaks of *pre1-1 pre4-1* cultures. We attribute this to the formation of abnormally elongated cells after sustained incubation. At all time points examined, *pre1-1 pre4-1 sit4-51* triple-mutant cells showed the same 1 N/2 N DNA ratios as measured for the *sit4-51* single-mutant cells. (Figure 3). On the basis of these data we conclude that *sit4-51 pre1-1 pre4-1* mutant cells, like wild type, *sit4* single-mutant, and even *pre1-1 pre4-1* double-mutant cells, are able to induce a G_1 arrest when they run out of nutrients. However, after 36 hr of incubation on minimal medium, strong morphological changes became visible specifically for the *sit4-51 pre1-1 pre4-1* triple-mutant cells. The majority of the cells appeared to be greatly enlarged, had lost their normal oval shape, and contained abnormal large vacuoles (Figure 4), which often collapsed when cells were exposed to even slight

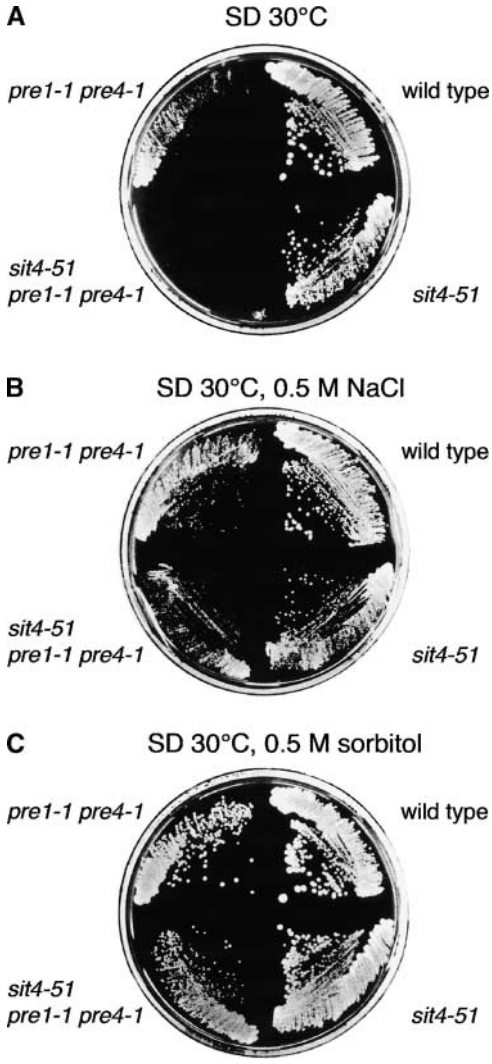

FIGURE 5.—The growth defect of *sit4-51 pre1-1 pre4-1* mutants on SD medium is rescued by high concentrations of salt or sorbitol. Wild-type (WCG4a), *sit4-51* single-mutant (YTS-100), *pre1-1 pre4-1* double-mutant (YHI29/14), and *sit4-51 pre1-1 pre4-1* triple-mutant (YTS102) cells were streaked on plates with SD (A), SD containing 0.5 M sodium chloride (B), or SD containing 0.5 M sorbitol (C) and incubated at 30° for 50 hr.

mechanical stress (*e.g.*, application of coverslips; data not shown). This morphology led us to the idea that under lethal conditions mutant cells may be prone to unregulated water uptake from the medium and, therefore, to be extremely sensitive to low osmotic pressure. To test this idea, cells were transferred onto SD agar media containing high concentrations of salt (NaCl >0.3 M or KCl >0.5 M) or sorbitol (>0.5 M). Indeed, these conditions of high osmotic pressure restored growth of *sit4-51 pre1-1 pre4-1* cells (Figure 5). Cells cured by high salt or sorbitol concentrations had normal size and shape (Figure 4). Taken together, these data indicate that the growth defects of mutants impaired in Sit4 and proteasome function are based on an osmosensitivity occurring under limiting nutrients.

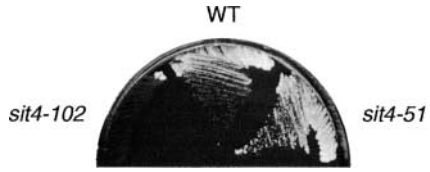

FIGURE 6.—*sit4-102* and *sit4-51* mutants behave differently against rapamycin. Wild-type cells (WCG4a), *sit4-51* (YTS100), and *sit4-102* mutants (YSH2) were streaked on YPD agar plates (30°) containing 50 μg/ml rapamycin.

***sit4-51* and *sit4-102* mutants show different behavior after rapamycin-induced inhibition of the TOR pathway:** Mutations *sit4-102* (SUTTON *et al.* 1991a) and *sit4-51* both cause temperature sensitivity and synthetic effects when combined with proteasomal mutations. However, when the TOR pathway is blocked (HALL 1996; THOMAS and HALL 1997) by the presence of the TOR specific inhibitor rapamycin, *sit4-51* and *sit4-102* mutants behave differently. As already known, *sit4-102* cells are hypersensitive against rapamycin. In contrast, compared to wild-type cells, *sit4-51* mutants exhibit significant resistance against the inhibitor (Figure 6).

Sit4 does not regulate the proteasome: A possible explanation for the Sit4-proteasome interaction may be that the proteasome is regulated by a Sit4-mediated dephosphorylation step. In agreement with this idea it is known that proteasomes from different species contain subunits that are phosphorylated (ARRIGO and MEHLEN 1993; ETLINGER *et al.* 1993; CASTANO *et al.* 1996; MASON *et al.* 1996). Additionally, potential phosphorylation sites have been identified in some yeast 20S proteasome subunits (HEINEMEYER *et al.* 1994). To test whether Sit4 phosphatase is involved in regulation of proteasomal activity, we compared the *in vitro* peptide-cleaving activities of 20S proteasomes partially purified from *sit4-51* mutants (YTS100) and isogenic wild-type cells, but we did not observe any deviations from 20S proteasomal peptidase activity profiles (Figure 7). To substantiate these results, we also tested the *in vivo* degradation rates of well-defined 20S proteasomal substrates in *sit4-51* mutant and wild-type cells. The kinetics of glucose-induced degradation of FBPase (SCHORK *et al.* 1994, 1995) was not altered in YTS100 cells (*sit4-51*) compared to wild-type cells (Figure 8, A and B). Moreover, presence of the *sit4-51* mutation did not influence FBPase stability in the *pre1-1 pre4-1* proteasome mutant background (Figure 8C). Also, the short-lived substrates of the N-end-rule pathway, Leu-β-Gal and Arg-β-Gal (BACHMAIR *et al.* 1986), as well as the short-lived Ub-Pro-β-Gal protein, were degraded at wild-type rates in *sit4-51* cells (data not shown). These data demonstrate that neither the *in vitro* peptidase activity of the 20S proteasome nor the *in vivo* proteolytic activity of the 26S proteasome are influenced by the *sit4-51* mutation. These data, in addition, indicate that Sit4 does not influence the cellular concentration of proteasomes.

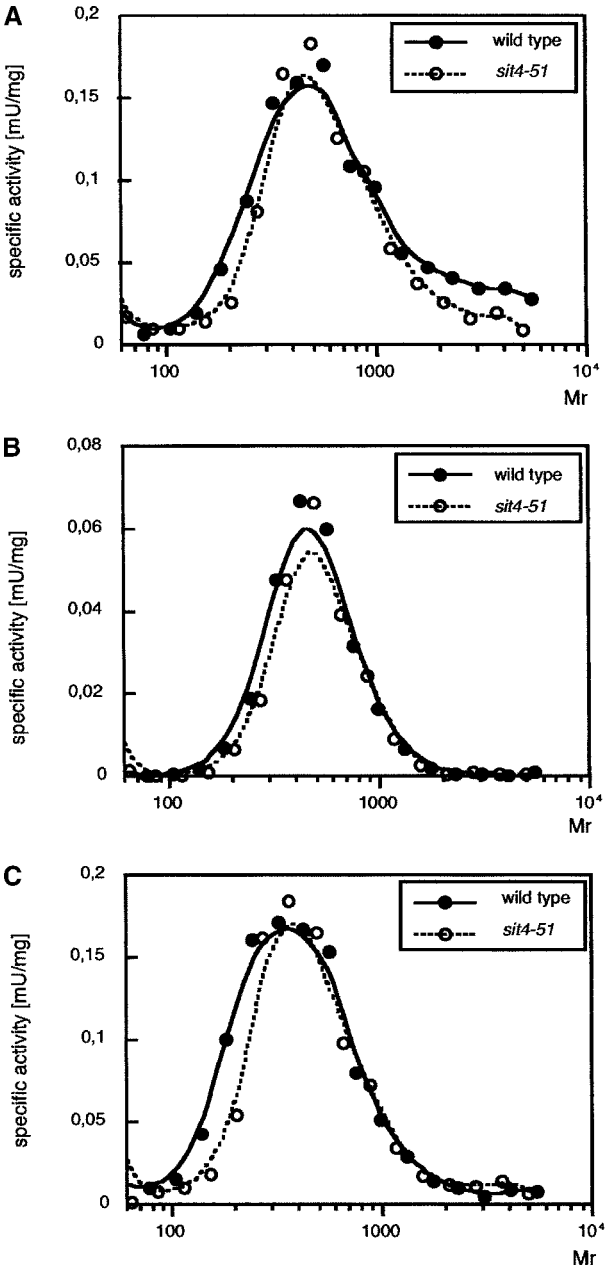

FIGURE 7.—*In vitro* 20S proteasome peptide-cleaving activities are not altered in *sit4-51* mutant cells. Crude extracts of cells grown to exponential phase (30°) were fractionated by gel filtration on sepharose CL4B and peptide-cleaving activities were determined using artificial substrates: (A) The chymotrypsin-like activity was determined using Suc-Leu-Leu-Val-Tyr-AMC; (B) the trypsin-like activity was determined using Cbz-Ala-Arg-Arg-MoβNA; and (C) the PGPH activity was determined using Cbz-Leu-Leu-Glu-βNA.

Sit4 is not a proteasomal substrate: The *sit4-51* and *sit4-102* mutations are recessive and are therefore believed to result in loss of Sit4 function. In such a case proteolytic stabilization of the *sit4-51* and *sit4-102* gene products is expected to cause suppressor, and not synthetic, effects when combined with mutations that impair proteasome function. Therefore, the genetic data

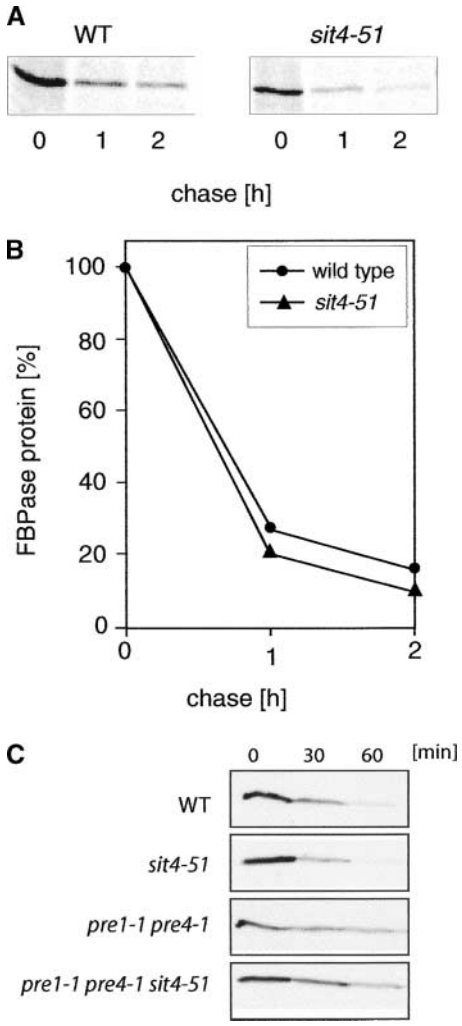

FIGURE 8.—Fructose-1,6-bisphosphatase is degraded at wild-type rates in *sit4-51* mutants. (A) Pulse-chase analysis of FBPase degradation in wild-type (WCG4a) and *sit4-51* (YTS100) mutant cells. Cells were pulse labeled during derepression of FBPase in ethanol-containing medium and chased with the addition of glucose followed by extraction, immunoprecipitation, and SDS-PAGE. (B) Quantification of A. (C) FBPase turnover during catabolite inactivation was followed in wild-type (WCG4a), *sit4-51* single-mutant (YTS100), *pre1-1 pre4-1* double-mutant (YHI29/14), and *sit4-51 pre1-1 pre4-1* triple-mutant (YTS102) cells by immunoblotting using the method of SCHORK *et al.* (1995).

indicate that Sit4 is not a target for proteasome-mediated degradation. To confirm this idea we tested whether Sit4 is a stable protein and not a substrate of the proteasome. Plasmid-encoded Sit4 containing an immunoepitope at the carboxyl terminus (Sit4-HA) or amino terminus (HA-Sit4; SUTTON *et al.* 1991a) was expressed in wild-type and proteasome mutant strains that contained a deletion of the chromosomal *SIT4* gene. Therefore, the plasmid-encoded HA-tagged *SIT4* genes comprised a single source of Sit4 protein in these cells. Both N-terminally and C-terminally HA-tagged Sit4 (Figure 9; data not shown) were found to be long lived in

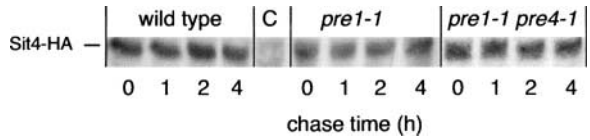

FIGURE 9.—*Sit4* is proteolytically stable. Wild-type (YMHO27), *pre1-1* (YMHO29), and *pre1-1 pre4-1* (YMHO33) mutant cells expressing plasmid (*LEU2 CEN*)-derived C-terminally epitope-tagged Sit4-HA protein as the only source of Sit4 were grown to logarithmic phase at 30°. Protein synthesis was blocked by the addition of cycloheximide (0.5 mg/ml) and Sit4-HA protein monitored at different chase times by immunoblotting using anti-HA antibodies. C, negative control; wild-type cells (WCG4α) containing no *SIT4-HA*-encoding plasmid.

wild-type cells. As expected, there was no difference in Sit4 stability in proteasome mutant strains (Figure 9). Due to the following facts we can exclude that the immunoepitopes influenced the proteolytic stability of the tagged Sit4 protein: (1) Concerning proteolytic stability, both epitope-tagged versions, Sit4-HA and HA-Sit4, behaved in the same manner; (2) both epitope-tagged versions of Sit4 used for determination of Sit4 stability were proven to be fully functional (SUTTON *et al.* 1991a); and (3) in contrast to *pre1-1 pre4-1* cells containing a *sit4-51* or *sit4-102* mutation, proteasomal mutants expressing either Sit4-HA or HA-Sit4 showed normal growth on minimal medium. This result evidences that the immunoepitope does not influence the functional interaction between Sit4 and the proteasome. Taken together, the data clearly show that Sit4 is a stable protein and not a target of proteasome-mediated destruction.

Sit4 function is linked to Rad6- and Cdc34-dependent ubiquitination: Sit4-related synthetic effects are specific for impaired proteasome-mediated proteolysis. No effects were found when the *sit4* mutations were combined with defective vacuolar proteolysis due to mutated proteinase A (data not shown). In most cases proteasome-dependent degradation requires targeting of substrate proteins by ubiquitination. If Sit4 phosphatase function is linked to defined proteasome-mediated degradation pathways, synthetic effects should be found when *sit4* mutations are combined with mutations causing defects in certain ubiquitination pathways. To test this idea, we generated *sit4* mutant strains that harbored mutations in genes coding for ubiquitin-conjugating (E2) enzymes. We tested the complete set of yeast E2 genes. Striking synthetic effects were found when *sit4* mutations were combined with the *ubc3/cdc34-1* or the *ubc2*Δ/*rad6*Δ mutation (Table 3; see also Figure 10D). No synthetic effects were detected with mutations of the other *S. cerevisiae* Ubc enzymes. No detectable synthetic effect was seen even for a *sit4-51 ubc4*Δ *ubc6*Δ *ubc7*Δ quadruple mutant.

On SD medium at 30°, *sit4-51 cdc34-1* double mutants showed very poor growth, whereas *sit4-102 cdc34-1* dou-

ble mutants exhibited synthetic lethality. Under such conditions *sit4-102 cdc34-1* cells appeared to be significantly enlarged, highly resembling the morphological phenotype of *sit4-51* mutants that contained *pre1-1 pre4-1* mutations. Moreover, as for *sit4 pre* mutants, impaired growth of *sit4-51 cdc34-1* double mutants, as well as synthetic defects of *sit4-102 cdc34-1* double mutants on SD medium, was cured by applying high osmotic pressure (1 M sorbitol; data not shown).

The strongest synthetic effect was observed when we combined *sit4-51* with a deletion of the *RAD6* gene. *sit4-51 rad6*Δ double mutants could not be directly made by dissection of tetrads obtained from heterozygous diploids. Spores containing the *sit4-51 rad6*Δ double mutation stopped growth at a size of ~30 cells even when grown on YPD medium (data not shown), indicating that *sit4-51 rad6*Δ mutants are able to germinate but cannot continue growth under these conditions. Therefore, *sit4-51 rad6*Δ spores that in addition contained a complementing *SIT4*-encoding *LEU2* plasmid (pCK2) were generated. After germination these spores were grown to colonies with normal size. After further growth on nonselective medium, clones that had lost the *SIT4*-encoding plasmid during mitosis, thereby yielding *sit4-51 rad6*Δ double mutants (strain YSH13), were detected. To prove whether these *sit4-51 rad6*Δ*::HIS3* cells had acquired a suppressor mutation that enabled their growth on rich medium, strain YSH13 was backcrossed to a *sit4-51* single-mutant strain. After sporulation tetrads were dissected and analyzed. As expected, in each tetrad two *sit4-51* single-mutant clones were found. However, in addition to nonviable *rad6 sit4* cells, histidine prototrophic *sit4-51 rad6* double-mutant spore clones that grew up to colonies were obtained. These *sit4-51 rad6* double-mutant clones grew considerably slower than the *sit4-51* single mutants. These results clearly prove that combination of *sit4-51* with *rad6*Δ causes genuine synthetic lethality even on rich medium.

The suppressor mutation present in YSH13 cells does not cure the synthetic growth defects of *sit4-51 rad6*Δ cells on minimal medium. YSH13 cells exhibited synthetic lethality on minimal medium at 23° and even at 18° (data not shown), representing the strongest synthetic effect of all mutants inspected in this work. Moreover, under these conditions *sit4-51 rad6*Δ cells arrested with 1N DNA content and developed the expected morphology of enlarged round cells (data not shown). Lethality of *sit4-51 rad6*Δ cells on SD medium could not be cured by high osmotic pressure (1 M sorbitol or 1.5 M KCl; data not shown) but rather by supplementation with the same set of single amino acids (see next section). On the basis of these findings we suggest that the suppressor mutation enabling growth of YSH13 cells on rich medium does not have a significant effect on the phenotypic behavior of YSH13 cells on minimal medium.

One prominent function of Rad6 is targeting of substrates of the N-end-rule pathway (Dohmen *et al.* 1991; Varshavsky 1997). In this pathway Ubr1 functions as the E3 enzyme required for substrate recognition (Bartel *et al.* 1990; Varshavsky 1996). No synthetic effect was observed when *sit4* mutations were combined with a deletion of *UBR1* (data not shown), proving that the proteasome-related function of Sit4 is independent of Ubr1-mediated pathways. Taken together, these data evidence that Sit4 function is linked to the proteasome system via Ubc2- and Ubc3-mediated pathways, but exclude Ubr1 as a contributor to that function.

TABLE 3

Synthetic effects of *sit4 ubc* mutants on SD medium

Strains	Growth at 30°
*rad6*Δ *sit4-51*	sl
cdc34-1 sit4-51	Slow growth
cdc34-1 sit4-102	sl
*ubc1*Δ *sit4-51*	No effect
*ubc4*Δ *sit4-51*	No effect
*ubc5*Δ *sit4-51*	No effect
*ubc6*Δ *sit4-51*	No effect
*ubc7*Δ *sit4-51*	No effect
*ubc8*Δ *sit4-51*	No effect
*ubc10*Δ *sit4-51*	No effect
*ubc11*Δ *sit4-51*	No effect
*ubc13*Δ *sit4-51*	No effect
*ubc4*Δ *ubc6*Δ *ubc7*Δ *sit4-51*	No effect

sit4-51 mutants containing different mutations of ubiquitin-conjugating (E2) enzymes were analyzed for synthetic effects on SD medium at 30°. Double mutants were made as described in MATERIALS AND METHODS. sl, synthetic lethality (no visible colonies).

The *sit4*-induced synthetic growth defect is suppressed by the presence of certain amino acids: *sit4 pre*- and *sit4 ubc*-induced synthetic effects were observed mainly on minimal medium. We were interested to know whether viability of cells defective in the Sit4 phosphatase and the ubiquitin-proteasome system depended on the general availability of nutrients or on the presence of certain amino acids. To answer this question, growth of *sit4 pre* or *sit4 ubc* mutants was tested on agar media that, in addition to the amino acids necessary for complementation of auxotrophic mutations, were supplemented with a single amino acid. Interestingly, addition of certain amino acids led to suppression of *sit4 pre*- or *sit4 ubc*-induced growth defects. The amino acids tested could be sorted into three classes depending on their ability to restore viability of the mutants. Addition of asparagine and serine caused strong suppression of *sit4 pre*- or *sit4 ubc*-induced growth defects. These amino acids could even rescue the *sit4-51 rad6*Δ-induced synthetic growth defects of YSH13 cells (Figure 10A, Table 4). The second class of amino acids (Ala, Ile, Phe, Thr) exhibited lesser suppressor effects. These amino acids cured only synthetic defects of mutants harboring

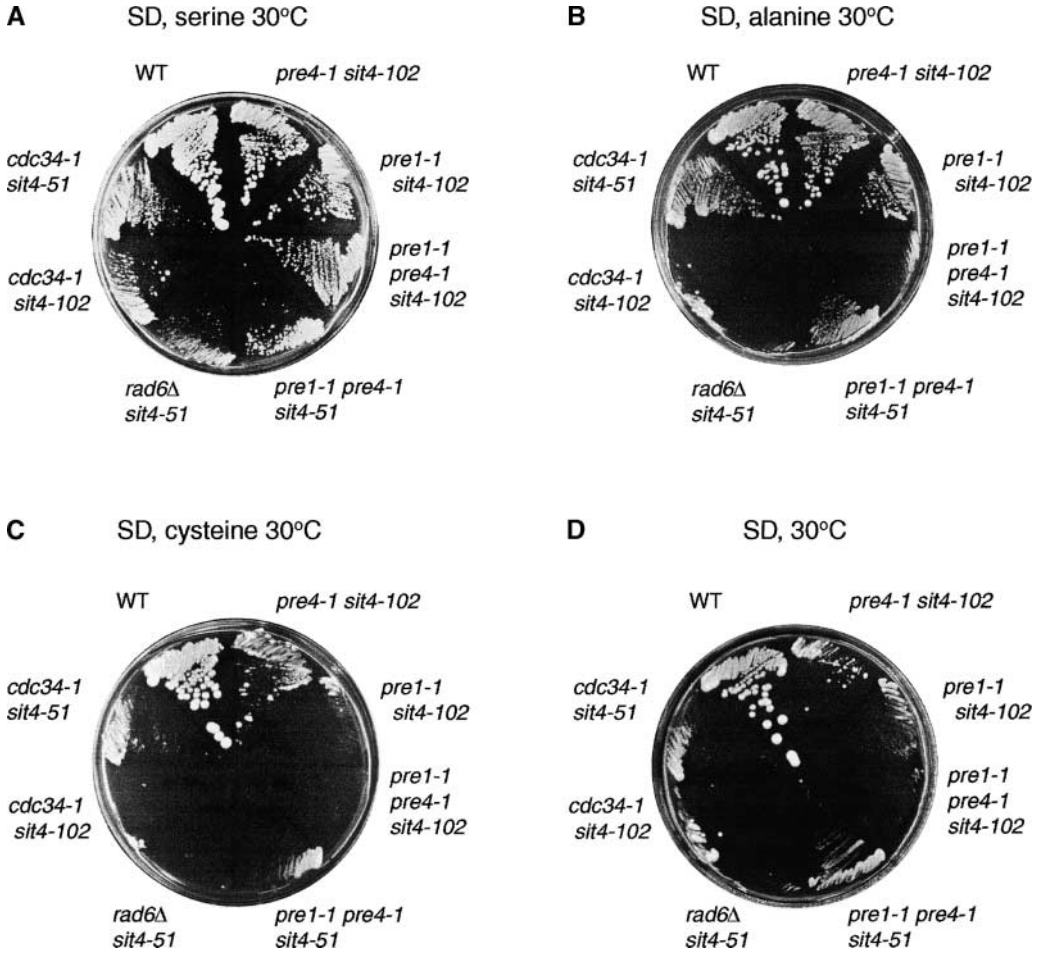

FIGURE 10.—Synthetic defects of *sit4 pre* and *sit4 ubc* mutants are suppressed by the addition of certain amino acids to the medium. Wild-type (WCG4a), *pre4-1 sit4-102* (YSH5), *pre1-1 sit4-102* (YSH4), *pre1-1 pre4-1 sit4-102* (YSH3), *pre1-1 pre4-1 sit4-51* (YTS102), *rad6Δ sit4-51* (YSH13), *cdc34-1 sit4-102* (YSH19), and *cdc34-1 sit4-51* (YSH11) cells were streaked on minimal media (SD ura$^+$, leu$^+$, his$^+$) that contained serine (A), alanine (B), or cysteine (C). Control plate without additional amino acids (D).

combinations of *sit4* and *pre* or *ubc* mutations that cause weak synthetic effects (for instance, *sit4-102 pre1-1* or *sit4-51 cdc34-1* mutations; Figure 10B, Table 4). The third class of amino acids led to almost no suppression of *sit4 pre*- or *sit4 ubc*-induced lethality. Growth of *sit4 pre* or *sit4 ubc* mutants was not restored even when SD medium was supplemented with mixtures of several class III amino acids (Table 4). Taken together, these data clearly demonstrate that the viability of cells bearing defects of the Sit4 phosphatase and the ubiquitin-proteasome system depends on the presence of certain amino acids. No direct relationship was found between the biosynthetic pathways of class I and class II amino acids that restored growth of the mutants.

DISCUSSION

***sit4* mutations cause synthetic growth defects when combined with proteasomal mutations:** We isolated mutants that showed lethality in combination with proteolytically impaired proteasomes. Complementation of one of the mutants using a plasmid-based library yielded two genes, *SIT4* and *SSD1*. The mutation conferring synthetic lethality was found to reside within the *SIT4* gene, whereas *SSD1* functioned as a low-copy suppressor. We suggest that *sit4* mutations conferring synthetic effects with impaired proteasomes lead to a general deficiency of Sit4 protein phosphatase function and not to a defect that is restricted to the proteasome-associated function of Sit4. This conclusion is based on the following observations: (1) Synthetic effects with proteasomal mutations are not limited to the *sit4-51* allele but can also be induced with the previously described *sit4-102* allele, which leads to multiple defects in Sit4-related functions (SUTTON *et al.* 1991a); (2) *sit4-51*-induced synthetic lethality, as well as *sit4-51*- and *sit4-102*-induced temperature sensitivity, were suppressible by low-copy expression of a *SSD1-v* gene, which is capable of rescuing cells from complete loss of Sit4 function; and (3) cysteine 51, which in the mutated *Sit4-51* protein is replaced by tyrosine, is conserved in many other protein phosphatases. This residue resides within a region that is highly conserved among protein phosphatases and even human calcineurin (Figure 2). The cysteine residue of the calcineurin chain A corresponding to cysteine 51 of Sit4 is located in the center of this structurally well-defined phosphatase in the vicinity of the active site. On the basis of these findings we suggest that the *sit4-51* mutation might affect Sit4 phosphatase activity.

The *sit4-102* mutation induced slightly stronger syn-

TABLE 4

Suppression of *sit4 pre-* and *sit4 ubc*-induced synthetic defects by the presence of additional amino acids in the medium

Supplements added	Growth
Ala	+
Arg	−
Asn	++
Asp	−
Cys	−
Gln	−
Glu	−
Gly	−
Ile	+
Met	−
Phe	+
Pro	−
Ser	++
Thr	+
Tyr	−
Val	−
Ino	−
Met Arg Val	−
Cys Gln Glu	−
Cys Gln Pro Val Arg	−

Amino acids were classified according to their capability to restore growth of *sit4 pre* and *sit4 ubc* mutants: ++, strong suppression as shown in Figure 10A; +, weak suppression as shown in Figure 10B; −, no suppression as shown in Figure 10C. Single amino acids and inositol were added at concentrations used in SC medium (120 μg/ml).

thetic phenotypes than *sit4-51* did, indicating that this mutation may affect Sit4 function to a larger extent. Interestingly, although *sit4-51* and *sit4-102* cells showed similar effects with defects of the ubiquitin-proteasome system, they responded differently to inhibition of the TOR-signaling pathway. *sit4-51* caused resistance against rapamycin, whereas *sit4-102* caused hypersensitivity against this drug. Therefore, the two mutations seem to influence differently the TOR-related function of Sit4. Moreover, on the basis of these results we can exclude a connection between the ubiquitin-proteasome-related function of Sit4 described in this study and the TOR-signaling pathway.

Sit4 function is specifically linked to deficient proteasomal proteolysis. No synthetic effects were observed when the *sit4-51* mutation was combined with a general defect in vacuolar proteolysis. *sit4-51* and *sit4-102* mutations cause synthetic growth defects when combined with *pre1-1 pre4-1* double mutations or with a *pre1-1* single mutation. Both *pre1-1 pre4-1* and *pre4-1* lead to significant defects in proteasome-mediated protein degradation. Surprisingly, even moderate synthetic effects were found when *sit4* was combined with *pre4-1*. So far, neither a defect of proteasomal substrate degradation nor any other cellular phenotype has been identified in mutants defective in proteasomal PGPH activity including *pre4-1* mutants (Hilt *et al.* 1993; Heinemeyer *et al.* 1997; Gueckel *et al.* 1998). Therefore, it was thought that lack of the PGPH activity does not cause impairment of *in vivo* proteasomal protein degradation and is dispensable for proteasomal *in vivo* function (Hilt *et al.* 1993; Heinemeyer *et al.* 1997; Gueckel *et al.* 1998). This view is now challenged by the synthetic effect observed in *pre4-1 sit4* mutants.

How is Sit4 linked to proteasome function? Different models might explain the link between the Sit4 phosphatase and the ubiquitin-proteasome system: (1) Sit4 may regulate the activity or the concentration of the proteasome; (2) Sit4 may be a substrate of proteasome-mediated degradation; (3) peptides produced by proteasomal degradation may provide a nutritional signal that feeds into a Sit4-mediated pathway; and (4) Sit4 and the proteasome may share a common target.

Several findings lead to the exclusion of models 1 and 2. The *sit4-51* mutation did not cause any alteration of the 20S proteasomal peptidase activities *in vitro*. In addition, well-defined model substrates of the proteasome pathway were degraded at wild-type rates in *sit4-51* mutant cells, demonstrating that the *sit4-51* mutation does not influence the *in vivo* activity of the proteasome. Therefore, we conclude that Sit4 is not implicated in controlling the activity or the concentration of the proteasome. We also demonstrated clearly that the Sit4 phosphatase is proteolytically stable. Hence, Sit4 is not a target of proteasome-mediated degradation. Thus, we suggest that Sit4 does not directly interact with the proteasome.

Could Sit4 respond to a nutritional signal that depends on peptides produced by proteasome-mediated protein destruction? Such a model is supported by the clear dependence of the synthetic effects of the *sit4 pre1-1 pre4-1* mutant on the availability of certain amino acids in the growth medium. However, there are also strong arguments against this model. Under starvation conditions, proteins are turned over mainly by vacuolar proteolysis (Teichert *et al.* 1989; Lang *et al.* 2000). Therefore, at least under such conditions it is the vacuole and not the proteasome system that is the major endogenous source for peptides and amino acids. However, no synthetic effects were observed when the vacuole-dependent pathway of protein degradation was blocked in a *sit4* mutant. Moreover, if peptides generated by proteasomal degradation were the source of an internal signal acting on Sit4, one would expect induction of this signal not to be limited to defects of Rad6- and Cdc34-dependent ubiquitination. Nevertheless, the possibility exists that Sit4 phosphatase and the proteasome system may execute a concerted function in sensing of external nutrients and our data may support such a model.

The finding that *sit4*-induced synthetic effects are restricted to defined ubiquitination pathways indicates that Sit4 phosphatase is functionally connected to a

protein that was degraded via the proteasome in a Rad6/Cdc34-dependent way. Because *sit4*-related synthetic effects were found in *rad6* null mutants, Sit4 phosphatase can be excluded from contribution to Rad6-mediated substrate targeting; in such a case epistatic, but not synthetic, effects should have been observed. Hence, at least for the Rad6-dependent pathway, we can exclude the requirement of Sit4-dependent dephosphorylation of a proteasomal substrate as a signal for its proteolytic destruction.

Only weak synthetic effects were observed when *sit4-51* or *sit4-102* mutations were expressed together with the conditional *cdc34-1* mutation. In this case, the *sit4*-induced synthetic effects were measured at temperatures permissive for growth of the *cdc34-1* cells. Under these conditions, cells are expected to possess residual Cdc34 activity. Consequently, at these temperatures, *cdc34-1*-related synthetic effects are expected to be weak.

Even though each has specific functions—Rad6/Ubc2 is involved in DNA repair, whereas Cdc34/Ubc3 has an essential role in the cell cycle—both ubiquitin-conjugating enzymes, Rad6 and Cdc34, are closely related. They exhibit strong sequence similarity (Haas and Siepmann 1997) and both localize to the nucleus. These data suggest that Rad6 and Cdc34 may also share common cellular functions. Indeed, it was reported that both enzymes (the *S. cerevisiae* and the human homologs) exhibit overlapping functions in substrate targeting (Kornitzer *et al.* 1994; Pagano *et al.* 1995; Tam *et al.* 1997; Pati *et al.* 1999). Therefore, we suggest that Rad6 and Cdc34 are involved in ubiquitination of a defined substrate protein, functionally connected to Sit4 phosphatase.

Sit4-induced synthetic effects depended on nutrient availability and osmotic conditions. Interestingly, Ubr1, which acts as an E3 enzyme contributing to Rad6-dependent ubiquitination, is required for peptide uptake (Alagramam *et al.* 1995; Byrd *et al.* 1998; Turner *et al.* 2000) and linked to osmoregulation (Ota and Varshavsky 1993; Posas *et al.* 1996). Thus, it seemed probable that Sit4 function was related to Ubr1-mediated degradation. However, this model is clearly excluded because no synthetic effects were observed when *sit4* mutations were combined with a *UBR1* deletion.

Models of Sit4-proteasome interaction: How could Sit4 and the proteasome mechanistically act on a common target protein? Both systems may redundantly contribute to the inactivation of a common target. Alternatively, Sit4 may control the cellular level of a common target by influencing its expression. Mutation of Sit4 protein was found to result in both induction and repression of gene expression. In light of these findings, a promising model for the Sit4-proteasome interaction is that *sit4* mutations cause ectopic expression or expression to abnormally high levels of a protein whose concentration is controlled by proteasomal degradation. This model is supported by the fact that the synthetic growth defect of *sit4-51 pre* mutants was restored by expression of *SSD1-v*. Due to the capability of *SSD1-v* alleles to restore correct expression of G_1 cyclins (Sutton *et al.* 1991a) and because Ssd1 can bind mRNA, we can speculate that *SSD1-v* alleles may cause correction of *sit4*-derived alteration of gene expression.

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