

The yeast nuclear import receptor is required for mitosis

(nucleocytoplasmic transport/importin)

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ABSTRACT The nuclear import system is highly conserved among eukaryotes. Here we report the effects of a conditional mutation in *SRP1*, which encodes a *Saccharomyces cerevisiae* homolog of the vertebrate nuclear import receptor importin. Importin was isolated as a factor required for the initial targeting step of a nuclear import substrate to the nuclear envelope in a mammalian *in vitro* assay. We show that yeast Srp1 is similarly required for protein import. In addition, Srp1 is also required for the execution of mitosis: we demonstrate that cells containing a conditional mutation of *SRP1* arrest with a G₂/M phenotype in a manner analogous to classic *cdc* mutants. This defect may be due to the failure of the mutant to degrade the mitotic cyclin Clb2 and other proteins required for mitosis. The requirement of a nuclear import receptor for cell cycle-regulated proteolysis implies that import of cell cycle regulators into the nucleus is critical for cell cycle progression.

The import of proteins into eukaryotic nuclei consists of two separable steps: the binding of import substrate to the nuclear envelope and the subsequent translocation of the substrate across the nuclear pore complex (NPC) into the nucleoplasm (1, 2). The recognition of proteins for nuclear localization is mediated by the interaction of short signal sequences (nuclear localization sequence; NLS) within the targeted protein (3) with specific receptors. One approach to identify receptors for nuclear import has been to purify proteins that bind to NLS peptides. The major NLS binding protein in animals, fungi, and plants is an ≈55- to 70-kDa protein that has been identified by chemical cross-linking and blot-overlay assays (4, 5). The activity of these proteins is necessary for the binding step of the import reaction as determined by specific antibody inhibition of binding *in vitro* (6) and by reconstitution of a partially fractionated import reaction based on permeabilized mammalian cells with addition of purified NLS-binding protein (7). Furthermore, these proteins are highly phosphorylated, and their phosphorylation is required for NLS binding *in vitro* (8).

Another approach to understanding the import machinery has been to purify the cytosolic factors required for protein import in permeabilized cells. A protein required for targeting a nuclear import substrate to the nuclear envelope *in vitro* has recently been isolated and cloned by this strategy (9). This 60-kDa protein, importin, is absolutely required for the accumulation of import substrate on the nuclear envelope and functions as a NLS-binding protein *in vitro* (10, 11). Importin was shown to be 44% identical to Srp1, a previously identified protein from *Saccharomyces cerevisiae* (12). Mutations in *SRP1* have pleiotropic effects, including suppression of conditional mutations in RNA polymerase I (12), defects in nucleolar structure (13), and synthetic lethality with mutations in the

nuclear pore component gene, *NUPI* (14). Furthermore, Srp1 is physically associated with Nup1 and Nup2, a Nup1-related NPC constituent (14).

In this work we demonstrate that Srp1/importin is essential for nuclear protein import in yeast both *in vivo* and *in vitro*, consistent with its role as a NLS receptor. Surprisingly, conditional *srp1* mutants arrest in the cell cycle during the G₂/M phase. Moreover, degradation of the mitotic cyclin Clb2 is impaired in *srp1-31* cells, suggesting that the import of a critical cell cycle regulator necessary for mitotic degradation is especially sensitive to defects in nuclear import.

MATERIALS AND METHODS

Yeast Strains and Genetic Techniques. All strains in this study are derivatives of W303-1a. We replaced the *SRP1* chromosomal locus with the *srp1-31* mutant allele (13) to avoid copy number effects. Unlike the plasmid-borne version, the integrated *srp1-31* allele has a recessive, extreme temperature-sensitive growth defect. Four independent strains of *srp1-31* were constructed by plasmid integration and pop-out (15), and each isolate had identical growth properties. For cell cycle synchronization experiments, a *sst1Δ::hisG* mutation was introduced into each strain to increase sensitivity to α -factor.

To test genetic interaction with the cyclin-dependent kinase, a strain containing *srp1-31* and a *SRP1 URA3 CEN* plasmid was crossed with congenic strains bearing either the *cdc28-1N* or *cdc28-4* mutations. Tetrads were dissected, and the genotypes of the resultant spores were determined by complementation tests and a PCR-based assay for the *SRP1* genotype. The *srp1-31* mutation destroys an *Xba* I restriction site; this change was detected by amplification of a 630-nt fragment of the *SRP1* gene with oligonucleotide primers of the sequence CTGCA-GATGAACCTTCGTCGTC and GTCCACGTAGCGGTC-CTGATC, followed by restriction digestion and gel electrophoresis.

Microscopy. Immunofluorescence on yeast spheroplasts was done as described (16), except that formaldehyde fixation was performed for 60 min at room temperature. Monoclonal antibody 9C4 (17) was detected with CY3-labeled goat anti-mouse IgG (Jackson ImmunoResearch). To observe microtubules, we used YOL1/34 (Accurate Chemicals) as the primary antibody and goat anti-rat IgG conjugated with Texas Red (Jackson ImmunoResearch) as the secondary antibody.

***In vitro* Assay for Nuclear Import.** Components of the *in vitro* assay were prepared as described (18). *In vitro* assays were done with a cytosol concentration of 2 mg/ml at 30°C. The experiment was repeated three times, and for each sample >175 cells were scored for binding and import of the substrate.

Determination of Clb2 Stability. Steady-state levels of ectopically expressed Clb2 were determined by immunoblot. Derivatives of *SRP1* and *srp1-31* strains containing the *GALI::CLB2*

gene integrated at the *LEU2* locus were constructed by transformation with *YipG2::CLB2* (19). Protein concentrations in extracts (20) were determined using the bicinchoninic reagent (Pierce) and normalized before electrophoresis.

For the pulse-chase experiment, the *GAL1::CLB2-lacZ* fusion was constructed from a PCR-derived fragment containing residue -6 to the last codon of the *CLB2* gene cloned into the *Bam*HI and *Eco*RI sites of pKB64 (21). This plasmid was introduced in *SRP1* and *srp1-31* strains, and pulse-chase analysis was done as described (21).

RESULTS

Srp1 Is Necessary for Nuclear Import. To establish a functional role for Srp1, we first examined the nuclear import properties of the *srp1-31* mutant using an *in vitro* permeabilized cell assay (18). Because Srp1 protein is present in the permeabilized cell preparation and the cytosolic fraction (Fig. 1A), both fractions were prepared from mutant and wild-type cells. Mutant Srp1 was also present in both fractions after temperature shift, indicating that the *srp1-31* defect is not due to decreased expression or instability. Binding of fluorescent substrate to the nuclear envelope and import into the nucleus are both affected in *srp1-31* mutants (Fig. 1B). Import competence decreases in *srp1-31* mutants with time at 37°C. After 6 hr at 37°C, *srp1-31* mutant loses >95% of its ability to import the substrate into nuclei. Mixing mutant cells with wild-type cytosol or vice versa resulted in intermediate levels of import, suggesting that both soluble and nuclear envelope-bound Srp1 contributes to import. To confirm the observed *in vitro* import defect, we examined the localization of several nuclear proteins *in vivo* by immunofluorescence. We found localization defects for some, but not all, nuclear proteins tested. The nucleolar protein recognized by the 9C4 antibody (17) accumulates aberrantly in the cytoplasm of *srp1-31* cells at the nonpermissive temperature (Fig. 1C). However, the Npl3 protein (17) and a histone- β -galactosidase (β -Gal) fusion (23) are only moderately mislocalized, and the Nop1 protein (24) is properly localized under these conditions (data not shown). In contrast, the *srp1-31* mutation has no effect on mRNA export, as determined by *in situ* hybridization to poly(A)⁺ RNA (data not shown).

A *srp1* Mutant Arrests in Mitosis. *srp1-31* strains show a uniform G₂/M arrest phenotype at the nonpermissive temperature (Fig. 2). After 6 hr of incubation at 37°C, \approx 85% of cells have large buds with a single nucleus at the bud neck and a short bipolar spindle consistent with a block just before or during mitosis (Fig. 2A). DNA content analysis of these cells shows that >90% have replicated DNA (data not shown), consistent with a defect in the cell cycle after S phase. Furthermore, when synchronized in G₁ and then released into the nonpermissive temperature, *srp1-31* cells arrest with replicated DNA in the first cycle after the shift (Fig. 2B). Initiation of S phase is delayed by 30–45 min in *srp1-31* cells. The nuclear envelope, nuclear pores, and the mitotic spindles of arrested *srp1-31* cells appear identical by transmission electron microscopy to those found in large budded *SRP1* cells (data not shown), indicating that the cell cycle defect is not a consequence or cause of global defects in nuclear envelope structure. The G₂/M arrest phenotype is not unique to the *srp1-31* allele; another temperature-sensitive allele, *srp1-49*, also accumulates cells with replicated DNA. However, *srp1-31* has both the most dramatic effect on nuclear import and the most uniform cell cycle defect of the conditional alleles isolated. In strains containing a deletion of *SRP1*, whose survival depends upon expression of a galactose-inducible *SRP1* gene, depletion of the protein by glucose repression leads to a similar large budded arrest phenotype. However, Srp1 is an extremely stable protein, so the phenotype is only manifested after long periods of growth on glucose.

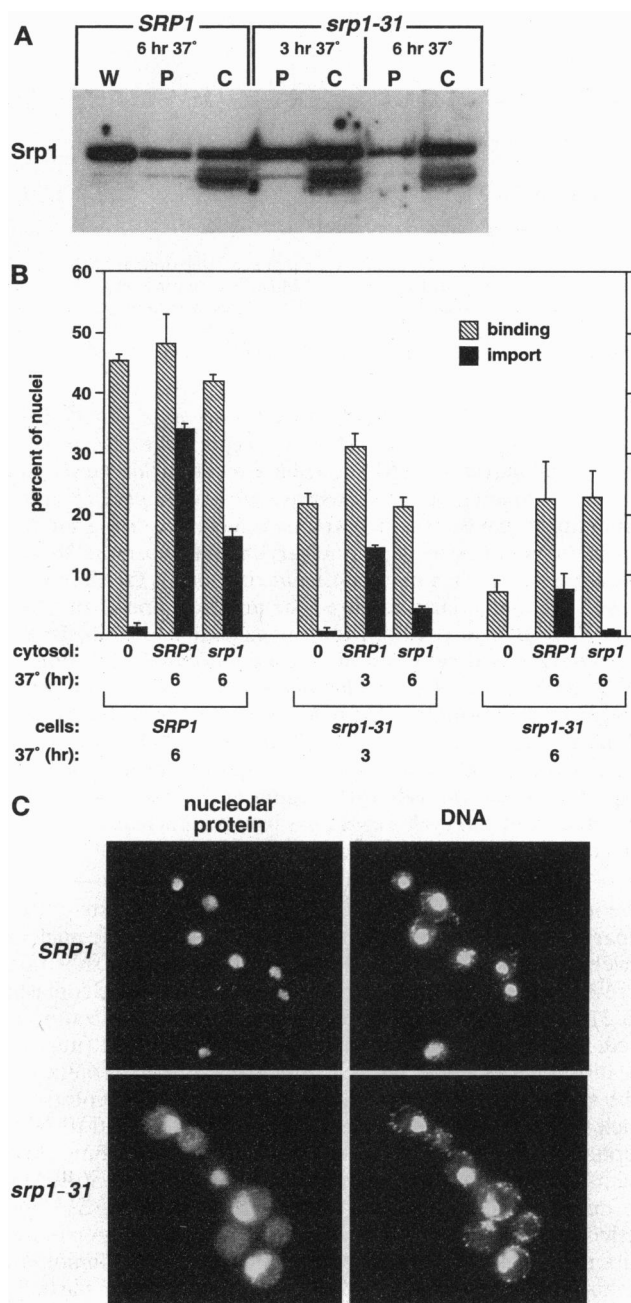


FIG. 1. Srp1 is essential for nuclear import. (A) Srp1 is present in both permeabilized cells and the cytosolic fraction of the *in vitro* nuclear import assay. Permeabilized cells and cytosol were prepared from *SRP1* and *srp1-31* cells grown at 25°C and 37°C for 3 and 6 hr. An immunoblot of components used in the *in vitro* assay prepared from *SRP1* and *srp1-31* cells grown at 37°C for the indicated times was probed with anti-Srp1 antiserum (12). W, whole cells; P, permeabilized cells; C, cytosol. Approximately equal numbers of cells were loaded in each lane. The lower band observed in cytosol preparations is probably a degradation product. (B) *srp1-31* blocks the import of nuclear proteins *in vitro*. Cell equivalents of permeabilized cells and cytosol were mixed along with the import substrate, a rhodamine-labeled human serum albumin-simian virus 40 NLS conjugate, and then import was observed by fluorescence microscopy. In contrast to *in vitro* assays using permeabilized mammalian cells (22), some binding of substrate to the yeast nuclear envelope occurs in the absence of added cytosolic proteins (18). (C) *In vivo* mislocalization of a nucleolar protein in the *srp1-31* mutant. Immunofluorescence with monoclonal antibody 9C4 (17) was performed on *SRP1* and *srp1-31* cells that had been shifted to 37°C for 6 hr in yeast extract/peptone/dextrose. Each frame is an identical exposure.

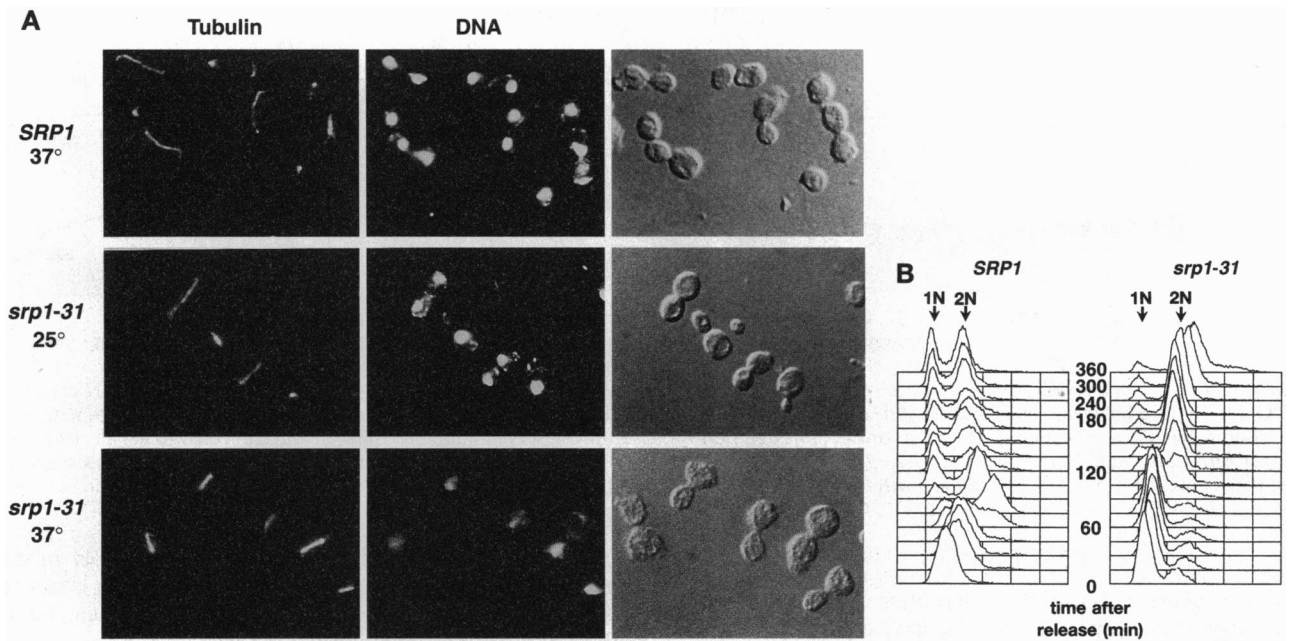


FIG. 2. A temperature-sensitive mutation in *SRP1* leads to arrest at the G_2/M phase of the cell cycle. (A) Tubulin localization in *srp1-31* mutants. *SRP1* and *srp1-31* strains were prepared for immunofluorescence 6 hr after shift to 37°C, and then microtubules were visualized with the YOL1/34 antibody. (B) DNA replication after synchronization by α -factor mating pheromone. *SRP1* and *srp1-31* cultures were synchronized in G_1 by treatment with 3 μ M α -factor in yeast extract/peptone/dextrose medium at 25°C for 2.5 hr. Cultures were then released from pheromone arrest into fresh medium at 37°C. Samples were withdrawn at 15-min intervals, then fixed, and analyzed by flow cytometry (25). Histograms of fluorescence intensity versus cell number are shown. 1N, unreplicated DNA; 2N, replicated DNA.

Cyclin Proteolysis Is Defective in *srp1-31* Mutants. The simplest explanation for the G_2/M arrest caused by the *srp1-31* mutation is that an event required for progression through mitosis depends on the import of a cell cycle regulator into the nucleus and is thereby sensitive to a block in Srp1-mediated transport. A good candidate is the wave of protein degradation that is required for mitosis and that persists through G_1 (26). Therefore, we compared the levels of Clb2 (a mitotic cyclin degraded during anaphase and throughout G_1 , whose degradation is required to exit mitosis (27, 28) in *SRP1* and *srp1-31* cells. Steady-state levels of Clb2 were tested in G_1 -arrested cells because any mutant that arrests at G_2/M will have high levels of Clb2, whereas only mutants that specifically affect mitotic cyclin regulation should affect the degradation of Clb2 in G_1 . Clb2 ectopically expressed from the *GAL1* promoter is present in at least 20-fold higher levels in *srp1-31* mutants arrested in α -factor as compared with *SRP1* (Fig. 3 Upper). The difference in steady-state level of Clb2 protein is not a result of increased expression of *CLB2* RNA (data not shown) or incomplete arrest of the *srp1-31* mutant strain (Fig. 3 Lower).

The increased levels of Clb2 are due to a defect in cyclin proteolysis: pulse-chase analysis revealed a dramatic increase in the half-life of a Clb2- β Gal fusion in the *srp1-31* mutant strain arrested in G_1 at 37°C as compared to *SRP1* (Fig. 4). In the *srp1-31* mutant, the stability of Clb2- β Gal in G_1 is comparable to the apparent half-life of Clb2- β Gal in cycling *SRP1* cells. The half-life of another unstable protein, Gcn4- β Gal, is not significantly affected in the *srp1-31* mutant (data not shown).

***SRP1* Interacts Genetically with a Mitotic Arrest Allele of *CDC28*.** Some yeast mutants defective in enzymes involved in protein degradation, such as *UBC9*, *PRG1*, *CIM3*, and *CIM5*, have cell cycle-arrest phenotypes similar to that of *srp1-31* (30–32). *CIM3* and *CIM5* were isolated as mutants that are lethal in combination with *cdc28-1N*, an allele of the cyclin-dependent kinase that causes arrest at G_2/M . These putative components of the 26S protease accumulate Clb2 and Clb3 proteins at the nonpermissive temperature. Because *srp1-31*

also appears to be involved in cyclin degradation, we tested for possible genetic interactions between *SRP1* and *CDC28* (Fig. 5). We found that *srp1-31* is synthetically lethal with *cdc28-1N*, but not with *cdc28-4*, which arrests in G_1 . Moreover, *srp1-31* is not lethal in combination with other mutants that arrest in G_2/M , such as *tub2-401*. These data suggest a specific interaction between Srp1 and the G_2/M kinase.

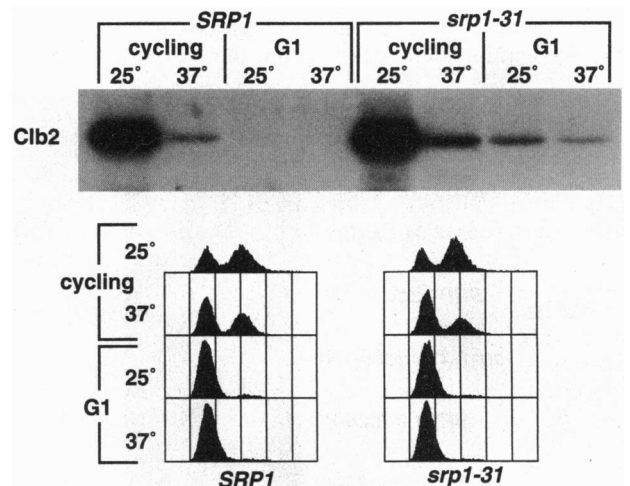


FIG. 3. Accumulation of Clb2 in α -factor-arrested *srp1-31* cells ectopically expressing *CLB2* from the *GAL1* promoter. Cycling and α -factor-arrested cells containing a *GAL1::CLB2* gene were induced with galactose at 25°C. One hour after galactose addition, samples were split, and half were shifted to 37°C. After 2 hr further incubation, total protein and RNA were prepared, and flow cytometry analysis was done. (Upper) An immunoblot of equal amounts of total protein probed with polyclonal rabbit anti-Clb2 (29). (Lower) The flow cytometry profile of each sample at the time of harvest. Galactose induction of *GAL1::CLB2* is much more efficient at 25°C; therefore the Clb2 level in arrested cells should be compared with that of cycling cells grown under the same temperature regimen.

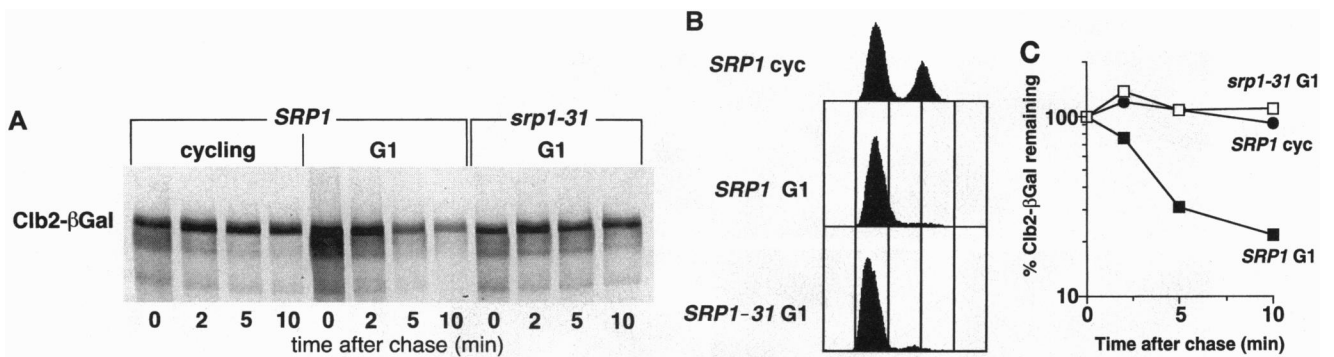


FIG. 4. Clb2- β -Gal is stabilized in *srp1-31* mutants during G₁. Cycling and α -factor-arrested *SRP1* cells and α -factor-arrested *srp1-31* cells grown at 25°C were induced by galactose addition. After 40 min, cultures were shifted to 37°C for 80 min, pulse-labeled for 2 min with [³⁵S]methionine, and chased with excess unlabeled methionine for the indicated periods. Clb2- β -Gal was immunoprecipitated and subjected to gel electrophoresis (21). (A) Autoradiograph of immunoprecipitated samples. (B) The flow cytometry profile of each strain at the time of labeling. (C) Comparison of the decay rates of Clb2- β -Gal from each culture was determined by quantifying the amount of labeled immunoprecipitate in each lane of the gel in A with a Fuji phosphorimager device. cyc, Cycling.

DISCUSSION

The effects of the *srp1-31* mutant on protein uptake *in vitro* and *in vivo* suggest that Srp1 and importin have equivalent roles in nuclear import. This conclusion is supported by their sequence similarity and the ability of Srp1 to bind to NLSs (11). Furthermore, Srp1 is likely to be identical to NBP70, a previously isolated yeast NLS-binding protein (6) because Srp1 purified from *Escherichia coli* is recognized by an antibody raised against NBP70 (G.S., unpublished observation). The *srp1-31* mutation (Ser-116 \rightarrow Phe) is in an invariant residue among all five published importin homologs. Therefore, it may be useful to introduce this mutation into importins from other species as a probe for structure-function analysis of this protein family.

The genetic and physical interactions between Srp1 and XFXFG nucleoporins (14) suggest that the targeting step of nuclear import requires the interaction of Srp1/importin-substrate complexes with these components of the NPC. It is not known whether the interaction between substrate and receptor occurs in the cytoplasm and is followed by docking or if the recognition step occurs within the NPC. Although we

found that both the soluble and insoluble pools of Srp1 participate in import *in vitro*, most Srp1 protein in intact cells is associated with the nuclear envelope and nuclear interior [(12) and J.D.J.L., unpublished observations].

In addition to its nuclear import defect, the *srp1-31* mutant arrests in the cell cycle with a G₂/M terminal phenotype. We found that the *srp1-31* mutant does not properly execute the proteolysis of the B-type cyclin Clb2 that normally occurs during mitosis and persists during G₁. Clb2 stability is similarly altered by a mutation in *CSE1*, a gene required for normal mitotic segregation of chromosomes (33, 34). Because *SRP1* has been isolated as a multicopy suppressor of *cse1*, these proteins may function together in the import process.

We suggest that the G₂/M arrest observed in the *srp1-31* mutant is due to the inability of the mutant strain to degrade and thereby inactivate key mitotic regulators, including Clb2. The *srp1-31* mutant also specifically interacts with the *cdc28-1N* mutation of the cyclin-dependent kinase that is defective in the G₂/M stage. Therefore, in the *srp1-31 cdc28-1N* double mutant at 25°C, the defective kinase and the stabilized mitotic regulators may synergistically prevent exit from mitosis. Accumulation of Clb2 is unlikely to be the sole cause of the arrest phenotype because Clb2 stabilized by the deletion of its cyclin destruction box leads to a later, anaphase arrest phenotype (27). Rather, the G₂/M arrest of *srp1-31* is probably a consequence of the inability to destroy multiple proteins whose degradation is coordinately regulated with Clb2, such as other B-type cyclins and the proposed sister chromatid adhesion factor (35).

Because Srp1 functions as the nuclear import receptor, the mechanism of arrest in *srp1-31* cells probably involves the failure to import a nuclear protein required for mitosis. The similar arrest phenotypes and common defects in cyclin degradation make components or regulators of the mitotic degradation program appealing candidates for the nuclear import substrate(s) whose transport is limiting in the *srp1-31* mutant. It is possible that under normal conditions, regulated import of this substrate is a trigger for the execution of mitosis.

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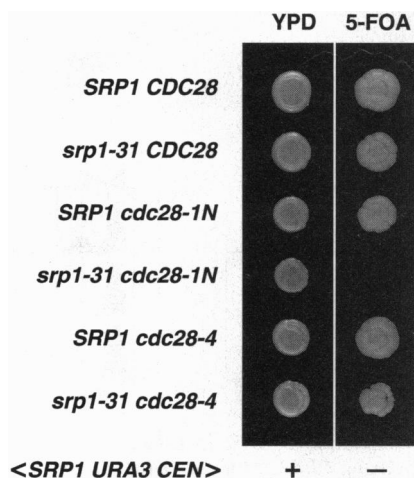


FIG. 5. Synthetic lethality between *srp1-31* and *cdc28-1N*. Saturated liquid cultures of strains of the indicated genotypes containing a *SRP1 URA3 CEN* plasmid (pDP168) were spotted onto medium permissive for the presence of the plasmid yeast extract/peptone/dextrose (YPD) or medium that selects against the presence of the plasmid (synthetic complete containing 5-fluoroorotic acid at 1 mg/ml, 5-FOA) at 25°C. YPD and 5-FOA plates were photographed after 2 and 3 days, respectively. Identical results were obtained with reciprocal crosses with a *CDC28 CEN URA3* plasmid.

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