LAS1 Is an Essential Nuclear Protein Involved in Cell Morphogenesis and Cell Surface Growth

Andrea I. Doseff*,† and Kim T. Arndt*

**Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724-2212 and †Graduate Program in Genetics, State University of New York, Stony Brook, New York 11794*

Manuscript received May 9, 1995
Accepted for publication August 7, 1995

ABSTRACT

Saccharomyces cerevisiae mutations that cause a requirement for *SSD1-v* for viability were isolated, yielding one new gene, *LAS1*, and three previously identified genes, *SIT4*, *BCK1/SLK1*, and *SMP3*. Three of these genes, *LAS1*, *SIT4*, and *BCK1/SLK1*, encode proteins that have roles in bud formation or morphogenesis. *LAS1* is essential and loss of LAS1 function causes the cells to arrest as 80% unbudded cells and 20% large budded cells that accumulate many vesicles at the mother-daughter neck. Overexpression of LAS1 results in extra cell surface projections in the mother cell, alterations in actin and SPA2 localization, and the accumulation of electron-dense structures along the periphery of both the mother cell and the bud. The nuclear localization of LAS1 suggests a role of LAS1 for regulating bud formation and morphogenesis via the expression of components that function directly in these processes.

FOR the budding yeast *Saccharomyces cerevisiae*, nutrient and growth signals are integrated during late G1 to give rise to the execution of Start. After Start is executed, the cells are committed to divide. The execution of Start requires the activation of CDC28/G1 cyclin kinase complexes. *CLN1* and *CLN2*, which encode two G1 cyclins that bind to CDC28, are transcribed during late G1 by a process that normally requires CDC28, CLN function, protein synthesis, and cell growth signals (CROSS and TINKELENBERG 1991; DIRICK and NASMYTH 1991; MARINI and REED 1992). The rate at which G1 cyclin levels increase in late G1 is determined in large part by the growth rate of the cells: more rapidly growing cells accumulate CLN1 and CLN2 at faster rates than slowly growing cells. Also, newly formed G1 daughter cells are smaller than mother cells and need to grow to a critical cell size in late G1 to execute Start.

Bud initiation is dependent on the execution of Start and requires protein synthesis (LEW and REED 1993), suggesting that bud formation is coupled to cell growth. Before the new bud is visible, some of the components implicated in bud site selection and bud formation, such as an ordered array of 10-nm filaments (KIM *et al.* 1991), are already localized at the site of future bud emergence. Bud formation also involves rearrangements of the cytoskeleton and the secretory machinery (KILMARTIN and ADAMS 1984). Actin cables extend into the bud, and cortical actin patches accumulate in the bud. It is generally assumed that the cytoskeleton controls the secretory machinery so that cell membrane and cell wall components are directed to the site of cell surface growth (FIELD and SCHEKMAN 1980). How these morphological changes are temporally and spatially regulated and how they are coordinated with growth signals and other cell cycle events is poorly understood.

Bud initiation is connected to G1 cyclin levels and G1 CDC28 kinase activity. First, actin polarization to the site of future bud formation can be triggered by CLN/CDC28 activation in the absence of *de novo* protein synthesis, raising the possibility that CDC28 may directly phosphorylate substrates regulating actin polarization (LEW and REED 1993). Second, *mpk1/slt2* mutations are lethal in combination with *cdc28* mutations that cause a G1 arrest (MAZZONI *et al.* 1993). *MPK1/SLT2* encodes a mitogen-associated protein (MAP) kinase homologue that functions downstream of BCK1/SLK1 in the protein kinase C pathway (LEE *et al.* 1993). The budding yeast protein kinase C pathway has been implicated in cell growth: at the nonpermissive temperature *pkc1*, *bck1/slk1*, or *mpk1* mutant cells lyse (COSTIGAN *et al.* 1992; LEE and LEVIN, 1992; LEVIN and BARTLETT-HEUBUSCH, 1992; LEE *et al.* 1993), possibly due to a defect in cell wall formation (SEE ROEMER *et al.* 1994). Third, a *bud2* mutation, which alters the pattern of bud site selection but is not normally essential for bud formation, causes lethality in combination with low G1 cyclin levels (BENTON *et al.* 1993; CVRCKOVA and NASMYTH 1993). These findings suggest that bud initiation and growth are intimately connected to Start.

SIT4 is a type 1/type 2A-related protein phosphatase that is required for the late G1 expression of *SWI4*, *CLN1*, and *CLN2*, which function for the execution of Start (SUTTON *et al.* 1991; FERNANDEZ-SARABIA *et al.* 1992). *SWI4* encodes a transcription factor that activates *CLN1* and *CLN2* transcription during late G1 (ANDREWS

Corresponding author: Kim T. Arndt, Cold Spring Harbor Laboratory, 1 Bungtown Rd., Delbrück Bldg., Cold Spring Harbor, NY 11724-2212. E-mail: arndt@cshl.org

TABLE 1

Yeast strains

Strain	Genotype	Source
CY1485	*MAT***a** Δ*ssd1::LEU2 ade2-1 ade3::HisG ura3-1 leu2-3,112 his3-11 trp1-1* ⟨*ADE3/TRP1/SSD1-v/CEN*⟩	This study
CY1488	same as CY1485, but *MATα*	This study
CY2804	CY1485 with chromosomal *las1-12*	This study
CY2807	*MAT***a** Δ*ssd1::LEU2* Δ*las1::HIS3 ade2-1 ade3::HisG ura3-1 leu2-3, 112 his3-11 trp1-1* ⟨*pGAL1:LAS1/*YCp50⟩ ⟨*ADE3/TRP1/SSD1-v/CEN*⟩	This study
CY2808	CY1485 plus ⟨*LAS1/*YCp50⟩	This study
CY2809	CY1485 plus ⟨*LAS1:HA/*YCp50⟩	This study
CY2810	*MAT***a**/*MATα* Δ*las1::HIS3/LAS1* Δ*ssd1::LEU2/*Δ*ssd1::LEU2 ade2-1/ade2-1 ade3::HisG/ade3::HisG ura3-1/ura3-1 leu2-3,112/leu2-3,111 his3-11/his3-11 trp1-1/trp1-1* ⟨*ADE3/TRP1/SSD1-v/CEN*⟩	This study
CY2813	*MATα* Δ*sit4::HIS3 ssd1-d1 his3 trp1 leu2 lys2 ura3 ade2* ⟨*TRP1/CEN*⟩ ⟨*sit4-102/LEU2/CEN*⟩	This study
CY2814	*MATα* Δ*sit4::HIS3 ssd1-d1 his3 trp1 leu2 lys2 ura3 ade2* ⟨*S. pombe ADH:CLN2/TRP1/CEN*⟩ ⟨*sit4-102/LEU2/CEN*⟩	This study
CY3820	CY3830 plus ⟨*LAS1/*YEp24⟩	This study
CY3826	CY3830 plus ⟨*LAS1:HA/*YEp24⟩	This study
CY3830	*MAT***a**/MATα Δ*ssd1::LEU2/*Δ*ssd1::LEU2 ade2-1/ade2-1 ade3::HisG/ade3::HisG ura3-1/ura3-1 leu2-3, 112/leu2-3, 112 his3-11/his3 trp1-1* ⟨*ADE3/TRP1/SSD1-v/CEN*⟩	This study
CY4401	CY2804 plus ⟨YCp50⟩	This study
CY4402	CY2804 plus ⟨*LAS1/*YCp50⟩	This study
CY4403	CY1485 plus ⟨*pGAL1:LAS1/*YCp50⟩	This study
CY4404	CY1485 plus ⟨*pGAL1/*YCp50⟩	This study

and HERSKOWITZ 1989; NASMYTH and DIRICK 1991; OGAS *et al.* 1991). In addition to the role of SIT4 for G1 cyclin expression, SIT4 is also required for bud initiation (FERNANDEZ-SARABIA *et al.* 1992). Possibly, SIT4 transmits nutrient/growth signals to induce the expression of G1 cyclins and the expression of genes needed for bud formation.

The phenotype of *sit4* cells depends on the polymorphic *SSD1* locus. If the cells have a *ssd1-d* or Δ*ssd1* allele, Δ*sit4* cells are inviable and *sit4-102* cells arrest at the nonpermissive temperature as unbudded G1 cells with 1n DNA content (SUTTON *et al.* 1991). By contrast, if the cells have a *SSD1-v* allele, Δ*sit4* cells are viable (but with a slow growth rate phenotype and a greatly expanded G1 phase) and *sit4-102* cells are not temperature sensitive. Deletion of *SSD1-v* results in only small phenotypic alterations if *SIT4* is present. The DNA sequence of *SSD1-v* predicts a 140-kD protein (SUTTON *et al.* 1991; WILSON *et al.* 1991) with similarity to the essential dis3 protein of *Schizosaccharomyces pombe* (KINOSHITA *et al.* 1991). *dis3* mutations cause a mitotic arrest under nonpermissive conditions and interact genetically with mutations in $dis2^+$, which encodes the catalytic subunit of a type 1 protein phosphatase. Both SSD1-v protein and dis3 protein have weak but significant similarity to the *Escherichia coli* and *Shigella flexneri* vacB proteins (which have similarity to the exoribonuclease II family of proteins that hydrolyze single-stranded RNA in the 3′ to 5′ direction) and to *E. coli* exoribonuclease II [Swissprotein data base accession numbers P21499, P30851, and P30850, respectively (K. T. ARNDT, unpublished results)]. Interestingly, mutations in the Shigella *vacB* gene reduce, via a posttranscriptional effect, the levels of certain virulence proteins (TOBE *et al.* 1992). It is not currently known if SSD1-v has exoribonucleases II activity, but it is intriguing to speculate that SSD1-v might also function via a posttranscriptional mechanism for the expression of certain gene products.

Although the mechanism by which SSD1 functions is not known, SSD1 (like SIT4) has been implicated in processes related to cell growth and morphogenesis. In addition to suppressing the lethality of Δ*sit4 ssd1-d* strains, *SSD1-v* also suppresses the slow growth rate and temperature-sensitive phenotype of Δ*swi4 ssd1-d* strains (CVRCKOVA and NASMYTH 1993; K. T. ARNDT, unpublished results). Related to this finding, *SSD1-v* suppresses the slow growth rate phenotype of Δ*cln1* Δ*cln2 ssd1-d* strains (CVRCKOVA and NASMYTH, 1993; K. T. ARNDT, unpublished results). Therefore, *sit4* mutations, *swi4* mutations, and *cln1 cln2* mutations all result in low G1 cyclin levels and are suppressed by *SSD1-v*. In relation to cell growth and morphogenesis, *SSD1-v* can partially suppress some of the phenotypic alterations caused by deletion of *BEM2/IPL2*, a gene implicated in bud emergence (KIM *et al.* 1994) or by hyperactivation of protein kinase A (SUTTON *et al.* 1991; WILSON *et al.* 1991). In budding yeast, protein kinase A is activated via the RAS pathway and regulates cell proliferation (BROACH 1991). In addition, *SSD1-v* suppresses mutations in *BCK1/SLK1* (COSTIGAN *et al.* 1992) and mutations in *MPK1* (LEE *et al.* 1993), which function in the protein kinase C pathway. Therefore, SSD1-v is involved in cell growth and morphogenesis.

To isolate genes whose products function in the SIT4 pathway(s) for G1 cyclin expression and bud formation, we initiated a genetic screen to obtain mutations that, like *sit4* mutations, require *SSD1-v* for viability. Here, we report on the isolation of *LAS1*. We show that LAS1

is an essential nuclear protein that is critically involved in cell surface growth and morphogenesis. The phenotypes and genetic interactions between LAS1, SIT4, and SSD1 suggest that these proteins have interconnected roles in bud formation and cell morphogenesis.

MATERIALS AND METHODS

Strains and growth conditions: The yeast strains are listed in Table 1. Yeast cultures were grown either in YEP (1% yeast extract, 2% Bacto-peptone) medium or in SC medium containing adenine and uracil at 0.1 g/l, leucine at 0.2 g/l, and the other amino acids at 0.1 g/l (ROSE *et al.* 1990). The indicated carbon sources were at 2% final. For plasmid selection, the appropriate amino acid or uracil was omitted. SC medium with 67 mg/liter adenine (SC/low adenine) was used for the colony color sectoring assay.

Mutant isolation: The *las* mutants were isolated as cells that require *SSD1-v* for growth. The colony color sectoring assay (KOSHLAND *et al.* 1985; KRANZ and HOLM 1990) was used to monitor the presence or absence of *SSD1-v*. On SC/low adenine plates, *ade2 ade3* strains give white colonies while *ade2 ADE3* strains give red colonies. Because *SSD1-v* is not required for a normal growth rate when *SIT4* is present, *ade2 ade3* Δ*ssd1* strains containing a *ADE3/TRP1/SSD1-v/CEN* plasmid can grow normally in the absence of the plasmid, giving rise to red colonies containing many white sectors. Stationary cultures of strains CY1485 and CY1488 were incubated for 60 min with 30 μl/ml of EMS (M0880, Sigma). Cells were diluted and plated onto SC/low adenine plates and incubated at 30° for 3–4 days. As judged by microscopic examination of the plate surface 2 days after plating, ~25% of the EMS-mutagenized cells did not give rise to a colony. Red colonies or colonies with small white sectors were picked and rechecked for a non- or reduced-sectoring phenotype. Diploid double mutants were generated by mating the corresponding *MATa* and *MATα* mutants. Haploid mutants yielding nonsectoring diploids were scored as having mutations in the same complementation group. The dependence of the nonsectoring phenotype on the presence of *SSD1-v* was tested by transformation with a second copy of *SSD1-v* on a *URA3/CEN* plasmid. Only mutants whose sectoring phenotype was restored by the second copy of *SSD1-v* were used for subsequent analysis.

Isolation of the *LAS1*, *LAS2*, and *LAS3* genes: For the *las1*, *las2*, and *las3* complementation groups, at least one member was temperature sensitive for growth. When the temperature-sensitive *las1*, *las2*, or *las3* mutants were crossed to a wild-type *LAS1* strain, the temperature-sensitive phenotype segregated 2/2 and was 100% linked (20 tetrads for each cross) to the inability to grow in the absence of *SSD1-v*. The *LAS1*, *LAS2*, and *LAS3* genes were isolated by introducing a YCp50 genomic library (ROSE *et al.* 1987) into the temperature-sensitive *las1*, *las2*, or *las3* mutants, respectively. The transformants were plated onto SC/low adenine minus uracil plates and tested for both their temperature-sensitive and sectoring phenotypes. In all cases, the putative *LAS* gene complemented both the temperature-sensitive and the nonsectoring phenotype. Strains containing a *URA3*-marked chromosomal locus were prepared for each *las*-complementing yeast DNA sequence and crossed to the corresponding *las1*, *las2*, or *las3* mutant. The Ura^+ progeny always had a Ts^+ phenotype while the Ura^- always had a Ts^- phenotype (in 20 tetrads for each cross), confirming that the complementing yeast DNA sequences contained the *LAS1*, *LAS2*, or *LAS3* gene. Using deletions and subclones of the original complementing plasmids, we determined the minimal regions of the *LAS1*-, *LAS2*- and *LAS3*-containing plasmids that were necessary for complementation. The data bases were searched with ~300 bases of DNA sequence from within the regions of *LAS2* and *LAS3* that were required for complementation. These searches showed that the sequenced region of *LAS2* corresponds to DNA sequences contained completely within the open reading frame of *SMP3* (IRIE *et al.* 1991a) and that the sequenced region of *LAS3* corresponds to DNA sequences contained completely within the coding region of *BCK1/SLK1* (COSTIGAN *et al.* 1992; LEE and LEVIN 1992).

A 3.5-kb *Xba*I-*Sna*BI fragment containing the *LAS1* gene was sufficient for full *las1* complementation and was cloned in both orientations into pUC119. Unidirectional nested deletion series were prepared, and the *LAS1* gene was sequenced on both strands using Sequenase (USB). This DNA sequence contained an open reading frame capable of encoding a 502-amino acid protein. Frameshift mutations at the *Nco*I site (codon 9) or the *Bam*HI site (codon 101) within this open reading frame eliminated *las1* complementation.

A deletion allele of *LAS1*: Oligonucleotide-directed mutagenesis (KUNKEL 1985) was used to replace codons 94–498 of *LAS1* (of 502 total codons) with a *Bgl*II site. A 1.8-kb *Bam*HI fragment containing the *HIS3* gene was placed into the new *Bgl*II site, yielding plasmid pCB1987. Four different Δ*las1::HIS3* diploid transformants (the *las1* deletion was confirmed by Southern analysis) were sporulated and tetrads were dissected. In >40 tetrads, each tetrad had two viable His^- and no viable His^+ haploid progeny.

Epitope tagging of LAS1: Oligonucleotide-directed mutagenesis (KUNKEL 1985) was used to create a *Not*I restriction site just upstream of the termination codon of *LAS1*. A 111-bp *Not*I restriction fragment encoding three tandem copies of the hemagglutinin epitope HA (TYERS *et al.* 1992) was inserted into the newly created *LAS1 Not*I site. The *LAS1:HA* gene, containing ~2 kb of DNA sequences upstream of the predicted *LAS1* ATG initiation codon, was cloned into YCp50 and YEp24. The *LAS1:HA* gene on YCp50 was able to fully complement the nonsectoring and temperature-sensitive phenotype of the *las1-12* mutant. In addition, the *LAS1:HA* gene on YCp50 rescued the viability and gave a wild-type growth rate to Δ*las1::HIS3* cells.

Expression of *LAS1* from the *GAL1* promoter: We prepared two different *pGAL1:LAS1* expression plasmids. For the first construct (pCB2006), a *Xba*I-*Sna*BI restriction fragment containing *LAS1* was cloned in the proper orientation into the *Bam*HI site of a *pGAL1*/YCp50 expression plasmid. The *Xba*I site is at −29 bp (relative to the A of the predicted ATG initiation codon) and an out of frame ATG (at −19 bp) exists between the *Xba*I site and the predicted ATG initiation codon. For the second *pGAL1:LAS1* expression plasmid (pCB2291), a *Hin*dIII restriction site was created 9 bp upstream of the predicted *LAS1* ATG initiation codon (sequences from −9 to the predicted ATG initiation codon have no additional ATG). When equivalent constructs contained *LAS1:HA*, both *pGAL1:LAS1* expression plasmids expressed similar levels of HA epitope-tagged LAS1 protein (data not shown). Moreover, both *pGAL1:LAS1* expression plasmids gave similar phenotypic effects.

To obtain a strain that conditionally expresses LAS1, a Δ*las1/LAS1* diploid strain (CY2810) was transformed with the *pGAL1:LAS1* plasmid (pCB2006). Tetrads were dissected onto YEP-galactose plates, and Δ*las1::HIS3* haploids strains containing the *pGAL1:LAS1* plasmid were recovered. The Δ*las1::HIS3* {*pGAL1:LAS1*/YCp50} strains cannot grow on glucose-containing medium, where expression of *pGAL1:LAS1* is repressed.

Budding index and cell size determination: The cells were sonicated (5 sec) for the determination of the budding index and the percentage of large budded cells. Cells were sonicated and the size was determined using a model ZM Coulter channelyzer.

Electron microscopy: Yeast cells, without sonication, were attached to poly-L-lysine-coated Thermanox coverslips (RMC, Tucson, AZ). The samples were impact frozen (temperature was approximately −193°) with a Life Cell CF100 impact freezer (Life Cell Corp., The Woodlands, TX) and transferred into a cryo-vial containing absolute acetone and 1% OsO_4. Frozen cells were freeze substituted at −80° for 3 days and then at −20° for 1 day. Samples were allowed to come to room temperature over a period of 6 hr. The samples were then washed three times for 10 min each with acetone, followed by two 10-min washes in propylene oxide and Spurrs resin (Polysciences Inc., Warrington, PA). After overnight infiltration, the samples were put into 100% Spurrs Resin (three 2-hr incubations). After polymerization overnight at 60°, 80-nm sections were cut with a diamond knife on a Reichert-Jung Ultracut E and then counterstained with uranyl acetate and lead citrate. At least 200 cells per sample were viewed on a Hitachi H7000 TEM at 75KV. Further details of this protocol may be obtained from DAVID SPECTOR (Cold Spring Harbor Laboratory).

Fluorescence microscopy: Immunolocalization of LAS1:HA was done as previously described (LJUNGDAHL *et al.* 1992). Cells, at 1×10^7 cells/ml in SC minus uracil medium, were diluted in YEPD medium to 1×10^6 cells/ml. After 6 hr, freshly prepared 40% formaldehyde (from paraformaldehyde) was added to a final concentration of 4%. After 15 min at room temperature, the cultures were fixed overnight on ice. Cultures were harvested and washed twice with solution A (0.1 M potassium phosphate buffer pH 7.5 in 1.2 M sorbitol). Cells were resuspended in solution A containing 30 mM β-mercaptoethanol. Spheroplasts were produced by incubating the cells for 15 min at 30° with 0.1 mg/ml oxalyticase (Enzogenetics, Corvallis, OR). Cell wall digestion was stopped by addition of 1 ml of cold solution A, followed by two washes with the same solution. Cells were pipetted onto polylysine-coated slides. After 15 min, the suspension was gently aspirated away and the slides were incubated at 24° for 15 min. The cells were washed twice with solution A, once with 100% methanol (incubated for 5 min at −20°) and five more times with solution A. Next, the cells were covered with incubation solution (solution A containing 4% nonfat dry milk) and incubated for 2 hr at 30° in a humid chamber. Then, the cells were covered with the primary antibody solution [a 1:50 dilution of anti-HA epitope 12CA5 ascites (FIELD *et al.* 1988) in incubation solution]. After 2 hr at 30°, the cells were washed five times in solution A (each wash incubated for 15 min). The cells were then covered with the secondary antibody solution: fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IG (Jackson Labs., West Grove, PA) at a 1:100 dilution in incubation buffer. After 2 hr at 24° in the dark, the cells were washed three times with solution A. Next, the cells were stained for DNA by incubation with 1 mg/ml of 4′,6-diamidino-2-phenylindole (DAPI) for 10 min. Finally, the cells were washed three times with solution A and mounted in 1% p-phenylenediamine (Sigma).

Cells used to study the immunolocalization of SPA2 and actin distribution were fixed as described (PRINGLE *et al.* 1989). Actin cables and patches were visualized by staining with 20 units/ml of rhodamine phalloidin for 2 hr (Molecular Probes Inc., Eugene, OR). Then, the cells were washed twice for 5 min with PBS buffer (40 mM K_2HPO_4, 10 mM KH_2PO_4 and 0.15 M NaCl) and mounted in FluorSave Reagent (Calbiochem, La Jolla, CA). To localize SPA2, spheroplasts were prepared by digesting the cell wall with zymolyase 100T (ICN, Costa Mesa, CA). Cells were first incubated for 2 hr with anti-SPA2 polyclonal antibody (preabsorbed with an extract prepared from a Δ*spa2* strain) diluted 1:3000 in PBS-BSA (PBS containing 10 mg/ml of BSA). After four 5-min washes with PBS, the cells were incubated for 2 hr with the secondary antibody: FITC-conjugated donkey anti-rabbit IG (Amersham, UK) at a dilution of 1:1000 in PBS-BSA. After this incubation, the cells were washed twice with PBS, and DNA staining with DAPI was done as described above. Cells were viewed with a Zeiss Axiophot microscope using a 100× objective. T-max P3200 Kodak film was used for photography.

Preparation of total protein extracts and Western immunoblots: Cellular extracts and Western immunoblots were prepared as described previously (SUTTON *et al.* 1991). A fraction of the same cultures used for subcellular fractionation experiments were harvested by centrifugation and washed in ice cold lysis buffer (100 mM Tris-HCl pH 7.5, 200 mM NaCl, 1 mM EDTA, 5% glycerol, 0.5 mM dithiothreitol). Cells were resuspended in 250 ml of lysis buffer containing 1 mM phenylmethylsulfonyl fluoride and 1.2 μg per ml of each of leupeptin, antipain, chymostatin, and pepstatin (Sigma). The cells were lysed by vortexing five times for 20 sec in the presence of glass beads. An additional 250 μl of RIPA buffer (50 mM Tris-HCl pH 7.5, 1% Triton, 0.5% Na-deoxycholate, 0.1% SDS, 200 mM NaCl) was added, and the cells were vortexed twice for 20 sec. The liquid was pipetted from the glass beads and centrifuged at 16,000 × *g* for 5 min to remove cell debris. Protein concentrations were determined using the Bio-Rad assay (Bio-Rad Lab., CA). An equal volume of 2× protein gel-loading buffer was added to the extracts. Samples were heated for 5 min at 95°, centrifuged for 3 min at 16,000 × *g*, and electrophoresed through an 8% SDS-polyacrylamide gel. The proteins were then analyzed by Western immunoblotting.

Subcellular fractionation: Subcellular fractionation was basically performed as described by LUE and KORNBERG (1987). Briefly, 40 ml of cells in late exponential phase were harvested by centrifugation and resuspended in 50 mM Tris-HCl (pH 7.5), 30 mM dithiotreitol, and they were shaken slowly at 30° for 15 min. After this incubation, the cells were resuspended in YEPD medium containing 1.2 M sorbitol (1 ml/g of cells). Cell walls were digested with zymolyase 100T (1.5 mg/g of cells). Digestion was stopped by the addition of ice cold YEPD containing 1.2 M sorbitol. The spheroplasts were recovered by centrifugation at 3000 × *g* and lysed in 4 ml of 18% (wt/vol) Ficoll (Pharmacia), 10 mM Tris-HCl (pH 7.5), 20 mM KCl, 5 mM $MgCl_2$, 3 mM dithiotreitol, 1 mM EDTA, 0.5 mM spermidine, 0.15 mM spermine, 1 mM phenylmethylsulfonyl fluoride, 1.2 μg/ml each of leupeptin, antipain, chymostatin, and pepstatin (Sigma) using a 7 ml Dounce homogenizer. After lysis, debris were separated by six 5-min centrifugations at 3000 × *g*. Nuclei-containing fractions were separated from cytoplasmic-containing fractions by centrifugation at 25,000 × *g* using a GS600 rotor for 30 min. The nuclear fraction was resuspended in lysis buffer. The samples were then treated as the total protein extracts (see above section). Proteins were separated through an 8% SDS-polyacrylamide gel and immunoblotted.

RESULTS

Isolation of *las* mutants and characterization of *LAS* genes: To better understand the role of SIT4 for bud formation, we isolated mutations that, like *sit4* mutations, result in lethality in the absence of the *SSD1-v* gene. In otherwise wild-type cells, the absence of *SSD1-v* results in only subtle phenotypic alterations and the cells have a wild-type growth rate (SUTTON *et al.* 1991). Mutations that result in a requirement for *SSD1-v* should occur in genes required for a functional SIT4 pathway and also probably in genes functioning in non-SIT4 pathways. To isolate mutants that require *SSD1-v*, we used the colony color sector assay (KOSHLAND *et al.*

1985; KRANZ and HOLM 1990). From a screen of 160,000 colonies derived from mutagenized cells, we isolated 75 mutants that could not grow in the absence of *SSD1-v* (see MATERIALS AND METHODS). Thirty-two of these mutants were placed into seven complementation groups termed *LAS1* through *LAS7* (lethal in the absence of *SSD1-v*). Each complementation group had at least three members. Some of the mutants did not fall into a particular complementation group, possibly because the screen was not carried out to saturation. Group 7 mutants, composed of four members, most likely have mutations in *SIT4* because the nonsectoring phenotype of these mutants was fully complemented by a low copy number plasmid containing the *SIT4* gene. The isolation of *sit4* mutants in our screen confirms that the screen should allow us to isolate additional mutations that might affect the function of the SIT4 pathway.

For the *las1, las2,* and *las3* complementation groups, at least one member of each group was temperature sensitive for growth. Moreover, the temperature-sensitive phenotype was 100% linked to the inability to grow in the absence of *SSD1-v* (see MATERIALS AND METHODS). The *LAS1, LAS2,* and *LAS3* genes were isolated by their ability to complement the respective *las* mutant for their temperature-sensitive phenotype (see MATERIALS AND METHODS). These genes also restored the ability to grow in the absence of *SSD1-v*. For each gene, targeted integration and linkage analysis were used to show that the complementing DNA fragment corresponded to the wild-type locus (see MATERIALS AND METHODS). *LAS2* and *LAS3* are previously identified genes. *LAS3* corresponds to *BCK1/SLK1* (see MATERIALS AND METHODS), which agrees with previous findings that *SSD1-v* can suppress *bck1/slk1* mutations (COSTIGAN *et al.* 1992). *BCK1/SLK1* encodes a protein kinase that functions downstream of protein kinase C, possibly to activate MPK1, a MAP kinase homologue (COSTIGAN *et al.* 1992; LEE *et al.* 1993; LEE and LEVIN 1992; COSTIGAN and SNYDER 1994). Cells lacking *BCK1/SLK1* can grow at 30°, but at higher temperatures the cells lyse. The cell lysis defect and the aberrant morphology of *bck1/slk1* mutants suggest that *BCK1/SLK1* plays a role in cell morphogenesis and cell growth.

LAS2 corresponds to *SMP3* (see MATERIALS AND METHODS). *smp3* mutations were originally isolated by their ability to cause an increased mitotic stability of heterologous plasmids (IRIE *et al.* 1991a). *SMP3* encodes an essential hydrophobic protein with no strong homology to other sequences in the data bases. Interestingly, *LAS2* and *LAS3* genetically interact: overexpression of *BCK1/SLK1* (*LAS3*) is able to suppress the temperature-sensitive phenotype of a *smp3* (*las2*) mutant (IRIE *et al.* 1991b). Also, *SMP3* (*LAS2*) interacts genetically with *SIT4:* a *las2-28* Δ*sit4* (*SSD1-v*) double mutant grows slightly slower than the single *las1-28* or Δ*sit4* mutants at 24° but is inviable at 30° (both single mutants are viable at 30°) (data not shown). Although it is not known how SMP3 functions relative to the PKC1/MPK1 pathway for polarized cell growth, *BCK1/SLK1* and *SMP3* define two of our seven major complementation groups of mutants that require *SSD1-v*. This finding further connects the function of SMP3 and BCK1/SLK1.

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MIPPRIVPWR DFAELEELKL WFYPKSKGTI   30
EDKRQRAVQR VQSYRLKGSQ YLPHVVDSTA
QITCAVLLDE KEACLGVHQD SIPIRLSYVM   90
ALIRFVNGLL DPTQQSQFAI PLHTLAAKIG
LPSWFVDLRH WGTHERDLPG LEMLRWAANE  150
ALSWLYDHYW NDEELEDDRD DDDDDDDTGY
GYRRNDKLEK YMESLTKTLD KWKRLRNEFL  210
EYKWVWENAN DSLITSSNFS GDNLVNYDAE
KRKSSHASSS ETMIRENLRQ WQELWKLSIY  270
HNVVLEKFFN NYDPLLLKVL MLNLNNFDWK
VIEWVARNYR TQQDDSNITT ILKRKFNAWK  330
ELQKRLLDVI INNLNNKNFK NKWQNWEKLI
DENASYLILY FCQSMLAKLE TEKITGNSWR  390
NKKRRKQIDS TVEIEAKLKE NIDNLSLRFN
EGEIKLYDFI PAEKDSVPLK KEVSPALKAD  450
TNDILGDLAS LKQRMSSFGT VGKKNKQEEN
RATPVKNWSR VQNWKPKPFG VL          502
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FIGURE 1.—The predicted amino acid sequence of LAS1. The predicted amino acid sequence is shown (accession #U09670). The double-underline region indicates the acidic region of LAS1. *LAS1* corresponds to open reading frame YKR063c on the *S. cerevisiae* chromosome XI sequence (DUJON *et al.* 1994).

The *LAS1* complementation group was the largest and was comprised of seven independent mutants. *LAS1* is located on the right arm of chromosome *XI,* as determined by hybridization of filters containing most of the yeast genome in lambda phage clones (clone #3893) (OLSON *et al.* 1986) and the tight linkage of *LAS1* to *SIS2,* which is located on chromosome *XI* (DI COMO *et al.* 1995). The DNA sequence of *LAS1* predicts a 502-amino acid protein of 59 kD with a small acidic region (Figure 1). LAS1 had no strong homology with other proteins in the current data bases. The remainder of this paper will focus on *LAS1.*

LAS1 encodes an essential protein: To study the role of the *LAS1* gene, we prepared a deletion allele of *LAS1* (see MATERIALS AND METHODS). Tetrads from *LAS1/*Δ*las1::HIS3* diploids (in either Δ*ssd1,* CY2810, or *SSD1-v* genetic backgrounds) gave rise to two viable His$^+$ (wild type) and two inviable (predicted Δ*las1::HIS3*) colonies. The Δ*las1::HIS3* spores germinated, but after three to four divisions they arrested as a mixture of ~60% unbudded and 40% large budded cells. Furthermore, *pGAL1:LAS1* strains are not able to grow on glucose-containing medium, where the transcription of *LAS1* is repressed (data not shown). Therefore, *LAS1* is essential for cell viability.

Inactivation of LAS1 results in unbudded cells and large budded cells that accumulate vesicles at the mother-daughter neck: We examined the effects due to a deficiency in LAS1 under three conditions: *las1-12*

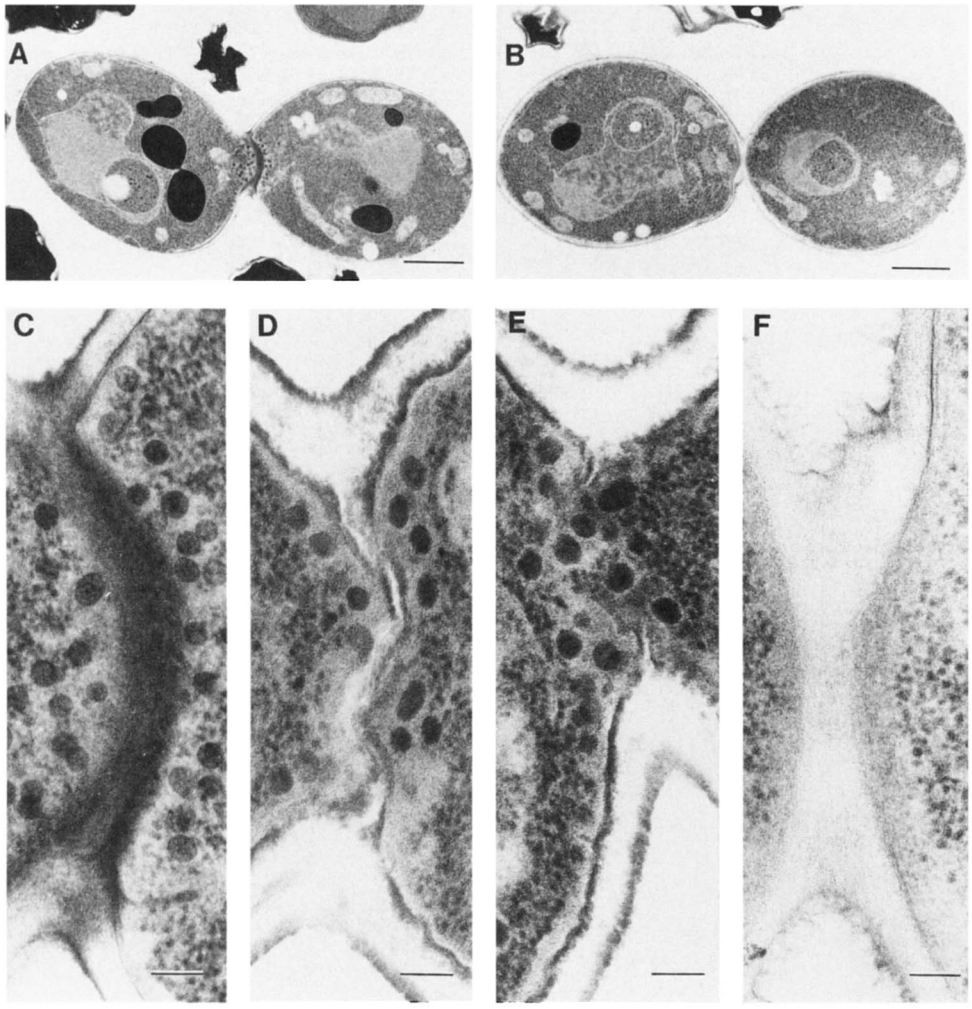

FIGURE 2.—Large budded cells deficient for LAS1 accumulate vesicles at the mother-daughter neck. Electron microscopy of *las1-12* and *LAS1* cells grown in YEPD medium. (A and C) *las1-12* cells (CY4401) grown at the semi-permissive temperature (30°). (D and E) *las1-12* cells grown for 17 hr at the nonpermissive temperature (37°). (B and F) *LAS1* cells (CY4402) grown at 30°. Cells were prepared for electron microscopy using freeze substitution as described in MATERIALS AND METHODS. Bars: A and B, 1 μm; C–F, 0.1 μm.

cells grown at 30°, *las1-12* cells grown at 37°, and cells depleted of LAS1. Compared to isogenic *LAS1* cells (CY4402), *las1-12* cells (CY4401) gave rise, when streaked onto YEPD plates, to slightly smaller colonies at 24° and to much smaller colonies at 30° (a semi-permissive temperature), and they were not able to grow at 37° (the nonpermissive temperature). Addition of 0.5 M sorbitol to the growth medium did not cure the temperature-sensitive phenotype of *las1-12* mutants, but it did cure (as previously shown: LEE and LEVIN, 1992; LEVIN and BARTLETT-HEUBUSCH, 1992; LEE *et al.* 1993) the temperature-sensitive phenotype of *pkc1*, *bck1/slk1*, and *mpk1* mutants (data not shown).

At 30°, an exponentially growing *las1-12* culture had an enrichment both in large budded cells (22%, compared to 10% for *LAS1* cells) and in unbudded cells (48%, compared to 23% for *LAS1* cells). Moreover, for the large budded *las1-12* cells many of the daughters were as large as the mother cell. We use the term "large budded cell" here merely to indicate that, in light microscopy views, the mother cell had a large daughter attached to it. These large budded cells may be pre- or postcytokinesis. Electron microscopy showed that the large budded *las1-12* cells accumulated many vesicles at the mother-daughter neck, within both the mother and the daughter cells (Figure 2, A and C). This enrichment of vesicles at the mother-daughter neck was not observed for *las1-12* cells grown at 24° or in isogenic *LAS1* cells grown at 30° (Figure 2B). These vesicles might be secretory vesicles that would normally deliver cell membrane and cell wall components to the surface of the cell. The enrichment in large budded *las1-12* cells containing many vesicles suggests that the ability to form a cell wall between the mother and daughter cells is defective in the partial absence of LAS1 function. However, most of the large budded *las1-12* cells did have cell membranes between the mother and daughter, suggesting that the large budded *las1-12* cells initiated and possibly completed cytokinesis.

We also examined *las1-12* cells shifted to the nonpermissive temperature of 37°. The *las1-12* cells did not immediately arrest but divided about six times during 17 hr at 37° (the cells were kept below an $O.D._{600}$ of 0.8). During longer incubations at 37°, the *las1-12* cells did not continue to divide but did slightly increase in size (from 51 fL at 17 hr to 61 fL at 24 hr). After 17 hr at 37°, the arrested *las1-12* culture had 80% of the population as unbudded cells and 20% as large budded cells (budding index determined after sonication). Moreover, ~80% of the cells had a 1n DNA content

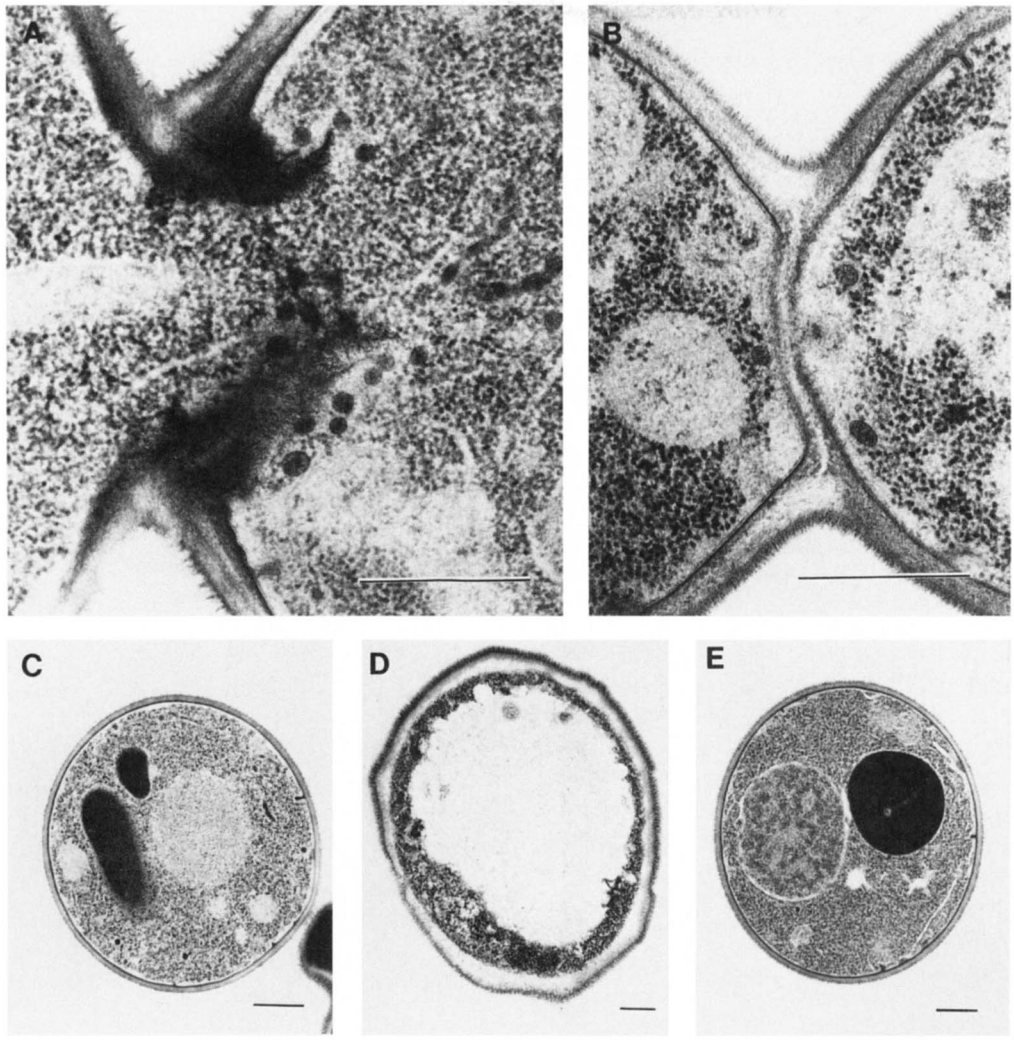

FIGURE 3.—Depletion of LAS1 produces unbudded cells and large budded cells that accumulate vesicles at the mother-daughter neck. Wild-type cells (CY4404) and cells with *pGAL1:LAS1* on a low copy number plasmid as the only source of LAS1 (CY2807) were grown in YEP-galactose medium. At time 0, glucose was added to repress the *GAL1* promoter. After 17 hr, the cells were collected and prepared for electron microscopy using freeze-substitution (see MATERIALS AND METHODS). (A) *pGAL1:LAS1* cells with an incomplete neck and a large accumulation of vesicles. (B) Wild-type cells with a trilaminar mother-daughter neck. Two different phenotypes were observed in unbudded cells depleted for LAS1: (C) unbudded cells resembling unbudded wild-type cells (E) and (D) cells with a disorganized cytoplasmic organization. Bars, 0.5 μm.

(determined by flow cytometry), suggesting that the unbudded cells had a 1n DNA content and were in G1. All the large budded cells had a nucleus in both the mother and daughter cell and accumulated many vesicles at the mother-daughter neck (Figure 2, D and E). These vesicles appear identical to the vesicles in *las1-12* cells grown at 30° (Figure 2, A and C). In contrast to the large budded cells, a large enrichment of vesicles was not observed in the unbudded *las1-12* cells at 37° (out of 200 cell sections observed, eight had three or four vesicles and the remainder had none, data not shown).

The arrested *las1-12* cells were mostly viable because 90% of the cells gave rise to a colony when shifted back to 24°. To determine if the arrested *las1-12* cells (80% of which are unbudded G1 cells) require the execution of Start for the initiation of a bud, an arrested *las1-12* culture (37° for 17 hr) was divided in half and α factor was added to one of the cultures. After 15 min at 37°, both cultures were shifted to the permissive temperature of 24°. The *las1-12* cells that were not treated with α factor formed a bud after ~135 min at 24°. In contrast, *las1-12* cells treated with α factor failed to form a bud even 6 hr after the shift to 24°. These findings suggest that when LAS1 function is restored, the unbudded G1 cells must execute at least some functions of Start to form a bud.

To study the effects due to the depletion of LAS1, glucose was added to *pGAL1:LAS1* cells (CY2807) and isogenic wild-type cells (CY4404) growing exponentially in galactose medium (glucose represses the *GAL1* promoter). To estimate the rate at which LAS1 protein depletes under these conditions, parallel experiments were also performed with a *pGAL1:LAS1:HA* strain and a *LAS1:HA* strain. After the addition of glucose, the LAS1:HA protein levels in the *pGAL1:LAS1:HA* strain

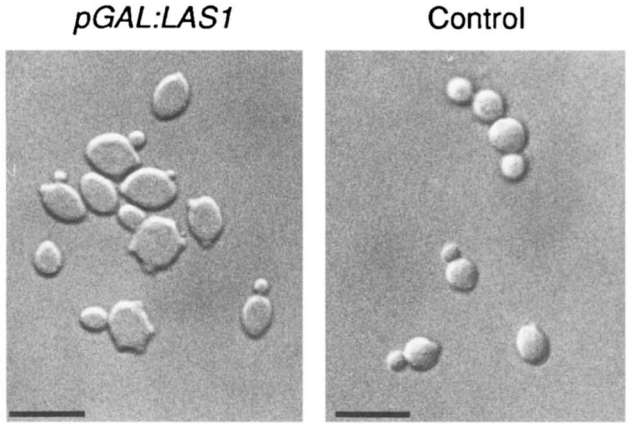

FIGURE 4.—Overexpression of LAS1 results in extra surface projections. Cells overexpressing LAS1 (*pGAL1:LAS1,* CY2807) and wild-type cells (control, CY4404) were grown exponentially in galactose-containing medium (SC-galactose). The cells were fixed as described in MATERIALS AND METHODS and viewed by Nomarski optics. Bar, 8 μm. The same projections, but only in 15% of the population, were observed when *pGAL1:LAS1* was expressed in cells containing a chromosomal copy of LAS1 (CY4403).

decreased by 50% about every 2 hr, so that after 5 hr the LAS1:HA protein levels in the *pGAL1:LAS1:HA* strain decreased to a level similar to that when *LAS1:HA* was expressed from its normal promoter and by 17 hr were not detectable (data not shown). After 17 hr in glucose, both the *pGAL1:LAS1* culture (CY2807) and the *pGAL1:LAS1:HA* culture arrested with 80% unbudded and 20% large budded cells (budding index determined after sonication). Like the wild-type cells, the large budded LAS1-depleted cells had a thick septum around the periphery of the mother-daughter neck (Figure 3, A and B). In contrast to wild-type cells, the septa in the large budded LAS1-depleted cells are incomplete in the central region. In addition, a large number of vesicles were found in the neck region (Figure 3A). The unbudded LAS1-depleted cells had two different appearances. About 60% of the unbudded cells had an ultrastructure similar to unbudded wild-type cells (Figure 3, C and E). The remainder of the unbudded cells (40% of the unbudded population, ~30% of total cells) had a disorganized cytoplasm ultrastructure and a thicker cell wall (Figure 3D). These cells might possibly be dead cells because 17 hr after the addition of glucose to the *pGAL:LAS1* culture, ~20% of the cells were not able to form colonies when plated onto YEP galactose plates.

Overexpression of LAS1 causes extra cell surface projections: To determine the effects due to overexpression of LAS1, we analyzed *pGAL1:LAS1* cells growing on galactose medium. Western analysis showed that *pGAL1:LAS1:HA* cells had about fivefold more HA epitope-tagged LAS1 than when *LAS1:HA* was expressed from the normal *LAS1* promoter (data not shown). The *pGAL1:LAS1* cells (CY2807, with the non-HA tagged LAS1) growing on galactose medium accumulated cells (45% of the total population) that had extra surface projections. These extra projections were seen only on the mother cells and were not observed on the growing buds (Figure 4). In addition, the projections only reach a certain size, after which they stop growing. These effects due to the expression of LAS1 from the *GAL1* promoter are most likely due to the overexpression of LAS1 and not the expression of LAS1 at abnormal stages of the cell cycle because the steady-state levels of *LAS1* RNA do not vary during the cell cycle (data not shown). Also, the formation of these projections by overexpressed LAS1 does not require *SSD1-v* because similar numbers of projections were observed in isogenic Δ*ssd1* and *SSD1-v* cells overexpressing LAS1 (data not shown).

To determine if the formation of the projections might require the execution of Start, *pGAL1:LAS1* cells (also containing chromosomal *LAS1,* CY4403) were grown in raffinose medium and arrested with α factor (which arrests the cells in G1 before Start). The α factor-arrested culture was divided into two halves, and the α factor was washed away from one of the halves. Then, both the α factor- and the non-α factor-containing cultures were divided, and galactose was added to one of them to induce LAS1 overexpression. Only cells released from the α factor arrest were able to form projections when LAS1 was overexpressed. These findings suggest that either projection formation requires the absence of the mating signals (which would normally direct secretion to the schmoo tip) or that projection formation requires the execution of Start.

To determine the time course of projection formation during the cell cycle, we microscopically monitored (over the course of a few hours) 10 small unbudded *pGAL:LAS1* cells growing on the surface of a YEP galactose plate. The small unbudded cells were probably G1 daughter cells because they were small and because they initially had no visible cell surface projections (daughter buds still attached to the mother cell do not have projections). The appearance of the projections on these small unbudded cells was sequential. The cells would first form one visible projection. Then, the first projection would cease to increase in size and a second visible projection would be formed. This process continued until one of the projections (the latest one formed) would continue to get larger and would eventually become the bud. These budded cells had, on average, three visible smaller projections that did not continue to increase in size. Moreover, once a growing bud was apparent, there was no further increase in the number of projections.

To further investigate the nature of these extra cell surface projections, we examined the distribution of some of the components involved in bud formation and growth. The actin cytoskeleton can be visualized as two structures: actin cables that are aligned along the mother-bud axis and extend into the bud, and cortical actin patches that accumulate at the site where the new

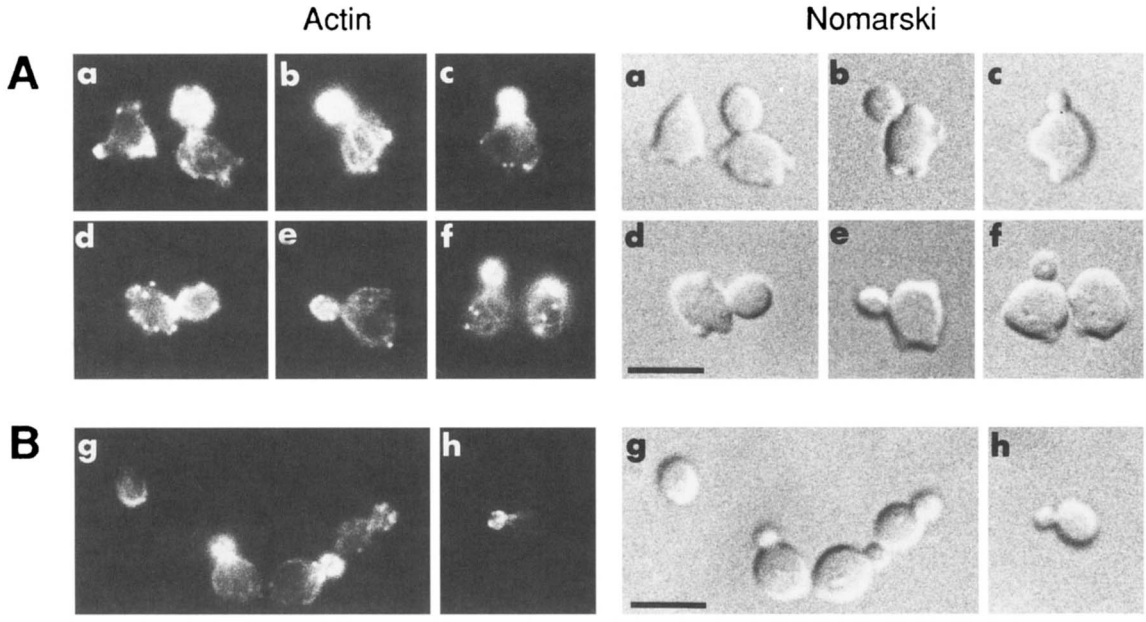

FIGURE 5.—Actin distribution in LAS1-overexpressing cells. (A) *pGAL1:LAS1* cells (CY2807) or (B) wild-type cells (CY4404) were grown in YEP-galactose medium at 30°. The cells were fixed and stained with rhodamine-conjugated phalloidin as described in MATERIALS AND METHODS. Left panels: Phalloidin visualization for actin. Right panels: The same cells viewed by Nomarski optics. Bar, 8 μm.

bud will form and that later concentrate in the tip of the growing bud (ADAMS and PRINGLE 1984; KILMARTIN and ADAMS 1984). In cells overexpressing LAS1, actin cables were found not only extending into the bud, but also into some of the projections (Figure 5 A, subpanel b). Moreover, the cells overexpressing LAS1 also had a less ordered distribution and an apparent increase in the number of cortical actin patches: the cortical actin structures accumulated not only in the bud, but were also oriented toward and sometimes localized at the projections (Figure 5A). Actin patches have been postulated to play a role in the insertion of vesicles containing cell wall and membrane components into the growing cell surface (FIELD and SCHEKMAN 1980; MULHOLLAND *et al.* 1994). Possibly, actin cables direct secretory vesicles to the site of cell surface growth, such as a growing bud. Therefore, the association of actin structures with the projections suggests that the projections are due to areas of cell surface growth.

In wild-type cells, the SPA2 protein localizes to the place where the new bud will form and, once the bud has formed, localizes as a patch at the tip of the emerging bud (SNYDER 1989; GEHRUNG and SNYDER 1990). In mating cells, SPA2 localizes to the tip of the schmoo. These findings suggest that the SPA2 protein localizes at or near sites of cell growth. In contrast to wild-type cells, cells overexpressing LAS1 have SPA2 localized to more than one spot in each cell (Figure 6A). Some of the SPA2 spots colocalize with the projections while other spots are found in places where projections are not seen, at least in light microscopy views (Figure 6A). In cells overexpressing LAS1, the localization of SPA2 to more than one site is not accompanied by an increase in the cellular levels of the SPA2 protein, as determined by Western analysis for SPA2 protein in whole cell extracts and by Northern analysis for *SPA2* RNA levels (data not shown). Moreover, SPA2 is not required for the formation of the projections because isogenic Δ*spa2* and *SPA2* cells containing *pGAL1:LAS1* gave equivalent numbers of projections (data not shown). The extra cell surface projections on the mother cell, the altered distribution of actin structures, and the altered localization of SPA2 protein indicate that overexpression of LAS1 alters the normal localization of directed cell growth.

The cells overexpressing LAS1 were also examined by electron microscopy. Remarkably, the LAS1-overexpressing cells accumulated a large number of electron dense structures that were concentrated around the periphery of the cell, including the projections (Figure 7, A–C). One possibility is that overexpression of LAS1 results in overexpression of components involved in cell surface growth to such a degree that all of the cell surface growth can no longer be localized to just one site. Extra projections are formed and unfused cell surface growth components accumulate around the periphery of the cells. Alternatively, the effects due to overexpression of LAS1 might be directly due to delocalization of the sites of cell surface growth, without an increase in the flux of cell surface growth components through the secretory pathway. For this explanation, the electron dense structures could be due to delocalized cell surface growth accompanied by a defect in the ability to properly fuse the vesicles with the cell membrane.

Overexpression of LAS1 can partially suppress the

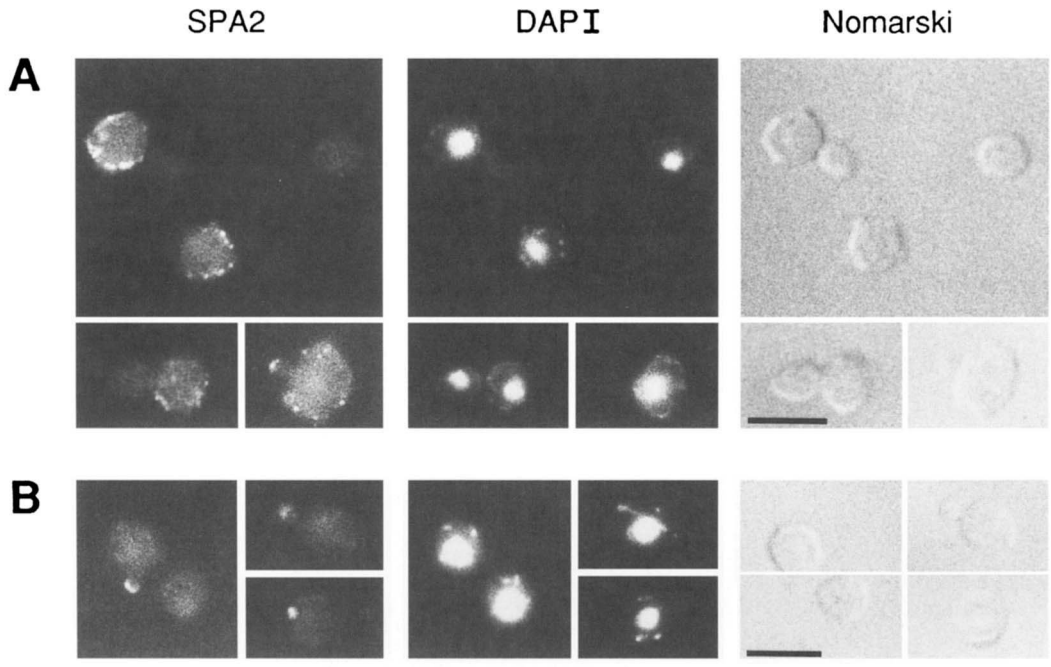

FIGURE 6.—Overexpression of LAS1 alters the localization of SPA2. (A) *pGAL1:LAS1* cells (CY2807) or (B) wild-type cells (CY4404) were grown in YEP-galactose medium at 30°. The cells were fixed and stained with anti-SPA2 antibody (obtained from M. SNYDER), and the DNA was stained with DAPI as described in MATERIALS AND METHODS. Left panels: Indirect immunofluorescence to visualize SPA2. Middle panels: DAPI staining of the same cells to visualize the DNA. Right panels: Visualization of the same cells by Nomarski optics. Bar, 8 μm.

bud emergence defect of *sit4* mutants, if CLN2 is provided from a SIT4-independent promoter: The SIT4 protein phosphatase is required during G1 for the expression of the G1 cyclins CLN1 and CLN2, which function for the execution of Start (SUTTON *et al.* 1991; FERNANDEZ-SARABIA *et al.* 1992). When shifted to the nonpermissive temperature, *sit4-102* cells arrest primarily as unbudded cells with 1n DNA content. However, expression of *CLN2* from a SIT4-independent promoter (*S. pombe ADH* promoter) in *sit4-102* cells allows DNA replication at the nonpermissive temperature, but bud formation is still mostly blocked (FERNANDEZ-SARABIA *et al.* 1992).

To investigate a possible connection between LAS1 and SIT4 in bud formation, we analyzed the effect of overexpressing LAS1 in either *sit4-102* or *sit4-102* (*S.pombe ADH:CLN2*) cells. Overexpression of LAS1 (*LAS1* on a high copy number plasmid) had no effect on the arrest phenotype of the *sit4-102* cells: both cultures arrested primarily as unbudded cells (Table 2) with a 1n DNA content (data not shown). By contrast, overexpression of LAS1 in *sit4-102* cells containing *CLN2* expressed from the *S. pombe ADH* promoter (a SIT4-independent promoter) resulted in a modest, but reproducible and statistically significant, increase in the percentage of budded cells at the nonpermissive temperature (Table 2). However, overexpression of LAS1 was not able to restore viability to these cells. A similar *S. pombe ADH:CLN2*-dependent increase in the percentage of budded cells was also observed in *sit4-102* cells overexpressing CDC24, which is known to be involved in bud initiation (SLOAT *et al.* 1981) (Table 2). Therefore, overexpression of either LAS1 or CDC24 can weakly promote bud formation in the absence of normal SIT4 function. Moreover, the bud-promoting activity due to overexpressed LAS1 requires SIT4-independent expression of *CLN2*, which implies that the execution of Start is required.

Genetic interactions of *LAS1* with G1 cyclins and bud morphogenesis: We tested the ability of increased levels of G1 cyclins to suppress the inability of *las1-12* cells to grow in the absence of *SSD1-v*. High copy number *SWI4* or *CLN2* and low copy number *S. pombe ADH:CLN2* (which gives levels of CLN2 similar to wild type, but from a SIT4-independent promoter), *S. cerevisiae ADH:CLN2* (which overexpresses CLN2 10- to 20-fold), or *S. pombe ADH:SWI4* (which gives ~5- to 10-fold higher levels of *SWI4* RNA than wild-type *SWI4*) all restored the ability of *las1-12* cells to grow in the absence of *SSD1-v*. None of these plasmids suppressed the temperature-sensitive phenotype of *las1-12* cells or allowed Δ*las1* cells to be viable. The higher levels of G1 cyclins could either bypass the need for *SSD1-v* in *las1-12* cells, increase the activity of las1-12 protein, or give conditions where the cell can survive with less (but not no) LAS1 activity.

We also determined the effect due to lower G1 cyclin levels in *las1-12* cells. For the crosses in this section, 40 tetrads were dissected and the growth phenotype of 10 segregants of a given genotype were determined. SWI4 and SWI6 are required for normal levels of G1 cyclins. Both *las1-12* Δ*swi4 SSD1-v* strains and *las1-12* Δ*swi6 SSD1-v* strains grew very slowly at 24° and were inviable

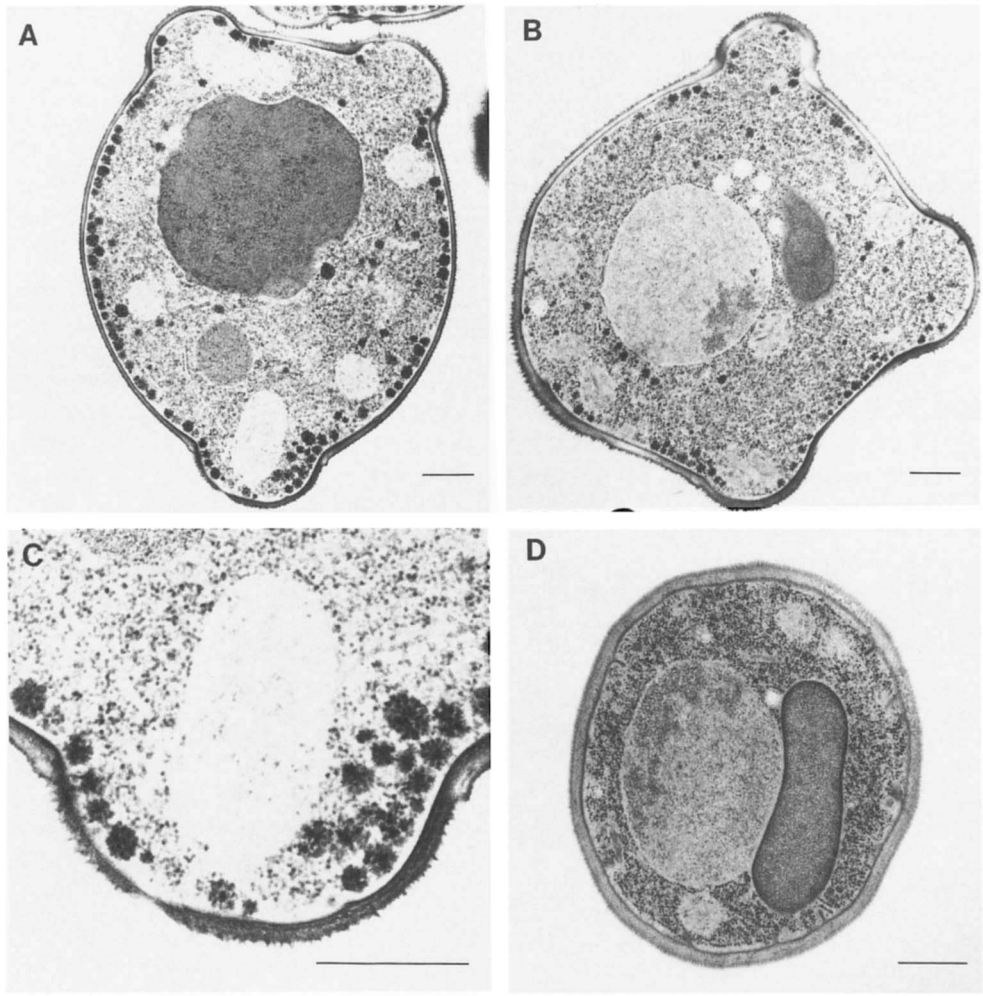

FIGURE 7.—Cells overexpressing LAS1 accumulate electron-dense structures. *pGAL1:LAS1* cells (CY2807) were grown in YEP galactose medium and were prepared for electron microscopy by freeze substitution as described in MATERIALS AND METHODS. (A and B) About half of the cells overexpressing LAS1 have projections and large numbers of electron-dense structures beneath the plasma membrane. (C) Higher magnification of a region of the cell shown in A. (D) Wild-type cells (CY4404). Bar, 0.5 μm.

at 30°. Therefore, normal LAS1 function becomes critical when G1 cyclin levels are low, which might be due to the connection of G1 cyclin function with bud formation and growth (BENTON *et al.* 1993; CVRCKOVA and NASMYTH 1993; MAZZONI *et al.* 1993). *SIT4* is also required for the normal expression of *SWI4* and of the *CLN1* and *CLN2* G1 cyclins and independently for bud initiation (FERNANDEZ-SARABIA *et al.* 1992). From a cross of a Δ*sit4 SSD1-v* strain to a *las1-12* strain, the Δ*sit4 las1-12 SSD1-v* progeny were able to grow slowly at 24° (the permissive temperature for *las1-12* cells) but were inviable at 30° (a semi-permissive temperature for *las1-12* cells). Neither SIT4 nor *SSD1-v* is required for the transcription of *LAS1* (data not shown). Therefore, ei-

TABLE 2

LAS1 promotes bud formation in *sit4-102* mutants only in the presence of heterologous expression of CLN2

	sit4-102		*sit4-102 S. pombe ADH:CLN2*	
	24°	38°	24°	38°
YEp24	67.2 ± 5.0 (6)	25.8 ± 3.5 (6)	61.3 ± 6.4 (3)	27.7 ± 3.8 (3)
LAS1/YEp24	65.0 ± 2.7 (6)	22.5 ± 5.9 (6)	69.7 ± 5.5 (3)	46.7 ± 4.2 (3)
CDC24/YEp24	64.6 ± 6.5 (5)	26.6 ± 1.5 (5)	68.5 ± 3.5 (2)	42.3 ± 1.1 (2)

The percentage of budded cells (±SD) was determined by counting at least 200 cells for each sample. *sit4-102* cells (CY2813) and *sit4-102 S. pombe ADH:CLN2* cells (CY2814) were transformed with either YEp24, *LAS1*/YEp24, or *CDC24*/YEp24. The transformants were grown at 24° in SC minus uracil, leucine, and tryptophan medium, inoculated into YEPD medium, grown for one generation, and shifted to the nonpermissive temperature. Cells were collected just before and 4½ h after the shift to 38° and fixed for 16 h at 4° in 70% ethanol. The number of independent cultures for each condition is given in parenthesis.

ther SIT4 and LAS1 function in parallel pathways where loss of the normal function of both pathways results in inviability, or SIT4 and LAS1 function within the same pathway where the activity of the las1-12 protein is so reduced in a *sit4* mutant that the cells are inviable. Further connecting LAS1 and SIT4 to bud initiation/morphogenesis is that *las1-12 Δbem2/ipl2 (SSD1-v)* strains grew very slowly at 24° and were inviable at 30°, while *Δsit4 Δbem2/ipl2 (SSD1-v)* strains were inviable at either 24° or 30°. *BEM2* is involved in polarity establishment and bud emergence (BENDER and PRINGLE 1991; HEALY *et al.* 1991).

LAS1 localizes to the nucleus: The effects of LAS1 on bud initiation/morphogenesis could be due to LAS1 performing a direct role in localized cell growth. If LAS1 functions in this way, we would expect LAS1 to be present in a cytoplasmic compartment. Alternatively, LAS1 might function as a regulator of cell growth, such as for the normal expression of some factor(s) that in turn functions for cell growth and morphogenesis. To distinguish between these possibilities, we determined the cellular localization of HA epitope-tagged LAS1 protein (see MATERIALS AND METHODS). The gene encoding the epitope-tagged LAS1 protein fully complements for *LAS1*.

The cellular distribution of LAS1:HA was initially determined by subcellular fractionation. Extracts of isogenic wild-type cells, containing either LAS1:HA (CY2809) or LAS1 (CY2808), were fractionated into a crude nuclear pellet and a cytoplasmic supernatant using the procedure of LUE and KORNBERG (1987) (see MATERIALS AND METHODS). Western blot analysis of total cell extracts and the nuclear and cytoplasmic fractions was performed using the 12CA5 ascites (FIELD *et al.* 1988), which recognizes the HA epitope. A protein of 62 kD, which is similar to the predicted size of the LAS1:HA protein, was detected only in the nuclear fractions from the cells containing the epitope-tagged LAS1 (Figure 8).

Indirect immunofluorescence methods were used to determine the subcellular localization of LAS1:HA. We used a diploid strain (CY3830) carrying either *LAS1:HA* or *LAS1* on either low or high copy number plasmids. In the cells containing high copy number *LAS1:HA*, LAS1:HA-specific immunofluorescence colocalized with the DAPI staining, which visualizes the nucleus (Figure 9A). The finding of LAS1:HA in the nucleus of unbudded, small budded, and large budded cells suggests that LAS1:HA is localized to the nucleus during most, if not all, stages of the cell cycle. Nuclear localization of LAS1:HA was also observed with *LAS1:HA* on a low copy number plasmid but the signal was very weak (data not shown). The control cells containing the untagged LAS1 protein showed weak background staining over the entire cell (Figure 9B). This background staining is probably due to a protein of ~50 kD that crossreacts with the 12CA5 antibody in Western analysis of yeast extracts (see Figure 8). Therefore, both the fractionation and indirect immunofluorescence studies show that LAS1:HA localizes to the nucleus, suggesting that LAS1 might regulate the expression of some factor(s) that in turn is involved in bud formation and cell surface growth.

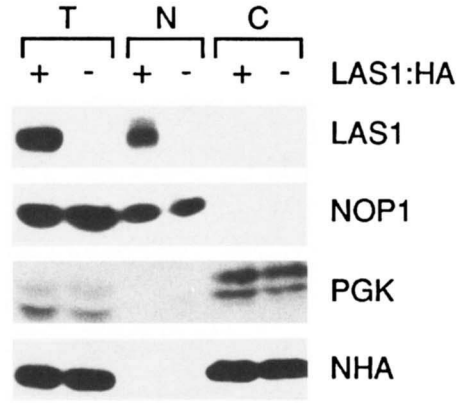

FIGURE 8.—LAS1:HA is present in the nuclear fraction. Cells expressing LAS1:HA (+) (CY2809) or nontagged LAS1 (−) (CY2808) were fractionated as previously described (LUE and KORNBERG 1987). Total cell extracts were prepared as described in SUTTON *et al.* 1991. Equivalent amounts of total (T), nuclear (N) and cytoplasmic (C) fractions from the same number of cells were run on an 8% SDS-polyacrylamide gel and analyzed by Western immunoblotting. The antibodies used were 12CA5 ascites (directed against the hemagglutinin epitope), anti-NOP1 monoclonal antibody A66 (ARIS and BLOBEL 1989) as a nuclear marker and anti-PGK polyclonal antibody as cytoplasmic marker. NHA corresponds to a protein that cross reacts with the 12CA5 antibody.

DISCUSSION

The effects caused by the loss of LAS1 function or the gain of LAS1 function suggest that LAS1 functions for bud formation and cell morphogenesis. Both *las1-12* cells at the nonpermissive temperature and cells depleted of LAS1 arrest with 80% unbudded and 20% large budded cells. These terminal phenotypes indicate that LAS1 function might be required at more than one point in the cell cycle. Normally, materials required for the formation of the plasma membrane and the new cell wall are delivered via secretory vesicles to areas of active surface growth. At cytokinesis these components form the primary and secondary septa, allowing the separation of mother and daughter cells (SHAW *et al.* 1991; CABIB *et al.* 1993). In contrast to wild-type cells, the large budded *las1* cells accumulate high numbers of vesicles at the mother-daughter neck. Vesicles similar in appearance to those that accumulate at the mother-daughter neck in large budded *las1* cells have been reported for *mpk1/slt2* mutants [affecting polarized cell growth (MAZZONI *et al.* 1993)] and for actin and myosin mutants [affecting the cytoskeleton (NOVICK and BOTSTEIN 1985; JOHNSTON *et al.* 1991; GOVINDAN *et al.* 1995)]. However, for these mutants the vesicles accumulate throughout the cytoplasm and occur in both budded cells and unbudded cells.

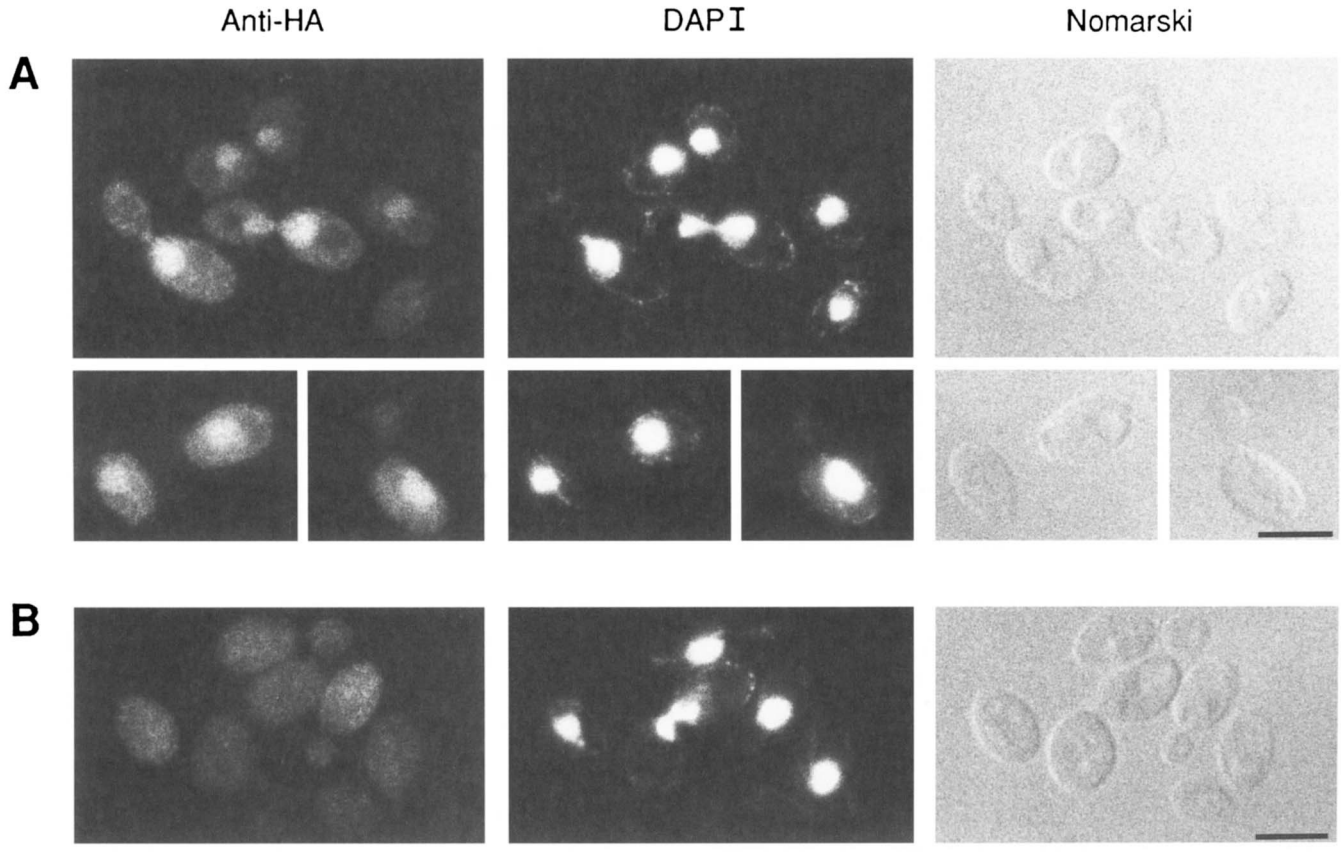

FIGURE 9.—Immunolocalization of epitope-tagged LAS1 to the nuclei. (A) Strain CY3826 containing a multicopy plasmid with *LAS1:HA* and (B) strain CY3820 containing a multicopy plasmid with nontagged *LAS1* were fixed and prepared for indirect immunofluorescence using anti-hemagglutinin 12CA5 ascites as described in MATERIALS AND METHODS. The left panels show localization of LAS1:HA using anti-hemagglutinin (anti-HA) monoclonal antibody. Middle panel, DAPI staining of DNA in the same cells. Right panel, the same cells viewed with Nomarski optic. Bar, 8 μm.

The accumulation of vesicles at the mother-daughter neck in large budded *las1* cells suggests that secretion is being properly directed to the normal site of cell surface growth. That only some of the cells in the population are large budded suggests that the secretory vesicles eventually fuse, allowing mother-daughter cell separation and increasing the population of unbudded cells. In contrast to the large budded *las1-12* cells, the unbudded *las1-12* cells did not show an accumulation of vesicles. That these unbudded *las1-12* arrested cells are viable when LAS1 function is restored indicates that they are blocked at the unbudded stage because of a lack of LAS1 function, not because they are inviable products of an aberrant cytokinesis. Possibly, the unbudded *las1-12*-arrested cells are defective in the formation of vesicles required for polarized cell growth.

Overexpression of LAS1 causes an increase in electron-dense structures that accumulate near the plasma membrane in both the mother cell and the bud (see Figure 7). These structures, which have a variable size and no apparent membrane at their edges, differ in appearance from the vesicles that accumulate in large budded *las1* cells (Figures 2 and 3). Possibly, the electron-dense structures due to LAS1 overexpression might be remnants of secretory vesicles that failed to fuse properly with the plasma membrane. Improper fusion of secretory vesicles might result either from too high a flux of secretory vesicles to sites of cell surface growth or from the abnormal localization or the abnormal functioning of the sites of cell surface growth (such as due to defects in bud site selection or defects in the cytoskeleton). Overexpression of LAS1 produces morphological alterations: in addition to the main bud, the mother cells have extra surface projections that are possibly related to the accumulation of the electron-dense structures. The phenotype of extra surface projections in cells overexpressing LAS1 is similar to that observed in some of the cells overexpressing both CDC24 and CDC42 [but not CDC24 alone or CDC42 alone (ZIMAN and JOHNSON 1994)]. That *pGAL1:LAS1* cells have normal levels of *CDC24* and *CDC42* RNA (A. DOSEFF, unpublished results) suggests that the effects due to the overexpression of LAS1 are not due to the overexpression of *CDC24* or *CDC42*.

Overexpression of LAS1 or CDC24 increases the percentage of budded cells at the *sit4-102* arrest. This bud promoting activity of LAS1 or CDC24 is dependent on the expression of *CLN2* from a SIT4-independent promoter. However, overexpression of LAS1 or CDC24 is not able to restore viability to the *sit4-102 ssd1-d* cells at

37°, suggesting either that high copy number *LAS1* or *CDC24* can only partially overcome the *sit4* bud initiation defect or that SIT4 has an additional essential function (besides G1 cyclin expression and bud formation) that cannot be overcome by overexpressing LAS1 or CDC24.

The ability of high copy number *LAS1* to partially overcome the bud initiation defect of *sit4* mutants, the primarily unbudded terminal phenotype of *las1-12*-arrested cells, and the effects due to the overexpression of LAS1 suggest a role of LAS1 in bud initiation and morphogenesis. Normal cell morphogenesis in *S. cerevisiae* involves an asymmetric pattern of growth, with components being delivered to the site of bud growth via vectorial transport of secretory vesicles to the cell surface (FIELD and SCHEKMAN 1980). Actin distribution changes during the cell cycle. Actin patches that are clustered in the newly forming bud have been associated with deposition of cell wall materials and therefore with active areas of growth (KILMARTIN and ADAMS 1984). That LAS1 is localized in the nucleus suggests that the effects of LAS1 on cell surface growth and morphogenesis are not due to a direct role of LAS1 in the mechanistics of actin rearrangement or secretory vesicle production and transport. Instead, LAS1 may regulate the expression of some component(s) that functions more directly in cell morphogenesis or the process of polarized cell growth. However, we have not yet been able to identify a putative target gene whose transcription might be induced by LAS1 (the RNA levels of *CDC24, CDC42, CDC10, CDC12, BUD2, BEM1, BEM2, SPA2, SWI4, CLN1, CLN2, HCS26, RNR1, SSD1,* and *SIT4* are not induced by *pGAL1:LAS1*) (A. DOSEFF, unpublished results). A mechanism by which the nuclear-localized LAS1 could regulate the expression of target genes is if LAS1 would function as a transcription factor. Although LAS1 has an acidic region (Figure 1), which is typical of certain transcription factors, a lexA-LAS1 fusion protein (the *lexA:LAS1* complemented for *LAS1*) was not able to activate transcription from lexA DNA-binding sites (A. DOSEFF, unpublished results). If LAS1 does regulate the expression of target genes, LAS1 could function by a mechanism that does not require a transcriptional activation domain.

Our screen for mutants that require *SSD1-v* for viability or a normal growth rate resulted in the isolation of four genes that are implicated in cell growth and morphogenesis: *LAS1, SMP3 (LAS2), BCK1/SLK1 (LAS3),* and *SIT4.* In this report, we have presented the identification and characterization of a new gene, *LAS1,* that encodes an essential nuclear protein involved in cell growth and morphogenesis. We originally isolated mutations that require *SSD1-v* for viability to obtain genes that function in the SIT4 pathway for bud formation. Indeed, preliminary experiments indicate that LAS1 is a phosphoprotein whose phosphorylation is increased in *sit4* mutants (A. DOSEFF, unpublished results). The determination of the mechanism by which LAS1 functions, including if LAS1 is a substrate of SIT4, should provide new insights into how cell growth and morphogenesis is regulated.

We especially thank BOB DERBY and DAVID SPECTOR for preparing specimens for electron microscopy. We thank M. SNYDER for *spa2* strains and anti-Spa2 antibody, J. THORNER for anti-PGK antibody, J. ARIS for anti-Nop1 antibody, G. TOKIWA for the triple HA construct, and A. BENDER for the *CDC24* plasmid. We also thank P. BURFEIND for the flow cytometry analysis, J. DUFFY, M. OKLER and P. RENNA for figure preparation and photography, and E. GROTEWOLD, M. LUKE, A. SUTTON, C. DI COMO and T. ZHONG for comments in the manuscript. A.D. would like to especially thank E. GROTEWOLD and A. SUTTON for useful and enjoyable discussions. This research was supported by National Institutes of Health grant GM-45179 to K.A.

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Communicating editor: A. G. HINNEBUSCH