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The Saccharomyces cerevisiae Homologue of Human Wiskott–Aldrich Syndrome Protein Las17p Interacts with the Arp2/3 Complex

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> Yeast Las17 protein is homologous to the Wiskott–Aldrich Syndrome protein, which is implicated in severe immunodeficiency. Las17p/Bee1p has been shown to be important for actin patch assembly and actin polymerization. Here we show that Las17p interacts with the Arp2/3 complex. *LAS17* is an allele-specific multicopy suppressor of *ARP2* and *ARP3* mutations; overexpression restores both actin patch organization and endocytosis defects in *ARP2* temperature-sensitive (ts) cells. Six of seven *ARP2* ts mutants and at least one *ARP3* ts mutant are synthetically lethal with *las17* Δ ts confirming functional interaction with the Arp2/3 complex. Further characterization of *las17* Δ cells showed that receptor-mediated internalization of α factor by the Ste2 receptor is severely defective. The polarity of normal bipolar bud site selection is lost. Las17-gfp remains localized in cortical patches in vivo independently of polymerized actin and is required for the polarized localization of Arp2/3 as well as actin. Coimmunoprecipitation of Arp2p with Las17p indicates that Las17p interacts directly with the complex. Two hybrid results also suggest that Las17p interacts with actin, verprolin, Rvs167p and several other proteins including Src homology 3 (SH3) domain proteins, suggesting that Las17p may integrate signals from different regulatory cascades destined for the Arp2/3p complex and the actin cytoskeleton.

INTRODUCTION

Arp2p and Arp3p are essential ubiquitous proteins of the actin-related family localized in actin-rich cortical structures (McCollum *et al.*, 1996; Moreau *et al.*, 1996; Mullins *et al.*, 1997; Welch *et al.*, 1997b; Winter *et al.*, 1997). Analysis of yeast mutations shed light on their roles in the cortical cytoskeleton. Delocalization of actin patches and a slight accumulation of membranous vesicles resulted from a temperature-sensitive (ts) mutation in *S. cerevisiae* Arp2p (Moreau *et al.*, 1996). This mutant was defective for dye uptake and for internalization of a membrane transporter, uracil permease (Moreau *et al.*, 1997). A fission yeast cold-sensitive

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ARP3 mutation caused disorganization of actin patches and defects in polarized growth (McCollum *et al.*, 1996). A ts mutation in *Saccharomyces cerevisiae* Arp3p impaired actin patch motility and caused accumulation of actin aggregates in the bud (Winter *et al.*, 1997). Arp2p and Arp3p were first isolated by purification of a profilin-binding complex from *Acanthamoeba*, which contained five additional peptides, p40, p35, p19, p18, and p14 (Machesky *et al.*, 1994). A homologous complex, purified from human platelets, localized at the leading edge of moving lamellipodia (Welch *et al.*, 1997b). Purification of the Arp2/3 complex from *S. cerevisiae* (Winter *et al.*, 1997) showed that it was homologous to the *Acanthamoeba* and human complexes with the exception that one subunit, p40 or Sop2p (Balasubramanian *et al.*, 1996), did not copurify (Winter *et al.*, 1997).

Structural modeling of Arp2p and Arp3p led to the hypothesis that Arp2p and Arp3p formed a heterodimer capable of nucleating actin polymerization at the pointed end of actin filaments (Kelleher *et al.*, 1995), whereas electronic microscopy of purified *Acanthamoeba* Arp2/3 complex showed the complex bound to the sides of actin filaments in

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vitro (Mullins et al., 1997). Arp2/3 complex promotes actin polymerization at the surface of the intracellular pathogen Listeria monocytogenes by recruiting host proteins through a surface protein, ActA (Welch et al., 1997). The complex then nucleates actin filaments, generating propulsive force. However, Arp2/3 complex is also localized all along actin tails of moving bacteria and not just at actin polymerization sites on the Listeria cell surface (Welch et al., 1998). Recent evidence indicated that the Arp2/3 complex alone was a weak nucleator, whereas ActA alone was not. Their combined action had a dramatic synergistic effect on nucleation of actin filaments (Welch et al., 1998). Localization, nucleation, and cross-linking studies in Acanthamoeba have led to the proposition that the Arp2/3 complex promotes polymerization at the leading edge of membranes by attaching newly formed filaments to preexisting structures. Thus, as actin filaments grow at their membrane-proximal ends to drive motility, the bound Arp2/3 complex would be translocated away from the membrane (Mullins et al., 1998b). More recently, in addition to nucleating actin at the barbed end of filaments, the Arp2/3 complex was shown to have high-affinity pointed end capping activity to induce branched networks of filaments in vitro (Mullins et al., 1998a).

A novel yeast protein, Las17p/Bee1p, was shown to be a component of actin patches. Yeast cells deleted for the Wiskott-Aldrich Syndrome protein (WASP) homologue concentrated aberrant actin aggregates in the bud and were defective in actin patch assembly, budding, and cytokinesis (Li, 1997). Moreover, biochemical studies using permeabilized cells and rhodamine-labeled actin suggested that Las17p/ Bee1p was required for actin polymerization and assembly in vitro (Lechler and Li, 1997). Las17p is proline-rich and shows homology to human WASP, mutations in which cause severe immunodeficiency (Amman and Hong, 1989). WAS patients show cytoskeletal abnormalities of lymphocytes and platelets (Molina et al., 1992), failure of B cells to respond to polysaccharide antigens, and defective chemotaxis in neutrophils (Ochs et al., 1980), suggesting that WASP is important in cytoskeletal reorganization necessary for the immunological response. WASP colocalized with polymerized actin in human cells and bound specifically to activated CDC42Hs (Apenström et al., 1996; Kolluri et al., 1996; Symons et al., 1996). A more ubiquitously expressed homologue, N-WASP, was shown to bind actin, calmodulin, and Ash/Grb2p (abundant Src homology/growth factor receptor-bound protein 2), an Src homology 2/Src homology 3 (SH3) domain protein implicated in signal transduction from membrane receptors to effectors of the cytoskeleton (Miki et al., 1996). Overexpression of either WASP or N-WASP in mammalian cells induced formation of actin clusters that colocalized with the overexpressed protein, indicating a role in actin polymerization (Miki et al., 1996; Symons et al., 1996). The vertebrate WASP family member Scar1/ WAVE, like yeast Las17p, does not contain a recognizable GTPase binding site, but overexpression induced clustered actin filaments, demonstrating its involvement in actin reorganization (Miki et al., 1998). WASP and Scar1 have now been shown to interact with actin via their WASP homology 2 (WH2) domains and with the p21 subunit of the Arp2/3 complex via their C-terminal acidic domains. Overexpression of these C-terminal acidic fragments caused delocalization of the Arp2/3 complex with concomitant loss of actin spots and lamellipodia (Machesky and Insall, 1998).

We previously showed that in yeast, increased *LAS17* expression suppressed *arp2-1* growth thermosensitivity (Madania *et al.*, 1997). Here we present genetic, cellular, and biochemical evidence that Las17p functions with the Arp2/3 complex and raise the possibility that Las17p may be part of signaling pathways directed to the Arp2/3 complex and actin.

MATERIALS AND METHODS

Plasmids, Strains, Gene Banks, and Genetic Manipulations

Plasmids and yeast strains used in this study are listed in Tables 1 and 2. Strains are derived from YPH and FY strains, which are S288C derivatives. Genomic DNA banks used were constructed in 2μ plasmids: Sau3A-derived fragments in pFL44 vector (from F. Lacroute), pGAD vector (from P. Bartel) (Fields and Song, 1989), multiple enzyme-derived fragments in pGAD424 (from P. James) (James *et al.*, 1996), or mechanically derived fragments cloned in pACTII (from P. Legrain) (Fromont-Racine *et al.*, 1997). The cDNA library used in two-hybrid screening was in pGAD-GH (from M. White). DNA manipulations were carried out by standard techniques (Sambrook *et al.*, 1989). Yeast cell cultures and genetic manipulations were carried out essentially as described in by Guthrie and Fink (1991). Yeast cells were transformed by the LiAc method using single-stranded carrier DNA and DMSO (Schiestl and Gietz, 1989; Hill *et al.*, 1991).

LAS17 Gene Deletion

Using PCR targeting with short flanking homology (Wach et al., 1994) the complete LAS17 coding region was deleted in three different diploid yeast strains: YPH501, FY1679, and W303 (Table 2). Oligonucleotides used to amplify the kanMX4 fragment on pFA6akanMX4 were 5'-AATTACAGTTCGTTACTTTAAGTGTTGATAG-GCGTGATTTAATGCTGCAGGTCGACGGATC-3' and 5'-ACATA-TTTTCTATAACAGTAGTTTCATCTTTGTTTGCATTCCATCGAT-GAATTCGAGCTC-3'. Recombinants bearing the kanMX4 marker were selected on YPD plates containing G418 at 200 μ g/ml. Correct integration of the kanMX4 cassette was verified by PCR analysis of genomic DNA. Haploid *las17*∆ strains (YMW17K1 and YMW17K2) were obtained by sporulation and dissection of heterozygous diploid disruptants; they appeared fully rescued at 37°C by plasmid-borne wild-type LAS17. The same methodology and oligonucleotides allowed us to create las17A::HIS3 strains using plasmid pFA6a-HIS3MX6 as template. FDW174H is the resulting $las17\Delta$::HIS3 diploid strain.

Chromosomal Integration of Tagged ARP2 and ARP3 ts Alleles

Seven *ARP2* (*arp2-1* to *arp2-7*) ts mutations, initially obtained by PCR mutagenesis on plasmid pYCW207 (Moreau *et al.*, 1996), were integrated at the *ARP2* locus. The *ARP2* gene was tagged with *URA3* by cloning an *XbaI–PvuII* fragment containing the *URA3* gene from pJJ244 (Jones and Prakash, 1990) blunt into the *SspI* site 130 bp downstream of the *ARP2* stop codon in plasmid pBES18 (Moreau *et al.*, 1996). This gave rise to pIMW18-U. Mutant alleles (*arp2-1* to *arp2-7*) were exised as 1.38-kb *SacI–SspI* fragments from the mutant bank in pYCW207, purified, and ligated into the *SacI–SaII* (blunted) sites of pBMW18-U. Wild-type and resulting mutant plasmids were digested with *SacI–KpnI*, and the 3.4-kb fragments containing the *ARP2* ts alleles with the downstream *URA3* marker were purified, transformed into YPH499, and selected on SC – Ura. Ura⁺ clones were tested for thermosensitive growth then for correct integration

Table 1. Plasmids used in this study

Plasmid	Description
pIMW18-U	pUC18 containing ARP2 tagged with URA3 (this study)
pIMW300	pUC18 containing ARP3 as an 1.85-kb BamH1 insert (this study)
pYCW204	pUN60, CENIV, URA3, SUP11, ARP2 (Moreau et al., 1996)
pCMW306	pUN60, CENIV, URA3, SUP11, ARP3, constructed by ligating the SalI–BamHI fragment of pIMW300 into the same sites of pUN60 (this study)
pCMW304	pRS314 (Sikorski and Hieter, 1989), ARP3:LEU2 (this study)
pYCW207	pUN90, CENIV, HIS3, ARP2 (Moreau et al., 1996)
pYEW170	pFL44, $2\mu D$, URA3, LAS17 and flanking regions as a 4-kb BamHl insert (this study)
pAS2	$2\mu D$, TRP1, ADHpro-NLS-GAL4BD, HA, CYH2, bait vector (Durfee <i>et al.</i> , 1993)
pAMW172	pAS2, GAL4BD fused to full-length LAS17 (this study)
pDAB7	pAS2 derived, CEN, GAL4BD fused to full-length ACT1 (Amberg et al., 1995)
pACT2	$2\mu D$, LEU2, ADHpro-NLS-GAL4AD, HA, prey vector (Durfee <i>et al.</i> , 1993)
pAMW253	pACT2, GAL4AD fused to intronless full length ARP2 (this study)
pAMW315	pACT2, GAL4AD fused to full length ARP3 (this study)
pAMW173	pACT2, GAL4AD fused to full length LAS17 (this study)
PGAD.GH	$2\mu D$, LEU2, ADHpro-NLS-GAL4AD, prey vector (Chien <i>et al.</i> , 1991)
pAMW200	pGAD.GH, ARP2 (this study)
pAMW171	PGAD.GH, LAS17 (2μ overexpression, this study)
pFA6a-kanMX4	pFA6a, cassette for resistance to G418 (Wach et al., 1994)
pFA6a-HIS3MX6	pFA6a, his3 complementing heterologous HIS5 ORF from S. pombe (Wach et al., 1997)
pFA6a GFPS65T-kanMX6	Same as pFA6a-kanMX4, containing GFP (S65T) upstream of the kanMX6 module (Wach et al., 1997)

of the *URA3* cassette at the *ARP2* locus by PCR analysis of genomic DNA (our unpublished results). Haploid-tagged wild-type and mutants were mated with YPH500, and diploids were sporulated. In all 20 tetrads dissected for each cross, two ts *URA3* and two non-ts *ura3* spores were obtained (Table 2, YMW201U and YMW211U–YMW271U). In a similar manner, wild-type *ARP3* and the *arp3-14* mutant allele were tagged with the *LEU2* gene (Madania, 1998).

Construction of GFP Fusions with LAS17, ARP2, and ARP3

Stop codons of the *LAS17*, *ARP2*, and *ARP3* genes were replaced with the coding sequence of green fluorescent protein (GFP) by short flanking homology PCR targeting using the kanMX6 marker (Wach *et al.*, 1994). For *LAS17:GFP*, pFA6a-GFPS65TkanMX6 was used as template to amplify a 2.5-kb PCR fragment with oligonucleotides 5'-TAAACTGGGAGCTCATGACGATATGGACAATGG-TGATGATTGGGCAGCGAGTAAAGGAGAAGAACTTT-3' and 5'-ACATATTTTCTATAACAGTAGTTTCATCTTTGTTTGCATTCCA-TCGATGAATTCGAGCTC-3' as primers. This fragment was transformed into diploid FY1679, and G418-resistant recombinants were verified for correct integration by PCR across both borders. Sporulation and dissection of a heterozygous G418-resistant diploid revealed a 2:2 segregation of the kanMX6 marker, which coincided with GFP fluorescence. Strain FMW173GK (homozygous for *LAS17:GFP*) was constructed by mating a and α spores.

For GFP fusion with *ARP2*, the pFA6a-GFPS65TkanMX6 plasmid was modified to add a 5× glycine-alanine linker following the ORF and then used as template to amplify a 2.5-kb PCR fragment with oligonucleotides 5'-TGGCAAGAAGCGGGGCCATCTGCAATGA-CTAAATTTGGTCCAAGAGGGATCCGGAGCAGGTGCT-3' and 5'-TCCATTTCCATTTTCATTCGTAGATTTAAACTTTTTATTAAT TCTTTTGTCGATGAATTCGAGCTCGTTT-3' as primers. A *ARP2*: *GFP* haploid strain was constructed and verified as above, and then a spores were mated to make strain FDW23GK. Growth of these strains was slow and temperature sensitive. To obtain *ARP2* strains with these fusion proteins that grew better, the GFP of modified pFA6a-GFPS65TkanMX6 plasmid was converted to *yEGFP* (Cormack *et al.*, 1997) and integrated into one allele of the diploid $las17\Delta$ strain, FDW174H, to create strain FDW180HK.

Similarly, to make a functional GFP fusion with *ARP3*, the stop codon of one allele of the *ARP3* gene was replaced by *yEGFP* using oligonucleotides 5-TATGGTCCGGAAATTGTTAGAAATTTCAG-CCTTTTCAACATGGTTGGATCCGGAGCAGGTGCT-3' (*ARP3* in frame with *GAGAGAGAGAGAEGP*) and 5'-AGGGGGCGTTCAGTTATTTGCATTTGTATTTGTACCTTTTTGTACCTTATAAGATCGATG - AATTCGAGCTGCTTT-3' as primers, and the resulting fragment was transformed into strain FY1679 to create strain FDW34GK and into FDW174H to create strain FDW190HK.

Cell Fixation, Phalloidin and Antibody Staining, and GFP Observation

Early log phase cells were fixed and stained as previously described (Pringle et al., 1991), except that incubation with phalloidin was for 1 h with shaking at 4°C in the dark using 1 µM TRITC-labeled phalloidin (Sigma, St. Louis, MO) or 1 µM Alexa-594 phalloidin (Molecular Probes, Eugene, OR). Fixed cells were sometimes kept at 4°C for a maximum of 3 d before staining. For GFP observation, fixation time was reduced to 15 min. For in vivo observations of GFP fusion proteins, cells were grown in liquid YPD at 25°C to early log phase and then harvested, resuspended in 1 M sorbitol, and immediately observated using a GFP bandpass filter (excitation, 460-500; dichroic mirror, 505; emission, 510-560). Antibody staining of spheroplasts was as previously described (Moreau et al., 1996) with C4 monoclonal mouse immunoglobuliln G1 (IgG1) anti-actin antibody (Boehringer Mannheim, Indianapolis, IN) and polyclonal rabbit anti-GFP antibody (a gift from K. Sawin, London, United Kingdom). Preparations were analyzed and photographed using an Optiphot-2 microscope (Nikon, Melville, NY) equipped with fluorescent optics. Photographs were taken using 400ASA film and then scanned (300 dots per inch final resolution) or direct acquisitions were made with a Photonics (Milpitas, CA) Science charge-coupled device Coolview camera equipped with the Gel Grab 2.02 software program.

Table 2. Yeast strains used in this study

Strain	Genotype	Source
YPH499	MAT a , ade2-101, his3- Δ 200, leu2- Δ 1, lys2-801, trp1- Δ 63, ura3-52	P. Hieter
YPH500	MAT a , ade2-101, his3- Δ 200, leu2- Δ 1, lys2-801, trp1- Δ 63, ura3-52	P. Hieter
YPH501	$MATa$, $ade2-101$, $his3-\Delta200$, $leu2-\Delta1$, $lys2-801$, $trp1-\Delta63$, $ura3-52$	P. Hieter
	MATa, ade_{2-101} , $his_{3-\Delta 200}$, $leu_{2-\Delta 1}$, ly_{2-801} , $trp_{1-\Delta 63}$, ura_{3-52}	
YMW11	$MATa$, $arp2::LEU 2$, $ade2-101$, $his3\Delta200$, $leu2-\Delta1$, $lys2-801$, $trp1-\Delta63$, $ura3-52$ (pYCW204)	Moreau <i>et al.</i> (1996)
YMW81	MATa, arp2-1(H330L), ade2-101, his3- Δ 200, leu2- Δ 1, lys2-801, trp1- Δ 63, ura3-52	Moreau <i>et al.</i> (1996)
YMW201U	MATa, ARP2: URA3, ade2-101, his3- Δ 200, leu2-1, lys2-801, trp1- Δ 63, ura3-52	This study
YMW211U	MATa, $arp2-1(H330L)$: URA3, $ade2-101$, $his3-\Delta200$, $leu2-\Delta1$, $lys2-80$, $trp1-\Delta63$, $ura3-52$	This study
YMW221U	MATa, arp2-2(G19D): URA3, ade2-101, his3-Δ200, leu2-Δ1, lys2-801, trp1-Δ63, ura3-52	This study
YMW231U	MATa, arp2-3(E256K): URA3, ade2-101, his3- Δ 200, leu2- Δ 1, lys2-801, trp1- Δ 63, ura3-52	This study
YMW241U	MATa, arp2-4(L280P): URA3, ade2-101, his3- Δ 200, leu2- Δ 1, lys2-801, trp1- Δ 63, ura3-52	This study
YMW251U	MATa, arp2-5(L316S): URA3, ade2-101, his3-Δ200, leu2-Δ1, lys2-801, trp1-Δ63, ura3-52	This study
YMW261U	MATa, arp2-6(G35S Y146N): URA3, ade2-101, his3- Δ 200, leu2- Δ 1, lys2-801, trp1- Δ 63, ura3-52	This study
YMW271U	MATa, arp2-7(F127S F203Y): URA3, ade2-101, his3-4200, leu2-41, lus2-801, trp1-463, ura3-52	This study
YMW300T	MATa, $arp_{3\Delta}$:: TRP1, ade2-101, his3- Δ 200, leu2- Δ 1, lus2-801, trp1- Δ 63 ura3-52	This study
	$MATa, ARP3, ade2-10, his3-\Delta 200, leu2-\Delta 1 lus2-801 trp1-\Delta 63 ura3-52$	
YMW301T	$MATa, arp_{3}\Delta$:: TRP1 ade2-101 his_3- Δ 200, leu2- Δ 1, lus_2-801, trp1- Δ 63, ura_3-52 (+pCMW 306)	This study
YMW311L	MATa, ARP3: LEU2, ade2-101, his3-200, leu2-21, lus2-801, trp1-263, ura3-52	This study
YMW321L	MATa, arp3-14 (P33L aa 48-108 Δ): LEU2, ade2-101, his3- Δ 200, leu2- Δ 1, lus2-801, trp1- Δ 63, ura3-52	This study
YMW171K	MATa, $las17\Delta$::kanMX4, $ade2-101$, $his3-\Delta200$, $leu2-\Delta1$, $lus2-801$, $tro1-\Delta63$, $ura3-52$	This study
YMW172K	MATa, $las17\Delta$:: $kanMX4$, $ade2-101$, $his3-\Delta200$, $leu2-\Delta1$, $lus2-801$, $trb1-\Delta63$, $ura3-52$	This study
YMW173K	MATa, $las17\Delta$:: $kanMX4$, $ade2-101$, $his3-\Delta200$, $leu2-\Delta1$, $lus2-801$, $trv1-\Delta63$, $ura3-52$	This study
	$MATa$, $las17\Delta$:: $kanMX4$, $ade2-101$, $his3-\Delta200$, $leu2-\Delta1$, $lus2-801$, $trv1-\Delta63$, $ura3-52$	
YMW175K	MATa, ura_3-52 , $his_3-\Delta 200$, $leu_2-\Delta 1$, $arp_2\Delta$:: LEU2, $trp_1-\Delta 63$ LAS17-PROTA: $kanMX6$ ($pYEW248$)	This study
RH4207	MATa, $las17\Delta$:: kanMX4, har1:: LYS2, ade2-101, his3- Δ 200, leu2- Δ 1, trn1- Δ 63, ura3-52	This study
FY1679	$MATa, his3-\Delta 200, leu2-\Delta 1, trn1-\Delta 63, ura3-52, GAL2$	B. Dujon/F. Winston
	MATa, HIS3, LEU2, TRP1, ura3-52, GAL2	(EUROFAN strain)
FDW23GK	MATa, ARP2-S65TGFP: kanMX6, his3-2200, leu2-21, trp1-263, ura3-52, GAL2	This study
121120010	MATa, ARP2-S65TGEP: kanMX6, his3-A200, LEU2, TRP1, ura3-52, GAL2	The stady
FDW24GK	MATa, ARP2, his3-0.200, leu2-0.1, tro1-0.63, ura3-52, GAL2	This study
121121010	MATa, ARP2-EGEP: kanMX6, HIS3, LEU2, TRP1, ura3-52, GAL2	The stady
FDW34GK	MATa ARP3 his3-0.200 lev2-0.1 trn1-0.63 ura3-52 GAL2	This study
1 D WO IGI	MATa A RP3-EGEP: kan MX6 HIS3 LEU2 TRP1 ura3-52 GAL2	This study
FMW17GK	MATa LASIZ-GEP kan MX4 his $3-\lambda 200$ lev $-\lambda 1$ tra $-\lambda 63$ ura $3-52$ GAI 2	This study
	MATa LASIT-GEP-kanMX4 HIS3 LELI2 trn1-\63 ura3-52 GAL2	This study
FDW174H	MATa los17A::HISMX6 his3-A200 LEU2 TRP1 ura3-52 GAL2	This study
101111	MATa Jost7A::HISMX6 his3-2000 LEU2 TRP1 ura3-52 GAL2	This study
FDW180HK	MATa las17A::HISMX6 ARP2 his3-A200 LE12 TRP1 ura3-52 GAL2	This study
12111001110	MATa las17A::HISMX6 ARP2-EGEP:kanMX6 his3-A200 LEU2 TRP1 ura3-52 GAL2	This study
FDW190HK	MATa las17A::HISMX6 ARP3 his3-A200 LF12 TRP1 ura3-52 GAL2	This study
1 D W 1901 IIX	MATa las17A::HISMX6 ARP3-EGEP:kanMX6 his3-A200 LEU2 TRP1 ura3-52 GAL2	This study
W303	MATa his $A1$ here $A12$ here $A12$ here $A23$ here $A23$ here $A23$ here $A12$ here	P Philippsen
11000	MATa his 3.1 leu 3.112 trn 1.289 ura 3.52	FUROFAN strain
V187	MATa coldA collocation b_{12} ($p_1 = 0.00$) and $c_2 = 0.01$ ($p_2 = 3.112$) $p_1 = 3.52$ ($P_1 = 3.52$) $P_2 = 0.52$	Durfee <i>et al.</i> (1993)
1107	transformed by the ERVI weast DNA genomic library	Iames et al. (1996)
Y190	MATa val4 Λ val80 Λ his 3 trn-901 ade2-101 lev2-3 112 ura3-52 IIR A3. CAI 1(IIAS)-lac7	Iames et al (1996)
11/0	$I VS^{2} GAI 1(IIAS) - HIS3 cubr$	Juneo et ut. (1990)
CG1945	MATa val4-542 val80-538 his3 trn-901 ade2-101 leu2-3 112 ura3-52 IIRA3. CALA(3x17more)_	P Legrain
01/10	CYC1(TATA)-lacZIYS2::GAL1(IIAS)-GAL1(TATA)-HIS3 cuhr	Froment-Racine et al (1997)
	Creating, molecter Grief (Grief Grief (Grief) Grief (Grief)	roment fuence et ut. (1997)

YMW strains and the RH strain are isogenic derivatives of YPH strains. Strain names beginning with F are isogenic FY1679 derivatives. Both of these series of strains are derivatives of S288C.

Latrunculin Treatment of GFP-labeled Cells

After growth to midlog phase at 25°C in liquid YPD, cells were concentrated to 10⁸ cells/ml. Latrunculin-A (Molecular Probes) in DMSO (from a 10 mM stock to give 100 μ M final concentration) or an equivalent volume of DMSO was added to a 100 μ l aliquot for each condition tested. Cells were fixed by adding 10 vol of YPD containing 3.7% formaldehyde and incubating with gentle shaking for 15 min.

Lucifer Yellow and FM4-64 Staining and $\alpha\text{-Factor}$ Uptake

Lucifer yellow (LY)-carbohydrazine (Fluka, Buchs, Switzerland) uptake, an indication of fluid phase endocytosis, was done as described by Dulic *et al.* (1991). Staining with the lipophilic dye FM4-64 [*N*-(3-triethylammoniumpropyl)-4-(*p*-diethylaminophenylhexatrienyl) pyridinium dibromide] to follow uptake into membranes was as described by Vida and Emr (1995). ³⁵S-Alpha factor uptake assays were performed as described (Dulic *et al.*, 1991). All strains were preincubated at 37°C for 15 min before the addition of alpha factor. The samples were processed as described above. All uptake assays were performed twice.

Immunoprecipitation of Las17p and Arp2p

To tag Las17p with protein A, the stop codon of the *LAS17* gene was replaced by the coding frame of the IgG binding domain of protein A using the kanMX6 selection marker as a PCR-synthesized fragment with short flanking homology on either side of the *LAS17* stop codon. pYM7 was the template source of protein A (Knop *et al.*, 1999), amplified with the same primers as for the *LAS17:GFP* fusion. The resulting 2.1-kb PCR fragment was transformed into the diploid strain FY1679, which was sporulated. G418-resistant progeny were verified for correct tag insertion by PCR. One of these haploids was crossed with a haploid *arp2A* rescued by hemagglutinin (HA)-tagged Arp2p (YMW15 + pYEW250) (Moreau *et al.*, 1996), and a recombinant haploid derivative was selected (strain YMW175K).

Whole-cell extracts were made by the liquid nitrogen grinding in 50 mM HEPES, pH 7.5, 100 mM KCl, 3 mM MgCl₂, 1 mM EGTA, 0.5% Triton X-100 buffer (Li, 1997) and resuspended at 10 mg/ml after clarification by 30 min of centrifugation at 20,000 × g. Eighty microliters of Las17-prot A extract and nontagged extract were incubated for 1 h at 4°C with 40 μ l of IgG-Sepharose (6 Fast Flow; Pharmacia, Piscataway, NJ) suspension. Immunoprecipitation and washes were with starting buffer. Eluted proteins were precipitated with 10% trichloroacteic acid and resuspended in loading buffer. Samples were separated by 10% PAGE and blotted to nitrocellulose. Blots were incubated with appropriate primary and secondary antibodies and then developped with a Super Signal (Pierce, Rockford, IL) chemiluminescent kit for HRP.

IgG to reveal Las17-prot A was purified from nonimmunized rabbit serum by passage over a protein A-Sepharose column (Pharmacia CL-4B). Production and characterization of anti-Arp2p antibodies has been previously described (Moreau *et al.*, 1996).

Construction of GAL4 Fusion Plasmids for Two-Hybrid Interactions

LAS17 was amplified with oligonucleotides 5'-GGCGTGATTTAC-CATGGGACTCC-3' (with an NcoI site at the ATG of LAS17) and 5'-CATCTTCTCGAGCATTCCATTACCAA-3' (with an XhoI site just after the stop codon) using pAMW171 as template. A 1.9-kb NcoI–XhoI PCR fragment was cloned into the NcoI–SaII sites of pAS2 in fusion with GAL4 binding domain (GAL4BD) to give pBMW172 and into the NcoI–XhoI sites of pACTII in fusion with GAL4 activation domain (GAL4AD) to give pAMW173. Both pBMW172 and pAMW173 were able to rescue YMW171K (las17 Δ) at 25 and 37°C (our unpublished data). All GAL4 fusions constructed were systematically controlled by sequencing the fusion site. Two-hybrid GAL4AD constructions coding for wild-type Cdc42p and its activated form (Cdc42p^{G12V}) were kindly provided by E. Leberer (Leberer et al., 1997) and by the Pringle laboratory (Cvrckova et al., 1995).

Two-Hybrid Screens with LAS17 as Bait

cDNA Screen. Y190 strain (Durfee *et al.*, 1993) containing pBMW172 (*GAL4BD-LAS17*) was transformed with a yeast cDNA library in pGAD-GH (kindly provided by Michael White, University of Texas Southwestern Medical School, Dallas, TX) and plated on SC – Trp, Leu, and His supplemented with 35 mM 3-aminotriazole (3-AT). After incubation for 1 wk at 30°C, 309 colonies among 200,000 initial transformants were patched onto SC – Trp, Leu, and His 35 mM 3-AT plates and allowed to grow at 30°C for 2 d. These clones were then tested in a 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) filter assay, and clones that turned blue were further ana-

lyzed. Plasmids were rescued and reintroduced into Y190 and Y190 containing pBMW172 and tested in the X-Gal colony filter assay. One hundred sixty plasmids giving a signal with pBMW172 were sequenced with oligonucleotides 5'-GGAATCACTACAGGGAT-G-3' to control in-frame fusions and with 5'-GAAGTGAACTT-GCGGGG-3' to determine the size of the fused protein. After elimination of redundant fusions, 23 different positive clones representing 10 different genes were identified and tested in β -galactosidase liquid culture assays.

Genomic DNA Screen. Yeast strain Y187 was transformed by standard procedures with DNA amplified from an aliquot of the FRYL library (Fromont-Racine *et al.*, 1997). Ten million yeast transformants were collected and pooled, and aliquots were stored at -80° C. To screen by mating, a tube containing 10^{7} transformants was equilibrated in fresh YPD then mixed with 2×10^{7} log phase CG1945 cells previously transformed with plasmid pBMW172 (*GAL4BD-LAS17*) and incubated for 6 h. Cells representing 1.15×10^{6} diploids were plated on SC - Trp, Leu, and His supplemented with 35 mM 3-AT and incubated at 30°C for 7 d. The clones thus obtained were extracted and sequenced.

X-Gal Colony Filter Assay and β -Galactosidase Assay

β-Galactosidase activity of simple or double transformants of Y190 was assessed on nitrocellulose filters as described by Breeden and Nasmyth (1985). To quantitate β-galactosidase activity, freshly streaked Y190 double transformants were inoculated into 2.5 ml of liquid SC – Trp and Leu and allowed to grow overnight to OD₆₀₀ nm of ~1. β-Galactosidase activity was measured and expressed in Miller units (Kandels-Lewis and Seraphin, 1993).

RESULTS

LAS17 Is an Allele-specific Multicopy Suppressor of ARP2 ts Mutations

To identify proteins that interact with Arp2p, we screened yeast multicopy genomic libraries for clones whose expression was able to rescue the temperature sensitivity of the *arp2-1* mutation in strain YMW81. In a preliminary screen using the Lacroute genomic bank we obtained several clones, the smallest of which contained a plasmid, pYEW170, with an insert of 4 kb covering several genes. Using the Fields and Bartel YL1/2/3 genomic bank, 24 plasmids were able to support growth at 37°C. Besides the *ARP2* gene, seven groups of inserts were identified. Sequencing of insert borders revealed that all seven clones contained a full-length *LAS17* coding sequence. Because the inserts of the two shortest clones did not overlap any adjacent coding regions, we concluded that *LAS17* was responsible for suppression.

Suppression of previously isolated ts mutations *arp2-1* to *arp2-7* (Moreau *et al.*, 1996) was tested to find out whether *LAS17* suppression was allele specific. Sequence changes in the *ARP2* gene were determined and are shown in Table 2. WT and mutant fragments tagged with the *URA3* gene were integrated into strain YPH499 at the *ARP2* locus (see MA-TERIALS AND METHODS), giving rise to strains YMW211U to YMW271U. We have previously shown that the *arp2-1* ts mutation causes delocalization of polarized cortical actin patches at 37°C (Moreau *et al.*, 1996). When strains YMW221U to YMW271U were stained with phalloidin, actin distribution appeared normal in most cells of



arp2-2, arp2-4, arp2-5, and *arp2-7* at 25°C, whereas *arp2-3* and *arp2-6* cells showed loss of actin organization at this temperature (Figure 1A). All mutants had lost the polarized distribution of actin patches after 2 h at the restrictive temperature. Actin clumps or aggregates in the bud or at the mother–daughter neck and an enlarged bud neck were ob-

served in budding cells of each of the ts mutants. Surprisingly, none of these mutant actin phenotypes was qualitatively different from those observed for the original *arp2-1* mutant. A similar ts phenotype also resulted from fusion of green fluorescent protein to the carboxyl terminus of the *ARP2* coding sequence (see MATERIALS AND METHODS).

left panels were photographed with a GFP bandpass filter, and the right panels were photographed with a rhodamine filter. Bar, 5 μ m.



Figure 2. LAS17 overexpression suppresses certain *ARP2* ts mutants. Seven *ARP2* ts strains (*arp2-1* to 2-7/YMW211U–YMW271U) and wild-type (YMW201U) described in Table 2 were transformed with pGAD (vector) (A), pAMW171 (*LAS17* in pGAD) (B), and pAMW200 (*ARP2* in pGAD) (C). Transformants were streaked on SC – Ura and Leu plates and incubated at 37°C for 4 d. Note that the *arp2-5* ts mutant grows at 37°C on minimal medium but is more ts on complete medium.

Examination of the *ARP2-GFP* strain did not reveal any Arp2-gfp fluorescence in the many phalloidin-staining actin aggregates present in buds or in mother–bud necks, whereas both actin and Arp2p were present in the remaining nonpolarized patches (Figure 1B).

These ARP2 mutants were used to test allele specificity of LAS17 suppression. When ARP2 strains were transformed by a multicopy LAS17 plasmid, pAMW171, the arp2-2 mutant as well as the arp2-1 mutant initially used in the selection were rescued at 37°C. The thermosensitivity of the other mutants was not relieved (Figure 2). This clearly shows that suppression is specific to certain point mutations and suggests that a specific interaction could be involved. Major mutant phenotypes such as disorganization of the actin cytoskeleton and deficient endocytosis were examined in LAS17-suppressed strains YMW211U, YMW221U, and YMW241U. LAS17 overexpression suppressed actin cytoskeleton defects in the arp2-1 mutant and partially suppressed defects of the arp2-2 mutant at 37°C, in good correspondence with growth recovery. Figure 3A shows that even partial suppression in the arp2-2 mutant is clearly visible; the majority of arp2-2 ts cells containing overexpressed LAS17 no longer showed aberrant clumped actin in the bud at 37°C (Figure 3A, b, lower panel). Actin patches were less concentrated in the buds than in rescued cells (Figure 3A, c, lower panel). Suppression of endocytosis as judged by the ability of cells to take up and deliver the fluorescent neutral dye Lucifer yellow-carbohydrazine to the vacuole by fluid phase endocytosis (Riezman, 1985) followed the same pattern of allele specificity as growth and actin defects. Even at permissive growth temperature after extended times (60 min here vs. 10 min normally), uptake was inhibited in the arp2-2 mutant (Figure 3B, a, compared with the rescued mutant, c). The LAS17 overexpression allowed LY uptake and accumulation in the vacuole in the *arp2-2* mutant (Figure 3B, b) although slightly less efficiently than for the arp2-1 allele (our unpublished data). Investigation of the arp2-4 allele revealed that the mutant was also endocytosis deficient but that LAS17 overexpression did not rescue this deficiency.

LAS17 Overexpression Can Also Rescue Conditional Mutations in the ARP3 Gene

Because Arp2p and Arp3p are part of the same macromolecular complex (Machesky et al., 1994; Welch et al., 1997b; Winter et al., 1997), we questioned whether LAS17 overexpression would also suppress the phenotype of ARP3 conditional mutations. We have isolated recessive ARP3 ts alleles on plasmids in an $arp3\Delta$ strain (Madania, 1998). The capacity of LAS17 overexpression to rescue growth at nonpermissive temperatures for six different mutants, arp3-9 to arp3-14, was tested. Only one ts mutant, arp3-9, was well suppressed at 37°C. At 36°C LAS17 overexpression allowed good growth of arp3-9, arp3-10, and arp3-13 mutants and some growth of arp3-12 mutant, whereas growth of arp3-11 and arp3-14 mutants was not rescued (Figure 4). Thus, LAS17 is also an allele-specific suppressor of ARP3 mutants indicating that Las17p interaction with the Arp2/3 complex is important for at least one essential function.

Synthetic Enhancement of LAS17 Deletion with ARP2 and ARP3 Mutants

To gain further information on Las17p interactions with Arp2p and Arp3p, we looked for synthetic enhancement between $las17\Delta$ and ARP2 or ARP3 mutants. The $las17\Delta$ strain YMW172K was crossed with seven URA3-tagged ARP2 and one LEU2-tagged ARP3 integrated mutants (see MATERIALS AND METHODS). Viable haploid spores after meiosis were tested for G418 resistance (kanMX4 marker for *las17* Δ) and for uracil and leucine prototrophy (Table 3). As demonstrated by the absence of viable double mutants, six of seven different ARP2 ts mutants, arp2-1, arp2-2, arp2-3, *arp2-4, arp2-5, and arp2-6, are lethal in the absence of LAS17.* Analysis of a cross between strain YMW321L bearing arp3-14:LEU2 and strain YMW171K bearing $las17\Delta$ revealed no viable kanR Leu⁺ spores (Table 3). These synthetic lethality results between $las17\Delta$ and ARP2 and ARP3 ts mutants confirmed the importance of Las17p in the vital functions of Arp2p and Arp3p, prompting us to further investigate the cellular functions and interactions of Las17p.



Figure 3. Suppression of *arp2-2* actin and endocytosis phenotypes by *LAS17*. Strain YMW221U (bearing the *arp2-2:URA3* ts allele) was transformed with vector (a), with pAMW171 (*LAS17 2µ*) (b), and with pAMW200 (*ARP2* in pGAD) (c). (A) Transformants were grown at 25°C in liquid YPD to log phase and shifted to 37°C for 2 h. Cells were fixed, stained with TRITC-phalloidin, and photographed as described in MATERIALS AND METHODS. Bar, 5 µm. (B) Transformants were grown in liquid YPD at 25°C to log phase and incubated for 1 h with LY as described in MATERIALS AND METHODS. Cells were photographed using an FITC filter (LY) or Nomarski optics (differential interference contrast [DIC]).



Figure 4. LAS17 allele-specific suppression of *ARP3* ts mutants. The *arp3* Δ (YMW301T) strain rescued by five different *ARP3* ts alleles (*arp3*-9 to *arp3*-13 and *ARP3*) and strain YMW321L (bearing the *arp3*-14: *LEU2* allele) were transformed by vector (a), pYEW170 (*LAS17*, 2 μ) (b), or pCMW304 rescue plasmid (*ARP3:LEU2*) (c). Freshly cultured transformants were spotted onto YNB – Leu and Ura plates, which were incubated at 25°C for 5 d or at 36°C for 3 d before being photographed.

Phenotypes of las17 Δ Mutant Cells

Deletion of *LAS17* in the S288C strain background has been reported to cause temperature-sensitive growth, budding, and cytokinesis defects with aberrant actin bundles and lack of patches in the buds (Li, 1997). *las17* Δ cells showed misshapen unpinched bud necks and actin cortical patch defects as described (Li, 1997), except that actin-containing patches were visible in both the bud and the mother cell, as has been since reported (Karpova *et al.*, 1998). We found that deletion of *LAS17* results in lethality or temperature sensitivity, depending on the strain background. The phenotype was more severe in the W303 background than in the YPH or FY1679 background. Testing of *las17* Δ ts cells in the presence of osmoregulators showed that they were acutely osmosensitive. Growth was completely inhibited at 25°C with 0.30 M NaCl or 1.8 M sorbitol added to complete medium.

Because LAS17 overexpression suppressed defective endocytosis in certain ARP2 and ARP3 mutants, we also investigated endocytosis in $las17\Delta$ cells using a variety of methods. Three different reporter systems were used to reveal different aspects of endocytosis; LY for fluid phase endocytosis, the lipophilic vital stain FM4-64 to follow membrane internalization (Vida and Emr, 1995), and α -factor pheromone uptake to follow ligand-dependent receptor-mediated (Ste2p) endocytosis (Singer and Riezman, 1990; Riezman, 1993). Naqvi et al. (1998) have recently reported deficient LY endocytosis by $las17\Delta$ cells. Our independent results showed severe deficiency at the permisive growth temperature with no visible accumulation in the vacuole. We also examined strain YMW171K after incubation with FM4-64 to follow the intracellular fate of the dye. Very little dye was taken up by mutant relative to wild-type cells (Figure 5A). Nevertheless, trafficking through membrane compartments without accumulation in intermediary (endosomal) structures in addition to accumulation of weak fluorescence in membranes around the vacuole suggests that the dye progressed to its normal destination. Thus, the pathway from the plasma membrane to the vacuole appears to function normally.

Pheromone recognition and receptor internalization are the first steps in receptor-mediated endocytosis. The ability of cells to bind and internalize α -factor is constant throughout the cell cycle, allowing measurement of ligand-dependent internalization of the Ste2p receptor in growing cells (Zanolari and Riezman, 1991). To increase sensitivity to α -factor, strain YMW171K and its parent were first disrupted for the *BAR1* gene (MacKay *et al.*, 1988). Radioactive uptake of biosynthetically labeled α -factor across the plasma membrane was then quantified. Figure 5B shows that in cells grown at 24°C, tested before and after shift up to 37°C, α -factor binds to *las17* Δ cells. However, receptor-mediated endocytosis is severely deficient at 24°C, and uptake is indistinguishable from background at 37°C. Taken together

Table 3. Sythetic lethality of $las17\Delta$ with ARP2 and ARP3 mutant alleles								
Cross	No. tetrads	No. spores Ura3 ⁺ kanR	No. spores Leu2 ⁺ kanR	No. viable spores	No. dead spores			
las17 $\Delta imes$ arp2-1	35	28		0	28			
$las17\Delta \times arp2-2$	18	15		0	15			
$las17\Delta \times arp2-3$	17	19		0	19			
$las17\Delta \times arp2-4$	17	16		0	16			
$las17\Delta \times arp2-5$	17	15		0	15			
$las17\Delta \times arp2-6$	15	12		0	12			
$las17\Delta \times arp2-7$	15	14		14	0			
$las17\Delta \times ARP2$	17	13		12	1			
$las17\Delta imes arp3-14$	44		37	0	37			
las17 $\Delta imes ARP3$	45		53	48	5			

Strain YMW172K (MAT α *las*17) was crossed with strains YMW201U (*ARP2:URA3*) and YMW211U to YMW271U (*arp2ts:URA3*). Strain YMW172K (MAT α *las*17 Δ ::*kanMX4*) was crossed with strains YMW311L (MATa *ARP3:LEU2*) and YMW321L (MATa *arp3-14:LEU2*). Diploids were sporulated, tetrads were dissected, and viable spores were tested for G418 resistance and for growth on SC – Ura at 25°C. The genotype of dead spores was inferred from that of viable spores in each tetrad tested. For each cross the number of tetrads was analyzed, and the number of viable and dead spores among these double mutants is indicated.



Figure 5. Endocytosis defects in *las*17 Δ cells. (A) FM4-64 uptake by *las*17 Δ cells. YMW171K (*las*17 Δ) and YPH499 (WT) cells were grown to log phase in liquid YPD at 25°C and then concentrated and incubated for 15 min in 20 μ M FM4-64 followed by a 45-min chase in fresh YPD. Cells were photographed using an FITC filter (top panel) or DIC optics (bottom panel). Bar, 5 μ m. (B) Alpha factor internalization by *las*17 Δ cells. *las*17 Δ strain RH 4207 and its equivalent wild type were preincubated at 37°C for 15 min before the addition of ³⁵S-alpha factor. The samples were processed as described by Dulic *et al.* (1991). Results are from one of two independent experiments, which gave nearly identical results. •, WT, 24°C; •, MT, 37°C; □, *las*17 Δ , 37°C.

these results allow us to conclude that Las17p is essential for the internalization step of endocytosis.

Las17p Is Also a Determinant for Polarity Development

The actin cytoskeleton is required for the diploid-specific bipolar budding pattern normally seen in wild-type cells, and mutations in a number of cortical patch components affect this pattern (Rodriguez and Paterson, 1990; Sivadon *et al.*, 1995; Vaduva *et al.*, 1997; Yang *et al.*, 1997). When the bipolar budding pattern was examined, most *las17* Δ diploid cells had randomly located bud scars, whereas wild-type cells showed a normal bipolar budding pattern (Figure 6). Thus, the choice of the bud site, an initial step in the determination of a growth polarity, appears to be the primary defect rather than bud emergence or growth.

Las17-gfp Localizes to Cortical Patches Independently of Polymerized Actin

Las17p has been shown to coprecipitate with G-actin and is important for in vitro reconstitution of cortical actin assembly sites (Lechler and Li, 1997; Li, 1997). To visualize Las17p in living cells, an in situ fusion with GFP was constructed to replace *LAS17* (see MATERIALS AND METHODS). *LAS17: GFP* spores grew well at all temperatures, confirming functionality. GFP fluorescence in strain FMW17GK localized in cortical patch structures concentrated at sites of bud emergence, in small buds and at the bud neck before cytokinesis. When *LAS17:GFP* cells were fixed and stained with phalloidin, actin and Las17-gfp patches colocalized (control cells; Figure 7). This in vivo colocalization confirms that established using an overexpressed Myc-tagged Las17/Bee1p (Li, 1997). We then examined the consequences of disrupting the (polymerized) actin cytoskeleton with latrunculin-A on Las17p localization. The drug latrunculin-A binds G-actin (Coué *et al.*, 1987) and has been shown to rapidly disrupt the yeast actin cytoskeleton by inhibiting actin filament assembly (Ayscough *et al.*, 1997). FMW17GK cells expressing Las17-gfp were treated for 5, 15, or 30 min with 100 μ M latrunculin-A and then briefly fixed, washed, and stained with phalloidin. Actin filaments were no longer detectable by the 5 min time point, whereas Las17-gfp remained localized in cortical patch-like structures at presumtive bud sites,

∆las17/∆las17 LAS17/LAS17

Figure 6. Bipolar budding defects in $las17\Delta/las17\Delta$ cells. Strain

Figure 6. Bipolar budding defects in $las17\Delta/las17\Delta$ cells. Strain FMW173K was grown overnight in YPD at 25°C. Cells were fixed and stained with calcofluor, washed, and observed in the microscope with a UV filter. Bar, 5 μ m.



Figure 7. Functional Las17-gfp remains localized independently of actin. (A) Localization of Las17p at 25°C after treatment of cells with latrunculin-A. Strain YMW173G homozygous for *LAS17:GFP* was grown in liquid YPD to log phase at 25°C and incubated with latrunculin-A (100 μ M final concentration) in DMSO or an equivalent volume of DMSO. Images show cells fixed after 30 min exposure to latrunculin-A, which were briefly fixed and then stained with Alexa-phalloidin. Bar, 5 μ m. (B) Cells were grown as in A, fixed for 1 h, and then digested with zymolyase to make spheroplasts. Spheroplasts were labeled with rabbit anti-GFP IgG (left panels) and mouse monoclonal anti-actin IgG (right panels), which were revealed by goat anti-rabbit FITC and goat anti-mouse Cyanine 3 secondary antibodies using appropriate filters. No overlap between filter channels was detected in control exposures.

in small buds, and at the mother–daughter bud neck of dividing cells. This localization remained up to 30 min after latrunculin-A treatment (Figure 7A), although cells were slightly larger and more rounded than at the 5-min time point, indicating anisotropic cell growth. Las17p localization is thus maintained in the absence of actin cytoskeletal structures needed for polarized growth. Because Las17p might bind actin monomers or dimers not revealed by phalloidin staining, we also examined latrunculin-A-treated cells using anti-actin antibodies. After 30 min of latrunculin-A treatment, control cells (Figure 7B, upper panels) showed normal polarized distribution of both actin and Las17-gfp. How-

ever, no concentration of actin was detected in patch structures, whereas anti-GFP antibodies clearly revealed Las17gfp concentrated in polarized patches (Figure 7B, lower panels) in the majority of cells.

Las17p Is Necessary for the Polarized Localization of Arp2p and Arp3p

We then addressed the question of whether Las17p was necessary for polarized localization of Arp2p and Arp3p. Because Arp2-gfp and Arp3-gfp fusion proteins as unique source of Arp caused ts growth in haploids (our unpublished observations), we tagged one allele in a diploid strain to render these fusion proteins functional. ARP2:EGFP and ARP:EGFP were integrated on one allele of a $las17\Delta/las17\Delta$ strain (FDW174H) giving rise to strains FDW180HK and FDW190HK, respectively. Diploid strains were no longer ts, and both Arp2-egfp (Figure 8A, strain FDW24GK) and Arp3-egfp (Figure 8B, strain FDW34GK) were localized in a polarized manner. In the absence of Las17p, Arp2-egfp and Arp3-egfp patches were clearly visible at the cortex (Figure 8, A and B, lower panels) but were not organized in a polarized manner. This depolarization of Arp2p patches was also observed (using Arp2p antibody) after latrunculin-A treatment (Ayscough et al., 1997). Loss of patch organization indicates that Las17p is a necessary polarity determinant for the localization of the Arp2/3 complex as well as for actin patches as shown above.

Does Las17p Interact Physically with the Arp2/3 Complex ?

Because LAS17 showed genetic and functional interactions with ARP2 and ARP3, we asked whether Las17p could interact directly with Arp2p and/or Arp3p using the twohybrid system. Constructions are described in MATERIALS AND METHODS. Las17p bait showed activation with actin as expected, but significant Las17p interaction was not detected with Arp2p or Arp3p fusions (Table 4). In light of specific genetic interactions between Las17p and Arp2p and Arp3p, we pursued the possibility of direct interaction between Las17p and the Arp2/3 complex by immunoprecipitation of Las17p from crude extracts. The LAS17 gene was tagged in situ with a protein A-coding DNA fragment as described in MATERIALS AND METHODS. This strain was then crossed to an $arp2\Delta$ strain complemented by 3HAC'ARP2 on pYEW248 (Moreau et al., 1996) and recombinant doubly tagged spores were selected, giving rise to strain YMW175K.

To immunoprecipitate Las17p, protein A-Sepharose beads were incubated with IgG and then with whole cell extract from strain YMW175K as described in MATERIALS AND METHODS. Las17-prot A precipitated from the supernatant was visualized after SDS gel electrophoresis on blots with IgG (Figure 9A). Arp2p clearly precipitated with Las17-prot A after multiple washes (Figure 9B). Neither Las17-prot A nor Arp2p precipitated in a parallel control experiment from a nontagged wild-type extract (Figure 9, A and B, lane C). Analysis of the same fractions with specific anti-Arc35p/ End9p antibodies (Brodbeck-Schärer, unpublished results) confirmed the presence of this Arp2/3 complex subunit in the immunoprecipitate with Arp2p. This result confirms that



Figure 8. Loss of Arp2p and Arp3p polarity in *las17* Δ strains. (A) Localization of Arp2-gfp in wild-type and *las17* Δ /*las17* Δ cells. One allele of *ARP2* was replaced by an *ARP2:EGFP* (Cormack *et al.*, 1997) allele in FY1679 (WT) and in strain YMW173K (*las17* Δ /*las17* Δ) as described in MATERIALS AND METHODS. Cells were grown at 25°C in YPD, harvested, resuspended in 1 M sorbitol, and immediately put on slides for microscopic examination. Images were recorded using a GFP filter. (B) Localization of Arp3-gfp in wild-type and *las17* Δ /*las17* Δ cells. Experimental conditions were as in A. Bar, 5 µm.

the genetic data presented above represents specific physical interaction between Las17p and the Arp2/3 complex.

The human homologue of Las17p, WASP, regulates actin polymerization and cytoskeleton rearrangements and has been shown to interact directly with activated CDC42Hs (Apenström *et al.*, 1996; Miki *et al.*, 1996; Symons *et al.*, 1996). We were intrigued to know whether Las17p interacted di $\label{eq:table_$

GAL4-BD (D domain) β-ga activity (Mi	GAL4-BD (DNA binding domain) β-galactosidase activity (Miller units)		
Las17p ^{1–643} (pBMW172)	Act1p ¹⁻³⁷⁵ (pDAB7)		
$\begin{array}{c} 00.1 \pm 0.02 \\ 3.5 \pm 0.5 \\ \leq 0.1 \\ \leq 0.1 \\ \leq 0.1 \\ 1.0 \pm 0.1 \\ 2.0 \pm 0.1 \\ 50 \pm 1 \\ 54 \pm 5 \\ 122 \pm 3 \\ 16 \pm 2 \\ 62 \pm 9 \\ 29 \pm 7 \\ 58 \pm 5 \\ 96 \pm 11 \\ 15 \pm 4 \\ 6 \pm 0.2 \\ 24 \pm 1 \end{array}$	$\begin{array}{c} 0.2 \pm 0.06 \\ 1.4 \pm 0.07 \\ \leq 0.2 \\ \leq 0.2 \\ 1.2 \pm 0.3 \\ 1.1 \pm 0.1 \\ 1.0 \pm 0.1 \\ 0.7 \pm 0.2 \\ \leq 0.2$		
8 ± 1	≤0.2		
	$\begin{array}{c} {\rm GAL4-BD\ (D)}\\ {\rm domain\ }\beta\text{-ga}\\ {\rm activity\ (Mi)}\\ \hline\\ {\rm Las17p^{1-643}}\\ {\rm (pBMW172)}\\ \hline\\ 00.1\pm0.02\\ 3.5\pm0.5\\ \leq 0.1\\ \leq 0.1\\ 1.0\pm0.1\\ 2.0\pm0.1\\ 50\pm1\\ 54\pm5\\ 122\pm3\\ 16\pm2\\ 62\pm9\\ 29\pm7\\ 58\pm5\\ 96\pm11\\ 15\pm4\\ 6\pm0.2\\ 34\pm1\\ 8\pm1\\ \end{array}$		

Vectors encoding fusions with the GAL4-(AD) were transformed into Y190 cells expressing GAL4-DB fusions with Las17p (pBMW172) or Act1p (pDAB7) and plated on SC – Trp and Leu plates. At least three colonies from each transformation were cultivated overnight in liquid selective medium (SC – Trp and Leu). β -Galactosidase activities were measured as described in MATERI-ALS AND METHODS. Values shown \pm SD. Most double transformants that did not show blue color in the filter test were considered as approximately equal (\leq) to background and were not assayed in liquid cultures. Plasmids marked with an asterisk were found in the FYRL genomic library; nonmarked entries are from the White cDNA library. Redundancy of the AD plasmids found is shown in parentheses. Not all candidates found are shown; interactions of the strongest and shortest fusions of each candidate are shown; longest fusions for Rvs167p and Vrp1p are also shown.

rectly with Cdc42p in *S. cerevisiae* because the Cdc42p consensus binding sequence is absent. To test these interactions in the two-hybrid system, a Las17p bait was tested for interaction with wild-type and activated Cdc42p (Cvrckova *et al.*, 1995; Leberer *et al.*, 1997), in addition to profilin and actin (Amberg *et al.*, 1995) as known interactants. No significant interactions with wild-type or activated Cdc42p were detected with these constructions.

To learn more about the interactions of Las17p, we used the two-hybrid system to screen a yeast cDNA library and a genomic DNA library for potential Las17p interacting proteins (see MATERIALS AND METHODS). In these screens (neither of which represent saturation) we found clones representing three known cytoskeletal proteins actin, verprolin, and Rvs167p (reduced viability upon starvation), and six proteins of unknown function, named LSB for Las seventeen binding proteins: Lsb1p (YGR136W), Lsb2p (YPR154w), Lsb3p (YFR024c-A), Lsb4p/Ysc84p (YHR016c), Lsb5p (YCL034w), and Lsb6p (YJL100w). Plasmids express-



Figure 9. Coimmunoprecipitation of Las17p and Arp2p (Arp2/3 complex). Las17-prot A was immunoprecipitated from crude extracts of strain YMW175K, which contained a chromosomally integrated allele of protein A-tagged Las17p and plasmid-borne HA tagged Arp2p (described in MATERIALS AND METHODS). After precipitation with TCA, cell extract and each step of the immunoprecipitation were subjected to 10% SDS-PAGE and blotted to nitrocellulose. E, extract; S, supernatant; L1, wash 1; L2, wash 2; L3, wash 3; P, immunoprecipitate; C, control immunoprecipitate, mock IP from crude extract of the YMW175K parental strain without protein A tag. (A) Las17-protA detected with purified rabbit IgG. (B) HA-tagged Arp2p detected with mouse monoclonal anti-HA IgG.

ing these fusions were purified and transformed into strain Y190 expressing either Gal4DB-Las17p (pBMW172) or Gal4DB-Act1p (pDAB7). Results of β -galactosidase assays for the most informative in-phase clones are summarized in Table 4.

Of the known proteins found, a fusion containing actin with 27 additional aa at the amino terminus was found, similar to an extended actin (pAIP70) found in a screen against actin itself (Amberg et al., 1995). Las17p showed strong two-hybrid interaction with multiple derivatives of the SH3 domain protein Rvs167p. Nine different truncations (from the cDNA library) gave strong interaction with the Las17p bait. Rvs167p, originally identified along with Rvs161p as being important for recovery from starvation, affects cytoskeletal organization (Bauer et al., 1993; Sivadon et al., 1995). The smallest interacting fusion (83 aa) contains only the SH3 domain plus 21 aa of the GPA (for rich in Gly, Pro, and Ala) domain, whereas the strongest interacting fusion (239-482) contains the SH3 domain plus the GPA domain. In addition, four more N-terminally extended clones, beginning at aa 220, 191, 183, and 69, showed interaction with actin, as was reported by Amberg et al. (pAIP38 aa 189-549), whereas shorter clones did not. Strikingly, for verprolin (Vrp1p/End5p), eight different genomic fragments (and one cDNA fragment) all contained the C-terminal extremity of the protein. The smallest fusion contained only the C-terminal 36 aa, and the strongest interacting fusion had 327 aa. These results "map" a major Las17p interaction with Vrp1p to the Vrp1p C-terminus.

Among the unknown candidates, sequence comparisons revealed that Lsb1p, Lsb2p, Lsb3p, and Lsb4/Ysc84 contain SH3 domains with high similarity to the SH3 domain of Rvs167p and to that of human Grb2p. Lsb1p/V25 (241 aa) and Lsb2p (215 aa) contain an N-terminal SH3 domain followed by a proline-rich acidic region. With 64% identity to each other they form a strongly interacting minimal family. Except for the SH3 domain, no significant homologies for Lsb1p/Lsb2p were detected with other proteins in databases. Lsb1p has been characterized in a comparative mRNA study as being highly expressed in high-density cultures and upon diauxic shift, but deletion of the gene showed no particular phenotype (Wang et al., 1997). Lsb3p (451 aa) and Ysc84p/Lsb4p (468 aa) are 67% identical to each other, forming another minimal family with a C-terminal SH3 domain. BLAST searches and sequence alignment revealed homology between these two proteins and an SH3domain containing protein of unknown function from mouse (340 aa, cDNA clone ge:D85926). YSC84/LSB4 was studied as an example of a divergently transcribed gene whose expression increased 5- to 10-fold during sporulation. However, its deletion caused no apparent phenotype for vegetative growth, progression into meiosis, or sporulation (Rocco et al., 1993). These SH3 domain proteins are currently under investigation. Candidates Lsb5p and Lsb6p are unknown, but both have possible homologues in other organisms. Lsb5p (265 aa) is similar to a hypothetical Schizosaccharomyces pombe protein. Lsb6p (607 aa) is similar to a hypothetical Caenorhabditis elegans protein C56A3.8. Although the existence of possible homologues in other species suggests that these ORFs code for functional proteins, the biological relevance to Las17p remains to be established.

DISCUSSION

LAS17 Is Important for the Function of the Arp2/3 Complex

The phylogenetic conservation of the Arp2/3 complex and its localization to actin-rich cortical regions of cells suggest that it fulfills a common essential function in the cortical actin cytoskeleton. The Arp2/3 complex functions to bind and cross-link actin filaments (Mullins *et al.*, 1997, 1998b) and to nucleate actin polymerization required to produce movement at the cell surface (Mullins *et al.*, 1998b; Welch *et al.*, 1997a, 1998). Las17p/Bee1p has been shown to be involved in the actin cytoskeleton (Li, 1997) and in actin assembly in vitro (Lechler and Li, 1997). We addressed here the question of how Las17p produces its effects on actin organization. Our genetic evidence shows that Las17p plays an important role in the functioning of Arp2 and Arp3 proteins, thus linking it to the function(s) of the Arp2/3 complex. First, *LAS17* is an allele-specific suppressor of the temperature-sensitive growth of *ARP2* and *ARP3* mutants. Defects in polarity of actin distribution and in fluid phase endocytosis were also suppressed. That is, no separation of function between actin defects and deficient endocytosis was apparent. Second, synthetic lethality of *las17* Δ with six of seven *ARP2* mutants and with an *ARP3* mutant indicates that these proteins affect the same cellular processes. Taken together, these results would tell us that Las17p plays an important role in the functions of the Arp2/3 complex but do not differentiate between stabilization of Arp2/3 complex interactions from regulation of actin-polymerizing function and/or signaling mechanisms mediated by Las17p.

LAS17 Δ and ts Mutations in ARP2 and ARP3 Cause Similar Phenotypes

Deletion of LAS17 perturbs the organization of the actin cytoskeleton at permissive growth temperature (Li, 1997; this study) and is lethal at 37°C or is essential at all temperatures depending on the strain context. We have found a striking similarity between the $las17\Delta$ phenotype and the phenotypes of ARP2 ts mutants. In addition to depolarization of actin patches, many $las17\Delta$ cells show actin aggregates in the bud and at the bud neck of large budded cells as is seen in *ARP* mutant cells. An independent study showed that aberrant aggregates in $las17\Delta$ cells, as do cables, contain Sac6p but lack normal patch components cofilin and Cap2p (Li, 1997). Nor do aggregates appear to contain Arp2p or Arp3p. Interestingly, Arp2p-gfp still colocalized with residual actin patches (Figure 1B; Dumoulin, unpublished observations). Recent microscopic studies have confirmed that cables and patches depend on each other for assembly and function (Karpova et al., 1998). The fact that overexpression of LAS17 suppresses the formation of such structures in *arp2-1* and *arp2-2* mutants implicates Las17p and the Arp2/3 complex together in filament organization. Abnormally enlarged necks between mother and daughter cells are a further common defect of $las17\Delta$ and certain ARP2 mutant cells, suggesting that Las17p and Arp2p may both be important for cytokinesis and/or cell division. This is consistent with the previously shown genetic interactions between *arp2-1* and the septin cdc10-1 mutations (Moreau et al., 1996). In further characterization of $las17\Delta$ cells, deficient α factor uptake lets us conclude that Las17p is essential for liganddependent receptor-mediated internalization in addition to fluid phase endocytosis, whereas later steps of the endocytosis pathway are apparently functional. Internalization was severely deficient at 24°C, at which cells continued to divide, as we have previously described for arp2-1 cells (Munn et al., 1995; Moreau et al., 1997). This step of endocytosis has also been shown to require actin and fimbrin and many other cytoskeletal proteins, including End3p, Sla2p/End4p, Vrp1p/End5 Rvs161/End6p, and Rvs167p (Raths et al., 1993; Munn and Riezman, 1994; Munn et al., 1995).

We also investigated the in vivo relationship of Las17p to polarity and actin organization. Loss of the bipolar budding pattern in growing diploid cells points to a possible role for Las17p in establishment of cell growth polarity. Furthermore, in the presence of latrunculin-A, functional Las17-gfp, probably with other proteins, remains in cortical patches in a polarized distribution in the absence of any detectable concentration of filamentous or total actin. Las17p must therefore be upstream of actin with respect to the flow of

polarity information. Interestingly, certain other proteins important for polarity establishment, such as Cdc42p and Bem1p (Ayscough et al., 1997) as well as verprolin (Vaduva et al., 1997), also show polarized localization independently of polymerized actin in virtually all cells, whereas others such as Bud6/Aip3p, Myo2p, and calmodulin appear to maintain only partial polarity. Still other proteins that are important for the actin cytoskeleton and secretion, such as Arp2p, Aip1p, Sec4p, Sec8p, and Smy1p are dependent on actin structures for their polarized localization (Ayscough et al., 1997). The fact that $las17\Delta$ strains show increased sensitivity to latrunculin-A in growth tests (our unpublished results), in addition to being necessary for actin, Arp2p, and Arp3p localization, suggests that Las17p normally plays a role in polarized localization and in stabilization of the actin-polymerizing complex at the cortex. When the actin filament cytoskeleton is destroyed by latrunculin-A treatment, despite nonpolarized growth of cells (in the absence of increase of cell numbers), proteins such as Las17p and verprolin remain polarized, suggesting that the flow of polarity information necessary for polarized growth may also require interaction with actin filaments for these proteins to function with the Arp2/3 complex. Although Las17p is not essential for growth in all conditions, it is clearly a major determinant in multiple cellular processes implicating the actin cytoskeleton.

Elucidating Las17p Functions through its Interactions

Genetic evidence for functional interaction between $las17\Delta$ and ARP2 and ARP3 mutations is extended biochemically to indicate physical interaction with the coimmunoprecipitation of Arp2p and Las17p from crude extracts. How does high-copy expression of LAS17 overcome specific ARP2 and ARP3 ts mutations? Las17p might interact directly with Arp2p or Arp3p despite the fact that we were unable to detect interactions between Las17p and Arp2p or Arp3p in two-hybrid tests. In this case, Arp2p or Arp3p might bind less tightly to Las17p, but higher expression of Las17p could compensate weak binding. Another interpretation of our results is that intermediary protein components are necessary to allow functional interaction of Las17p with the Arp2/3 complex. Las17p might interact with the Arp2/3 complex through contact with actin, with other subunits, or both. In vitro protein interaction between Las17p and actin was demonstrated by coimmunoprecipitation (Li, 1997), and the two proteins interact directly in the two-hybrid system. Direct interaction between bovine N-WASP and actin has also been shown in vitro. The WH2 domain or "verprolin homology" and the "cofilin homology" domains of N-WASP participated in actin binding (Miki et al., 1996). Similar direct physical interactions with actin were confirmed for both WASP and SCAR (Machesky and Insall, 1998). Because the WH2 domain is rather well conserved in the C-terminal region of the yeast homologue, it likely to be the region of Las17p that interacts with actin:

GRGALLDQIRQ..GIQLNKT (WASP_human Swiss prot) GRDALLDQIRQ..GIQL.KS (N-WASP; DDBJ accession D88460) GRDALLASIRGAGGIGALRKV (La17_yeast; Swiss prot).

SCAR and WASP were recently found to regulate the actin cytoskeleton through the Arp2/3 complex. The C-

terminal WA fragment (WH2 and acidic domains) of both the WASP and the SCAR homologues bind the p21-Arc subunit (equivalent to *S. cerevisiae* Arc18p) of the Arp2/3 complex (Machesky and Insall, 1998). The *ARP2* allele-specific interactions we found with *las17* Δ are not inconsistent with a similar interaction in *S. cerevisiae*, because certain mutations could affect small subunit binding indirectly. However, we did not detect two-hybrid interactions between a full-length Las17p and Arp2p, Arp3p, Arc35p, Arc40p, or any of the small subunits Arc19p, Arc18p, or Arc15p tested as activation fusions (Table 4; our unpublished results).

During the revision of this manuscript several in vitro studies pertinent to our results have been published. Las17p (GST-Bee1p) and the Arp2/3 complex have been coimmunoprecipitated in yeast, an independent demonstration of the physical interaction we report here between Las17-protA and the Arp2/3 complex. This interaction required both Arc19p and Arc15p (Winter et al., 1999). Simultaneous requirement of two or more subunits could be an explanation for the lack of two-hybrid interactions. Further knowledge of the Arp2/3 complex topology will be necessary to understand whether the mammalian and yeast results represent the same overall interactions. In addition to the activation of the S. cerevisiae Arp2/3 complex by Las17p in vitro (Winter et al., 1999), the direct role of WASP (Yarar et al., 1999) and SCAR (Machesky et al., 1999) in the activation of the in vitro nucleation activity of the Arp2/3 complex to generate new filament barbed ends has been elucidated in mammalian cells.

It has been shown previously that Cdc42, activated with guanosine 5'-3-O-(thio)triphosphate, induces actin polymerization in neutrophils (Zigmond et al., 1998). The Arp2/3 complex is one of the elements required for Cdc42-induced actin polymerization in Acanthamoeba (Mullins and Pollard, 1999) and Xenopus extracts (Ma et al., 1998). Now, it has also been shown that the stimulation of actin nucleation by N-WASP binding to the Arp2/3 complex requires both the actin binding domain and the Arp2/3 complex binding domain of N-WASP, as is the case for SCAR activation of the Arp2/3 complex (Machesky et al., 1999). Actin polymerization in the Xenopus system is enhanced by both Cdc42 and phosphatidylinositol-(4,5)-biphosphate, linking signal transduction pathway molecules to the stimulation of actin polymerization (Rohatgi et al., 1999). Evidence that the combined action of WASP family members and the Arp2/3 complex are also directly dependent on Cdc42 in vivo (Castellano et al., 1999) comes from experiments which used a modifed plasma membrane receptor capable of inducible local recruitment of Cdc42 (or WASP) to show that activated Cdc42 (or WASP) triggered actin polymerization and subsequent formation of protrusions in whole cells.

Las17p Shows Two-Hybrid Interaction with SH3 Domain Proteins

Previous studies showed that proline-rich stretches of various proteins bind to SH3 domains (Ren *et al.*, 1993). SH3 domains are small regions (\sim 60–80 aa) present in a large number of proteins showing similarity to a domain in c-src and v-src proto-oncogene proteins. The presence of several proline-rich stretches in Las17p suggests that it could interact directly with one or several SH3 domain proteins. In

two-hybrid screens with Las17p bait, five of the nine proteins we identified as Las17p interactants contained an SH3 domain. Although the strength of possible in vivo interactions between complete proteins cannot be implied directly from the activation values of two-hybrid interaction, clones representing the SH3 domain with or without other parts of these proteins interacted strongly with full-length Las17p. One of these SH3 domain proteins was Rvs167p, which contains three domains, BAR/RVS, GPA, and SH3. The SH3 domain with a few additional amino acids of the neighboring GPA domain was sufficient to show strong interaction with Las17p. Thus, in addition to the SH3 domain, Las17p-Rvs167p interaction may require participation of the GPA domain. Rvs167p is known to function with Rvs161p/End6p (Navarro et al., 1997), which was also fished out in a screen for endocytosis-defective mutants as mentioned above (Munn et al., 1995). In this screen we did not pick up Rvs161p (which is homologous to Rvs167p except for lack of the C-terminal SH3 domain) but it does interact with Las17p when tested directly (Recordon-Navarro, 1998), further suggesting that SH3 domains may not be solely responsible for interaction with Las17p. Rvs167p was previously found as an actin partner (Amberg et al., 1995). Our results show that only Rvs167p fusions containing at least part of the Nterminal domain of Rvs167p interact with actin in twohybrid tests (≥aa 220-482; Table 4; our unpublished observations).

We also found that Las17p interacts strongly with verprolin (Vrp1/End5p). Verprolin has been shown to be part of the actin cytoskeleton and, like Las17p, is important for endocytosis and is a polarity determinant that localizes to polarized cortical patches independently of polymerized actin (Munn et al., 1995; Vaduva et al., 1997). Results of an independent communication have been presented showing that the C-terminal half of Las17p binds verprolin (Naqvi et al., 1998). Our results show that interactions of several different fragments from a random genomic bank map the binding site of verprolin to the C terminus; a fragment of only 35 C-terminal amino acids is sufficient for strong binding to Las17p. This interaction is comparable with that of WASP (as bait) with the human homologue of verprolin, WIP (WASP-interacting protein), isolated in a two-hybrid screen. In two-hybrid tests a C-terminal 87-aa truncation of WIP no longer bound to WASP, in good agreement with our mapping of the verprolin-Las17 interaction. Overexpression of WIP affected cortical actin assembly, inducing the formation of actin-containing projections at the cell surface of human B cells (Ramesh et al., 1997).

Given that the *las17* Δ genetic interaction with *ARP2* and *ARP3* mutants is allele specific, and that Las17p and Arp2p interact physically, Las17p is shown to be an effector of the Arp2/3 complex. These results coincide with multiple demonstrations in many systems of WASP family members' interaction with the Arp2/3 complex. We have also shown that Las17p interacts directly with actin, Rvs167p, and verprolin by two-hybrid analyses. Finally, results of our two-hybrid screen suggest that Las17p might interact with at least six other unknown proteins, four of which are SH3 domain proteins. The fact that Lsb1p has been localized in the nucleus by transposon tagging of genes with increased expression in stationary phase and that *LSB4/YSC84* expression is increased 5–10 times during sporulation raises the

question of how Las17p might integrate signals from different developmental processes. Possible implication of these new proteins in the actin cytoskeleton or in signaling mechanisms are currently under investigation.

Whereas Arp2p and Arp3p bind as a complex to actin filaments (Mullins et al., 1997, 1998b), Las17p may bind only actin monomers, by analogy to SCAR and WASP (Machesky and Insall, 1998) and verprolin (Vaduva et al., 1997), which bind actin monomers in vitro. The absence of a concentration of actin in Las17p patches after latrunculin-A treatment does not eliminate the possibility that Las17p recruits the first monomers to cortical sites, but further studies are needed to clarify this possibility. The suppression of mutations in Arp2/3 complex components by LAS17 overexpression could then be explained by increasing monomeric actin at nucleation sites. The polarized localization of Las17p reflects its anchorage in areas of cell surface growth, hence its potential for involvement in both plasma membrane and intracellular signaling. An attractive possibility is that Las17p, in association with verprolin and Rvs167p (and perhaps with other proteins such as the Lsbp reported here), forms the essential part of a complex that remains anchored at the plasma membrane, receives diverse signals, and transmits them to the actin-polymerizing complex where Las17p plays a direct role in amplifying actin nucleation by the Arp2/3 complex.

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