Pxl1p, a Paxillin-like Protein in *Saccharomyces cerevisiae*, May Coordinate Cdc42p and Rho1p Functions during Polarized Growth

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Rho-family GTPases Cdc42p and Rho1p play critical roles in the budding process of the yeast *Saccharomyces cerevisiae*. However, it is not clear how the functions of these GTPases are coordinated temporally and spatially during this process. Based on its ability to suppress *cdc42*-Ts mutants when overexpressed, a novel gene *PXL1* was identified. Pxl1p resembles mammalian paxillin, which is involved in integrating various signaling events at focal adhesion. Both proteins share amino acid sequence homology and structural organization. When expressed in yeast, chicken paxillin localizes to the sites of polarized growth as Pxl1p does. In addition, the LIM domains in both proteins are the primary determinant for targeting the proteins to the cortical sites in their native cells. These data strongly suggest that Pxl1p is the “ancient paxillin” in yeast. Deletion of *PXL1* does not produce any obvious phenotype. However, Pxl1p directly binds to Rho1p-GDP in vitro, and inhibits the growth of *rho1-2* and *rho1-3* mutants in a dosage-dependent manner. The opposite effects of overexpressed Pxl1p on *cdc42* and *rho1* mutants suggest that the functions of Cdc42p and Rho1p may be coordinately regulated during budding and that Pxl1p may be involved in this coordination.

INTRODUCTION

Rho-family GTPases regulate cytoskeletal remodeling, vesicular trafficking, and cell cycle progression in eukaryotic cells. The budding yeast *Saccharomyces cerevisiae* genome encodes six Rho-family members, Cdc42p and Rho1p–Rho5p, all of which are involved in some aspects of cell polarization (Cabib *et al.*, 1998; Johnson, 1999; Pruyne and Bretscher, 2000; Roumanie *et al.*, 2001; Schmitz *et al.*, 2002). Cdc42p plays an essential role in the establishment of cell polarity by regulating actin and septin organization and secretion (Johnson, 1999; Adamo *et al.*, 2001; Zhang *et al.*, 2001; Gladfelter *et al.*, 2002; Caviston *et al.*, 2003). Temperature-sensitive (Ts) *cdc42* mutant, such as *cdc42-1* (Adams *et al.*, 1990), is unable to polarize cell growth to the presumptive bud site at the restrictive temperature. As a result, cells grow isotropically and arrest with large, round, and unbudded cell morphology. Thus, Cdc42p is thought to function at the very early stage of the budding process through a host of its effectors including the p21-activated kinases (PAKs), Ste20p, Cla4p, and Skm1p; the formin, Bni1p; the IQGAP, Iqg1p/Cyk1p; the exocyst component, Sec3p; and a pair of yeast-specific, CRIB-containing proteins, Gic1p/Gic2p (Cvrckova *et al.*, 1995; Brown *et al.*, 1997; Chen *et al.*, 1997; Evangelista *et al.*, 1997; Osman and Cerione, 1998; Holly and Blumer, 1999; Weiss *et al.*, 2000; Zhang *et al.*, 2001).

Rho1p also plays an essential role in the budding process. Conditional inactivation of Rho1p function by Ts mutations or depletion results in major defects in cell wall integrity and also in polarized actin organization and secretion (Yamochi *et al.*, 1994; Drgonova *et al.*, 1996; Qadota *et al.*, 1996; Guo *et al.*, 2001). As a result, *rho1* mutants are arrested at early stages of the budding process, with either unbudded or small-budded cell morphology that is often associated with cell lysis. The functions of Rho1p in the budding process are mediated by some of its effectors including Bni1p; Sec3p; the protein kinase C, Pkc1p; and the cell-wall remodeling enzymes, Fks1p/Fks2p (Mazur *et al.*, 1995; Nonaka *et al.*, 1995; Kohno *et al.*, 1996; Imamura *et al.*, 1997; Andrews and Stark, 2000; Guo *et al.*, 2001; Dijkgraaf *et al.*, 2002). Even though Cdc42p and Rho1p are involved in related processes during budding and share some effectors, it is not clear how the functions of these two GTPases are coordinated temporally and spatially during polarized growth to ensure successful budding.

A number of proteins that regulate cell proliferation, differentiation, and cell motility, contain LIM (Lin-11, Isl-1, Mec-3) domains, which generally comprise ~50–60 amino acids and are thought to mediate protein-protein interactions (Dawid *et al.*, 1998). Through the chelation of one zinc molecule by four amino acids including cysteine, histidine, or aspartic acid, each LIM domain forms two zinc finger structures separated by two amino acids, [CXXCX_{16–23}HXX(H/C)]-XX-[CXXCX_{16–23}CXX(D/H/C)] (X is any amino acid; Freyd *et al.*, 1990; Michelsen *et al.*, 1993). In cultured fibroblasts, many LIM proteins in the zyxin/paxillin family, including zyxin, paxillin, Hic-5, and LPP, all target to focal adhesions, which are membrane attachment

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Table 1. *Saccharomyces cerevisiae* strains used in this study

Name	Genotype	Source
YEF473	a/ α <i>his3/his3 leu2/leu2 lys2/lys2 trp1/trp1 ura3/ura3</i>	Bi and Pringle (1996)
YEF473A	a <i>his3 leu2 lys2 trp1 ura3</i>	Bi and Pringle (1996)
YEF2258	a <i>his3 leu2 lys2 trp1 ura3 cdc42-201</i>	Zhang <i>et al.</i> (2001)
JPC241	a <i>his3 leu2 lys2 trp1 ura3 cdc42^{G60D}</i>	Caviston <i>et al.</i> (2002)
JPC261	As YEF473 except <i>pXl1Δ::kanMX6/PXL1</i>	This study
JPC262	As YEF473 except <i>PXL1-GFP-kanMX6/PXL1</i>	This study
JPC265	a <i>his3 leu2 trp1 ura3 lys2 pXl1Δ::kanMX6</i>	This study
JPC266	α <i>his3 leu2 trp1 ura3 lys2 pXl1Δ::kanMX6</i>	This study
JPC267	a <i>his3 leu2 trp1 ura3 lys2 PXL1-GFP-kanMX6</i>	This study
JPC272	a <i>his3 leu2 trp1 ura3 lys2 pXl1Δ::His3MX6</i>	This study
JPC273	α <i>his3 leu2 trp1 ura3 lys2 pXl1Δ::His3MX6</i>	This study
NY2284	α <i>ura3 leu2 trp1 his3 ade2 lys2 rho1::HIS3 ade3::[pRHO1-RHO1:LEU2]</i>	Guo <i>et al.</i> (2001)
NY2285	a <i>ura3 leu2 trp1 his3 ade2 lys2 rho1::HIS3 ade3::[pRHO1-rho1-2:LEU2]</i>	Guo <i>et al.</i> (2001)
NY2286	α <i>ura3 leu2 trp1 his3 ade2 lys2 rho1::HIS3 ade3::[pRHO1-rho1-3:LEU2]</i>	Guo <i>et al.</i> (2001)
NY2287	a <i>ura3 leu2 trp1 his3 ade2 lys2 rho1::HIS3 ade3::[pRHO1-rho1-5:LEU2]</i>	Guo <i>et al.</i> (2001)

sites to the extracellular matrix, and participate in integrin signaling (Turner, 2000a, 2000b).

We have identified a novel LIM domain-containing protein, Pxl1p in *S. cerevisiae*, from our genetic screens. Overexpression of *PXL1* on high-copy plasmids suppressed the Ts growth of *cdc42* mutants, suggesting Pxl1p may function in Cdc42p signaling pathway. We also show that Pxl1p directly binds to Rho1p-GDP in vitro and downregulates Rho1p function. Thus, our findings suggest that the functions of Cdc42p and Rho1p may be coordinately regulated during budding, which may involve the function of Pxl1p.

MATERIALS AND METHODS

Strains and Growth Conditions

Yeast strains used in this study are listed in Table 1. Standard culture media and genetic techniques were used (Guthrie and Fink, 1991). *Escherichia coli* strains DH12S (Life Technologies, Gaithersburg, MD) and BL21 (DE3; Novagen, Madison, WI) were used as a plasmid host and for protein expression, respectively.

Construction of Plasmids

Plasmids used in this study include YEp24 (2 μ , *URA3*), YEp352 (2 μ , *URA3*), pFA6a-kanMX6, pFA6a-His3MX6, and pFA6a-GFP (S65T, F64L)-kanMX6 (Longtine *et al.*, 1998), pUG36 (*CEN*, *URA3*, *pMET25-yEGFP3*; kindly supplied by Dr. J. H. Hegemann). YEp24-PXL1 was isolated from the genetic screen. *PXL1* full-length and various fragments were amplified from the YEp24-PXL1 plasmid by PCR and subsequently cloned into pUG36 vector. Details for these constructs are available upon request. A *Bam*HI-*Eco*RI fragment containing the full-length chicken paxillin (1–559) and a *Pst*I-*Eco*RI fragment containing the C-terminal portion of paxillin (309–559) from plasmid pcDNA3-paxillin (Brown *et al.*, 1996) were cloned into pUG36. The N-terminal portion of paxillin (1–325) was amplified by PCR as a *Bam*HI-*Eco*RI fragment and cloned into pUG36. YEp24-GAL-PXL1 was constructed by tagging of *PXL1* in YEp24 with the *kanMX6-pGAL1* module by a PCR-mediated method (Longtine *et al.*, 1998). To construct pMAL-c2X-PXL1 that expresses an MBP-Pxl1p fusion protein, a 2.1-kb DNA fragment containing *PXL1* gene flanked by two *Sal*I sites was generated by PCR, and inserted into *Sal*I-digested pMAL-c2X vector (New England Biolabs, Beverly, MA). The constructs expressing GST-Cdc42p and GST-Rho1p fusion proteins were kindly provided by Dr. Wei Guo (University of Pennsylvania; Guo *et al.*, 2001; Zhang *et al.*, 2001). Galactose-inducible overexpression of Cdc42p and Rho1p was achieved by using YEp352-GAL-CDC42 and pYES2-RHO1 plasmids. pYES2-RHO1 was constructed by cloning PCR-amplified *Bam*HI-*Eco*RI fragment of *RHO1* into pYES2 (Invitrogen, Carlsbad, CA).

Construction of Yeast Strains

To make strains carrying *pXl1 Δ* , one copy of *PXL1* in strain YEF473 was replaced by a *kanMX6* module by a PCR-based method (Longtine *et al.*, 1998). The resulting diploid strain JPC261 was sporulated and tetrads were dissected to generate the haploid strains JPC265 and JPC266. The *KanMX6* module in strains JPC265 and JPC266 was later substituted with a *His3MX6* module to

generate JPC272 and JPC273, respectively. To construct a C-terminal *PXL1-GFP* fusion on the chromosome, one copy of *PXL1* in strain YEF473 was tagged with GFP (S65T, F64L)-kanMX6 module (Longtine *et al.*, 1998). The resulting diploid strain JPC262 was sporulated and tetrads were dissected to generate JPC267.

Genetic Screens

In the first screen, yeast strain JPC241 (a *cdc42^{G60D}*) was transformed with a YEp24-based genomic DNA library (Carlson and Botstein, 1982) and grown on SC-Ura plates at 35°C. From ~82,000 transformants screened, 75 suppressor-carrying plasmids were isolated and determined to carry two known genes, *CDC42* (71 $\times$) and *MSB1* (2 $\times$), and one novel gene, *PXL1* (2 $\times$). Among the latter group, further subcloning and deletion analysis showed that *PXL1* was solely responsible for the suppression. In the second screen, strain YEF2258 (a *cdc42-201*) was transformed with the same DNA library and grown on SC-Ura plates containing 1 M Sorbitol at 35.5°C. From ~65,000 transformants, 10 suppressor-carrying plasmids were isolated and determined to carry *CDC42* (3 $\times$), *MSB1* (3 $\times$), *YHR149C* (2 $\times$), *MSB3* (1 $\times$), and *PXL1* (1 $\times$).

Microscopy and Immunoblotting

For localization of GFP-tagged proteins, cells were grown exponentially in YPD or SC-Ura media at 24°C. Differential interference contrast (DIC) and fluorescence microscopy were performed using Nikon Microscope ECLIPSE E800 (Nikon Corporation, Tokyo, Japan) with a 60 $\times$ Plan Apo objective. The images were acquired using Phase 3 Imaging Systems (Glen, PA).

For immunoblotting, the mouse monoclonal anti-GFP antibody (BABCO, Richmond, CA) and horseradish peroxidase-conjugated goat anti-mouse IgG antibody (Roche Molecular Biochemicals, Indianapolis, IN) were used. Proteins were detected with the ECL Western blotting detection reagents.

In Vitro Protein-binding Assay

The recombinant MBP-Pxl1p, GST-Cdc42p and GST-Rho1p fusion proteins were purified from *E. coli* strain BL21. GST fusion proteins of Cdc42p and Rho1p were first stripped off nucleotide and then loaded with either GDP or GTP- γ -S. To do this, 15 μ g of GST alone or a GST fusion protein was first incubated in the preloading buffer (20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM EDTA, 5 mM DTT, and 10 mM GDP or GTP- γ -S) at 30°C for 30 min, followed by the addition of MgCl_2 to a final concentration of 25 mM, and the reaction was incubated at 24°C for 15 min. After nucleotide loading, GST fusion proteins were added to the Amylose beads containing 5–10 μ g of MBP-Pxl1p fusion protein. After incubation at 4°C for 2 h, samples were washed five times with washing buffer (20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10 mM MgCl_2 , 5 mM DTT, and 0.1% Triton X-100), and the bound proteins were eluted from the beads with SDS sample buffer. Samples were analyzed by a 12% SDS-PAGE, and proteins were detected by Coomassie blue staining and immunoblotting with an anti-GST antibody.

RESULTS

Isolation of PXL1 as a Multicopy Suppressor of *cdc42-Ts* Alleles

To identify additional components involved in Cdc42p signaling pathway, we performed two independent genetic

Figure 1. Multicopy *PXL1* suppresses two temperature-sensitive *cdc42* mutants. Yeast strains JPC241 (*cdc42^{G60D}*) and YEF2258 (*cdc42-201*) were transformed with YEp24 empty vector, or YEp24 carrying *CDC42*, *PXL1*, or *MSB1*. Transformants were streaked onto SC-Ura plates and incubated at 24°C or 35.5°C for 3 days.

screens with two recessive, Ts alleles of *CDC42*, *cdc42-201* (Zhang *et al.*, 2001) and *cdc42^{G60D}* (Caviston *et al.*, 2002; see details of the screens in *Materials and Methods*). The *cdc42-201* allele encodes a protein with four amino acid changes (C6S, D31V, D65G, and F90G) and cells carrying this allele are elongated at the permissive temperature and are defective in the localization of the exocyst component Sec3p at the non-permissive temperature (Zhang *et al.*, 2001). In contrast, *cdc42^{G60D}* cells form multiple buds per mother per nuclear cycle at the permissive temperature and the mutant Cdc42p appears to be hyperactive (Caviston *et al.*, 2002). Because these *cdc42* alleles cause distinct morphological defects at the permissive temperature, we thought that we might be able to identify some allele-specific multicopy suppressors. Screens with both alleles led to the identification of a novel gene, *PXL1* (YKR090W). Multicopy *PXL1* or *MSB1*, another gene isolated from both screens and a known multicopy suppressor of loss-of-function *cdc24*-Ts and *cdc42*-Ts alleles (Bender and Pringle, 1989), suppressed both *cdc42-201* and *cdc42^{G60D}* mutants effectively at 35.5°C (Figure 1). The suppression by *MSB1* suggests that both *cdc42* mutants behave as loss-of-function alleles at the nonpermissive temperature. We also found that multicopy *PXL1* did not suppress the morphological defects of either *cdc42* mutant significantly at the permissive temperature (our unpublished data). In addition, multicopy *PXL1* failed to suppress temperature-sensitive growth phenotype of three other *cdc42*-Ts alleles, *cdc42-1* (Adams *et al.*, 1990), *cdc42^{V36G}* (Caviston *et al.*, 2003), and *cdc42-13* (Zhang *et al.*, 2001; our unpublished data), suggesting that the suppression of *cdc42* mutants by *PXL1* is allele-specific. Together, these results suggest that *PXL1* is somehow involved in a Cdc42p-mediated function.

Pxl1p Is a Novel LIM Domain-containing Protein

Pxl1p, a protein of 706 amino acids, contains two LIM domains at its C-terminus (Figure 2A). Database search revealed that Pxl1p displays amino acid sequence homology and similar structural organization with paxillin (Figure 2), an adaptor protein involved in focal adhesion signaling (Turner, 2000a). Pxl1p and chicken paxillin share 15% identity and 26% similarity over their entire length and both contain tandem LIM domains at their C-termini separated

by an eight-amino acids linker (Figure 2). There are two leucine-rich stretches in Pxl1p, ³⁴⁰VEDLSLDGL³⁴⁸ and ³⁷²VEQLIAQLDDVSL³⁸⁴, which resemble the LD motifs of paxillin (Figure 2B; Brown *et al.*, 1996, 1998; Tumbarello *et al.*, 2002). The strongest homology between these two proteins resides in their LIM domains (Figure 2C), with 30% identity. Both LIM1 (556–612) and LIM2 (621–672) of Pxl1p resemble the LIM3 of paxillin the most in terms of the composition of eight zinc-chelating amino acids (Figure 2C). Homologous proteins with C-terminal LIM domains are also present in other fungal, including SPBC4F6.12 in *Schizosaccharomyces pombe* and IPF9950 in *Candida albicans*.

Pxl1p Is Not Essential for Cell Viability and Localizes to the Sites of Polarized Growth

PXL1 is not essential for vegetative growth, as *pxl1Δ* cells are viable in our strain background and in the systematic deletion project (Saccharomyces Genome Database). In addition, we failed to detect any obvious morphological and growth abnormalities associated with *pxl1Δ* cells at temperatures of 20–37°C on plates with or without 1 M sorbitol (our unpublished data). Moreover, there was no enhanced polarity defect observed between *pxl1Δ* and deletion of a number of polarity genes including *msb1Δ*, *msb3Δ*, *msb4Δ*, *gic1Δ*, *gic2Δ*, *bni1Δ*, *pea2Δ*, *bud6Δ*, and *ste20Δ*.

To gain some hint on how Pxl1p might function in Cdc42p-mediated pathway, we examined the localization of Pxl1p in yeast cells. We tagged the chromosomal copy of *PXL1* with GFP to its C-terminus. The *PXL1*-GFP construct thus functioned as the sole source of *PXL1* gene under the control of its own promoter. Similar to Cdc42p and other polarity proteins, Pxl1p-GFP localized to the sites of polarized growth in a cell cycle-regulated manner (Figure 3A). It first appeared at the presumptive bud site. After bud emergence, it formed a cap at the tip of a small bud. As the bud enlarged, the Pxl1p-GFP cap gradually diffused and eventually became invisible. At the time of cytokinesis, Pxl1p-GFP appeared at the mother-bud neck.

In cultured fibroblasts, paxillin localizes to focal adhesions (Brown *et al.*, 1996). We expressed a GFP-paxillin fusion protein in yeast cells and found that paxillin displayed an essentially identical localization pattern with Pxl1p (Figure 3B). These findings suggest that the mechanisms underlying the targeting of Pxl1p and paxillin to discrete cortical regions of the cell may be conserved, and that certain similarity exists between the bud tip of a yeast cell and the focal adhesion of a mammalian cell, two seemingly different structures.

Identification of LIM Domains as the Primary Targeting Element in Pxl1p

To determine which portion of the protein is responsible for targeting Pxl1p to the sites of polarized growth, we generated N-terminal GFP-*PXL1* fusion constructs by cloning different fragments of *PXL1* into pUG36 vector and monitored the localization of these GFP-tagged proteins in *pxl1Δ* cells. All these fusion proteins were expressed well as determined by immunoblotting with an anti-GFP antibody (Figure 4A). The full-length GFP-Pxl1p, not any of its truncated derivatives, expressed from the *MET25* promoter in pUG36 was at least partially functional, because pUG36-*PXL1* complemented *cdc42-201* mutant at 30°C (Figure 4B), but not at 35.5°C. In contrast, GFP-tagged full-length or C-terminal portion of paxillin expressed from pUG36 plasmid failed to complement *cdc42-201* at 30°C (Figure 4B). The full-length GFP-Pxl1p expressed from pUG36-*PXL1* showed an identical localization pattern with the full-length GFP-Pxl1p ex-

Figure 2. Pxl1p is a LIM-domain protein and shares sequence homology with paxillin. (A) The schematic diagram of domain structure in Pxl1p and chicken paxillin, which contains five LD motifs and four LIM domains. (B) Alignment of the N-terminal portion of Pxl1p (37–555) with that of chicken paxillin (1–325) using MacVector software. Identical and similar residues are highlighted with bold letters in dark shade and regular letters in gray shade, respectively. The two lines above the sequence indicate LD motif-like sequences in Pxl1p. (C) Alignment of individual LIM domains from Pxl1p and chicken paxillin using MacVector software. Residues conserved in more than half of the aligned sequences are shaded. The eight residues that form the core of double zinc-fingers are indicated with the boxes.

pressed from its native promoter (Figure 4C). The N-terminal portion (1–555) of Pxl1p was capable of targeting itself to the sites of polarized growth. However, its targeting capacity was greatly reduced. In contrast, the C-terminal portion (548–706) of Pxl1p, particularly the two LIM domains (548–680) showed an identical localization pattern with the full-length protein (Figure 4, C and D). These results indicate that the primary element involved in targeting Pxl1p to the sites of polarized growth resides in the two LIM domains. When analyzed separately, each LIM domain showed much reduced targeting capacity, especially for LIM2 (Figure 4, C

and D), suggesting that the two LIM domains may work synergistically to promote maximal targeting.

Besides the localization to the cortical sites, a pool of full-length Pxl1p also localized to the nucleus in a few cells (our unpublished data; Figure 4D). The N-terminal portion of Pxl1p did not localize to the nucleus. In contrast, all the Pxl1p fragments containing the LIM domains, particularly LIM2 alone, displayed strong nuclear localization (Figure 4, D and F), suggesting that the nuclear localization of Pxl1p may also be mediated by its LIM domains.

Figure 3. Pxl1p and paxillin both localize to the sites of polarized growth. (A) Cells of strain JPC267 (*PXL1-GFP*) were grown in YPD media and incubated at 24°C for 12–16 h before visualization. (B) The localization of GFP-paxillin fusion protein in yeast cells. The pUG36 vector carrying full-length chicken paxillin was transformed into strain JPC272 (*pxl1Δ*). Cells were grown in SC-Ura media and visualized for GFP.

Because the C-terminal LIM domains of paxillin are the primary targeting element to focal adhesions in fibroblasts (Brown *et al.*, 1996; Thomas *et al.*, 1999), we asked whether these LIM domains also mediate the localization of paxillin to the sites of polarized growth in yeast. In contrast to the full-length protein, the LIM domains of paxillin mainly localized to the nucleus (Figure 4, E and F). Its targeting ability to the sites of polarized growth was greatly reduced (Figure 4E) and its localization pattern largely resembled that of the LIM2 of Pxl1p. It is not clear whether the reduced localization to the sites of polarized growth for the LIM2 of Pxl1p or the LIM domains of paxillin is due to their decreased affinity for a cortical protein or due to their increased nuclear targeting activity in the absence of other portions of the molecules. On the other hand, the N-terminal portion of paxillin targeted to the sites of polarized growth very efficiently (Figure 4E), suggesting that this portion of paxillin is capable of interacting with protein(s) localized at the sites of polarized growth.

Pxl1p Binds to Rho1p and Downregulates Its Function

Besides Pxl1p, the budding yeast genome encodes three additional LIM domain-containing proteins: Rga1p (Stevenson *et al.*, 1995; Chen *et al.*, 1996), Rga2p (Gladfelter *et al.*, 2002; Smith *et al.*, 2002), and Lrg1p (Muller *et al.*, 1994; Watanabe *et al.*, 2001). All three contains at least two LIM domains at their N-termini and a Rho GAP (GTPase-activating protein) domain at their C-termini, and they are indeed GAPs for Rho-family GTPases including Cdc42p and Rho1p (Chen *et al.*, 1996; Watanabe *et al.*, 2001; Gladfelter *et al.*, 2002; Smith *et al.*, 2002). Because some LIM domains are involved in protein dimerization (Sanchez-Garcia *et al.*, 1993; Xue *et al.*, 1993; Feuerstein *et al.*, 1994), we speculated that Pxl1p might regulate Cdc42p and Rho1p function through interacting with either Rga1p, Rga2p, or Lrg1p. However, we failed to detect any interactions between Pxl1p and Rga1p, Rga2p, or Lrg1p with two-hybrid assays. In addition, a GFP-Pxl1p fusion protein localized well in *rga1Δ rga2Δ lrg1Δ* cells (our unpublished data).

To understand the molecular basis for the observed genetic interaction between *PXL1* and *CDC42*, we asked whether Pxl1p binds directly to Cdc42p *in vitro*. We performed a protein-binding assay using purified MBP-Pxl1p and GST-Cdc42p expressed in *E. coli*. GST-Rho1p was included as a control. We did not observe any significant interaction between Pxl1p and Cdc42p that was stripped off nucleotide with EDTA or loaded with either GDP or GTP-γS (Figure 5A). Surprisingly, we found that Pxl1p bound preferentially to Rho1p-GDP, indicating that Pxl1p interacts directly with the inactive form of Rho1p.

To determine the functional consequences of the Pxl1p-Rho1p interaction, we examined the overexpression effect of

Pxl1p on the growth of several *rho1*-Ts mutants. Mild overexpression of *PXL1* under its own promoter carried on high-copy plasmids appeared to weakly inhibit the growth of *rho1-2* and *rho1-3* mutants (our unpublished data). When *PXL1* was placed under the control of a galactose-inducible promoter *pGAL1*, overexpression of Pxl1p strongly inhibited the growth of *rho1-2* and *rho1-3* mutants at 30°C, but not *rho1-5* and wild-type cells (Figure 5B). These findings suggest that Pxl1p downregulates Rho1p function by directly binding to its inactive form. However, it is not clear whether the suppression of *cdc42*-Ts mutants by Pxl1p is due to its inhibitory effect on the Rho1p function.

Functional Interactions between Cdc42p and Rho1p Signaling Pathways

The observations that overexpression of Pxl1p suppressed *cdc42*-Ts, but inhibited *rho1*-Ts mutants suggest that Cdc42p and Rho1p signaling pathways might be antagonistic to each other during budding and that Pxl1p is involved in coordinating this relationship between Cdc42p and Rho1p. If so, overexpression of Cdc42p might inhibit Rho1p function, and vice versa. Indeed, overexpression of Cdc42p from a galactose-inducible promoter inhibited the growth of *rho1-2* and *rho1-3* mutants at 30°C, a semipermissive temperature for both mutants (Figure 6A). Reciprocally, overexpression of Rho1p from a galactose-inducible promoter inhibited the growth of *cdc42-201* mutant, but not *cdc42^{G60D}* mutant or the wild-type strain at 24°C and at 30°C (Figure 6B). Because both the wild-type strain and the hyperactive *cdc42^{G60D}* mutant could tolerate increased expression of Rho1p at and below 30°C, these findings suggest that overexpression of Rho1p inhibits cell growth only when Cdc42p function was compromised.

We also found that overexpression of Cdc42p inhibited *pxl1Δ rho1-2* and *pxl1Δ rho1-3* mutants at 30°C, and that overexpression of Rho1p inhibited *pxl1Δ cdc42-201* mutants at 24°C or at 30°C (our unpublished data). These results suggest that Pxl1p is not the sole mediator between Cdc42p and Rho1p signaling pathways, which is consistent with the fact that deletion of *PXL1* did not produce any obvious phenotype.

DISCUSSION

Pxl1p Is a Zyxin/Paxillin-family Protein

Proteins that contain LIM domains can be roughly classified into five groups: LIM homeodomain proteins, LIM only proteins, LIM kinases, GTPase-activating proteins (GAPs), and zyxin/paxillin-family proteins. In *S. cerevisiae*, four proteins, Rga1p, Rga2p, Lrg1p, and Pxl1p, contain LIM domains. The first three are Rho GAPs and they have a similar

Figure 4. Pxl1p localizes to the sites of polarized growth through its C-terminal LIM domains. (A–D) The pUG36 vector carrying the full-length Pxl1p (FL, 1–706), N-terminal portion (N-term, 1–555), C-terminal portion (C-term, 548–706), two LIM domains (LIM1 + 2, 548–680), LIM1 (548–620), and LIM2 (613–680), along with the full-length chicken paxillin (FL, 1–559) or its C-terminal portion (C-term, 309–559) was transformed into JPC272 (*pxl1Δ*) cells. Cells were grown in SC-Ura media. (A) Expression of the GFP-fusion proteins. Cell lysates prepared from the strains described above were separated by a 10% SDS-PAGE and immunoblotted with an anti-GFP antibody. The position of different GFP-fusion proteins on the blot is indicated by an asterisk. (B) Overexpression of GFP-Pxl1p fusion protein suppresses *cdc42-201* mutant. Yeast strain YEF2258 (*cdc42-201*) carrying plasmids pUG36, pUG36-PXL1, pUG36-PXL1 N-term, pUG36-PXL1 C-term, pUG36-PXL1-LIM1 + 2, pUG36-PXL1-LIM1, pUG36-paxillin, and pUG36-paxillin C-term was streaked onto SC-Met-Ura plate and incubated at 30°C for 4 days. Control SC-Ura plate was incubated at 24°C for 4 days. (C) Localization of different GFP-tagged Pxl1p fragments. (D) A summary on the localization of GFP-tagged Pxl1p fragments. Strength in targeting capacity is indicated from strong (+++++) to very weak (+/-) or no detection (-). (E) Localization of paxillin N-term and paxillin C-term fused with GFP. (F) GFP-Pxl1p-LIM2 and GFP-paxillin C-term localize to the nucleus. Yeast strain JPC272 (*pxl1Δ*) carrying pUG36-PXL1-LIM2 and pUG36-paxillin C-term was fixed with formaldehyde and stained for DNA.

structural organization, with the LIM domains at their N-termini and the Rho GAP domain at their C-termini (Muller *et al.*, 1994; Stevenson *et al.*, 1995; Chen *et al.*, 1996; Watanabe *et al.*, 2001; Gladfelter *et al.*, 2002; Smith *et al.*, 2002). Several lines of evidence suggest that the novel Pxl1p belongs to the zyxin/paxillin family. First, the LIM domains of Pxl1p are located at its C-terminus, resembling zyxin/paxillin-family

proteins. Second, Pxl1p shares overall sequence homology with paxillin. Third, Pxl1p and paxillin display a similar localization pattern in yeast. Finally, like paxillin and Hic-5 (Brown *et al.*, 1996; Thomas *et al.*, 1999), the LIM domains of Pxl1p act as the major targeting element for its localization to the sites of polarized growth. This similarity in targeting paxillin-like proteins to a cortical site in yeast and mamma-

Figure 5. Physical and genetic interactions between Pxl1p and Rho1p. (A) In vitro binding assay was performed using MBP-Pxl1p, GST-Cdc42p, and GST-Rho1p. Equal amount of purified GST-Cdc42p, GST-Rho1p, or GST alone, was either stripped off nucleotide (–nt) or loaded with GDP or GTP- γ -S, and then incubated with MBP-Pxl1p bound to Amylose resin. After washing, flow-through and bound fractions were collected and detected by immunoblotting with an anti-GST antibody. (B) Overexpression of Pxl1p inhibits the growth of *rho1-2* and *rho1-3* cells. Wild-type (WT, NY2284), *rho1-2* (NY2285), *rho1-3* (NY2286), and *rho1-5* (NY2287) strains were transformed with plasmid YEp24 (Vec), or YEp24-GAL1-PXL1. Transformants were streaked onto SC-Ura plates containing dextrose (Dextrose) and SC-Ura plates containing 2% galactose plus 1% raffinose (Galactose), and incubated at 30°C for 3 days.

lian cells suggests that the yeast Pxl1p can be used as a model to address the important question in paxillin biology: how is paxillin initially recruited to the focal adhesion site?

Coordination between Cdc42p and Rho1p GTPases during Budding

We isolated *PXL1* as a multicopy suppressor of *cdc42-Ts* mutants from two independent screens. One interpretation for this genetic interaction is that Pxl1p is a direct regulator or downstream effector of Cdc42p. However, we failed to detect any significant interaction between Pxl1p and Cdc42p regardless of its nucleotide-bound state (Figure 5A). We also failed to detect any interactions by two-hybrid assays between Pxl1p or its N- and C-terminal fragments and Cdc42p, or a number of Cdc42p regulators such as its GAPs, Rga1p and Rga2p; the scaffold protein Bem1p; and its effectors such as the formin Bni1p; and PAKs Ste20p, Cla4p, and Skm1p (our unpublished data). In addition, overexpression of Pxl1p did not suppress *cdc42-Ts* mutants, *cdc42-4* and *cdc42-11*, which are defective in the guanine-nucleotide-exchange factor (GEF) activity for Cdc42p (Zheng *et al.*, 1994; our unpublished data), suggesting that the suppression of *cdc42* mutants by Pxl1p is not mediated by Cdc42p GEF. Thus, it remains an open question how Pxl1p mechanistically regulates Cdc42p signaling pathway.

We also showed here that Pxl1p binds to Rho1p-GDP in vitro and that overexpression of Pxl1p inhibited the growth of *rho1* mutants. Such a functional connection between Pxl1p and Rho1p is supported by additional genetic interactions observed between Pxl1p and other components of the Rho1p signaling pathway such as the Rho1p GAP, Bem2p (Zheng *et al.*, 1993), and the Rho1p effector, Fks1p (Drgonova *et al.*, 1996; Qadota *et al.*, 1996; Mackin *et al.*, 2004). Together, these results suggest that Pxl1p might coordinate Cdc42p and Rho1p signaling pathways by exerting opposite effects on them. These data also raise the possibility that Cdc42p and Rho1p might be functionally antagonistic during budding. Indeed, we observed that overexpression of Cdc42p inhibited the growth of some *rho1* mutants, and vice versa. Thus, it appears that Pxl1p might mediate this functional relationship between Cdc42p and Rho1p. However, Pxl1p cannot be the sole linker between these GTPases, because deletion of *PXL1* did not produce any obvious defect in polarized growth and, in addition, did not affect the mutual inhibition displayed by overexpression of Cdc42p or Rho1p in the relevant mutants. Currently, we are trying to identify genes

Figure 6. Antagonistic relationship between Cdc42p and Rho1p signaling pathways. (A) Overexpression of Cdc42p inhibits the growth of *rho1-Ts* mutants. Yeast strains NY2284 (WT), NY2285 (*rho1-2*) and NY2286 (*rho1-3*) carrying plasmid YEp352 (Vec) or YEp352-GAL-CDC42 were streaked onto SC-Ura plates containing dextrose (Dextrose) and SC-Ura plates containing 2% galactose and 1% raffinose (Galactose), and incubated at 30°C for 3 days. (B) Overexpression of Rho1p inhibits the growth of *cdc42-201*, but not *cdc42^{G60D}* mutant. Yeast strains YEF473A (WT), YEF2258 (*cdc42-201*), and JPC241 (*cdc42^{G60D}*) carrying plasmid pYES2 (Vec) or pYES2-RHO1 (GAL-RHO1) were streaked onto SC-Ura plates containing 2% galactose and 1% raffinose (Galactose), and incubated at 24 or 30°C for 3 days. Control SC-Ura plate containing dextrose (Dextrose) was incubated at 24°C for 3 days.

that act in parallel to *PXL1* in coordinating the functions of Cdc42p and Rho1p during budding.

Conditional inactivation of Cdc42p blocks bud emergence, resulting in large, round, and unbudded cells. Thus, Cdc42p is generally believed to act at the very early stage and be continuously required during the budding process (Adams *et al.*, 1990; Johnson, 1999; Gladfelter *et al.*, 2002). In contrast, conditional inactivation of Rho1p results in cell lethality with cells arrested predominantly as small-budded cells (Yamochi *et al.*, 1994). Thus, it appears that Cdc42p and Rho1p act sequentially during bud formation. However, this simple interpretation is complicated by the fact that certain alleles of *RHO1* also cause defects at bud emergence as well as in actin organization in a similar way as most *cdc42* mutants do (Guo *et al.*, 2001). In addition, Cdc42p and Rho1p share some effectors, such as Bni1p and Sec3p (Kohno *et al.*, 1996; Evangelista *et al.*, 1997; Guo *et al.*, 2001; Zhang *et al.*, 2001); thus, the relationship between Cdc42p and Rho1p may be more complex than previously envisioned. We speculate that Cdc42p and Rho1p may act cyclically to promote bud growth. Cdc42p may promote the initial membrane expansion and the loosening of the cell wall at the bud tip by controlling actin dynamics very much in the same way as Cdc42 and Rac promote the initial membrane expansion and/or cell spreading in animal cells. On the other hand, Rho1p might fix the expanded membrane at the bud tip by regulating cell wall synthesis through its effector Pkc1p and Fks1p/Fks2p and/or by controlling the delivery of cell wall materials to the bud tip via its effector Bni1p-nucleated actin cables. This hypothesis reconciles the "sequential" and "mutually inhibitory" relationships between Cdc42p and Rho1p. Further studies must be carried out to determine how Cdc42p and Rho1p are coordinated at the molecular level during bud formation.

Studies in mammalian cells also indicate a coordinated regulation of small GTPases in specific cellular functions. During cell spreading, the binding of integrins to the extracellular matrix triggers the rearrangement of the actin cytoskeleton by regulating the activity of Rho family GTPases, Cdc42, Rac1, and RhoA. A transient localized downregulation of RhoA activity is critical for the initial phase of cell adhesion (Ren *et al.*, 1999; Arthur *et al.*, 2000). A recent study showed that paxillin plays a role in the local suppression of RhoA activity by binding to p120RasGAP, which releases its normal binding partner p190RhoGAP to downregulate RhoA activity (Tsubouchi *et al.*, 2002). These and our studies suggest that the inhibitory function of paxillin and its yeast homologue, Pxl1p, on Rho GTPases appears to be an evolutionarily conserved feature, although the molecular details may differ.

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