**Schizosaccharomyces pombe** Pxl1 Is a Paxillin Homologue That Modulates Rho1 Activity and Participates in Cytokinesis

Mario Pinar, Pedro M. Coll, Sergio A. Rincón, and Pilar Pérez

Instituto de Microbiología Bioquímica, Consejo Superior de Investigaciones Científicas/Departamento de Microbiología y Genética, Universidad de Salamanca, Edificio Departamental, 37007 Salamanca, Spain

Submitted July 28, 2007; Revised January 17, 2008; Accepted January 29, 2008
Monitoring Editor: Daniel Lew

**Schizosaccharomyces pombe** Rho GTPases regulate actin cytoskeleton organization and cell integrity. We studied the fission yeast gene SPBC4F6.12 based on its ability to suppress the thermosensitivity of cdc42-1625 mutant strain. This gene, named *pxl1*, encodes a protein with three LIM domains that is similar to paxillin. Pxl1 does not interact with Cdc42 but it interacts with Rho1, and it negatively regulates this GTPase. Fission yeast Pxl1 forms a contractile ring in the cell division region and deletion of *pxl1* causes a delay in cell–cell separation, suggesting that it has a function in cytokinesis. Pxl1 N-terminal region is required and sufficient for its localization to the medial ring, whereas the LIM domains are necessary for its function. Pxl1 localization requires actin polymerization and the actomyosin ring, but it is independent of the septation initiation network (SIN) function. Moreover, Pxl1 colocalizes and interacts with Myo2, and Cdc15, suggesting that it is part of the actomyosin ring. Here, we show that in cells lacking Pxl1, the myosin ring is not correctly assembled and that actomyosin ring contraction is delayed. Together, these data suggest that Pxl1 modulates Rho1 GTPase signaling and plays a role in the formation and contraction of the actomyosin ring during cytokinesis.

**INTRODUCTION**

The Rho family of GTPases regulates a variety of morphologic events leading to actin cytoskeleton remodeling in all eukaryotic cells (Ridley, 2006). Additionally, Rho GTPases regulate cell wall biosynthesis and cell integrity in fungal cells (Levin, 2005; García et al., 2006; Park and Bi, 2007). **Schizosaccharomyces pombe** is a highly polarized yeast growing by elongation of its ends and dividing by medial fission. Its genome contains six genes coding for Rho GTPases (Miller and Johnson, 1994). Cdc42 is positively regulated by two GDP-GTP exchange factors (GEFs): Scd1, which is required to maintain apical growth (Chang et al., 1994); and Gef1, which plays a role in cytokinesis and in the switch to bipolar growth (Coll et al., 2003; 2007; Hirota et al., 2003). No negative regulator for Cdc42 has been described yet. *rho1* is also an essential gene that is required for maintenance of cell integrity and polarization of the actin cytoskeleton (Arellano et al., 1997). Rhof1 functions are mediated by at least three targets, the (1,3)-β-D-glucan synthase (Arellano et al., 1996) and the kinases Pck1 and Pck2 (Arellano et al., 1999b). Rhof1 GTPase is positively regulated by at least three GEFs: Rgf1 and Rgf2, involved in the formation of the cell wall; and Rgf3, involved in cytokinesis in fission yeast (Tajadura et al., 2004; Morrell-Falvey et al., 2005; Mutoh et al., 2005). Rhof1 is negatively regulated by at least three GAPs (GTPase-activating proteins): Rga1, Rga5, and Rga8 (Nakano et al., 2001; Calonge et al., 2003; Yang et al., 2003).

*S. pombe* cytokinesis involves the assembly of a contractile actomyosin medial ring attached to the membrane (Feierbach and Chang, 2001). The initial phase of cytokinesis is the establishment of the division site, which is defined by the position of the interphase nucleus, usually in the center of the cell, and requires the participation of the kinase Pom1, the Polo kinase Plo1, and Mid1, a protein with a PH domain (Burgess and Chang, 2005; Wolfe and Gould, 2005). Mid1, which is in the nucleus during interphase, moves to the cytoplasm at the beginning of mitosis, before the segregation of chromosomes, due to the action of Plo1 and establishes a broad band of small dots in the cell cortex that surrounds the nucleus. There Mid1 recruits the proteins that will form the medial actomyosin ring (Burgess and Chang, 2005; Wolfe and Gould, 2005). Conventional myosin II recruitment is one of the earliest events after spindle pole body separation. This myosin II is formed by hexamers with a pair of Myo2 heavy chains, a pair of Cdc4 essential light chains (Cdc4), and a pair of Rcl regulatory light chains (Motegi et al., 2000; Naqvi et al., 2000). *S. pombe* has another unconventional myosin II heavy chain, Myo3/Myp2, which is not required for the formation of the ring (Bezanilla and Pollard, 2000). The C-terminal region of Myo2 is sufficient for the myosin II accumulation to the broad band that is dependent on Mid1 and independent of F-actin (Motegi et al., 2004). Over a period of 10 min after myosin II accumulates, the IQGAP protein Rng2, the PCH protein Cdc15, and the formin Cdc12 accumulate in the dots of the broad band (Wu et al., 2003). This is followed quickly by condensation of the dots into a contractile ring in an F-actin–dependent manner that also requires the motor activity of Myo2 (Naqvi et al., 1999; Le Goff et al., 2000). It remains poorly understood how the myosin filaments assemble into a ring in association with...
actin and other proteins during late mitosis. Once the ring is formed, a set of proteins named septation initiation network (SN), triggers the contraction of the actomyosin ring coordinated with the synthesis of the primary and the secondary septa that will form the new cell wall (Krapp et al., 2004; Wolfe and Gould, 2005). Mutants in components of the SN form the ring correctly, but they do not contract the ring and deposit the septum material. The SN controls the formation of the septum, probably by means of the regulation of the glucan synthase Bgs1/Cps1, but the mechanism is not known (Le Goff et al., 1999). SIN proteins such as Cdc11 and Cdc14 are necessary for Bgs1 location (Cortes et al., 2002).

Nevertheless, any direct interaction of Bgs1 with the SIN complex or with the contractile ring has not been described. So far, Rho1 GTPase is the only known direct activator of the glucan synthase (Arellano et al., 1996).

In this article, we report the characterization of a S. pombe protein that is similar to animal cell pxl1 and to Saccharomyces cerevisiae Pxl1. Pxl1 is a LIM domain-containing adaptor protein localized to focal adhesions of adherent cells, in which it modulates RhoA activity, and it has been implicated in the regulation of cytoskeletal organization and cell motility (Turner, 2000; Brown and Turner, 2004; Carragher and Frame, 2004). S. cerevisiae Pxl1 is a LIM domain-containing protein that modulates Rhô1 activity, and it is required for selection and/or maintenance of polarized growth sites (Gao et al., 2004; Mackin et al., 2004). LIM motifs are cysteine- and histidine-rich, zinc-coordinating domains composed of two zinc fingers. They are protein–protein interaction motifs critically involved in processes such as gene expression, cytoskeleton organization, cell adhesion, cell motility, and signal transduction (Kadrmas and Beck, 2004). We show here that S. pombe pxl1 homologue, Pxl1, modulates Rhô1 activity in the same way that S. cerevisiae Pxl1 does, but it is not required for polarized growth at the cell poles. Instead, it participates in the actomyosin ring formation and constriction during cytokinesis.

**MATERIALS AND METHODS**

### Fission Yeast Strains, Media, and Techniques

Standard S. pombe media and genetic manipulations were used (Moreno et al., 1991). All the strains were isogenic to wild-type strains 972 h+ and 975 h+, and they are described in Supplemental Table 1. The strains were constructed by either tetrad dissection or random spore germination method. Cells were usually grown in rich medium (YES) or minimal medium (EMM) supplemented with the necessary requirements. Escherichia coli DH5α was used as host for propagation of plasmids. Cells were grown in LB medium supplemented with 50 μg/ml ampicillin when appropriate. Solid media contained 2% agar.

### Plasmids and Strains Construction

The pxl1+ promoter-containing vectors pREP41X, pREP81X, pREP41-GFP, and pREP1-GST (Forsburg and Sherman, 1997) were used for the overexpression of pxl1+ that was induced by growing the cells transformed with these plasmids in the absence of thiamine for 12 h. To delete pxl1+ from the S. pombe genome, the whole open reading frame was replaced with the kanMX6 gene or the ura4+ gene by PCR-based gene targeting as described previously (Bähler et al., 1998). Stable transformants were selected and sporulated. Dissected tetrads were screened by PCR or Southern blot for the appropriate gene replacement. Genomic versions of pxl1+ with green fluorescent protein (GFP), glutathione transferase (GST), cherry red fluorescent protein (RFP), and the hemagglutinin (HA) epitope coding sequences fused at the 5′ end of the open reading frame (ORF) were generated by cloning into a BlueScript plasmid. The EcoRI site of the 5′ pxl1+ flanking sequence, the corresponding tagging sequence, the pxl1+ ORF, and 460 base pairs of the 3′ pxl1+ flanking sequence. The resulting constructs were cloned into the integrative vector pJK148 that was then cut with NdeI and integrated at the leu1 locus of the leu1-32 ura4-D18 pxl1+ strain. Transformant clones were selected in EMM without leucine and screened by PCR for the appropriate gene integration. Genomic versions of truncated GFP-pxl1 were integrated at the leu1+ locus of the leu1-32 ura4-D18 pxl1Δ strain following the same strategy.

**Pull-Down and Immunoprecipitation**

Extracts from 5 × 10^8 cells expressing the different tagged proteins were obtained as described previously (Arellano et al., 1997), using 200 μl of lysis buffer (20 mM Tris-HCl, pH 8.0, 2 mM EDTA, 100 mM NaCl, and 0.5% NP-40) containing 100 μM p-aminophenyl methanesulfonyl fluoride, 2 μg/ml leupeptin, and 2 μg/ml aprotinin. Cell extracts (1 mg of total protein) were incubated with glutathione-Sepharose (GS) beads or with the corresponding antibody and protein A-Sepharose beads for 2–4 h at 4°C. The beads were washed four times with lysis buffer, and then they were resuspended in sample buffer. Proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred to Immobilon-P membranes (Millipore, Billerica, MA), and blotted to detect GST-, HA-, or GFP-fused epitopes with the corresponding antibodies and the enhanced chemiluminescence (ECL) detection kit (GE Healthcare, Buckinghamshire, United Kingdom). Total amount of protein was monitored in cell extracts aliquots and 30 μg of total protein was used directly for Western blot.

**In Vivo Analysis of Rho1 Activity**

The expression vector pGEX-RBD (Rho binding domain of rhokin) (Reid et al., 1996) was used to transform E. coli. The fusion protein was produced according to the manufacturer’s instructions and immobilized on glutathione-Sepharose 4B beads (GE Healthcare).

The amount of GTP-bound Rho1 was determined as described previously (Calonge et al., 2003) using a pull-down assay modified from (Ren et al., 1999).

**Microscopy Techniques**

For confocal staining, exponentially growing S. pombe cells were harvested, washed, and resuspended in a confocal solution (0.1 mg/ml) for 5 min at room temperature. After washing with water, cells were observed. Actin staining was performed with phallolidin-Alexa Fluor 488. To disintegrate F-actin, latrunculin A (Lat A) dissolved in dimethyl sulfoxide (DMSO) at 50 mM was added to S. pombe cultures to a final concentration of 50 μM.

Cell samples were observed using a DMXRA microscope (Leica, Wetzlar, Germany) equipped for Nomarski optics and epifluorescence, and photographed with a Sensys camera (Photometrics, Tucson, AZ). Confocal microscopy was performed on a Leica TCS SL microscope, and the images were analyzed with the Leica confocal software.

**RESULTS**

Pxl1 Suppresses the Thermosensitive Growth of cdc42-1625

We have constructed some thermosensitive S. pombe strains carrying different cdc42 mutant alleles with the purpose of identifying allele-specific multicytokinesis suppressors that might participate in the Cdc42 signaling pathway. A thermosensitive mutant, cdc42-1625, was previously found to be defective in actin cable formation and secretion (Martin et al., 2007; our unpublished data). cdc42-1625 thermosensitive growth was suppressed by mild overexpression of cdc42+, sdc1+, and gef1+ but not of shkl+ or shk2+, the two known Cdc42 effectors (Martin et al., 2007; Figure 1A). It was described previously that S. cerevisiae PXL1, which codes for a pxl1 homologue, was able to specifically suppress some loss-of-function cdc42 alleles defective in the localization of the exocyst component Sec3 (Gao et al., 2004). We therefore cloned SPB4C46.12, an ORF coding for a protein with similarity to S. cerevisiae Pxl1, and we tested whether overexpression of this ORF, named pxl1+, was able to suppress cdc42-1625 thermosensitive growth. As shown in Figure 1A, pxl1+ was able to rescue cdc42-1625 growth at 36°C, at the same level as sdc1+, and more efficiently than gef1+, because pxl1+ rescued the growth even when the promoter was.
S. pombe Pxl1 Interacts with Rho1 and Has a Function in Cytokinesis

LIM domains can interact specifically with other LIM domains and with many other protein domains mediating protein–protein interactions. Interestingly, the three other S. pombe proteins containing LIM domains, Rga1, Rga3, and Rga4, are Rho-GAPs (Nakano et al., 2001). We could not detect a direct interaction between Pxl1 and Cdc42 by co-immunoprecipitation, and it has been described that S. cerevisiae Pxl1 directly binds to Rho1 in vitro (Gao et al., 2004). To examine whether there was interaction between Pxl1 and Rho1, we performed coprecipitation experiments using extracts from cells carrying an HA epitope-tagged rho1Δ (Arellano et al., 1997) and GFP-pxl1Δ expressed from their own promoters. Cell extracts were immunoprecipitated with anti-GFP and protein A-Sepharose. As shown in Figure 2A, a band corresponding to HA-Rho1 was specifically detected. These results indicate that Pxl1 binds to Rho1, as does S. cerevisiae Pxl1 (Gao et al., 2004).

To further investigate the function of pxl1Δ, the ORF of this gene was replaced with KanMX6 or ura4Δ genes in a diploid strain. Sporulation of the heterozygotic diploid strain containing one disrupted allele of pxl1Δ yielded tetradts with four viable spores, indicating that pxl1Δ is not essential for vegetative growth. The cells did not show any growth defect. However, there was a high percentage of septating cells in the pxl1Δ culture (49%; n = 200) compared with wild-type (16%; n = 200), and some multiseptated cells were observed (8%; n = 200) (Figure 2D). We could also see some misplaced septa (Figure 2B, see arrow), and some cells slightly swollen in the middle region. The cell separation defect was more noticeable at 25°C than at 36°C (data not shown).

We next analyzed the level of GTP-bound Rho1 in wild-type and pxl1Δ cells. Cells lacking Pxl1 always showed higher level of GTP-bound Rho1 than wild-type cells as measured by pull-down from the extracts using GST-RBD that contains the rho1kin binding domain (Figure 2C). The increase in GTP-bound Rho1 observed in cells lacking Pxl1 was slightly lower than that observed in cells lacking Rga5, a Rho1-specific GAP whose deletion also causes increase in septating cells (Calonge et al., 2003). By contrast, no change in the GTP-bound level of Cdc42 was observed using GST-CRIB (data not shown). These results indicate that Pxl1 is a negative regulator of Rho1 in vivo. Moreover, taking into consideration that Pxl1 overexpression suppressed cdc42Δ-1625 Thermosensitivity and inhibited Rho1, it is tempting to propose that Cdc42 and Rho1 signaling pathways might be antagonistic to each other, as has been suggested previously for both S. cerevisiae (Gao et al., 2004) and S. pombe (Yang et al., 2003).

Rho1 signaling is required to maintain S. pombe cell integrity, regulating the cell wall biosynthesis, mainly β-(1-3)-glucan biosynthesis, and the actin organization (Arellano et al., 1999a). To further study the Pxl1 effect on Rho1 signaling pathway, we analyzed pxl1Δ rga5Δ cells. This double mutant strain had a higher percentage of septating cells (57%; n = 200) than the single mutants (49 and 25% in pxl1Δ and rga5Δ cultures, respectively) (Figure 2D), indicating additive defects. We also mildly overexpressed Rho1 or the two Rho1-GEFs, rfg3Δ and rfg1Δ that are involved in cytokinesis and general cell wall synthesis, respectively (García et al., 2006). rho1Δ or rfg3Δ overexpression in wild-type cells caused a slight increase in septating cells (from 16 to 18 and 19%, respectively; n = 200), and it also increased even more the percentage of septating cells in pxl1Δ cultures (from 49 to 62 and 63%, respectively; n = 200). Remarkably, rfg3Δ overexpression also caused a dramatic increase in pxl1Δ multiseptated cells (from 8% to 28%; n = 200), suggesting that it has an additive effect on the pxl1Δ phenotype. By contrast, overexpression of rfg1Δ caused no effect in either wild-type or pxl1Δ cultures (Figure 2D).

We also analyzed the effect of pxl1 deletion on the phenotypes of cells carrying mutant alleles of rfg3Δ and rfg1Δ. ehs2-1 cells, carrying an rfg3Δ Thermosensitive allele, do not grow at 37°C and are hypersensitive to NaF (Sánchez, unpublished data), similar to lad1-1 cells that contain another rfg3Δ mutant allele (Morrell-Falvey et al., 2005). Elimination of Px1Δ was able to suppress both ehs2-1 phenotypes (Figures 2, E and F), likely due to the increase in Rho1 activity. By contrast, the lack of Pxl1 did not suppress the hypersensitivity of rfg1Δ cells to Caspofungin, a specific β-(1-3)-glucan synthase inhibitor (data not shown). Because Rgf3 activates Rho1 during cytokinesis and Rgf1 during apical growth (García et al., 2006), together our results suggest that Pxl1 is negatively modulating Rho1 activity specifically during cytokinesis.
Pxl1 Forms a Contractile Ring at the Medial Region and Its Concentration Is Higher during Cytokinesis

To gain further insight into the Pxl1 function, we examined the localization of Pxl1 by tagging the genomic locus with the GFP gene fused in frame to the 5’ end of pxi1+. The fusion to the 3’ end was nonfunctional. GFP-Pxl1 localized as a ring that contracts during septation and disappeared when the septum was formed (Figure 3, A and B). The Pxl1 ring was always in the inner edge of the primary septum that was specifically stained with calcofluor.

To further characterize the Pxl1 ring assembly and contraction, we performed time-lapse videomicroscopy by using a strain carrying both GFP-Pxl1 and Cut11-GFP; the latter localizes to the nuclear envelope and spindle pole body (SPB) in living cells (West et al., 1998). GFP-Pxl1 seemed very faint in the medial region of the cell cortex after SPB duplication, and it formed a brighter ring in late anaphase, before septation. When the nuclei separated, Pxl1 SPB duplication, and it formed a brighter ring in late anaphase, before septation. When the nuclei separated, Pxl1 was present at low levels throughout the cell cycle (Figure 3D).

pxi1+ is part of a gene cluster whose expression is under control of the transcription factors Sep1 and Ace2, which activate the cytokinesis program (Rustici et al., 2004; Alonso-Nunez et al., 2005). The expression of these genes, including pxi1+, peaks during late mitosis (Rustici et al., 2004). Additionally, Pxl1 can be visualized only in cells during cytokinesis; therefore, we considered the possibility that Pxl1 protein levels might be regulated in a cell cycle-dependent manner. HA-tagged Pxl1 was analyzed by Western blot in a synchronous population of cdc25-22 thermosensitive mutant cells, which arrest in late G2 at restrictive temperature (36°C). Cells were synchronized at 36°C during cell division was monitored to assess the synchrony. The maximum percentage of septa was reached at 75 min. HA-Pxl1 was present at low levels throughout the cell cycle (Figure 3D).

Pxl1 N-Terminal Region Is Sufficient for the Localization to the Division Area and the Three LIM Domains Are Required for Pxl1 Function

To determine which portion of Pxl1 is responsible for targeting Pxl1 to the site of cell division, we generated different N-terminal GFP fusion constructs containing fragments of pxi1+ that were expressed in pxi1Δ cells under the endogenous pxi1+ promoter (Figure 4A, bottom). Interestingly, the concentration of Pxl1 in the cell was much higher than that of the other Pxl1 truncations, whose protein level was very low, despite the fact that they were all expressed from the pxi1+ promoter. These results suggest that the N-terminal region regulates Pxl1 level or that the level of Pxl1 is regulated when this protein is correctly localized.

Only the full-length GFP-Pxl1 completely suppressed the cytokinesis defect of pxi1Δ cells (Figure 4B). None of the truncated derivatives were fully functional, but there was a
partial complementation by the construct lacking only the C-terminal LIM domain (Pxl1\(H9004\)). The Pxl1 truncation containing the three LIM domains but lacking the N-terminal part of the protein (Pxl1\(N\)) did not localize, and it seemed dispersed throughout the cytoplasm. On the contrary, the N-terminal portion of Pxl1 lacking the three LIM domains (Pxl1\(1,2,3\)) localized to the division area, although it did not rescue the phenotype of \(pxl1\)/\(H9004\) cells (Figure 4C). We conclude that the N-terminal region of Pxl1 is necessary and sufficient for its localization into the contractile ring, and the LIM domains are necessary for its function. In contrast, the LIM domains of \(S\). \(cerevisiae\) Pxl1 are the primary determinants for targeting the protein to the cortical sites of the budding yeast cells (Gao \textit{et al.}, 2004; Mackin \textit{et al.}, 2004). Indeed, we cloned \(S\). \(cerevisiae\) Pxl1 into the \(S\). \(pombe\) pREP41-GFP expression plasmid and used it to transform \(pxl1\Delta\) cells, but \(S\). \(cerevisiae\) GFP-Pxl1 was not localized to the division area and it was unable to suppress the cytokinesis defect of \(pxl1\Delta\) cells (data not shown).

**Pxl1 Localization to the Division Area Is Dependent on the Actomyosin Ring but Not on the SIN Pathway**

We examined whether Pxl1 localization to the medial ring was dependent on actin polymerization by promoting the disassembly of actin by using 50 \(\mu\)M Lat A in G2 synchronized \(cdc25-22\) cells expressing GFP-pxl1\(^{+}\). Cells were arrested at 36°C for 4 h, and then they were returned to permissive temperature in the presence of Lat A or DMSO. After 20 min Pxl1 could already be observed forming a medial ring in DMSO-treated cells. By contrast, in Lat A-treated cells GFP-Pxl1 was visualized as punctuated membrane structures accumulating at the tips (Figure 5A). To see whether actin polymerization was also required to maintain Pxl1 in a medial ring, Lat A was added 40 min after switching the \(cdc25-22\) cells to permissive temperature. At that time GFP-Pxl1 was already localized to a ring, and cells were initiating septation, as assessed by calcofluor staining. GFP-Pxl1 disappeared from the division site in Lat A-treated cells in which the ring had already been formed, and it was observed again as punctuated structures at the poles (Figure 5B). Therefore, Pxl1 requires F-actin to localize and to maintain its localization in the medial ring.

To further investigate how Pxl1 localization is regulated, we analyzed GFP-Pxl1 in different septation mutants (Supplemental Figure 2). Cdc15 is an FCH domain-containing protein necessary for the actomyosin ring formation. \(cdc15-140\) cells are impaired in the assembly of the actomyosin ring when grown at 36°C (Carnahan and Gould, 2003). GFP-Pxl1 was not recruited to the medial region in \(cdc15-140\) cells grown at 36°C, and it was diffused in the cytoplasm (Supplemental Figure 2).
cdc12-112 is a thermosensitive allele of the gene coding for the essential Cdc12 formin. Cells carrying cdc12-112 do not form actomyosin rings at the restrictive temperature (Chang et al., 1997). GFP-Pxl1 did not form a ring in cdc12-112 cells grown at 36°C, but in some cells GFP spots were observed in the membrane at sites in which division should have occurred (Supplemental Figure 2). Interestingly, the same localization was observed for Cdc15-GFP in cdc12-112 mutant cells. cdc4-8 is a thermosensitive allele of the gene coding for the essential myosin light chain, and it does not form actomyosin rings at the restrictive temperature (McCollum et al., 1995). GFP-Pxl1 showed up as a bright cytoplasmic spot in the medial region of cdc4-8 cells grown at the restrictive temperature (Supplemental Figure 2). We conclude from these results that the actomyosin ring is necessary for the localization of Pxl1 to a ring at the division site.

To test whether Pxl1 localization required the SIN proteins, essential for the formation of the division septum after assembly of the actomyosin ring, we analyzed GFP-Pxl1 in cdc11-119 mutant cells. Cdc11 is a protein required for the localization of the known SIN components, except Sid4, to the spindle pole body. At the restrictive temperature, cdc11-119 mutant cells assemble the actomyosin ring but do not form a septum (Krapp et al., 2004). GFP-Pxl1 localized properly in cdc11-119 cells grown at the restrictive temperature (Supplemental Figure 2). These observations indicate that Pxl1 localization is not SIN pathway dependent.

Cdc16 is a negative regulator of the GTPase Spg1 that activates the SIN. At the restrictive temperature, cdc16-116 mutants form multiple well oriented septa in one cell (Furge et al., 1998). GFP-Pxl1 only localized to one of the multiple septa formed by cdc16-116 cells grown at the restrictive temperature (Supplemental Figure 2). Therefore, Pxl1 does not require the SIN inactivation to disappear from the division area.

Pxl1 Localizes with the Actomyosin Ring Proteins

To see whether Pxl1 is part of the actomyosin ring, we constructed a strain carrying Pxl1 tagged with cherry RFP at the N terminus, and we analyzed the simultaneous localization of cherry RFP-Pxl1 and other proteins belonging to the contractile ring tagged with GFP or YFP. We observed colocalization of GFP-Myo2 and cherry RFP-Pxl1 during ring constriction (Figure 6A). However, at the early steps of cytokinesis, when Myo2 was present as a broad band of dots, Pxl1 was not there, suggesting that Pxl1 arrives at the cell division area later. Cdc12 and Cdc15 also concentrated in small nodes around the equator over a period of 10 min after myosin II occurred, followed quickly by lateral condensation of the nodes into a contractile ring. We observed colocalization of cherryRFP-Pxl1 with either Cdc12-3YFP or GFP-Cdc15 during cytokinesis (Figure 6, B and C). However, we could observe some cells in early cytokinesis with Cdc12-3YFP that did not have cherryRFP-Pxl1 (Figure 6B, inset). We also observed that cherryRFP-Pxl1 formed a lateral dot, whereas GFP-Cdc15 was forming small nodes around the equator at early stages of cytokinesis (Figure 6C, inset). These results suggest that Pxl1 forms part of the actomyosin ring and is recruited to the medial region after myosin II and Cdc12, perhaps together with Cdc15.

To further characterize the interaction of Pxl1 with the ring proteins, we performed immunoprecipitation of extracts from cells expressing GST-Pxl1 and rlc1Δ-GFP at endogenous levels. We observed coprecipitation of Rlc1-GFP with GST-Pxl1 by using GS beads (Figure 6D). Addi-
tionally, we performed immunoprecipitation of extracts from cells carrying HA-pxl1/H11001 and GFP-cdc15/H11001 expressed at endogenous levels, and we also observed coimmunoprecipitation of GFP-Cdc15 with HA-Pxl1 by using anti-HA antibodies (Figure 6E). These results suggest that these proteins might form a complex during cytokinesis.

Genetic Interactions between pxl1/H11545 and Genes Regulating Cytokinesis

To better understand the molecular function of Pxl1, we looked for possible genetic interaction of pxl1/H9004 with other genes encoding proteins forming the actomyosin ring or participating in cytokinesis. Several double mutants of these genes and pxl1/H9004 were generated, and their growth at different temperatures was examined (Table 1 and Supplemental Figure 3). Mid1 interacts with Myo2 and anchors the myosin at the medial cortex (Motegi et al., 2004). There were synthetic fitness (slow growth) defects between pxl1/H9004 and mid1/H9004. Thus, pxl1/H9004 mid1/H9004 double mutant was not viable at 25°C (Supplemental Figure 3A), and the cells grew very slowly at 32°C. There was also a strong genetic interaction among pxl1/H9004, and the genes coding the components of myosin II. Thus, pxl1Δ rlc1Δ double mutant was not viable at 25°C (Supplemental Figure 3A), and the cells grew very slowly at 32°C. There was also a strong genetic interaction among pxl1/H9004, and the genes coding the components of myosin II. Thus, pxl1Δ rlc1Δ double mutant was not viable at 25°C, and the cells grew very slowly at 28 or 32°C (Supplemental Figure 3B). rlc1Δ, coding for the regulatory light chain of myosin II, is not essential, and the phenotype of rlc1Δ cells is a cytokinesis defect similar to that of cells lacking Pxl1 (Le Goff et al., 2000). In the same way, myo2-E1 mutant cells are thermosensitive, and they have a cytokinesis defect (Balasubramanian et al., 1998). myo2-E1 defects were drastically aggravated in cells lacking Pxl1. Thus, myo2-E1 pxl1Δ mutant cells grew slowly at 25°C, and they could not grow at temperatures of 28°C or higher (Table 1, Supplemental Figure 3B). It was not possible to obtain double mutant strains carrying pxl1Δ and cdc4-8, a thermosensitive allele of the gene coding for the myosin essential light
chain. The pxl1Δ cdc4-8 spores germinated and generated branched multiseptated cells that could not form colonies (Supplemental Figure 3D). These germinating spores were similar to myo2Δ spores, forming short filaments with septa that failed to cleave (Kitayama et al., 1997).

The IQGAP-related protein Rng2p is a component of the actomyosin ring, and it is required for ring formation after assembly of F-actin at the division site (Eng et al., 1998). It was not possible to obtain double mutant strains carrying rng2-D5 and pxl1Δ mutations. The germinated spores formed short filaments as pxl1Δ cdc4-8 and myo2Δ spores (Supplemental Figure 3D). There was also genetic interaction between pxl1Δ and cdc15Δ. Double mutant cells carrying pxl1Δ and the thermosensitive cdc15-140 allele did not grow at temperatures of 28°C or higher (Supplemental Figure 3B). Surprisingly, the absence of Px1 did not aggravate the growth phenotype of cdc12-112 cells; thus, the cdc12-112 pxl1Δ double mutant grew as well as cdc12-112 cells at 32°C (Supplemental Figure 3C). However, the percentage of septating and multiseptated cells was higher in cdc12-112 pxl1Δ than in pxl1Δ cells (data not shown). These results suggest that Px11 plays a role in actomyosin ring formation.

Interestingly, we also observed lethality in double mutant strains carrying pxl1Δ and any of the bgs1Δ-thermosensitive alleles, csp1-112, csp1-12, and csp1-191. By contrast, the lack of Px11 did not aggravate the growth defect of csp1-112, a bgs4-thermosensitive mutant (Table 1). Bgs1 and Bgs4 are the (1,3)β-D-glucan synthase catalytic subunits responsible for biosynthesis of the primary septum and cell wall, respectively (Cortes et al., 2005, 2007). These results suggest that Px11 and Bgs1 are contributing to some essential process in cytokinesis that might be related to primary septum synthesis, because that is the role of Bgs1 (Cortes et al., 2007), and septum synthesis is coordinated with the actomyosin ring contraction.

We did not observe synthetic lethality with myo3Δ; although the multiseptation phenotype of pxl1Δ myo3Δ was more severe than the parental phenotypes (Table 1). Similarly, the lack of Px11 had no effect on the growth of either mid2Δ or spn3Δ, but the double mutants showed additive septation defects (Table 1). Together, these results point toward Px11 collaborating with proteins that participate in the formation of the actomyosin ring and the primary septum but not with the proteins that act later in cytokinesis such as Mid2 or the septins.

### Table 1. Genetic interactions between pxl1Δ and genes regulating cytokinesis

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<tr>
<th>Strain</th>
<th>Gene product</th>
<th>Phenotype of double mutant with pxl1Δ</th>
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<tr>
<td>mid1Δ</td>
<td>Anillin</td>
<td>SG A</td>
</tr>
<tr>
<td>cdc4-8</td>
<td>Essential myosin light chain</td>
<td>SL A</td>
</tr>
<tr>
<td>rlc1Δ</td>
<td>Regulatory light chainA</td>
<td>SG A</td>
</tr>
<tr>
<td>myo2-E1</td>
<td>Myosin II heavy chain</td>
<td>SG A</td>
</tr>
<tr>
<td>rng2-D5</td>
<td>IQGAP</td>
<td>SL A</td>
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<tr>
<td>cdc15-140</td>
<td>FCH protein</td>
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<tr>
<td>cdc12-112</td>
<td>Formin</td>
<td>NE A</td>
</tr>
<tr>
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<td>Bgs1 glucan synthase subunit</td>
<td>SL A</td>
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<td>SL A</td>
</tr>
<tr>
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<td>Bgs4 glucan synthase subunit</td>
<td>NE NE</td>
</tr>
<tr>
<td>myo3Δ</td>
<td>Myosin II heavy chain</td>
<td>NE A</td>
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<tr>
<td>spn3Δ</td>
<td>Septin</td>
<td>NE A</td>
</tr>
<tr>
<td>mid2Δ</td>
<td>Anillin</td>
<td>NE A</td>
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Growth of each double mutant strain was compared with those of the corresponding parental strains. SL, synthetic lethal; SG, slow growth; A, aggravated; and NE, no effect.

**Figure 7.** Px11 is required for proper myosin II localization to the actomyosin ring. (A) Fluorescence microscopy of wild-type and pxl1Δ cells carrying GFP-myo2Δ-GFP, rl1-1Δ-GFP, and cdc4-4Δ-GFP endogenously expressed. (B) Confocal fluorescence images of GFP-Myo2 and Sad1-GFP in pxl1Δ cells. (C) Fluorescence microscopy of a calcofluor-stained pxl1Δ cell expressing rl1-1-GFP to see the septum formation while one of the myosin rings was not contracted. (D) Localization of Myo3-GFP in wild-type and pxl1Δ cells. Arrows point to irregular rings.

**Px11 Is Required for Proper Myosin II Organization into the Actomyosin Ring**

We have described that Pxl1 localization is actomyosin ring-dependent and that there is colocalization of myosin II and Pxl1 during ring contraction. Additionally there is strong genetic interaction between pxl1Δ and other genes related to the actomyosin ring formation. So we next compared the behavior of myosin II and other ring components in pxl1Δ and pxl1Δ strains. Fluorescence microscopy of cells endogenously expressing any of the myosin II chains GFP-Myo2 (and the SPB marker Sad1-GFP), Rlc1-GFP, or Cdc4-GFP showed that myosin II localized to the division area in cells lacking Px11, but the formation of the myosin contractile ring was not always correct in these cells (Figure 7). Cells carrying tagged myosin chains in a wild-type background do not have a visible phenotype. However, pxl1Δ cells carrying tagged myosin chains showed a stronger septation defect than pxl1Δ cells, suggesting that the tagged myosin chains are not fully functional. With all of them, we observed irregular myosin rings in pxl1Δ cells (47, 50, and 51% with...
GFP-Myo2, Rlc1-GFP, and Cdc4-GFP, respectively; n = 125) that were not present in wild-type cells. Most rings had disorganized filaments connected to them or floating nearby (Figure 7B). We could also see more than one ring (Figure 7 and Supplemental Movies S2, S4, and S6) in some cells (19, 18, and 22% with GFP-Myo2, Rlc1-GFP, and Cdc4-GFP, respectively; n = 125). In the cells in which there was more than one ring, only one of them contracted and formed a septum, whereas the other ring or rings remained noncontracted (Figure 7C and Supplemental Movies S2, S4, and S6). The absence of Px1 did not cause an increase in total amount of myosin II, as detected by Western blot of the light chains Cdc4 and Rlc1 (data not shown). Therefore, the phenotypes observed are probably due to a defect in myosin II organization and interaction with other ring proteins.

We observed an increased septation defect of pxl1Δ myo3Δ double mutant compared with the parental strains. Therefore, considering that Myo3/Myp2 is the other heavy chain of myosin II and also participates in cytokinesis, we analyzed Myo3-GFP localization in cells lacking Px1. Fluorescence microscopy of these cells showed a significant number of irregular rings (41%; n = 90) (Figure 7D), but we never saw more than one ring formed. There was also a considerably higher percentage of cells that accumulated Myo3-GFP at the end of cytokinesis (Figure 7D). This last effect could be caused, at least partially, by a delay in cell separation.

We could not see the localization of Cdc15 in pxl1Δ cells because targeted Cdc15 (either GFP-Cdc15 or Cdc15-GFP) was lethal in combination with the lack of Px1, suggesting that targeted Cdc15 is not totally functional and Px1 is essential in that situation. We then compared Cdc12-3YFP localization in pxl1Δ and pxl1Δ cells, and we did not observe any difference (Supplemental Figure 4A). Because the formin Cdc12 seemed properly organized in the actin ring during cytokinesis in cells lacking paxillin, we performed actin staining to see whether we could detect actin ring disorganization. Most actin rings in cells lacking Px1 were very similar to those in wild-type cells, although more disorganized patches were observed around the ring, and in very few cells (<1%) a double ring was detected (Supplemental Figure 4B, see inset, bottom right corner).

Because the main defect in pxl1Δ cells is an increase in the percentage of septating cells (45–50%), we also analyzed the localization of different proteins required for proper septum formation and for cell separation. In particular, we analyzed Bgs1p and Bgs4p, the (1,3)β-D-glucan synthase catalytic subunits, and both were correctly localized (Supplemental Figure 5). However, we observed that many cells have incomplete Bgs1-GFP (60 vs. 34% in wild-type cells) or Bgs4-GFP (40 vs. 18% in wild-type cells) fluorescent signal in the division area, suggesting that these cells are delayed in the process of septum formation. We also analyzed Spr1-GFP, one of the fission yeast septins that is essential for septin ring formation (An et al., 2004); Sec8-GFP from the exocist complex, involved in the targeting of enzymes responsible for septum cleavage (Wang et al., 2002); and the glucanases Eng1-GFP and Agn1-GFP, which are responsible for the degradation of the primary septum and the lateral cell wall, respectively (Martin-Cuadrado et al., 2003; Garcia et al., 2005). These four proteins all localized correctly to the septum area in pxl1Δ cells (Supplemental Figure 6). Therefore, our results suggest that the main defect of cells lacking Px1 is myosin II misorganization into the actomyosin ring.

**Pxl1 Collaborates in the Constriction of the Actomyosin Ring**

Myosin II is responsible for actomyosin ring contraction during animal cell cytokinesis (Matsumura, 2005), and it is essential for cytokinesis in fission yeast (Kitayama et al., 1997). Our results indicate that Pxl1 function could be related to myosin II organization; therefore, we tried to determine whether the delay observed in pxl1Δ cell separation was due to a delay in ring assembly or in ring constriction. Time-lapse confocal microscopy at 25°C of cells expressing both GFP-Myo2 and Sad1p-GFP was used to analyze the SPB localization and the ring constriction. As shown in Figure 8, A and B, the rate of ring closure was much slower in pxl1Δ cells than in a wild-type strain. Although the time for ring constriction was variable among different pxl1Δ cells, we calculated the average ring constriction rate at 25°C using cells from several independent experiments. Constriction was always slower in pxl1Δ (45 ± 15 nm/min; n = 20) than in wild-type cells (92 ± 10 nm/min n = 10). Additionally, the rings remained for a much longer time during the late stages of cytokinesis in pxl1Δ cells (Figure 8C). We also observed simultaneously the ring constriction and septum formation by calcofluor staining of cells carrying both GFP-Myo2 and Sad1-GFP, and in some cases, as with the left cell shown in Figure 8D, the myosin ring was formed but the septum formation was deferred at least 40 min. We can conclude that a delay in ring contraction could be the cause of the increased percentage of septating cells observed in pxl1Δ cell cultures. A similar septation phenotype was observed in rlc1Δ cells (Le Goff et al., 2000), in myo2-E1 mutant cells grown at the restrictive temperature (Balasubramanian et al., 1998), and in cells in which myo2+ was switched off (Kitayama et al., 1997).

**DISCUSSION**

The genome of *S. pombe* contains an ORF coding a protein that shares similarity with *S. cerevisiae* Pxl1 (Gao et al., 2004; Mackin et al., 2004) and paxillin from animal cells; therefore, we named it pxl1+. Mild overexpression of this gene was able to suppress the thermosensitive growth lethality of cdc42-1625 mutant with defects in actin cable formation and secretion (Martin et al., 2007). Coimmunoprecipitation of Px1 and Rho1, and the biochemical data suggest that Pxl1 acts as a negative regulator of Rho1, modulating the level of GTP-Rho1 in vivo. Thus, the lack of Px1 causes an increase in GTP-bound Rho1 slightly lower than that produced by the absence of Rga5, a specific Rho1 GAP. The genetic interaction with rga5+ shown in Figure 2, D–F also strongly supports the relevance of these results. In contrast, we could not see interaction between Px1 and Cdc42. Therefore, pxl1+ suppression of cdc42-1625 thermosensitivity might not be due to a direct activation of the Cdc42 signaling pathway altered in cdc42-1625, but rather to an indirect effect caused by a decrease in Rho1 signaling. Antagonistic cross-talk of these two GTPases was first reported in fibroblasts, in which Cdc42 and RhoA mutually inhibit each other (Nobes and Hall, 1995), and it has been described in various processes of animal cells (Sander et al., 1999).

Pxl1 does not have a clear RhoGAP domain; therefore, we explored the possibility that the interaction between Rho1 and Pxl1 was mediated through a Rho1GAP but two-hybrid assays performed using Px1 as bait and the three Rho1 GAPs, Rga1, Rga5 and Rga8 did not show any interaction. Additionally, there was coimmunoprecipitation of Rho1 and Px1 in rga5Δ and rga8Δ strains (data not shown), suggesting
that the interaction was not mediated by those Rho1 GAPs. 

*S. pombe* Pxl1 seems to behave similarly to budding yeast Pxl1 with respect to its interaction with Rho1 GTPase (Gao et al., 2004). However, *S. cerevisiae* Pxl1 was not able to suppress *pxl1Δ* phenotype when expressed in *S. pombe* (data not shown). There is a major difference in the localization of these two paxillin-like proteins: *S. cerevisiae* Pxl1 localizes to sites of polarized growth and LIM domains are required to target the protein to these sites; by contrast, *S. pombe* Pxl1 localizes to the area of cellular division and it is the N-terminal region of the molecule, not the LIM domains, that is necessary and sufficient to target the molecule to the division site. It is possible that the mechanism of Rho1 modulation is similar in both paxillin-like proteins, but the spatial regulation allows a different function of these molecules in budding and fission yeasts.

Pxl1 localization experiments indicate that it is a nonessential member of the contractile ring that is recruited to the division area after myosin II and Cdc12, immediately before the ring is formed. Additionally, Pxl1 interacts with Cdc15 and Rlc1. These results, supported by the genetic interactions with other septation genes, suggest a role of Pxl1 in actomyosin ring organization that is corroborated by fluorescence microscopy of the GFP-tagged myosin chains in *pxl1Δ* cells. It has been proposed that the interaction between the Myo2 fibers formed from the nodes in the broad medial band and the F-actin cables formed in the same area might pull nodes together and laterally condense the networks of Myo2 fibers and F-actin cables into a contractile ring. Myo2 is stabilized when the ring forms and in turn it contributes to actin polymerization. Pxl1, together with Cdc15, might aid in the coalescence of the myosin II and F-actin cables into a ring. Myo2 fibers connected to the ring can be observed in *pxl1Δ* cells, indicating that the coalescence has not been properly achieved. Moreover, the additional myosin ring formed in some *pxl1Δ* cells is noncontractile, suggesting that myosin is not stably associated to the actin ring in the absence of Pxl1.

The interactions between myosin II and actin in the ring are believed to generate the force that constricts animal cells to divide in two daughter cells (Matsumura, 2005). A lower concentration of myosin II properly organized in the actomyosin ring may cause a reduction in the contraction force, which in turn causes the delay or even the halt in the ring contraction that we have seen in some *pxl1Δ* cells during the time-lapse experiments. Other mutations affecting myosin II, such as *rlc1Δ*, *myo2-E1*, and *ring2-D5*, cause a similar phenotype (Balasubramanian et al., 1998; Le Goff et al., 2000; Mulvihill and Hyams, 2003), suggesting that myosin II activity is required for proper cytokinesis. Disassembly of the myosin rings is also slowed down in *pxl1Δ* cells, and a strong accumulation of Myo3 is observed. These effects

![Figure 8. Ring constriction during cytokinesis is delayed in *pxl1Δ* cells. Analysis of Myo2 ring contraction in wild-type and *pxl1Δ* cells grown at 25°C. (A) Time-lapse confocal fluorescence microscopy of GFP-Myo2 Sad1-GFP in wild-type and *pxl1Δ* cells. (B) Rate of ring constriction at 25°C of wild type (n = 10) and *pxl1Δ* cells (n = 20) calculated from the time-lapse confocal images in three independent experiments. (C and D) Time-lapse fluorescence images of different GFP-Myo2 Sad1-GFP in wild-type and *pxl1Δ* cells during cytokinesis. In D, cells were stained with calcofluor.](image-url)
might also cause additional delay in septation and cell separation. Zebra fish embryos with reduced myosin activity also exhibit at late stages of cytokinesis a stabilized contractile ring apparatus that suggests a role for myosin function in the disassembly of the contractile ring (Urven et al., 2006).

The actomyosin ring is not essential for cytokinesis in S. cerevisiae, which can still complete cytokinesis in its absence, possibly by localized cell wall synthesis (Bi et al., 1998). In fission yeast it is not known whether ring constriction is the force that triggers septum formation or if septum formation forces ring constriction and cytokinesis as has recently been proposed (Johnson et al., 2005). Indeed, bgs1Δ shut-off and deletion also causes a multiseptation phenotype (Cortes et al., 2007). The lethal interaction observed between pxl1Δ cells and several bgs1 mutant strains suggests that both ring constriction and primary septum formation might collaborate in the completion of S. pombe cytokinesis.

Whether Pxl1 modulation of Rho1 activity is related to Pxl1 function in actomyosin ring formation and contraction remains unknown. Further studies will be required to clarify the possible involvement of Rho1 in S. pombe contractile ring formation and cytokinesis.

ACKNOWLEDGMENTS

We thank J. C. Ribas, B. Santos, and H. Valdivieso for useful comments. We thank to D. Posner for language revision. We thank M. Balasubramanian (University of Singapore), F. Chang (Columbia University, New York, NY), S. Erdman (Syracuse University, Syracuse, NY), K. Gould (Vanderbilt University, Nashville, TN), T. Pollard (Yale University, New Haven, CT), J. C. Ribas (CSIC, Spain), Y. Sánchez (University of Salamanca, Spain), V. Simanis (ISREC, Switzerland), and R. Y. Tsien (University of California, San Diego) for generous gifts of strains, and plasmids. Thanks to D. Riveline and C. Castro for technical help with the microscopy. M. Pinar was supported by a fellowship from the Spanish Ministerio de Educación. This work was supported by grant BIO2004-0834 from the Comisión Interministerial de Ciencia y Tecnología, Spain.

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