Fission yeast Cyk3p is a transglutaminase-like protein that participates in cytokinesis and cell morphogenesis

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ABSTRACT

Cell morphogenesis is a complex process that relies on a diverse array of proteins and pathways. We have identified a transglutaminase-like protein (Cyk3p) that functions in fission yeast morphogenesis. The phenotype of a \textit{cyk3} knockout strain indicates a primary role for Cyk3p in cytokinesis. Correspondingly, Cyk3p localizes both to the actomyosin contractile ring and the division septum, promoting ring constriction, septation, and subsequent cell separation following ring disassembly. In addition, Cyk3p localizes to polarized growth sites and plays a role in cell shape determination, and it also appears to contribute to cell integrity during stationary phase, given its accumulation as dynamic puncta at the cortex of such cells. Our results and the conservation of Cyk3p across fungi point to a role in cell wall synthesis and remodeling. Cyk3p possesses a transglutaminase domain that is essential for function, even though it lacks the catalytic active site. In a wider sense, our work illustrates the physiological importance of inactive members of the transglutaminase family, which are found throughout eukaryotes. We suggest that the proposed evolution of animal transglutaminase cross-linking activity from ancestral bacterial thiol proteases was accompanied by the emergence of a subclass whose function does not depend on enzymatic activity.

INTRODUCTION

Transglutaminases (TGases) are a family of enzymes that catalyze intramolecular or intermolecular protein cross-linking through isopeptide bond formation between lysine (or polyamines) and glutamine residues. The activity of the enzymes plays an important role in various intracellular and extracellular processes. For example, keratinocyte TGase functions in the terminal differentiation of keratinocytes and formation of the cornified cell envelope (Rice and Green, 1978; Thacher and Rice, 1985); tissue TGase (TG2) is found in many cell types and is involved in inflammation, apoptosis, cell adhesion, and cancer and other human diseases (Fesus and Szondy, 2005; Mangala and Mehta, 2005; Mehta et al., 2010; Facchiano et al., 2006; Zemskov et al., 2006). Factor XIII probably represents the best-studied TGase; it participates in blood clotting, tissue repair, and wound healing (Pisano et al., 1968; Schwartz et al., 1973; Mosher and Schad, 1979; Sakata and Aoki, 1980; Knox et al., 1986).

A hallmark of these enzymes is a catalytic triad made up of conserved cysteine, histidine, and aspartate residues, each of which is essential for activity and defines the catalytic core (Figure 1A; Hettasch and Greenberg, 1994; Micanovic et al., 1994; Pedersen et al., 1994; Yee et al., 1994).

The increasing abundance of protein sequence information has revealed a new inactive class of TGases that possesses a conserved catalytic domain lacking the catalytic triad (Makarova et al., 1999). For example, a red blood cell protein (band 4.2) possesses a TGase domain that lacks the catalytic cysteine and histidine residues (Figure 1A) and consequently lacks enzyme activity (Korsgren et al., 1990; Cohen et al., 1993). Naturally occurring mutations in the human...
morphogenesis in fungi, and more broadly identifies an important cellular role for the inactive subclass of the TGase family.

RESULTS
Fission yeast Cyk3p functions in cytokinesis and morphogenesis
Like other known fungal members of this protein family (Korinek et al., 2000; Reijnst et al., 2010), fission yeast Cyk3p possesses an N-terminal SH3 domain (Figure 1B). In addition, we identified a TGase-like domain in the C-terminal half of the protein (Figure 1B), a domain preserved in Cyk3p sequences from other fungi. This 111–amino acid domain showed homology to TGase domains in S. cerevisiae Cyk3p (30% identical/51% similar) and animal TGases (e.g., 17%/41% vs. human factor XIII). The catalytic core of the TGase domain can be separated into three motifs centered on the conserved cysteine, histidine, and aspartic acid active-site residues that form the catalytic triad. Interestingly, the catalytic triads of the fungal Cyk3p proteins are all incomplete, containing the conserved histidine and aspartic acid residues in motifs II and III but lacking the active site cysteine in motif I (Figure 1A).

Deletion of cyk3 had no obvious effect on morphology or growth at 25–32°C. However, cyk3Δ cells exhibited temperature-sensitive defects in cell separation at 36°C, as reflected by the appearance of elongated cells with multiple septa and multiple nuclei (Figure 2A). In addition, cyk3Δ cells showed a greater tendency to adopt a rounded/swollen morphology, as opposed to the typical cigar shape of fission yeast (Figure 2A). Consistent with a significant role for Cyk3p in cytokinesis, the cyk3Δ mutation showed synthetic defects when combined with mutations in genes encoding known contractile ring components, such as Myo2p (myosin II), Cdc4p (essential light chain), Cdc12p (formin), and Cdc15p (F-BAR protein) (Figure 2, B and C, and Supplemental Figure S1). Although the contractile rings typically assembled in such double mutants, they showed obvious defects in cytokinesis (Figure 2D), suggesting a role for Cyk3p in ring constriction.

Surprisingly, unlike myo2-1 and many other cytokinetic mutations (Bezanilla et al., 1997; Motegi et al., 1997), cyk3Δ did not exhibit synergetic cytokinetic defects when combined with a myo2 null (Figures 2E and S1). Myp2p is a nonessential myosin II required for normal cytokinesis (Bezanilla et al., 1997; Motegi et al., 1997), and the lack of an additive phenotype suggests that Cyk3p and Myp2p may share a specific function in cytokinesis. Myp2p is known to associate with chitin synthase (Chs2p), an inactive form of the enzyme that localizes to the contractile ring and contributes to septum formation in fission yeast (Martin-Garcia et al., 2003; Martin-Garcia and Valdivieso, 2006). We therefore also examined a cyk3Δ chs2Δ double mutant. Interestingly, loss of Chs2p completely suppressed the cytokinetic defects associated with loss of Cyk3p (Figures 2F and S1), suggesting a functional relationship between these two proteins at the septum.

Cyk3p localizes to the contractile ring, division septum, and sites of polarized growth
We used gene replacement to generate single and triple chromosomal green fluorescent protein (GFP) fusions to examine the subcellular localization of endogenous Cyk3p. The fusion proteins were functional based on their ability to fully support Cyk3p function in a

**FIGURE 1:** Conserved protein domains of Cyk3p. (A) A sequence alignment comparing identities (black) and similarities (gray) among amino acids from four human transglutaminases and three Cyk3p homologues (Sp: fission yeast S. pombe; Sc, Ca: budding yeasts S. cerevisiae and C. albicans). The alignment centers on the active-site cysteine (motif I), histidine (II), and aspartate (III) residues (asterisks) forming the conserved catalytic triad in the transglutaminase core. The variable regions (dashed lines) between motifs I and II span 32 residues for the human transglutaminases (except TGase 3: 31 residues) and 14 residues for the Cyk3p proteins (except Sc: 12 residues); the variable regions between motifs II and III span 11 residues for human transglutaminases and 3 residues for the Cyk3p proteins. The arrowhead marks a cysteine residue (Cys554 in S. pombe) conserved among the Cyk3p proteins. (B) Position of the SH3 (amino acids 10-65) and transglutaminase-like (amino acids 485-595) domains in the 886-residue fission yeast Cyk3p sequence. The domains were identified using the Blastp program.

band 4.2 gene cause congenital spherocytic anemia characterized by sphere-shaped (rather than biconcave disk-shaped) cells that are more prone to hemolysis (Yawata, 1994; Bruce et al., 2002; Dahl et al., 2004). This phenotype highlights the physiological relevance of proteins with inactive TGase domains, and may reflect a structural role at the cell membrane in the case of band 4.2 (Satchwell et al., 2004). In addition, cyk3Δ cells shed light on the molecular mechanisms governing Cyk3p function. Localization of Cyk3p at the division site depends on its N-terminal SH3 domain (Jendretzki et al., 2009). The variable regions (dashed lines) between motifs I and II span 32 residues for the human transglutaminases (except TGase 3: 31 residues) and 14 residues for the Cyk3p proteins (except Sc: 12 residues); the variable regions between motifs II and III span 11 residues for human transglutaminases and 3 residues for the Cyk3p proteins. The arrowhead marks a cysteine residue (Cys554 in S. pombe) conserved among the Cyk3p proteins. (B) Position of the SH3 (amino acids 10-65) and transglutaminase-like (amino acids 485-595) domains in the 886-residue fission yeast Cyk3p sequence. The domains were identified using the Blastp program.
that apparently failed to separate (Figure 6B), as well as an increase increased percentages of septated cells (Figure 6A) and of cells of the contractile ring that was relatively faint, but it became brighter a short time later, when Cyk3p was accumulated at the old cell poles later in the cell cycle (Figure 3D). Although the bipolar tip localization was relatively faint when the tagged Cyk3p was expressed at endogenous levels, it became more conspicuous when Cyk3p-GFP was expressed from a multi-copy plasmid (Figure 3E).

In summary, Cyk3p localizes in a manner consistent with roles in cell morphogenesis during both division and polarized cell growth.

**Cyk3p is a component of the contractile ring and promotes ring dynamics**

Given its prominent localization at the actomyosin ring, we tested whether Cyk3p was truly a component of this structure. Fluorescence recovery after photobleaching (FRAP) studies have shown that ring components exchange rapidly and that myosin II Myo2p exchanges with different kinetics in nonconstricting and constricting rings (Sladewski et al., 2009; Stark et al., 2010). Examination of Cyk3p-GFP exchange revealed almost identical kinetics to those of Myo2p in both nonconstricting (t½ = 27 s vs. −37 s for Myo2p), and constricting (t½ = 12 s vs. −13 s for Myo2p) rings (Figure 4, A and B; Sladewski et al., 2009). Depolymerization of the actin cytoskeleton with latrunculin A blocked the formation of both myosin II and Cyk3p rings and led to a buildup of both proteins as ring precursors at the incipient medial division sites (Figure 4C). Taken together, these results suggest that Cyk3p is indeed a component of the actomyosin ring.

To test for a possible role of Cyk3p in contractile ring dynamics, we first monitored contractile ring behavior, using Ric1p-GFP as a marker, in relation to mitotic progression, as judged by the spindle-pole body (SBP) protein Sad1p-GFP (Figure 5A, left box). Using the beginning of SBP separation as a reference point, we found that ring assembly time and constriction rate were affected little or not at all in a cyk3Δ mutant grown at 25°C, whereas the preconstriction “dwell” time was significantly lengthened (Figure 5, A and B, and Table 1). Interestingly, the delay was paralleled by a delay in the completion of spindle elongation: although ring constriction began −8−9 min following spindle breakdown in both wild-type and cyk3Δ cells, spindle breakdown (like ring constriction) was delayed −6 min in the mutant relative to wild-type (Figure 5B). The reason for this delay is not clear, but it appeared to be due mostly to a slower spindle elongation during the phase of contractile ring assembly (Figure 5B).

The ability of cyk3Δ cells to grow at 36°C allowed a further in vivo analysis in which cells grown at 36°C were subsequently imaged at 23°C. In this case, the assembly, dwell, and constriction phases were all significantly affected, with the largest effect on the dwell time (Figure 5C and Table 1), probably because the absence of Cyk3p during growth at 36°C produced changes in the levels of Cyk3p-interacting proteins that could affect ring dynamics even after a return to lower growth temperature. In summary, although Cyk3p appears to be involved in all phases of contractile ring function, its most important role seems to be in promoting ring constriction.

**Cyk3p participates in cell separation following disassembly of the contractile ring**

The localization of Cyk3p to the septal region (Figure 3) and multi-septate phenotype of cyk3Δ mutants (Figure 2A) suggested Cyk3p might play a role in the maturation of the septum, its splitting at cell division, or both. Consistent with such a role(s), cyk3Δ cells showed increased percentages of septated cells (Figure 6A) and of cells that apparently failed to separate (Figure 6B), as well as an increase forming a tight spot at the center of the septum just before cell separation and remaining at the new cell poles following separation (Figure 3, A and D). This unipolar tip localization became bipolar as Cyk3p accumulated at the old cell poles later in the cell cycle (Figure 3D). Although the bipolar tip localization was relatively faint when the tagged Cyk3p was expressed at endogenous levels, it became more conspicuous when Cyk3p-GFP was expressed from a multi-copy plasmid (Figure 3E).

**FIGURE 2: Cyk3p functions in cytokinesis and polarized growth.** (A) Representative DIC images of wild-type (MLP 11) and cyk3Δ (MLP 3) cells following growth at 36°C in YE5S medium. Plots below provide quantification of morphological defects observed under these conditions. Left, percentages of multinucleate cells (following treatment with 4′,6-diamidino-2-phenylindole stain: 3+ nuclei/cell, □; n = 750) and of cells possessing >1 division septa, ■ (which account for the majority of multinucleate cells). Right, percentages of cells with a rounded/swollen shape (n = 750). (B) Representative cdc4-8 (TP 6) and cdc4-8 cyk3Δ (MLP 178) cells following growth at 27.5°C on YE5S medium. (C) Growth of wild-type, cyk3Δ, cdc4-8, and cdc4-8 cyk3Δ strains at 30°C. Five-micrometer cell suspensions of identical optical density were spotted along with five 10-fold serial dilutions onto a YE5S plates. The plate contained 5 µg/ml phloxin B, a pink dye that accumulates inside dead cells (note the pink color of the cdc4-8 and cdc4-8 cyk3Δ colonies). (D) Representative myo2-E1 (TP 73) and myo2-E1 cyk3Δ (MLP 17) cells following growth at 27.5°C on YE5S medium. Top panels, DIC images; bottom panels, Ric1p-GFP localization at rings (as denoted by arrowheads). (E) Representative myp2Δ (MLP 34) and myp2Δ cyk3Δ (MLP 35) cells following growth at 36°C on YE5S medium. (F) Representative cyk3Δ and cyk3Δ chs2Δ (LP 112) cells following growth at 36°C on YE5S medium. Counts of the percentages of multinucleate cells for the strains shown in (B), (D), (E), and (F) are provided in Figure S1.

cdc4-8 background. During vegetative growth, Cyk3p localized to three distinct sites: contractile rings, division septa, and cell tips (Figure 3A). The Cyk3p signal was most prominent at contractile rings. Time-lapse analysis revealed that Cyk3p joins the ring at the final stages of its assembly, is present at both rings and across growing septa during ring constriction, and remains as a band spanning the septum following ring disassembly (Figure 3B). This pattern was particularly clear when Cyk3p was colocalized with the myosin II regulatory light chain Ric1p, which assemble earlier and disappears following ring contraction (Figure 3C and Supplemental Movie S1). On contractile ring disassembly, Cyk3p localization at the septum was relatively faint, but it became brighter a short time later,
in the average time from septum completion to cell separation (Figure 6, C and D), during growth at 36°C. These effects were not seen at 25°C (Figure 6D), and even at 36°C, septum completion appeared to coincide with the completion of contractile ring constriction, as in wild-type cells (Figure 6C). Thus, in addition to a role in actomyosin ring constriction, Cyk3p also contributes to cell separation.

**Cyk3p concentrates in dynamic cortical puncta during stationary phase**

When cells expressing Cyk3p-GFP grew to stationary phase, they displayed a pattern of localization that appeared to be distinct from the division site and cell tip localization seen during vegetative growth. In particular, Cyk3p was found randomly throughout the cortex as discrete, dynamic puncta (Figure S2 and Movies S2 and S3). Although these puncta were similar in size and distribution to endocytic actin patches, Cyk3p did not colocalize with the actin-patch component Fim1p (fimbrin) during stationary phase (Figure S2), and the Cyk3p puncta had a considerably longer average lifetime (~4 min) than did Fim1p or actin patches (~20 s; Figure S2; Sirotkin et al., 2010). In addition, unlike typical endocytic patches, the Cyk3p puncta did not depend on actin (Figure S2) and often showed considerable lateral motility (Movies 2 and 3). These observations suggest that Cyk3p may play a more general role in cell-surface organization in addition to its roles at specific sites during the vegetative cell cycle.

**Overexpression of Cyk3p leads to defects in cytokinesis and cell shape**

To explore further the roles of Cyk3p in vegetative cells, we overexpressed it in otherwise wild-type cells. On overexpression, cells displayed gross morphological defects characterized by multinucleate cells with abnormal septa and rounded/swollen shapes that had lost the typical cigar shape of fission yeast (Figures 7 and S3). A combination of both phenotypes was evident in some cells (Figure 7), but time-lapse observations revealed that the phenotypes were often independent of one another (Figure S3), so that cell swelling was not simply a secondary effect of cytokinetic failure. These phenotypes paralleled those of cyk3Δ cells (Figure 2A), presumably reflecting roles for Cyk3p in both cytokinesis and cell shape determination.

**Cyk3p function depends on its transglutaminase domain but not its SH3 domain**

To gain insight into Cyk3p function, we created plasmids expressing proteins that lacked the SH3 domain or contained mutations in the transglutaminase domain (see Materials and Methods). Surprisingly, Cyk3p-SH3Δ appeared to retain full function during cytokinesis (Figure 8, A and B) and to support normal polarized growth (Figure 8A). Moreover, like wild-type, this truncated form localized normally (Figure 8C), produced cytokinetic defects and morphological abnormalities when overexpressed (Figure 8D), and was expressed at normal levels (Figure 8E). In contrast, mutation of the conserved aspartate residue of the catalytic triad (in the TGase core) led to a similar complete loss of Cyk3p function (Figure 8, A, B, and D), aspartate residue of the catalytic triad (in the TGase core) led to a seemingly complete loss of Cyk3p function (Figure 8A, B, and D), and expression at normal levels (Figure 8E). In contrast, mutation of the conserved aspartate residue of the catalytic triad (in the TGase core) led to a seemingly complete loss of Cyk3p function (Figure 8, A, B, and D), and expression at normal levels (Figure 8E). In contrast, mutation of the conserved aspartate residue of the catalytic triad (in the TGase core) led to a seemingly complete loss of Cyk3p function (Figure 8, A, B, and D), and expression at normal levels (Figure 8E).

**Stage, as marked with an asterisk in (C). (D) Time-lapse observations of Cyk3p-3GFP localization from 12 min after completion of ring constriction and septation, which is also 12 min before cell separation. (E) Cyk3p-GFP localization following expression from a multi-copy plasmid with cyk3-GFP under control of the weak-strength 81nmt1-inducible promoter. cyk3Δ cells were transformed and grown in EMM lacking uracil at 30°C. Scale bars (A–E): 4 μm.
Roles of an inactive transglutaminase

expressed at normal levels (Figure 8E). We further tested the importance of the TGase domain using another mutation (His-577-Ala) in the catalytic triad (Figure 1). This mutant protein was expressed ef- fectively, localized normally, and rescued the growth of a cdc4-8 cyk3Δ mutant (Figures 8E and S4). However, closer inspection revealed that Cyk3p-His-577-Ala retained only partial function: it was unable to fully relieve the morphological and cytokinetic defects associated with loss of Cyk3p, and it produced relatively mild morphological defects when overexpressed (Figure S4). In summary, the TGase domain, but not the SH3 domain, is critical for Cyk3p function in fission yeast.

**Cyk3p links the contractile ring and division septum during cytokinesis**

Contractile ring constriction and septum formation are coupled in yeast. The unique localization of Cyk3p in both rings and septa prompted us to further examine its roles in these structures. Because *S. cerevisiae* Cyk3p division site localization depends on the MEN pathway (Meitinger et al., 2010), we tested whether the homologous pathway (the septation initiation network, or SIN) governed Cyk3p localization in fission yeast. The SIN is essential for initiating septum formation and ring constriction (McCollum and Gould, 2001). Use of a temperature-sensitive sid2-250 mutant demonstrated that localization of Cyk3p to rings was not dependent on the SIN (Figure S5). Thus, as with other contractile ring proteins, the formation of Cyk3p rings is actin-dependent (Figure 4C) but does not rely on septum formation. This indicates that Cyk3p is a core component of the ring that must interface with the leading edge of the trailing septum during cytokinesis, presumably leading to its incorporation across the septum (Figure 3C).

We turned to electron microscopy (EM) to directly assess Cyk3p’s role in ring constriction and septation, and, in particular, how the contractile ring defects of cyk3Δ mutants affected growth of the septum. Although septation was generally normal in the absence of Cyk3p (Figure 9A, compare a–d with e–g; Figure 9C), a few cyk3Δ cells (3 of 114 cells examined) contained an apparently complete septum with a gap in the electron-translucent central layer (Figures 9Ah and 9C) that was never observed in wild-type cells. Actomyosin ring mutants also displayed defects in septum structures (Figure 9B, a, b, f, and g), which became much more severe in double mutants lacking cyk3Δ (Figure 9B, c–e and h–j). Although these double mutants were viable, their septa were typically abnormally thickened and often appeared misdirected across the division plane (Figure 9, B and C). Consistent with the genetic data indicating that Cyk3p functions in the same pathway as Myp2p (Figures 1E and S1), the EM analysis revealed little or no exacerbation of the mpy2Δ septation defect by loss of Cyk3p (Figures 9C and S6). Taken together with the localization of Cyk3p, these direct observations of the septa imply a role for Cyk3p in coupling ring constriction and septum growth during cytokinesis.

**DISCUSSION**

Our work highlights the role of a TGase-related protein in cell morphogenesis. Fission yeast Cyk3p participates in actomyosin ring constriction, septation, and cell separation during cytokinesis, and also plays a relatively minor role in cell shape and integrity during vegetative growth and stationary phase. Inactive TGases such as Cyk3p are found throughout eukaryotes and our results demonstrate that the TGase domains of such proteins can have key roles in the cell.

**Fission yeast Cyk3p functions in cytokinesis and cell wall remodeling**

Cyk3p functions in cytokinesis, promoting ring constriction and subsequent cell separation. Correspondingly, Cyk3p appears in two...
distinct structures, the actomyosin ring and the division septum. Cyk3p is an integral component of the ring and is incorporated late in ring assembly. Around this time cyk3Δ cells exhibit a cell cycle delay reflected by a brief stall in anaphase B and a delay in the initiation of ring constriction. While the defects are subtle, cyk3Δ mutants (like other cytokinetic mutants) invoke lethality in the absence of the Cdc14 family phosphatase Clp1p/Flp1p (Mishra et al., 2004). When ring integrity is compromised Clp1p activates a cytokinetic checkpoint, during which Clp1p activity is also mobilized to stabilize ring structures (Mishra et al., 2004). Thus actomyosin rings lacking Cyk3p rely on compensatory maintenance to support cytokinesis and growth.

Although we do not yet understand the molecular mechanisms by which Cyk3p carries out its role at the ring, they probably involve three other factors: the F-BAR protein Cdc15p, the nonessential myosin II Myp2p, and the chitin synthase Chs2p. Cyk3p coprecipitates with Cdc15p from cell lysates and (along with other ring components) was found to accumulate at the division site upon prema-
TABLE 1: Influence of Cyk3p on contractile ring dynamics.

| Strain | Assembly (min) | Dwell (min) | Constriction (μm/min) | Lifetime (min) |
|--------|---------------|-------------|-----------------------|---------------|
| 25°C   | 17.9 ± 3.1    | 15.0 ± 3.1  | 0.40 ± 0.05           | 42.5          |
| cyk3   | 18.6 ± 3.9    | 20.7 ± 5.7  | 0.35 ± 0.10           | 52.1          |
| 36°C   | 12.0 ± 1.7    | 12.6 ± 2.2  | 0.48 ± 0.05           | 35.5          |
| cyk3Δ  | 18.5 ± 4.8    | 27.2 ± 14.0 | 0.37 ± 0.10           | 56.9          |

* n = 50–73 rings/strain (25°C); n = 20 rings/strain (36°C). Altered ring properties (in wild-type vs. cyk3Δ cells) were confirmed by paired Student’s t tests (in which a p < 0.05 indicates a significant difference between data sets). Ring assembly (36°C): p = 0.0023; dwell (25°C): p < 0.0001; dwell (36°C): p = 0.0002; constriction (36°C): p = 0.0003.

The actomyosin ring disassembles once ring constriction and septum formation are completed (Rajagopalan et al., 2003). Completion of cytokinesis and cell separation occur a short time later upon septum maturation and subsequent digestion of the primary septum by the Eng1p and Agn1p endoglucanases (Martín-Cuadrado et al., 2003; Dekker et al., 2004). Cell separation was significantly slower in cyk3Δ cells, suggesting an important role throughout this process for Cyk3p, consistent with its accumulation across the trailing septum during ring constriction and its polarization at the center of the septum immediately prior to cell separation. Outside of cytokinesis, Cyk3p localized at growing cell tips and redistributed throughout the cortex during stationary phase. These localizations and other results suggest that Cyk3p is mobilized for polarized growth during cell elongation and maintenance of cortical integrity upon cell cycle arrest and entry into the dormant phase. One consistent feature of Cyk3p localization is its appearance at the cortex during cell wall remodeling, both in growing cells and during stationary phase (when reinforcement and thickening of the wall occur to maintain cell integrity; Herman, 2002; Rincon et al., 2006). Thus we hypothesize that Cyk3p is generally involved in facilitating cell wall synthesis and remodeling at relevant sites on the cortex.

**FIGURE 7:** Cyk3p overexpression perturbs cell shape. Wild-type (MLP 11) cells were transformed with plasmid nmt1prom (control) or nmt1prom–cyk3 (to overexpress Cyk3p from the full-strength nmt1 promoter). Cells were grown initially on EMM-ura plates containing 5 μg/ml thiamine, and were then resuspended in EMM-uracil liquid medium without thiamine and grown for 24 h to induce overexpression. Top, representative DIC images of control and Cyk3p-overexpressing cells. Bottom, such images were scored for the indicated abnormal cell morphologies (n = 500 for each count). Scale bars: 4 μm.
FIGURE 8: Cyk3p function depends on its transglutaminase domain but not its SH3 domain. (A and B) Rescue of the cdc4-8 cyk3Δ synthetic phenotype by plasmids expressing wild-type Cyk3p-GFP or Cyk3p-SH3-GFP but not by vector alone or a plasmid expressing Cyk3p-Asp-592-Ala-GFP. cdc4-8 cyk3Δ cells (MLP 178) were transformed with plasmid pGFP (vector control), cyk3-GFP, cyk3-SH3Δ-GFP, or cyk3-Asp-592-Ala-GFP (see Table 3 and Materials and Methods). (A) Representative fields of cells were imaged by DIC microscopy after growth in EMM-uracil liquid medium at 32°C (used because the restrictive temperature of the cdc4-8 cyk3Δ double mutant is higher in minimal than in rich medium). (B) Left, 5-μl aliquots of cell suspensions (of identical optical density) were spotted along with four 10-fold serial dilutions of each onto EMM-uracil plates containing 5 μg/ml phloxin B and grown at 30°C. Right, the cultures described in (A) were scored for cytokinetic defects (as percentages of multinucleate cells; n = 500). (C) Normal localization of wild-type and mutant forms of Cyk3p-GFP to contractile rings (arrowheads). cyk3Δ transformants were grown in EMM-uracil liquid medium at 25°C. The variable strength of the Cyk3p-GFP signal presumably reflects the variable copy number of fission yeast plasmids. (D) Effects of overexpressing mutant forms of Cyk3p. Wild-type strain MLP 11 was transformed with plasmid nmt1 promoter-cyk3Δ-SH3Δ or nmt1 promoter-cyk3Δ-Asp-592-Ala (Table 3), and were then grown and scored as described in Figure 7. Top, representative DIC images; bottom, quantification of morphological defects (n = 500). (E) Wild-type and mutant forms of Cyk3p-GFP are expressed at similar levels. cyk3Δ strain MLP 3 was transformed with plasmids expressing the indicated proteins and grown as in (C). Extracts were prepared, normalized for total protein, and evaluated by immunoblots using anti-GFP or anti-actin (as a loading control) antibodies. The indicated band has the appropriate apparent molecular weight (125 kDa) for a full-length Cyk3p-GFP fusion; as expected, the Cyk3p-SH3Δ-GFP fusion runs slightly faster. Scale bars: 4 μm.

the Cyk3p SH3 domain is not critical for function and does not share a redundant role with the SH3 domains of the F-BAR proteins Cdc15p and Imp2p. Irrespective of the status of Cyk3p, removal of the SH3 domains from both F-BAR proteins is enough to prevent recruitment of the C2-domain protein Fic1p to the division site (Roberts-Galbraith et al., 2009).

Although its SH3 domain is dispensable, the fission yeast Cyk3p TGase domain is required for function (an issue that does not appear to have been investigated for the S. cerevisiae protein). Each of the conserved active-site residues cysteine, histidine, and aspartic acid, which make up the catalytic triad at the core of TGases, is essential for enzymatic activity (Hettasch and Greenberg, 1994; Micanic et al., 1994; Pedersen et al., 1994; Yee et al., 1994), and mutagenesis of the corresponding aspartic acid (Asp-592) or histidine (His-577) residues of fission yeast Cyk3p led to complete or partial loss of function, respectively. Although these results demonstrate the importance of the TGase domain, it cannot function enzymatically because it lacks the cysteine residue to complete the catalytic triad (Figure 1A). The nearest cysteine residue (Cys-554) is 18 amino acids downstream from where the catalytic cysteine would be expected (Figure 1A). Nonetheless, this cysteine is conserved among Cyk3p homologues (Figure 1A) and could theoretically form a catalytic triad with His-577 and Asp-592. However, analysis of a Cys-554-Ala point mutant indicated that this was not the case. The Cys-554-Ala protein was expressed normally and behaved just like wild-type Cyk3p in vivo (Figures 8E and S4). In addition, the fact Cyk3p-His-577-Ala (but not -Asp-592-Ala) retains partial function (as opposed to complete loss or full function) also argues against an enzymatic role for the TGase domain in Cyk3p function.

The inactive nature of the Cyk3p TGase domain may reflect a structural/cytoskeletal role for this domain in cell wall remodeling, in which the charged His-577 and Asp-592 residues could help mediate electrostatic interactions between the TGase domain and Cyk3p binding partners. Similarly, a structural role has been proposed for band 4.2, the inactive TGase implicated in human disease that functions at the membrane of red blood cells (Satchwell et al., 2009). Alternatively, the inactive Cyk3p TGase domain may be a mimic that functions as a dominant-negative regulator of other TGases. In this case, the localization of Cyk3p to sites of cell wall remodeling may promote cell wall dynamics and plasticity by sequestering substrates and thus limiting their cross-linking and stabilization by active endogenous TGases. This hypothesis is supported by the observations that TGase-mediated cross-linking contributes to cell wall organization in S. cerevisiae (Iranzo et al., 2002), C. albicans (Ruiz-Herrera et al., 1999), and the green alga Chlamydomonas reinhardtii (Waffenschmidt et al., 1999). Inhibition of cell wall cross-linking by Cyk3p might explain why its overexpression leads to the loss of polarity and ballooning of cells that could accompany loss of rigidity/excessive plasticity within the cell wall.

In conclusion, our work on fission yeast Cyk3p highlights the importance of the inactive subclass of the TGases that are found throughout eukaryotes. Animal TGases are thought to have evolved from bacterial thiol proteases, because they utilize the same catalytic triad and share the same core structural fold (Pedersen et al., 1994; Yee et al., 1994). Thus, in addition to adaptations favoring substrate cross-linking (via a reversion of the proteolytic reaction), adaptations associated with loss of enzymatic activity also appear to have been selected during the course of evolution.
MATERIALS AND METHODS

Fission yeast strains, plasmids, and genetic methods

Strains were grown in EMM (Edinburgh minimal media) or YE5S (yeast extract plus supplements) medium, and standard genetic and cell biology protocols were used (Moreno et al., 1991). Table 2 lists the strains used in this study. Gene deletion mutants and strains expressing fusion proteins tagged with GFP, 3GFP, or Cherry were constructed using genomic integrations with the relevant kan<sup>R</sup> or nat<sup>R</sup> cassettes (Bahler et al., 1998). Cyk3p-GFP and Cyk3p-3GFP fusions were functional based on their ability to fully support Cyk3p function in a cdc4-8 background. Plasmids used in this study are listed in Table 3. The cyk3 open reading frame (ORF) was amplified from a genomic library using the primers: 5′ XhoI-cyk3 CTCGAG ATGTCCATTCCTAAA-CAACTACCATGC; 3′ NotI-cyk3 GCGGCCGCCAACCGCTGCCAGGTTGCATAGC. The ORF was ligated into XhoI/NotI-linearized pDS572a (overexpression vector with the nmt1 high-strength promoter) and pDS572-81 (nmt1-81 weak-strength promoter vector for plasmid-based complementation). A Cyk3p N-terminal truncation construct lacking amino acids 3–65 (spanning the entire SH3 domain and seven upstream residues) was made using the 5′ primer: XhoI-cyk3-SH3Δ: CTCGAG ATGAGTGATATTCCCACGGTACGACCTGGC. A Cyk3p Asp-592-Ala point mutant was constructed using overlap extension (Ho et al., 1989) to amplify a mutated 3′ 800–base pair fragment of the cyk3 ORF. Overlapping subfragments were generated using the following primers (and their 3′ complements): 1) 5′ Cys-554-Ala: GCACTAGATTTATGGGCTGAGGTTATCG and 5′ His-577-Ala: CCAGAGATATTAATATAAATGCTGCTTGGAATG. The fidelity of cyk3 sequences was confirmed by DNA sequencing.

FIGURE 9: EM analysis of septation with and without Cyk3p. (A) Deletion of cyk3 alone has a mild effect on septum morphology. Representative electron micrographs of wild-type (a–d) and cyk3Δ (e–h) cells grown at 27.5°C in YE5S medium are shown. Cells in the initial (a and e) and late (b, c, f, and g) stages of septation are shown, as are cells with complete septa (d and h). Scale bars: 0.5 μm. (B) Deletion of cyk3 exacerbates the septum morphology defects in cdc4-8 (a–e) and myo2-E1 (f–j) mutants. Cells were cultured as in (A); representative images are shown. Black scale bars: 0.5 μm; white scale bars (e and j): 2 μm. (C) Quantitative analysis of septum morphology in wild-type and mutant strains. Cells were grown in YE5S medium at 27.5°C, except for two cultures that were incubated at 37°C for 4 h following overnight growth at 25°C. The septa observed by EM were classified as follows: 1, an incomplete sharp septum with an electron-translucent (white) band reaching its leading edge; 2, an incomplete bulged septum with a white band reaching its leading edge; 3, an incomplete bulged septum with a white band(s) not reaching its leading edge; 4, an incomplete bulged septum with no white band; 5, a complete septum across the division plane; 6, a complete but deformed septum with interrupted white bands.
Microscopy

Differential interference contrast (DIC) and epifluorescence cell images were captured using a Nikon (Melville, NY) TE2000-E2 inverted microscope with motorized fluorescence filter turret and a Plan Apo 60×/1.45 numerical aperture (NA) objective. For fluorescence, an EXFO X-CITE 120 illuminator was utilized. NIS Elements software was used to control the microscope, two Uniblitz shutters, a Photometrics CoolSNAP HQ2 14-bit camera, and auto-focusing. Time-lapse movies of cells monitored Cyk3p and/or Rlc1p contractile ring dynamics (every 2–3 min for 2–3 h) and Cyk3p and/or Fim1p cortical patch lifetimes (by capturing images every 5–10 s for 5–10 min) using appropriate filters. For ring movies, autofocus performance was performed on the DIC channel before each image capture. Cell suspensions (3 μl) were mounted on flat 30-μl media pads (solidified by 1% agarose) prepared on the slide surface. VALAP (1:1:1 vaseline:lanolin:paraffin) was used to seal slides and coverslips. For simultaneous tracking of SPBs (Sad1p-GFP) and rings (Rlc1p-GFP), a Z-stack of six images (taken every 0.75 μm spanning the depth of the cell) was collected every 2–3 min for 90–120 min. Images were captured using Nikon (Melville, NY) ND software, and analysis of ring and patch dynamics was performed using Image J, Microsoft Excel, and KaleidaGraph software. Ring dynamics were quantified by assessing individual phases: assembly was time taken for Rlc1p-GFP to compact into a mature ring following its appearance as a broad band of nodes; constriction initiation was change in ring circumference until initiation of constriction; constriction was change in ring circumference over time. Dwell times and constriction initiation were discerned by plotting ring diameter over time for each ring, and constriction rates were derived from the slopes of these plots. FRAP experiments used confocal laser-scanning microscopy with a Zeiss LSM 510 META system equipped with an argon laser, META detector, and a Plan-Apo 100×/1.4 NA objective. Cells were mounted on 1% agarose pads (as described above) prior to microscopy at room temperature. A region of interest (ROI) was selected on Cyk3p-GFP rings for directed bleaching. Photobleaching iterations were performed briefly at high laser power, resulting in

| Strain | Genotype | Source |
|--------|----------|--------|
| TP 6   | h* leu1-32 his7-366 ade6-M216 cdc4-8 | M. Balasubramanian⁴ |
| TP 19  | h* leu1-32 ura4-D18 his7-366 ade6-M216 cdc12-112 | M. Balasubramanian⁴ |
| TP 30  | h* leu1-32 ura4-D18 his7-366 ade6-M210 cdc15-127 | M. Balasubramanian⁴ |
| TP 73  | h* leu1-32 ura4-D18 his7-366 ade6-M216 myo2-E1 | M. Balasubramanian⁴ |
| MLP 3  | h* leu1-32 ura4-D18 his7-366 ade6-M210 cyk3Δ::kan⁸ | This study |
| MLP 11 | h* leu1-32 ura4-D18 his7-366 ade6-M210 | This study |
| MLP 15 | h* leu1-32 ura4-D18 his7-366 ade6-M210 cyk3Δ::GFP::kan⁸ | This study |
| MLP 17 | h* leu1-32 ura4-D18 his7-366 ade6-M210 myo2-E1 cyk3Δ::kan⁸ | This study |
| MLP 18 | h* leu1-32 ura4-D18 his7-366 ade6-M216 cyk3Δ::kan⁸ | This study |
| MLP 34 | h* leu1-32 ura4-D18 his7-366 ade6-M210 myp2Δ::his7* | T. Pollard |
| MLP 35 | h* leu1-32 ura4-D18 his7-366 ade6-M210 myp2Δ::his7* cyk3Δ::kan⁸ | This study |
| MLP 178 | h* leu1-32 ura4-D18 his7-366 ade6-M210 cdc4-8 cyk3Δ::kan⁸ | This study |
| MLP 198 | h* leu1-32 ura4-D18 his7-366 ade6-M210 rlc1Δ::GFP::kan⁸ | This study |
| MLP 319 | h* leu1-32 ura4-D18 his7-366 ade6-M216 myo2-E1 rlc1Δ::GFP::kan⁸ | This study |
| MLP 323 | h* leu1-32 ura4-D18 his7-366 ade6 cdc12-112 cyk3Δ::kan⁸ | This study |
| MLP 326 | h* leu1-32 ura4-D18 his7-366 ade6 cdc15-127 cyk3Δ::kan⁸ | This study |
| MLY 572 | h* leu1-32 ura4-D18 ade6 his3-D1 rlc1Δ::GFP::kan⁸ sad1Δ::GFP::kan⁸ | This study |
| MLY 655 | h* leu1-32 ura4-D18 his7-366 ade6 cyk3Δ::kan⁸ rlc1Δ::GFP::kan⁸ | This study |
| MLY 657 | h* leu1-32 ura4-D18 ade6 his3-D1 cyk3Δ::kan⁸ rlc1Δ::GFP::kan⁸ sad1Δ::GFP::kan⁸ | This study |
| MLY 757 | h* leu1-32 ura4-D18 his3-D1 ade6 cyk3-3xGFP::kan⁸ rlc1-Cherry::nat⁸ | This study |
| LP 33  | h* leu1-32 ura4-D18 his3-D1 ade6 cyk3-3xGFP::kan⁸ fim1-Cherry::nat⁸ | This study |
| LP 37  | h* leu1-32 ura4-D18 his3-D1 ade6 cyk3-3xGFP::kan⁸ | This study |
| LP 69  | h* leu1-32 ura4-D18 his3-D1 ade6 sid2-250 cyk3-3xGFP::kan⁸ rlc1-Cherry::nat⁸ | This study |
| LP 109 | h* leu1-32 ura4-D18 his7-366 ade6-M216 myo2-E1 cyk3Δ::kan⁸ rlc1Δ::GFP::kan⁸ | This study |
| LP 112 | h* leu1-32 ura4-D18 his3-D1 ade6 chs2Δ::ura4⁴ cyk3Δ::kan⁸ | This study |
| LP 116 | h* leu1-32 ura4-D18 his3-D1 ade6 cdc25-22 cyk3-3xGFP::kan⁸ rlc1-Cherry::nat⁸ | This study |
| HVP 280 | h* leu1-32 ura4-D18 his3-D1 ade6 chs2Δ::ura4⁴ | Henar Valdivieso⁵ |

⁴Temasek Life Sciences Laboratory, Singapore.
⁵University of Salamanca, Spain.

TABLE 2: Fission yeast strains.
This study pDS572-81 (pDS572-81 harboring wild-type pDS572a (Source Comment Control ROI (derived from an unbleached ring in the same field of Graph software. Data sets for each trace were corrected for any Cyk3p-GFP signal versus time were plotted and fit using Kaleida perform data analysis (see Figure 4B legend). Recovery curves of latency analysis at low laser power, with images collected every 5 s ∼2 min). The criteria for determining whether Cyk3p rings were constricting or not lay in their diameters: if rings were clearly narrower than the cell diameter, we scored them as constricting; if they were the same diameter, we viewed them as nonconstricting. The criteria for determining whether Cyk3p rings were constricting or not lay in their diameters: if rings were clearly narrower than the cell diameter, we scored them as constricting; if they were the same diameter, we viewed them as nonconstricting. The LSM 510 software (version 4.2) was used to collect images and perform data analysis (see Figure 4B legend). Recovery curves of Cyk3p-GFP signal versus time were plotted and fit using KaleidaGraph software. Data sets for each trace were corrected for any additional bleaching encountered during time-lapse imaging by a control ROI (derived from an unbleached ring in the same field of cells). To facilitate curve fitting, zero signal intensity was set for each trace by subtracting any residual Cyk3p-GFP signal (detected at the first time point postbleach, 0 s) from all trace values. The t½ values (± SD) represent the mean generated from the fits of each individual FRAP experiment.

EM was performed as previously described (Nishihama et al., 2009), with minor modifications. Cells were cultured overnight in YESS medium at 25°C to mid-log phase, diluted to an OD 600 of 0.1, and then regrown at 27.5°C to an OD 600 of 0.5 (or at 37°C for 4 h). The cells were harvested, fixed with glutaraldehyde and potassium permanganate, stained with uranyl acetate, and embedded in LR White resin (Fluka, St. Louis, MO). Thin-section samples were post-stained with uranyl acetate and lead citrate and were observed using a JEM1230/JOEL microscope (Tokyo, Japan) equipped with an ORIUS SC1000A cooled-CCD camera (Gatan, Pleasanton, CA).

Western Blotting
Cyk3Δ cells harboring pGFP (vector alone), cyk3-GFP, cyk3-Asp-592-Ala-GFP, cyk3-His-577-Ala-GFP, cyk3-Cys-554-Ala-GFP, or cyk3-537Δ-GFP plasmids were grown to an OD 600 of 1 in 200 ml of EMM Ura– medium. Cells were then harvested and washed once in water and once in ice-cold lysis buffer (750 mM KCI, 25 mM Tris-HCl, pH 7.4, 4 mM MgCl₂, 20 mM Na₂PO₄, 2 mM ethylene glycol tetraacetic acid, and 0.1% Triton X-100). Pellets were resuspended in an equal volume of ice-cold lysis buffer with additives consisting of 1 mM dithiothreitol, 4 mM ATP, 2 mM phenylmethylsulfonyl fluoride, and complete EDTA-free protease inhibitors (Roche, Indianapolis, IN). From this point forward, all work was performed at 4°C and samples were stored on ice. Cells were lysed by glass bead beating with a Fastprep (MP Biochemicals, Solon, OH). Lysates were normalized for total protein using a Bradford mix (Bio-Rad, Hercules, CA), mixed 1:1 with 2X SDS–PAGE loading buffer, and boiled for 10 min. Samples were run on a 10% SDS–PAGE gel, transferred to nitrocellulose, and immunoblotted (Sambrook et al., 1989) using anti-GFP (Clontech, Mountain View, CA) and anti-actin (Chemicon, Temecula, CA) antibodies diluted 1:1000 in PBS containing 0.1% Tween-20. Horse radish peroxidase-conjugated secondary antibodies were used (diluted 1:3000).

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