The Cdc42p GTPase and Its Regulators Nrf1p and Scd1p Are Involved in Endocytic Trafficking in the Fission Yeast Schizosaccharomyces pombe*

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Nrf1p was first identified in a screen for negative regulators of the Cdc42p GTPase. Overexpression of Nrf1p resulted in dose-dependent lethality, with cells exhibiting an ellipsoidal morphology and abnormal vacuolar phenotypes including an increase in vacuolar fusion. Green fluorescent protein (GFP)-Cdc42p and GFP-Nrf1p colocalized to vacuolar membranes and GFP-Nrf1p vacuolar localization depended on Scd1p, the Schizosaccharomyces pombe homolog of the Cdc24p guanine nucleotide exchange factor. In this study, site-directed mutagenesis was conducted on Nrf1p to determine its functional domains. Mutations in the three putative transmembrane domains resulted in mislocalization of GFP-Nrf1p and an inability to induce lethality, suggesting a loss of function. Mutations in the second extramembranous loop of Nrf1p also resulted in a loss of function and altered the ability of GFP-Nrf1p to localize to vacuolar membranes. Analysis of Δnrf1D and Δscd1D mutants revealed defects in endocytosis. In addition, overexpression of constitutively active Cdc42pG12Vp resulted in an increase in endocytosis and an ability to rescue the endocytic defects in Δnrf1D and Δscd1D cells. These data are consistent with Nrf1p and Scd1p being necessary for efficient endocytosis, possibly through the regulation of Cdc42p.

Cdc42p is a Rho-like GTPase that is ubiquitously expressed in eukaryotes and has been implicated in many cellular processes including regulation of cellular polarity, transcriptional activation, and phagocytosis of bacteria into mammalian cells (1). Cdc42p acts as a binary switch, active in the GTP-bound state and inactive in the GDP-bound state. The nucleotide-bound state is regulated by guanine-nucleotide exchange factors that mediate the exchange of GDP for GTP, thereby activating Cdc42p and GTPase-activating proteins, which enhance the intrinsic GTPase activity of Cdc42p, thereby inactivating the protein (1).

In fission yeast Schizosaccharomyces pombe, Cdc42p is essential and regulates directed growth, with the Δcdc42 allele exhibiting a phenotype of small, round cells and activated alleles conferring a phenotype of large, round cells (2). Scd1p, a putative guanine-nucleotide exchange factor for S. pombe Cdc42p, shares amino acid identity with the DbI family of Rho-type guanine-nucleotide exchange factors including the Saccharomyces cerevisiae guanine-nucleotide exchange factor Cdc24p. A Δscd1D mutant is viable and exhibits a round cell morphology, suggesting that Scd1p plays a role in directed cell growth (3).

S. pombe Nrf1p is a 122-amino acid protein with three putative transmembrane domains that was previously identified in a screen for negative regulators of Cdc42p (4). A Δnrf1D mutant was viable; however, high level expression of Nrf1p was lethal, resulting in an ellipsoidal morphology and abnormal vacuolar phenotypes including vacuolar coalescence around the nucleus and subsequent fusion of the vacuoles. In S. pombe, vacuoles are numerous, with greater than 50 individual organelles sometimes present within a cell. Green fluorescent protein (GFP)-Nrf1p and GFP-Cdc42p colocalized to the plasma membrane, nuclear membrane, septum, and vacuolar membranes. The localization of GFP-Nrf1p to the vacuolar membrane and subsequent vacuolar coalescence and fusion depended on Scd1p (4).

To characterize the functional domains of Nrf1p, site-directed mutations were generated. Mutations generated in the three putative transmembrane domains affected GFP-Nrf1p localization, consistent with the prediction that these transmembrane domains were essential for membrane anchoring. Mutations created in predicted extramembranous domains of Nrf1p had varying effects on GFP-Nrf1p localization and function, with the second loop domain of Nrf1p having a role in targeting Nrf1p to the vacuolar membrane. Studies were also conducted to further examine the role of Scd1p in Nrf1p localization to the vacuole. These studies led to the discovery that Δscd1D and Δnrf1D mutants had a defect in endocytosis, and this defect could be reversed by expression of activated Cdc42p. Together, these data suggest that Cdc42p-dependent signaling pathways play a role in endocytosis in S. pombe.

MATERIALS AND METHODS

Strains, Media, and Growth Conditions—S. pombe cells were grown in yeast extract and supplements (YES) complex media or in Edinburgh minimal media (EMM) lacking uracil, leucine, or both (5). EMM and EMM agar were purchased from Bio101 (Vista, CA). Thiamine was added to S. pombe growth media at 5 μg/ml to repress transcription from the nmt1 promoter. The S. pombe strains are listed in Table I. Yeast transformations were performed as described previously (5, 6).

Plasmids and DNA Manipulations—Enzymes, polymerase chain reaction kits, and other reagents were purchased from standard commercial sources.

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The abbreviations used are: GFP, green fluorescent protein; YES, yeast extract and supplements; EMM, Edinburgh minimal media; DCFDA, 2′,7′-dichlorofluorescein diacetate; v-ATPase, vacuolar-ATPase subunit; Thi, thiamine.
and half of the cells were resuspended in H2O for carboxy-DCFDA-A8 (provided by A. Merla) at the GFP–tp319–13C ypt7–hSPSCD1U–GFP antibodies. 

Immunoblot Analysis—Total cellular protein was isolated from cells containing nmt1 promoter-driven GFP-Nrf1p fusion proteins. The cells were grown in EMM5-Leu-Thi liquid media to mid-log phase, collected, washed with H2O, resuspended in 100 μl of 1× phosphate-buffered saline, and spheroplasted at 37 °C in the presence of 5 μg/ml of lucifer yellow carbonyl hydrazine, and half (100 μl) of each sample was incubated at 32 °C with the other half kept on ice. After 90 min, the cultures were washed three times with ice-cold H2O, treated with zymolyase for 30 min at 37 °C, then lysed with acid-washed glass beads for 1 min. 400 μl of media was added to the beads, and the liquid was transferred to a fresh tube, then centrifuged for 15 min at 10,000 × g. The fluorescence (excitation, 426 nm; emission, 550 nm) was determined using a fluorimeter and quantified by comparison to a standard curve of lucifer yellow fluorescence.

**RESULTS**

**Nrf1p Has Three Transmembrane Domains—**GFP-Nrf1p was previously shown to localize to the plasma membrane, nuclear membrane, septum, and vacuolar membrane. To determine if the three predicted transmembrane domains in Nrf1p were functional, three amino acids in the middle of each putative domain were mutated to charged residues (Fig. 1A). These mutations (nrf1tm1, nrf1tm2, and nrf1tm3) were constructed in pREP3X-GFP-A8–nrf1–, a high level expression vector, and expressed as GFP-Nrf1p fusion proteins. Total protein was isolated from wild-type ED668 cells containing GFP-Nrf1p, GFP-Nrf1tm1p, GFP-Nrf1tm2p, or GFP-Nrf1tm3p, and all were expressed at similar levels (Fig. 1B). Although high level overexpression of GFP-Nrf1p was lethal (4), the three transmembrane mutant proteins did not confer lethality when overexpressed (Fig. 2A). The localization of these mutant proteins was abnormal with no vacuole localization, variable levels of diminished plasma and nuclear membrane localization, and a general increase in the presence of cytosolic GFP aggregates (Fig. 2B). These data suggest that all three of these domains

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are necessary for efficient localization of GFP-Nrf1p to plasma, nuclear, and especially vacuolar membranes, supporting the hypothesis that these are functional transmembrane domains.

The Second Loop of Nrf1p Was Necessary for Its Localization to the Vacuole—Site-directed mutagenesis was performed on the putative extramembranous loops of Nrf1p to determine which were necessary for function. The “positive inside” observation was followed, which predicts that the nontranslocated loops of a protein are enriched in positively charged residues compared with translocated loops (10). Three charged residues within a stretch of five amino acids in the predicted nontranslocated (i.e., cytoplasmic) loops were changed to alanine residues in pREP3X-GFP-A8-nrf1 (Fig. 1A). Total protein was isolated, and the level of expression of GFP-Nrf1 nt-1p, GFP-Nrf1L2–1p, GFP-Nrf1 L2–2p was comparable with wild-type GFP-Nrf1p (Fig. 1B). Overexpression of GFP-Nrf1 nt-1p was lethal, and localization of the protein appeared similar to GFP-Nrf1p, suggesting that these charged residues were not necessary for the proper localization of GFP-Nrf1p or its lethality when overexpressed. Overexpression of GFP-Nrf1 L2–2p also did not confer lethality, and no fluorescence was localized to the vacuole, although the plasma membrane and nuclear membrane localization appeared enhanced compared with overexpression of wild-type GFP-Nrf1p (Fig. 3, A and B). These data indicate that this portion of the second extramembranous loop was necessary for efficient localization to the vacuoles and was also necessary for the protein to cause a lethal defect when overexpressed. Overexpression of GFP-Nrf1 L2–2–p also did not confer lethality, but it localized similarly to wild-type GFP-Nrf1p (Fig. 3, A and B). These data indicate that the nrf1 L2–2 mutation affects the ability of Nrf1p to cause a lethal phenotype when overexpressed; however, it does not appear to affect the localization of the protein in wild-type cells.

Overexpression of nrf1 Mutations in Dscd1 Cells—The localization of Nrf1p to vacuoles and subsequent coalescence of vacuoles around the nucleus and vacuolar fusion depended on Scd1p (4). To determine if Scd1p may affect the localization of the GFP-Nrf1 mutant proteins, we expressed them in Dscd1 cells. Overexpression of wild-type GFP-Nrf1p or GFP-Nrf1nt-1p was lethal in Dscd1 cells; however, overexpression of the other GFP-Nrf1 mutant proteins did not affect the viability of this strain (data not shown). GFP-Nrf1nt-1p was localized to the plasma and nuclear membranes but was not observed in the vacuolar membranes similarly to wild-type GFP-Nrf1p (Fig. 4). The three transmembrane mutant proteins GFP-Nrf1tm1p, GFP-Nrf1tm2p, and GFP-Nrf1 tm3p all showed a similar localization pattern with no vacuole localization and variable levels of diminished plasma membrane and nuclear membrane localization, comparable with the localization pattern in wild-type cells (data not shown). Localization of GFP-Nrf1tm2–1p was as predicted, with localization to the plasma and nuclear membranes only (Fig. 4). However, GFP-Nrf1tm2–2–p localization was similar to that observed in wild-type cells, with localization to the plasma membrane, nuclear membrane, and the vacuolar membranes, indicating a bypass of the Scd1p requirement for vacuolar membrane localization (Fig. 4). These data further implicate the second extramembranous loop of Nrf1p in the localization of GFP-Nrf1p to the vacuolar membranes and suggest that there may exist a Scd1p-independent mechanism for Nrf1p localization.
Sced1p and Nrf1p Affect Endocytosis—These above-mentioned localization results suggested that Nrf1p may first be targeted to the plasma membrane and subsequently localize to the vacuolar membrane through endocytosis, implicating Scd1p and/or Nrf1 in the endocytosis pathway. To examine this possibility, lucifer yellow uptake was assayed in wild-type, \( \Delta \)scd1, and \( \Delta \)nrf1 cells as well as \( \Delta \)ypt7 cells, which are deficient in vacuolar fusion and, hence, endocytosis. \( \Delta \)scd1 cells showed a decrease in endocytosis similar to what was observed with \( \Delta \)ypt7 cells, whereas \( \Delta \)nrf1 cells showed a lesser deficiency in uptake of lucifer yellow (Fig. 4A). These data are consistent with Scd1p and Nrf1p having a positive function in endocytosis.

To determine whether endocytosis in wild-type or \( \Delta \)scd1 cells could be affected by overexpression of Nrf1p or the Nrf1 mutant proteins, the \( \Delta \)nrf1 mutations described previously were inserted into pREP1-\( \Delta \)nrf1+, a high level expression plasmid. As with overexpression of GFP-Nfr1p fusion proteins, overexpression of the wild-type Nrf1p or Nrf1\( \Delta 12\)p from these plasmids led to lethality after extended induction periods in both strains, whereas overexpression of the other mutant proteins did not (data not shown). Overexpression of wild-type Nrf1p or Nrf1\( \Delta 12\)p caused a significant decrease in lucifer yellow uptake in wild-type cells under conditions where the cells were still viable, whereas overexpression of the other mutant proteins led to at least as much lucifer yellow uptake as the vector control (Fig. 4B). These data suggest that overexpression of functional Nrf1p leads to a decrease in endocytosis. Overexpression of Nrf1p or any of the mutant proteins did not affect lucifer yellow uptake in the \( \Delta \)scd1 strain (data not shown). Overexpression of Nrf1\( 1.2\)p, which localizes to the vacuole in \( \Delta \)scd1 cells, did not increase endocytosis in these cells, suggesting that targeting of Nrf1p to the vacuole (at least of this mutant protein) could occur independent of Scd1p-dependent endocytosis.

Scd1p and Nrf1p Effects on Endocytosis Appear to Be Dependent on Cdc42p—To examine whether Cdc42p may be involved in endocytosis, dominant-activated Cdc42\( ^{G12V}\)p and dominant-negative Cdc42\( ^{T17N}\)p were overexpressed in wild-type cells, and the ability to uptake lucifer yellow was analyzed. Overexpression of dominant-activated Cdc42\( ^{G12V}\)p produced a 3–5-fold increase in lucifer yellow uptake (Fig. 5A), with no vacuolar abnormalities or coalescence observed (data not shown). A modest increase in lucifer yellow uptake was also observed with overexpression of dominant-negative Cdc42\( ^{T17N}\)p (Fig. 5A). These results suggest that overexpression of Cdc42 mutant proteins can influence endocytosis. A small decrease in lucifer yellow uptake was also observed upon overexpression of kinase-inactive Pak1\( ^{K415R,K416R}\)p, suggesting that Pak1p may be involved in the endocytic process. These data raise the possibility that the endocytic defects observed in the \( \Delta \)scd1 and \( \Delta \)nrf1 mutants may be the result of decreased Cdc42p activity. To test this possibility, dominant-activated Cdc42\( ^{G12V}\)p was overexpressed in the \( \Delta \)scd1 and \( \Delta \)nrf1 strains. Overexpression of Cdc42\( ^{G12V}\)p rescued the endocytosis defect in both the \( \Delta \)scd1 and \( \Delta \)nrf1 strains (Fig. 5B) but not the defect observed in cells overexpressing Nrf1p (data not shown), suggesting that the endocytosis defect observed in these strains was due to a decrease in Cdc42p activity (see “Discussion”).

The Vacuolar Coalescence Observed upon Nrf1p Overexpression Was Independent of the Mitogen-activated Protein Kinase Pmk1p/Spm1p but Was Dependent on Ypt7p—Vacuoles fuse in response to osmotic stress (i.e. placement in H2O), and this fusion has been shown to be dependent on the Pmk1p/Spm1p mitogen-activated protein kinase cascade as well as the conserved Rab-like GTPase Ypt7p, which is necessary for vacuolar fusion in S. cerevisiae (11, 12). To determine whether the vacuolar coalescence around the nucleus and subsequent vacuole fusion observed in cells overexpressing Nrf1p act by a similar
mechanism, Nrf1p was overexpressed in Δpmk1 and Δypt7 mutant strains. As previously shown in wild-type cells, overexpression of Nrf1p was lethal in these strains (data not shown). The vacuolar morphology was observed by carboxy-DCFDA staining and confirmed by subsequent localization of GFP-Nrf1p. The Δpmk1 strain showed the abnormal vacuolar phenotypes associated with Nrf1p overexpression, but no vacuolar changes were observed in the Δypt7 cells (Table II). GFP-Nrf1p localization appeared normal in the Δpmk1 cells, but the Δypt7 strain showed localization only to the plasma membrane (data not shown). These data suggested that the localization of Nrf1p to the vacuole and the subsequent abnormal vacuolar morphology was independent of Pmk1p but was dependent on Ypt7p.

Nrf1p and Scd1p Were Not Necessary for Vacuolar Fusion Induced by Osmotic Stress—To determine whether Scd1p or Nrf1p may be necessary for vacuolar fusion induced by osmotic stress, Δscd1 and Δnrf1 cells were grown in YES media, stained with carboxy-DCFDA, and shifted into H₂O to observe the vacuolar morphology (Fig. 7). Fusion was observed in Δscd1 cells as well as Δnrf1 cells, suggesting that the vacuolar fusion induced by osmotic stress is independent of Scd1p and Nrf1p. These data are consistent with the vacuolar fusion induced by overexpression of Nrf1p occurring through a separate mechanism.

**FIG. 6. Overexpression of constitutively activated Cdc42<sup>G12V</sup>p suppressed the Δscd1 and Δnrf1 endocytosis defects.** A, the indicated cdc42 and pak1 alleles were transformed into ED668 cells and selected on EMMS-Leu–Thi media. Individual transformants were grown in liquid EMMS-Leu–Thi media at 30 °C for 18–24 h then harvested and assayed for the ability to uptake lucifer yellow. B, pREP1-cdc42<sup>G12V</sup>p was transformed into ED668, Δscd1, and Δnrf1 cells and selected on EMMS-Leu–Thi media. Individual transformants were grown in liquid EMMS-Leu–Thi media at 30 °C for 18–24 h then harvested and assayed for the ability to uptake lucifer yellow. WT, wild type.

**FIG. 7. Nrf1p and Scd1p were not required for osmotically induced vacuolar fusion.** ED668, Δscd1, and Δnrf1 cells were grown in YES media to mid-log phase, harvested, then stained with carboxy-DCFDA. Half of the cells were resuspended in YES, and the other half were resuspended in H₂O and examined microscopically for carboxy-DCFDA staining. Scale bars = 10 μm.

**TABLE II**

| Strain      | Plasmid       | % normal | % abnormal* |
|-------------|---------------|----------|-------------|
| ED668       | pREP1         |          |             |
| ED668       | pREP1-nrf1    |          |             |
| Δscd1       | pREP1         | 100      | 0           |
| Δscd1       | pREP1-nrf1    | 99       | 1           |
| Δypt7       | pREP1         | 100      | 0           |
| Δypt7       | pREP1-nrf1    | 100      | 0           |
| Δpmk1       | pREP1         | 96       | 4           |
| Δpmk1       | pREP1-nrf1    | 68       | 32          |

* Represents cells with vacuoles coalesced around the nucleus. n = 200.

**DISCUSSION**

Nrf1p proteins that contained mutations in the putative transmembrane domains designed to perturb the predicted hydropathy of this domain no longer conferred a lethal phenotype and were mislocalized, with a decrease in membrane localization and an increase in GFP aggregates, suggesting that these domains were necessary for efficient targeting to membranes. The nrf1<sup>Δ<sup>1-1</sup></sup> mutation did not affect the GFP localization or lethality conferred by overexpression, whereas mutations in the predicted second extramembranous loop did, suggesting that this region of the protein was involved in localization of GFP-Nrf1p to the vacuolar membrane. Both GFP-nrf1<sup>Δ<sup>2-2</sup></sup>p and GFP-nrf1<sup>Δ<sup>2-2</sup></sup>p did not confer lethality when overexpressed, and GFP-nrf1<sup>Δ<sup>2-2</sup></sup>p did not localize to the vacuolar membrane, whereas GFP-nrf1<sup>Δ<sup>2-2</sup></sup>p localized to the vacuolar membrane independent of Scd1p.

Both deletion and overexpression of nrf1p led to similar defects in endocytosis. This seemingly paradoxical result is reminiscent of results with Cdc24p in *S. cerevisiae* in which both deletion and overexpression of Cdc24p led to a loss-of-function phenotype (13). One explanation could be that loss of a protein and excess amounts of a protein both adversely affect the stability and/or function of a multiprotein complex essential for the process in question. Deletion of scd1 also led to a decrease in endocytosis, whereas overexpression of dominant-activated Cdc42<sup>T17N</sup>p led to a 3–5-fold increase in endocytosis and rescued the defect in Δscd1 and Δnrf1 cells. These data implicate the Cdc42p-dependent signaling pathway in the endocytic process in *S. pombe*. Overexpression of dominant-negative Cdc42<sup>T17N</sup>p also led to a modest increase in endocytosis, sug-
gesting that this allele may not function negatively in the endocytosis pathway. In mammalian cell lysates, endocytic vesicles have been shown to move at the ends of actin tails, and the Cdc42p effector, Wiskott-Aldrich syndrome protein (WASP), has been shown to be involved in the nucleation of actin and subsequent propulsion of these vesicles (14–16). Two Rho-type GTPase inhibitors, ToxB and RhoGDI, interfered with this process, and purification of a soluble factor capable of rescuing the ToxB defect was identified as a Cdc42p-RhoGDI complex (15). These data are consistent with the Cdc42p-dependent signaling pathway being involved in the trafficking of endocytic vesicles.

In mammalian cells, Cdc42p localizes to Golgi membranes and has been shown to regulate targeted secretion to the basolateral membrane in polarized epithelial cells (17, 18). Microinjection of plasmids encoding dominant-negative Cdc42p17N resulted in a mistargeting of proteins normally destined for the basolateral membrane as well as a mistargeting of basolateral endocytosed proteins. Microinjection of activated Cdc42pQ61L-encoding plasmids resulted in a complete loss of plasma-membrane polarity (18). Cdc42p has also been shown to interact with the COPI coatamer complex, specifically through the γCOP subunit (19). COPI subunits have been purified from isolated endosomes, suggesting that COPI is involved in targeting in the endocytic pathway as well (20, 21). The cdc42f2S17L-transforming mutant showed a defect in COPI recruitment to vesicles; however, a triple Cdc42pF2S17L,K183S,K184S mutant protein that could no longer interact with γCOP resulted in a lack of transformation ability (19). This mutant protein contained two altered C-terminal lysine residues necessary for COPI binding, suggesting that Cdc42p interaction with COPI was necessary for its ability to transform cells. Altogether, these data implicate mammalian Cdc42p2 in both exocytic and endocytic trafficking, and the data herein implicate S. pombe Cdc42p in the endocytic pathway, suggesting a conserved role for this GTPase in these processes.

In S. pombe, vacuoles are numerous, with greater than 50 individual organelles sometimes present within a cell. Under hypo-osmotic stress conditions, the vacuoles can fuse into one individual organelle sometimes present within a cell. Under critical reading of this manuscript.

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