Functional analysis of RhoGDI inhibitory activity on vacuole membrane fusion

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INTRODUCTION

Rho GTPases act as molecular switches to regulate many cellular processes such as cytoskeletal rearrangement, cell motility and membrane trafficking [1–3]. Three classes of proteins regulate the switch mechanism of Rho GTPases: Rho GEFs (guanine-nucleotide-exchange factors) [4], Rho GAPs (GTPase-activating proteins), and RhoGDIs (GDP-dissociation inhibitors). Rho GEFs promote GTPase activation by facilitating the exchange of GDP for GTP, whereas GAPs stimulate intrinsic GTPase activity, leading to GTPase inactivation. RhoGDIs are the natural inhibitors of Rho GTPase, which contains several distinct regulatory activities (reviewed in [5,6]). They bind to GDP-bound Rho GTPases and inhibit GDP dissociation, thereby maintaining them in an inactive form and preventing GEF-mediated activation. RhoGDIs can also bind to GDP-bound Rho GTPases and inhibit GAP-stimulated GTP hydrolysis. In this context, RhoGDIs have been shown to prevent the binding of effector proteins. RhoGDI forms a high affinity soluble complex with Rho GTPases, whereby the C-terminal lipid tail of Rho proteins are sequestered in a hydrophobic pocket formed by an immunoglobulin-like β-sandwich at the C-terminus of RhoGDI [5]. This confers the ability of RhoGDIs to extract Rho proteins from membranes, and thus regulate cycling between the membrane and cytosol. Given these multiple functions, RhoGDIs are clearly important regulators of Rho GTPase signaling transduction.

Few studies have investigated the regulatory role of the sole RhoGDI in budding yeast, Rdi1p. Overexpression of RDI1 has been reported to cause growth arrest [7,8] or mild morphological defects [8,9] depending on the level of expression. In contrast, no major morphological abnormalities are apparent in RDI1 deletion mutants [7,8,10]. Rdi1p has been shown to interact with Cdc42p, Rho1p and Rho4p, but not Rho3p or Rho5p [8,10]. With respect to the role of Rdi1p in membrane trafficking, we have shown previously that GST (glutathione transferase)–Rdi1p can extract Cdc42p and Rho1p from vacuole membranes in vitro, which are then no longer fusion competent [11]. This defines a novel role for vesicle-bound Rho proteins in membrane fusion.

It is thought currently that Cdc42p and Rho1p regulate two sub-reactions of vesicle transport and fusion: the formation of cytoskeletal tracks that are required for spatial regulation of vesicle mobilization [4], and the activation of membrane-localized actin remodelling activity [12–14]. Using yeast homotypic vacuole fusion, we have shown that the latter process occurs during membrane docking and is dependent on the activation of both Cdc42p and Rho1p [11,12,14,15]. More recently, we have shown that cycles of Cdc42p activation are required to support multiple rounds of vacuole fusion in vivo [16]. In the present paper, we have characterized the effects of RDI1 gene-deletion and overexpression on cell morphology and vacuole membrane fusion. RDI1 deletion showed no effects, whereas overexpression resulted in several morphological defects including abnormal cell cycle, highly fragmented vacuole fragmentation and elevated levels of soluble Rho proteins. Cell-free assays showed impaired vacuole fusion and GTPase activation. We prepared a highly specific antibody for yeast Rdi1p, which we used to show that Rdi1p is predominantly free of Rho GTPases in the cytosol. Structure–function analysis defined the need for the C-terminal lipid-binding domain of vacuole membrane fusion.
Table 1  \textit{S. cerevisiae} strains used in the present study

<table>
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<th>Strain</th>
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<td>The present study</td>
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<tr>
<td>RDI1Δ</td>
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<td>KTY1</td>
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* KTY1 background; † KTY2 background.

The pocket of Rdi1p to bind effectively Cdc42p and Rho1p which was sufficient to inhibit membrane fusion.

**EXPERIMENTAL**

Yeast strains, growth and overexpression analysis

The yeast strains used in the present study are listed in Table 1. These strains were grown at 28°C in YPD medium [1% (w/v) yeast extract, 2% (w/v) peptone and 2% (w/v) dextrose] or YPDG [1% (w/v) yeast extract, 2% (w/v) peptone, 1% (w/v) dextrose and 1% (w/v) galactose] for the induction of Rdi1p overexpression. To generate the Rdi1p overexpression strains, RDI1Δ, the \textit{GALI} promoter and three HA (haemagglutinin antigen) epitopes were inserted at the 5’ end of the \textit{RDI1} open reading frame by homologous recombination using a PCR product. This product was generated with primers containing the 40 nt of sequence immediately upstream and downstream of the \textit{ATG} start codon and the 20 nt of sequence that anneals to the template plasmid pFA6a-HIS3MX6-PGAL1-3HA [17]. To create the \textit{RDI1} deletion strains rdi1Δ, DFY1 and DFY2, a HIS3MX6 DNA cassette flanked by 40 nt of homology with the 5’ and 3’ UTR (untranslated region) of \textit{RDI1} was generated by PCR using pFA6a-HIS3MX6 as a template [17] and then inserted into the \textit{RDI1} locus by homologous recombination. For qPCR (quantitative PCR), total yeast RNA was isolated by phenol/chloroform extraction of disrupted cells in nucleic acid isolation buffer [20 mM Tris/HCl, (pH 7.5), 150 mM NaCl, 0.1% SDS and 0.5% Triton X-100] containing 10 units/ml DNAse I and 10 units/ml RNAse Out™.

cDNA was generated from 1 μg of RNA using the qScript™ Flex cDNA synthesis kit according to the manufacturer’s instructions (Quanta Biosciences). qPCR reactions were conducted on a MX3005PTM thermocycler (Stratagene) using a PerfeCTa SYBR® Green supermix low Rox real-time PCR kit (Quanta Biosciences) using \textit{ACT1} and \textit{RDI1}-specific primers. qPCR products were quantified using the two standard curve method [18] with \textit{RDI1} mRNA normalized to actin mRNA. The primer sequences are listed in Supplementary Table S1 (at http://www.BiochemJ.org/bj/434/bj4340445add.htm).

Biochemical reagents and antibodies

Reagents were purchased from Sigma unless otherwise specified. GDP and GTPγS were dissolved in PS buffer [20 mM Pipes/KOH (pH 6.8) and 200 mM sorbitol] as 10 mM stock solutions. PIC (protease inhibitor cocktail) was made as a 60× stock solution (10 μg/ml leupeptin, 20 μg/ml pepstatin, 25 mM α-phenanthroline and 5 mM Pefabloc® SC). FRB (fusion reaction buffer; 125 mM KCl, 5 mM MgCl2, 10 μM CoA and 1× PIC) and ATPreg (ATP-regenerating system; 0.5 mM ATP, 0.5 mM MgCl2, 20 mM creatine phosphate and 0.5 mg/ml creatine kinase) were made as a 10X stock solutions in PS buffer. Rabbit anti-Rdi1p antibodies were generated in New Zealand white rabbits against full-length Rdi1p (Pacific Immunology). Antibodies against Cdc42p were purchased from Santa Cruz Biotechnology. Antibodies against Rho1p vacuole-associated proteins (Ypt7p, Vam3p, Nv1p, Vti1p, Vam2p, Vac8p and Vph1p) and vacuole lumenal enzymes CpY (carboxypeptidase-Y), ALP and PrA (proteinase A) have been described previously [4,12,19]. Immunoblots were quantified using fluorescently tagged secondary antibodies and an Odyssey image analysis system (LiCor).

**Protein preparation and subcellular fractionation**

Whole-cell lysates were prepared from cell cultures grown to <2.0 \textit{D}0. The cells were disrupted by vortexing with glass beads in lysis buffer [20 mM Pipes/KOH (pH 6.8), 60 mM KCl, 5 mM MgCl2, 0.1 mM DTT (dithiothreitol), 0.1 mM PMSF, 2× PIC and 0.5% Triton X-100]. The lysates were cleared by centrifugation at 20000 g for 30 min at 4°C. For subcellular fractionation and gel-filtration analysis, cells were homogenized in lysis buffer in the absence of detergent and PNS (postnuclear supernatants) were prepared by 1000 g centrifugation for 20 min at 4°C. PNS samples were incubated for 10 min at 30°C with 5 mM ATP in FRB and 3.5 μM GST–Rdi1p where indicated. The supernatants were centrifuged at 20000 g and subsequently at 55000 rev/min using a TLA 120.1 rotor (“100 000 g”) for 30 min at 4°C to prepare subcellular fractions. For gel filtration, 200 μl of supernatant was loaded on to a Superdex® 200 10/300 GL column and 1 ml fractions were collected using an AKTA Explorer FPLC (GE Healthcare).

GST-tagged Rho-activation probes were derived from the Cdc42p- and Rho1p-binding domains of human PAK1 (p21 activated kinase 1), GST–CBD (Cdc42-binding domain) and rhoetkin [GST–RBD (RhoA-binding domain)] as described previously [15]. Full-length and truncation constructs of Rdi1p were cloned into pGEX 4T1 using the primers listed in Supplementary Table S2 (at http://www.BiochemJ.org/bj/434/bj4340445add.htm). GST-tagged fusion proteins were expressed and purified from \textit{Escherichia coli} as described previously [11]. Recombinant Rdi1p was cut from GST–Rdi1p immobilized on glutathione beads by digestion with 10 units/ml thrombin for 2 h at 37°C in 20 mM Tris/HCl (pH 8.0) 100 mM NaCl and 2.5 mM CaCl2. Thrombin was removed with p-aminobenzamidine agarose and the untagged Rdi1p was subjected to buffer exchange in PS buffer using a G25 column (GE Healthcare). The recombinant Rdi1p recovered from the eluate fraction of thrombin-digested GST–Rdi1p is shown in Supplementary Figure S3 (at http://www.BiochemJ.org/bj/434/bj4340445add.htm).

**Vacuole isolation, membrane fusion and GTPase-activation reactions**

Vacuoles were isolated on Ficoll density gradients and \textit{in vitro} membrane fusion reactions were performed as described previously [20]. Standard fusion reactions contained 3.5 μg of vacuoles from each of the \textit{proALP} (i.e. KTY1/DFY1/DFY3) and \textit{Protease} (i.e. KTY2/DFY2/DFY4) tester strains in
RESULTS

Rdi1p overexpression alters cell morphology and increases cytoplasmic Cdc42p and Rho1p

We have shown recently that two Rho GTPases, Cdc42p and Rho1p, are sequentially activated during yeast vacuole membrane fusion [15]. Rdi1p (yeast RhoGDI) is the natural inhibitor of Rho GTPase. To investigate further the role of Rho GTPase activation in membrane fusion we examined the effect of deletion and overexpression of Rdi1p. For overexpression in the strain RDI1Δ, we inserted the GAL1 promoter and 3-α-epitopes at the start of the RDI1 gene. Growth in galactose resulted in a 12-fold increase in RDI1 mRNA (Figure 1A). To compare protein levels, antibodies were generated against full-length Rdi1p. Immunoblotting showed a 5-fold increase in 3HA–Rdi1p levels over endogenous Rdi1p (Figure 1B). The RDI1 deletion strain rdi1Δ, showed no mRNA or protein signals.

To characterize the effect of RDI1 deletion and overexpression on cell morphology, both cell division (budding) and actin patch distribution were examined. The proportion of unbledd, small budded and large budded cells in the WT and rdi1Δ strains were similar, whereas RDI1Δ cells exhibited an increase in unbledd cells (Figure 1C). RDI1Δ cells also consistently exhibited numerous actin patches in the mother cell, which was not observed in WT cells (Figure 1D, arrows). These observations are consistent with previous studies that have shown depolarization of actin following Rdi1p up-regulation, and suggest that Rdi1p perturbs a morphological checkpoint which blocks cell division and increases cell rounding [7–9].

RhoGDI is thought to maintain a cytosolic pool of Rho GTPase, which can be recruited rapidly to membranes [5,6]. We prepared subcellular fractions to investigate the effect of RDI1 deletion and overexpression on the levels of membrane-bound compared with soluble Rdi1p–Rho GTPase complexes. In WT cells Rdi1p was localized to the 100 000 g superant that represents cytosol, while Cdc42p and Rho1p were not found in this fraction (Figure 2A). Instead Cdc42p and Rho1p were localized to light membrane fractions including the 20 000 g superant and 100 000 g pellet; Rho1p was also localized to heavy membranes (Figure 2A, 20KgP). When Rdi1p was overexpressed, Cdc42p and Rho1p could be detected in the cytosol (Figure 2B, 100KgS).

Gel-filtration chromatography was used to examine the size of complexes in the 200 000 g and 100 000 g superant fractions. In WT cell lysates, Cdc42p and Rho1p eluted in high MW (molecular mass) fractions of 200 000 g superants (Figure 2C, upper panel), and were not found in fractions of 100 000 g superants (Figure 2D, upper panel). This indicates that Rho proteins are localized to large membrane-associated complexes. In contrast with Rho GTPases, the majority of Rdi1p was localized to low MW fractions of the approximate size of monomeric Rdi1p. A small portion of Rdi1p was detected in the high MW membrane complexes in the 200 000 g superant fractions (Figure 2C, upper panel). These observations suggest that, under WT conditions, the majority of Rdi1p exists in an unbound state in the cytosol, whereas Cdc42p and Rho1p are membrane-bound. Overexpression of Rdi1p resulted in the presence of low MW Cdc42p and Rho1p in fractions of both the 200 000 g and 100 000 g superants (Figures 2C and 2D, middle panels). These coincided with the overexpressed 3HA–Rdi1p. The modest increase in soluble GTPase suggests that only a small portion of Rho proteins are free to interact with Rdi1p. Indeed, we observed that cytosolic GTPases were more readily detected in WT cell lysates that were pre-treated with exogenous GST–Rdi1p, which confirms the presence of new Rdi1p–Rho GTPase soluble complexes (Figures 2C and 2D, bottom panels). These
findings are consistent with previous studies, which have shown that elevated Rdi1p facilitates the dissociation of Rho GTPases from membranes [8,24,25].

**Rdi1p impairs vacuole fusion and ALP sorting via the extraction of Cdc42p and Rho1p**

We next examined the effect of *RDI1* deletion and overexpression on vacuole membrane morphology using the lipophilic dye FM4-64. Normal morphology is one to three large vacuoles, which was observed for both WT and *rdi1Δ* cells (Figure 3A). In contrast, *RDI1*OE cells exhibited highly fragmented vacuoles (Figure 3A, *RDI1*OE). To determine if fragmentation was due to a defect in membrane fusion, cells were exposed to hypotonic stress which stimulates rapid homotypic vacuole fusion [26]. Vacuoles in the WT and *rdi1Δ* strains fused rapidly following hypotonic shock, whereas vacuoles in the *RDI1*OE strain remained highly fragmented (Figure 3A, left-hand panels). This suggests that increased Rdi1p expression directly impairs vacuole fusion.

To determine the mechanism through which Rdi1p overexpression inhibits vacuole fusion, we examined the levels of proteins known to be required for fusion [27]. Immunoblotting of whole cell lysates showed no difference between the WT, *rdi1Δ* and *RDI1*OE strains for all proteins examined (Figure 3B, left-hand panels). However, Cdc42p and Rho1p were reduced significantly in the *RDI1*OE strain when the protein levels on purified vacuoles were examined. The reduction in membrane-associated Rho GTPases was selective since there was no change in expression of the vacuolar Rab GTPase Ypt7p (Figure 3B, top panel). In contrast with a recent study which reported that Rho1p and Cdc42p were reduced in *rdi1Δ* cell extracts [28], we observed no decrease for either GTPase in *rdi1Δ* whole cell lysates or purified vacuoles. With the noted exceptions, we generally found no differences in protein levels for vacuolar SNAREs (soluble N-ethylmaleimide-sensitive fusion protein-attachment protein receptors), membrane and peripheral membrane proteins in *rdi1Δ* and *RDI1*OE cells (Figure 3B, left-hand panels). Similarly, the vacuolar lumenal enzymes CpY and PrA were unchanged. The exceptions were Vam3p and ALP, both type II vacuole membrane proteins; these proteins were reduced in vacuoles purified from *RDI1*OE cells (Figure 3B). To quantitatively assess ALP and PrA levels, solubilized vacuoles were analysed by enzyme assays. These analyses confirmed that vacuolar PrA levels were unaffected by Rdi1p expression, whereas ALP was reduced ∼40% following Rdi1p up-regulation (Supplementary Figure S1 at http://www.BiochemJ.org/bj/434/bj4340445add.htm). It has been shown previously that ALP and Vam3p are sorted via a unique pathway directly from the Golgi to the vacuole, whereas ‘CpY pathway’ cargoes (e.g. Prc1p and PrA) transit through an intermediate organelle termed the pre-vacuolar compartment [29,30]. Our results indicate that Rdi1p overexpression may selectively affect the ALP-sorting pathway.
To characterize further ALP sorting, we expressed GFP (green fluorescent protein)–ALP in the WT, rdi1Δ and RDI1 OE strains (Figure 4A). Cells were co-stained with FM4-64, which labels the vacuole membrane via endocytosis and therefore is independent of the ALP-sorting pathway. Confocal microscopy showed co-localization of the two markers in the WT and rdi1Δ strains on the vacuole (Figure 4B, top two panels). In the RDI1 OE strain, while there was significant co-localization of the markers on the vacuole, there was also numerous green puncta that did not co-localize with the vacuole marker (Figure 4B, bottom panel). These puncta showed a similar distribution as transitional ER (endoplasmic reticulum) or Golgi, where secretory cargo is sorted and exported [31,32]. These results provide evidence that Rdi1p overexpression reduces vacuolar ALP levels because of a delay in sorting via the ALP pathway.

Many yeast mutants have been shown to give rise to fragmented vacuoles. These include genes that are implicated directly in vacuole fusion (vam mutants) [23,33], as well as others involved in vacuole protein sorting (vps mutants) and maintenance of vacuole function [29]. To determine if vacuole fragmentation

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**Figure 2** Rdi1p overexpression increases cytosolic Cdc42p and Rho1p

Whole cell extracts (2.5 mg/ml) from the WT and RDI1 OE strains were subjected to sequential centrifugation for 30 min at 1000 g (PNS), 20,000 g and then 100,000 g. (A and B) Equal amounts of PNS, and supernatant and pellet subcellular fractions from 20000 g and 100000 g spins (20K g and 100K g respectively) were analysed by immunoblotting for the presence of Rdi1p, Cdc42p and Rho1p. (C and D) 20K g and 100K g fractions were subjected to gel-filtration chromatography. In one set of experiments, 3.5 μM GST–Rdi1p was incubated with WT extracts for 15 min prior to the centrifugation steps. 0.2 ml of supernatant was loaded on to a Superdex 200 10/300 GL column and 1 ml fractions were collected. Fractions 9–20 were immunoblotted to determine the elution profile of Cdc42p, Rho1p and Rdi1p. Note that the upper blot shows the elution profile of endogenous Rdi1p, the middle blot shows 3HA–Rdi1p and the bottom blot shows exogenously added GST–Rdi1p. The approximate MW (kDa) of the eluted fractions are indicated at the top. Predicted molecular masses: Rho1p, 23 kDa; Cdc42p, 21 kDa; Rdi1p, 23 kDa; Rdi1p–Rho1p complex, 46 kDa; Rdi1p–Cdc42p complex, 44 kDa. # denotes background bands in Rdi1p immunoblots; * denotes fractions where Cdc42p–Rho1p and Rdi1p co-elute.

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resulting from Rdi1p overexpression was related to any of these established vacuole mutant classes, we examined rdi1Δ and RDI1OE strains for sorting of the vacuolar lumenal enzyme CpY, which is sensitive to caffeine, a cellular toxin that is metabolized by active vacuoles [32] and vacuolar acidification. These were typical assays used in screening and characterization of vps and vam mutants [21,23,29–34]. In contrast with vps mutants, which secrete CpY [29], we did not detect any sorting defect for CpY in the RDI1OE and rdi1Δ strains (Supplementary Figure S2A at http://www.BiochemJ.org/bj/434/bj4340445add.htm). In addition, cellular growth was not affected by the presence of caffeine in either of these strains compared with WT, demonstrating that the vacuoles were metabolically active (Supplementary Figure S2B). Lastly, we examined vacuole acidification as determined by quinacrine uptake. Fragmented vacuoles of the RDI1OE strain exhibited similar vacuole acidification as the normal vacuoles of the WT and rdi1Δ strains (Supplementary Figure S2C). Collectively, these observations support the hypothesis that vacuole fragmentation resulting from Rdi1p overexpression is due strictly to impaired membrane fusion rather than defects in vacuole protein sorting or vacuole metabolic activities.

To evaluate further the role of Rdi1p in membrane fusion, the fusogenic capacity of vacuoles isolated from WT, rdi1Δ and RDI1OE strains was quantified using an established in vitro assay [27]. For this assay, two vacuole populations are isolated from different strains: ‘proALP’ vacuoles (pep4Δ, prb1Δ, PHO8; strain KTY1) lack vacuolar lumenal proteases and therefore accumulate inactive proALP; and ‘protease’ vacuoles (pho8Δ, PEP4, PRB1; strain KTY2) which contain active lumenal proteases, but lack ALP. Vacuole fusion occurs when proALP and protease vacuoles are incubated with ATP, salt and cytosol. The mixing of content results in the processing of proALP to its active form by proteases. Active ALP units are proportional to the extent of membrane fusion. We made rdi1Δ and RDI1OE ‘tester strains’ (see Table 1) to determine the role of Rdi1p in vacuole fusion. Rdi1p overexpression caused an approx. 5-fold reduction in vacuole fusion compared with WT; in contrast, vacuoles isolated from rdi1Δ exhibited modestly higher fusion compared with WT vacuoles (Figure 5A). However, since Rdi1p overexpression affected ALP sorting (Figure 4B), but not protease levels (Supplementary Figure S1B), it was possible that impaired fusion may reflect lower ALP levels. To normalize ALP levels, protease vacuoles from the WT, rdi1Δ and RDI1OE strains (KTY2, DFY1 and DFY3 respectively) were fused to WT proALP vacuoles (strain KTY1). In this assay, vacuoles isolated from the RDI1OE strain exhibited an approx. 3-fold reduction in fusion (Figure 5B). Lastly, we examined the effect of GST–Rdi1p and recombinant Rdi1p (untagged) proteins on the fusion of WT vacuoles. Both GST–Rdi1p and recombinant Rdi1p impaired vacuole fusion in a dose-dependent manner; cleavage of the GST increased the Rdi1p inhibitory activity (Figure 5C).

Rdi1p inhibits GTPase signalling by extracting GDP and GTP-bound Cdc42p and Rho1p

We next examined the activation of Cdc42p and Rho1p during vacuole membrane fusion using an assay developed previously in our laboratory [15]. This assay utilizes GST-tagged Rho activation

Figure 3  Rdi1p overexpression results in vacuole fragmentation and reduction of Cdc42p and Rho1p

(A) WT, rdi1Δ and RDI1OE cells were grown to ∼1.0 D600 in galactose-supplemented medium (YPDG), vacuoles were stained with FM4-64 and cells were imaged by fluorescence microscopy. Hypotonic stress was induced by 10-fold dilution in water 1–2 min prior to imaging. (B) Whole cell extracts (60 μg/lane) and purified vacuoles (6 μg/lane) from WT, rdi1Δ and RDI1OE strains were examined by immunoblotting for vacuole-associated proteins.

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Sorting of GFP–ALP is delayed when Rd1p is overexpressed

Figure 4: Strains were transformed with the plasmid pREG576 containing an in-frame fusion of GFP with the N-terminus of ALP, a type II membrane protein, and grown in galactose and supplemented with kanamycin. (A) Immunoblotting analysis of N-terminally tagged GFP–ALP in WT, Rd1 deletion (rd1Δ) and Rd1 overexpression (RDI1OE) strains. A control sample is WT with an empty vector expressing GFP (control). Levels of tagged and untagged ALP were analysed using anti-ALP antibodies and the presence of GFP–ALP was analysed using anti-GFP antibodies. 50 μg of whole cell lysate was run in each lane. (B) Confocal microscopy of WT, rd1Δ and RDI1OE strains expressing GFP–ALP. Vacuole membranes were stained using the endocytic dye FM4-64. Co-localization of GFP–ALP and FM4-64 was analysed by cross-sectional intensity profiles (right-hand panels, Y-axis=intensity in arbitrary units), as indicated by the dashed white arrows on merged images. Note that the Rd1p overexpression strain, RDI1OE, shows green puncta that are clearly not vacuolar.
Rdi1p structure–function analysis

Several crystal structures of Rho GTPase–RhoGDI complexes have been solved which have broadly defined two RhoGDI domains: an N-terminal α-helical domain that binds to the switch region of the Rho GTPase, and a C-terminal β-sheet sandwich-fold domain that forms a hydrophobic pocket which sequesters the Rho GTPase geranylgeranyl lipid tail [5,6]. We cloned truncated forms of Rdi1p that were missing all or parts of these domains as N-terminal GST-fusion proteins (Figures 8A and 8B, top panel). We examined the ability of these Rdi1p truncation proteins to interact with detergent-solubilized Rho proteins, to extract vacuole-bound Rho GTPases, and to inhibit vacuole fusion. The C-terminal β-sheet domain was required for efficient interaction with Rho proteins, while only full-length Rdi1p showed extraction activity and produced soluble Rho proteins from intact membranes (Figure 8B). The Rdi1p constructs also showed variable vacuole-binding activity (Figure 8C). Again, a complete C-terminal β-sheet domain was required for efficient vacuole binding. Pre-treatment of vacuoles with PrK (proteinase K) disrupted the binding of Rdi1A and Rdi1D, whereas Rdi1E binding was unaffected. Rdi1B exhibited weak vacuole-binding activity, but also appeared to be sensitive to PrK pre-treatment (Figure 8C). These observations suggest that the association of Rdi1p with vacuole membranes is mediated by a protein–protein interaction; however, removal of the α-helical domain (as in Rdi1E) results in a highly lipophilic protein.

We next examined Rdi1p truncations for their ability to extract vacuole-bound Rho proteins and inhibit vacuole fusion. Full-length, but not other Rdi1p fragments, showed a dose-dependent reduction of vacuolar Rho proteins (Figure 8D). Interestingly, inhibition of vacuole fusion by Rdi1p truncations did not show a strict requirement for Rho protein extraction. Although Rdi1A (full-length Rdi1p) showed the most significant inhibition and extraction, Rdi1D (Rdi1p−Δα1) also showed >40% inhibition of fusion (Figure 8E), but showed no Rho protein extraction activity (Figure 8D). Furthermore, membrane association of Rdi1p alone was not sufficient to impair vacuole fusion. Rdi1E, which binds vacuoles efficiently (Figure 8D), showed no inhibition of fusion (Figure 8E). Collectively these observations support the hypothesis that the portions of the N-terminal α-helical domain of Rdi1p which interact with the Rho switch region are required for inhibition of fusion, whereas the entire C-terminal β-sheet domain is required for efficient membrane binding and subsequent targeted association with Rho GTPases.

DISCUSSION

We have shown previously that the Rho GTPases Cdc42p and Rho1p are enriched on purified vacuole membranes and required for vacuole fusion [11]. Cdc42p and Rho1p are activated sequentially during in vitro vacuole fusion reactions [15] and mutations that perturb the Rho activation cycle block vacuole...
**Figure 6** Increased Rdi1p suppresses the activation of vacuolar Cdc42p and Rho1p.

Vacuoles isolated from the strains KTY1, DFY1 and DFY3 (WT, rdi1Δ and RDI1OE respectively) were incubated in FRB, 1 × ATPreg, cytosol and 40 μM GTPγS for 40 min at 30°C or on ice. Levels of activated Cdc42p (A) and Rho1p (B) were determined by association with immobilized GST–CBD and GST–RBD Rho-activation probes respectively. (C) WT proALP vacuoles (from strain KTY1) were incubated for 40 min at 30°C in the presence of increasing concentrations of purified Rdi1p and levels of activated Cdc42p and Rho1p were determined.

fusion in vivo [16]. In the present paper, we have studied how Rho GTPase function for membrane fusion is regulated by their natural inhibitor RhoGDI (Rdi1p in yeast). Overexpression of Rdi1p resulted in strains with highly fragmented vacuoles which we attributed to the depletion of vacuolar Cdc42p and Rho1p, but not other vacuole-associated proteins (Figures 3B and 5A). Although membrane depletion of Rho proteins was not absolute when Rdi1p was overexpressed, the remaining pools of Cdc42p and Rho1p were not activated during membrane fusion reactions (Figures 6A and 6B). This suggests that the basis for defects in vacuole assembly when Rdi1p is overexpressed is the loss of both proper Rho GTPase localization and activation.

RhoGDI has been shown to form complexes with GDP-bound Rho GTPases and prevent GDP dissociation. A number of models favour the idea that RhoGDI preferentially binds and extracts GDP-bound Rho GTPases [5,6,10,35], which is supported by observations that RhoGDI exhibits a higher affinity for GDP-bound GTPases in vitro [35,36]. However, the specificity for GDP-bound Rho is controversial with some reports showing similar [37,38] or higher [8] affinities for GTP-bound GTPases. In addition, active GTPase mutants have been shown to localize to the cytosol and interact with RhoGDI [39–41]. Structural data obtained for Cdc42p also supports the idea that GDP- and GTP-bound forms should exhibit similar, if not identical, binding affinities to RhoGDI since no significant conformational changes in the Cdc42 switch I and II were reported following nucleotide binding [42]. Collectively, these observations support that RhoGDI is capable of interacting with Rho GTPases irrespective of their nucleotide state.

Our analyses demonstrate that Rdi1p can bind efficiently to GTPγS-bound GTPases and prevent interactions with GTPase effectors (Figure 7). These findings suggest that Rdi1p can exert a negative regulatory effect to attenuate GTPase signalling on vacuolar membranes through the extraction of Rho GTPases, irrespective of their nucleotide state. As suggested by other studies, it is possible that the accessibility of GTP-bound Rho GTPases in vivo may be limited due to the recruitment of GTPase effectors and other downstream signalling components.
A GST-Rdi1p pulldown

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Figure 7: Rdi1p extracts GDP- and GTP-bound Cdc42p and Rho1p from vacuolar membranes and impairs the binding of GTPase effector domains

(A) WT vacuoles purified from strain KY1 were loaded with GDP or GTP\(_S\) by chemical nucleotide exchange, then incubated at 30°C for 40 min in FRB, 1 × ATPreg and 3.5 μM GST–Rdi1p. Vacuoles were centrifuged and GST–Rdi1p precipitated from supernatants and membrane pellets using glutathione beads. The beads were washed three times in PS buffer and bound GTPases were examined by immunoblotting. Quantification of band intensities is shown in the lower panels. (B) Chemical nucleotide exchange was performed on WT vacuoles as described in (A). Vacuoles were incubated at 30°C for 40 min in FRB, 1 × ATPreg and purified Rdi1p. Following incubation, GST–CBD or GST–RBD immobilized to glutathione beads were added to the reaction mixture and incubated for an additional 30 min at 4°C. Beads were washed three times in PS buffer and the levels of bound GTPases determined by immunoblotting. Band intensities were quantified and are shown as normalized signals relative to untreated GTP\(_S\) controls (0 μM Rdi1p).

What then, is the role of yeast Rdi1p? As suggested by recent FRAP (fluorescence recovery after photobleaching) and mathematical modeling data from Slaughter et al. [10], it is plausible that two independent pathways function to recycle Rho GTPases: (i) a fast pathway mediated by Rdi1p [4]; and (ii) a slower path that is dependent on vesicular transport. This model supports the idea that endogenous Rdi1p–Rho GTPase complexes are probably transient and dynamic structures, which is in agreement with our finding that elevated levels of active GTPases are inhibitory for fusion as long as they contained an N-terminal domain capable of interacting with the Rho switch regions (Figures 8D and 8E; compare Rdi1D and Rdi1E). This indicates that GTPase signalling is probably attenuated at the moment of contact with Rdi1p at the membrane via the occupation of the Rho switch regions. However, membrane association of Rdi1p precedes Rho protein interaction.

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[10,35]. This could result in higher accessibility of GDP-bound Rho GTPases to RhoGDI and thus the perception that GDP-bound Rho proteins are the preferential substrates. Interestingly, our analysis of Rdi1p truncations demonstrated that membrane extraction of Rho GTPases is not a strict requirement to impair GTPase-dependent vacuolar fusion. Rdi1p truncations that showed association with the vacuole membrane, but little extraction of GTPases, were inhibitory for fusion as long as they contained a N-terminal domain capable of interacting with the Rho switch regions (Figures 8D and 8E; compare Rdi1D and Rdi1E). This indicates that GTPase signalling is probably attenuated at the moment of contact with Rdi1p at the membrane via the occupation of the Rho switch regions. However, membrane association of Rdi1p precedes Rho protein interaction.

It has been suggested that a key regulatory role of RhoGDI is to structurally recognize and extract Rho GTPases, and thus maintain a cytosolic ‘reserve’ pool [5]. However, if RhoGDI shows little selectivity between GTP- and GDP-bound GTPases, what prevents the continual block of all Rho GTPase signalling? Our analysis of free compared with complexed Rdi1p via gelfiltration analysis showed the apparent lack of Cdc42p and Rho1p in the cytosol of WT cells (Figure 2). The majority of Rdi1p was monomeric and not bound to Rho GTPases in the cytosol. Only when Rdi1p abundance was increased significantly via overexpression or the addition of recombinant Rdi1p did we find soluble Rdi1p–Rho GTPase complexes (Figure 2C). These findings differ from previous studies which show that a portion of HA-tagged Cdc42p and Rho1p are localized to cytosolic fractions following high-speed centrifugation [8,24]. We were also able to detect 3HA-tagged versions of Cdc42p and Rho1p in high-speed supernatant fractions, whereas endogenous GTPases were not detectable (results not shown). Our findings are consistent with previous analyses of endogenous Cdc42p and Rho1p, which have shown that they are predominantly membrane-bound with only trace amounts detected in high-speed supernatants [43,44].
Figure 8 Structure–function analysis of Rdi1p

(A) Diagram showing the Rdi1p N-terminal α-helical domain which interacts with Rho protein switch regions and the C-terminal β-sheet domain which folds into a β-sandwich and sequesters the isoprenoid lipid modification. The different truncation constructs were made as GST-tagged proteins. (B) The upper panel shows the expression levels of the Rdi1p full length and truncation constructs. The middle panels show the interaction of the various truncations with Cdc42p and Rho1p in detergent extract via GST-pulldown. The lower panels show the ability of Rdi1p truncation constructs to solubilize Cdc42p and Rho1p from intact membranes. (C) Association of full-length Rdi1p and truncation constructs with vacuole membranes. The indicated concentrations of Rdi1p were incubated with 50 μg/ml of vacuoles in FRB and 0.5 mM PMSF at 30°C for 15 min. Vacuoles were reisolated by centrifugation and the levels of bound Rdi1p construct were examined by immunoblotting. To determine the protein dependence of Rdi1p association one reaction contained vacuoles that were pre-treated with 10 units/ml PrK for 10 min followed by the addition of 0.5 mM PMSF (+ PrK). (D) Vacuole samples from (C) were analysed for the presence of vacuole-bound Rho GTPases (Rho1p is shown, Cdc42p is not shown but was similar to Rho1p). (E) Vacuole fusion level when reactions are incubated in the presence of 5 μM full-length Rdi1p (Rdi1A) or truncation constructs.

A recent separate study by Boulter et al. [28] also made a similar observation that rdl1Δ cell extracts exhibited higher levels of activated Cdc42p and Rho1p relative to WT.

In summary, our results suggest that the inhibitory activity of RhoGDI on Rho GTPase signalling is not dependent on the nucleotide-bound state of Rho proteins. Our results suggest the presence of additional regulation of RhoGDI interaction with membranes or proteins prior to binding Rho proteins. Furthermore, we show that inhibitory interactions occur without the need for Rho protein extraction.
AUTHOR CONTRIBUTION
Michael Logan conceived and conducted the Rdi1p functional experiments, analysed the data and co-wrote the paper. Lynden Jones performed the fusion reactions, enzyme analysis and Rho activation assays. Daniel Forsberg constructed the RDI1 deletion and overexpression strains and performed microscopy. Alex Bodman cloned and purified GST–Rdi1p and truncation proteins, and performed the structure–function analysis. Alicia Baier conducted the Rdi1p gel-filtration analysis. Gary Elizven supervised the study, performed confocal microscopy and co-wrote the paper.

ACKNOWLEDGEMENTS
We thank Dr William Wickner (Dartmouth Medical School, Hanover, U.S.A.) and Dr Richard Rachubinski (University of Alberta, Edmonton, Canada) for providing antibodies used in this study.

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REFERENCES


SUPPLEMENTARY ONLINE DATA

Functional analysis of RhoGDI inhibitory activity on vacuole membrane fusion

Michael R. LOGAN, Lynden JONES, Daniel FORSBERG, Alex BODMAN, Alicia BAIER and Gary EITZEN¹

Department of Cell Biology, University of Alberta, Edmonton, Alberta T6G 2H7, Canada

Figure S1 The effect of RDI1 deletion and overexpression on vacuolar luminal enzyme levels

(A) Enzyme assay for vacuolar ALP. proALP vacuoles were purified from KTY1 (WT), DFY1 (rdi1Δ) and DFY3 (RDI1OE) strains. Vacuoles (5 μg in 50 μl) were incubated in FRB with 1 unit/μl proteinase K and 0.5 % Triton X-100 for 10 min at 30 °C to cleave the pro-domain and activate ALP. ALP activity was quantified using p-nitrophenyl phosphate as substrate which liberates the coloured product p-nitrophenyl in the presence of phosphatase activity. Signals were normalized to WT controls (n = 3 independent experiments).

(B) Enzyme assay for vacuolar PrA. PrA vacuoles were purified from KTY2 (WT), DFY2 (rdi1Δ) and DFY4 (RDI1OE) strains. PrA activity was quantified using oxidized insulin chain B as substrate which is cut into TCA (trichloroacetic acid) soluble fragments in the presence of protease. Peptide fragments were reacted with diazonium salt which forms a coloured product in the presence of histidine or tyrosine residues. Signals were normalized to WT controls (n = 3 independent experiments).

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Figure S2  The effect of RDI1 deletion and overexpression on vacuole functions

(A) CpY sorting assay. Missorting of CpY to the plasma membrane determined by colony filter assays as described in the Experimental section of the main paper. Results show that WT, rdi1Δ and RDI1OE strains are not defective compared with vacuole sorting mutants such as vam6Δ. (B) Colony growth assay. A 10-fold dilution series of WT, rdi1Δ and RDI1OE strains were plated on to YPDG medium and YPDG + 6 mM caffeine. Galactose-supplemented medium induces the expression of Rdi1p in the RDI1OE strain. Caffeine selectively impairs the growth of vacuole assembly mutants such as pep5Δ, but not RDI1 deletion and overexpression mutants. (C) Vacuole acidification assay. WT, rdi1Δ and RDI1OE strains were incubated with 200 μM quinacrine which accumulates in acidic compartments. After 5 min of incubation, cells were washed and imaged. Results show that RDI1 strains are not defective for vacuole acidification.
Table S1  Primers used in the Rdi1p study

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Figure S3  Rdi1p purification

GST–Rdi1p was expressed in E. coli (lane 4), bound to glutathione beads (lane 3) and full-length Rdi1p (lane 2) was eluted from beads by incubation with 10 units/ml thrombin in 20 mM Tris/Cl (pH 8.0), 100 mM NaCl, and 2.5 mM CaCl2. Rdi1p eluates were incubated with benzamidine-agarose to remove thrombin (lane 1). Shown is the analysis of equal fractions by SDS/PAGE and Coomassie Brilliant Blue stain. MW is on the left. Note that Rdi1p is slightly lower than the prominent GST background band in lane 4.

REFERENCE


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