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Cdc42p and Rho1p are sequentially activated and mechanistically linked to vacuole membrane fusion

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ABSTRACT

Small monomeric GTPases act as molecular switches, regulating many biological functions via activation of membrane localized signaling cascades. Activation of their switch function is controlled by GTP binding and hydrolysis. Two Rho GTPases, Cdc42p and Rho1p, are localized to the yeast vacuole where they regulate membrane fusion. Here, we define a method to directly examine vacuole membrane Cdc42p and Rho1p activation based on their affinity to probes derived from effectors. Cdc42p and Rho1p showed unique temporal activation which aligned with distinct subreactions of *in vitro* vacuole fusion. Cdc42p was rapidly activated in an ATP-independent manner while Rho1p activation was kinetically slower and required ATP. Inhibitors that are known to block vacuole membrane fusion were examined for their effect on Cdc42p and Rho1p activation. Rdi1p, which inhibits the dissociation of GDP from Rho proteins, blocked both Cdc42p and Rho1p activation. Ligands of $PI(4,5)P_2$ specifically inhibited Rho1p activation while pre-incubation with U73122, which targets Plc1p function, increased Rho1p activation. These results define unique activation mechanisms for Cdc42p and Rho1p, which may be linked to the vacuole membrane fusion mechanism.

Introduction

The Rho family of GTPases, Rho, Rac and Cdc42, are small monomeric GTPases that regulate many intracellular processes from membrane bound positions [1]. These proteins cycle between active and inactive states via GTP binding and hydrolysis. Activation results in the extension of an "effector-loop" which engages downstream effector proteins. Methods to examine the Rho activation state have been developed based on their affinity to effector proteins that specifically associate with the GTP-bound/activated state [2,3]. Since effector proteins are highly selective for defined Rho proteins, specific assays for their activation are possible.

The genome of *Saccharomyces cerevisiae* encodes six Rho GTPases, *RHO2–5* are non-essential while *RHO1* and *CDC42* are essential. Numerous subcellular localizations have been shown for Cdc42p and Rho1p. Cdc42p is primarily localized to the bud tip in yeast where it acts to spatially direct vesicular transport via actin nucleating activity [4]. Rho1p colocalizes with its effectors on the cell surface [5,6], and has been found on secretory vesicles [7], mitochondria [8] and peroxisomes [9]. We have shown that Cdc42p and Rho1p localize to the vacuole [10–12]. However, mechanisms that trigger the activation of Cdc42p and Rho1p at these sites are unknown.

Purified vacuoles undergo homotypic membrane fusion *in vitro*, when incubated in a basic buffer containing ATP and cytosol [13]. We have shown that both Cdc42p and Rho1p participate in the regulation of vacuole membrane fusion. Likewise, Rho proteins have been shown to support membrane fusion at the plasma membrane during exocytosis [14,15].

Here, we surveyed the known yeast Cdc42p and Rho1p effector proteins for their use as activation probes. Our objective was to develop a method to analyze Rho signaling on intracellular membranes. Both Rho1p and Cdc42p are localized to the vacuole membrane, therefore, this is an idea model membrane to develop methods that examine their co-incidental activation. We use Cdc42p and Rho1p activation assays to show their distinct mechanism of activation during *in vitro* membrane fusion, which were uniquely controlled, both temporally and by factors/conditions that stimulated their activation. Membrane fusion inhibitors link Rho activation and vacuole fusion pathways. Our results suggest that the Cdc42p and Rho1p activation mechanism are an integral part of the vacuole membrane fusion mechanism.

Abbreviations: ATPreg, ATP regenerating system; C1b, diacylglycerol binding domain of Pkc1p; ENTH, epsin NH_2 terminal homology domain; FRB, fusion reaction buffer; GST, glutathione *S* transferase; GST-CBD, GST-tagged probe derived from the Cdc42/Rac binding domains of PAK; GST-RBD, GST-tagged probe derived from the Rho-binding domains of Rhotekin; $PI(4,5)P_2$, phosphatidylinositol 4,5-bisphosphate.

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Materials and methods

Yeast strains and growth. The yeast strain, BJ5459 (*ura3–52*, *his3Δ200*, *trp1*, *lys2–801*, *pep4::HIS3*, *prb1Δ1.6R*) was used for vacuole isolation and mutant Cdc42p and Rho1p overexpression. Rho wild-type and mutant overexpression strains were prepared using Drop&Drag cloning [16] as previously described [12]. Plasmid-bearing strains were grown in YPDGk (1% yeast extract, 2% peptone, 1% dextrose, 1% galactose, 30 μg/ml kanamycin), which results in mutant Rho protein overexpression from the galactose inducible *GAL1* promoter.

Protein preparation. Rho activation probes were prepared as previously described [2,3]. Cdc42p, Rho1p and yeast specific Rho activation probes were prepared by cloning PCR fragments from CDC42 (encoding aa 2–187), RHO1 (encoding aa 2–205), STE20 (encoding aa 331–410), CLA4 (encoding aa 184–243), PKC1I (encoding aa 87–243) PKC1II (encoding aa 394–620), and BNI1 (encoding aa 90–343) into pGEX-4TI. Fusion proteins were expressed in *Escherichia coli* BL21. Cdc42p and Rho1p were purified as C-terminal truncation proteins (-CAAX) by GST-cleavage of bead-bound protein with 10 U/ml thrombin in 20 mM HEPES–KOH, 60 mM NaCl, 2 mM $CaCl_2$, pH 7.5. Cytosol was prepared from log-phase cells by vortexing cells for 10 min at 4 °C with glass beads using a ratio of 1 vol of glass beads to 2 vol of cells, resuspended at 1/100th the original culture volume in lysis buffer (20 mM PIPES–KOH, pH 6.8, 200 mM sorbitol, 1 mM Mg–ATP, 1 mM DTT, 2× PIC). Lysates were cleared by centrifugation at 25,000*g* for 30 min at 4 °C.

Reagents. Reagents were purchased from Sigma unless otherwise indicated. Apyrase VII (1 U/μl), neomycin (10 mM) and BAPTA (75 mM) were dissolved in PS-buffer (20 mM PIPES–KOH, pH 6.8, 200 mM sorbitol). U73122 and U73343 (Calbiochem) were dissolved in ethanol at 10 mM. Antibodies against Sec17p and Vam3p were purified as IgG fractions from rabbit sera and the proteins, GST-Rdi1p, Gdi1p, GST-ENTH and GST-C1b were expressed and purified from *E. coli* as previously described [10,17]. GST was removed from fusion proteins by incubation with 10 U/ml thrombin in 20 mM Tris–Cl, pH 8, 100 mM NaCl, 2.5 mM $CaCl_2$. Thrombin was subsequently removed with *p*-aminobenzamidine agarose and samples were dialyzed in PS-buffer with 125 mM KCl, 5 mM $MgCl_2$. 1× ATPreg (ATP regenerating system) was made as a 10× stock solution in PS-buffer (10× ATPreg = 5 mM ATP, 5 mM $MgCl_2$, 250 mM creatine phosphate, 2.5 mg/ml creatine kinase). Protease inhibitor cocktail (PIC) was made as a 60× stock solution (60× PIC = 12 μg/ml leupeptin, 24 μg/ml pepstatin, 25 mM *o*-phenanthroline, 6 mM Pefabloc SC).

Rho activation assays. Level of activated (GTP-bound) vacuolar or cytosolic GTPases were determined by affinity precipitation with Rho activation probes; GST-CBD for Cdc42p and GST-RBD for Rho1p. Specific nucleotide-bound states of recombinant, lysate or vacuolar GTPases were prepared by chemical nucleotide exchange. Briefly, samples were incubated for 5 min at 30 °C in the presence of 3 mM EDTA and 40 μM nucleotide (GDP, GTP or GTPγS), which facilitates nucleotide loading. Loading reactions were quenched by addition of 10 mM $MgCl_2$, which locks GTPases in specific nucleotide-bound states. To assay Rho activation, 1 μg of recombinant GTPases, 200 μg of lysate or 50 μg of vacuole and 30 μg of immobilized GST-CBD or GST-RBD were incubated in 400 μl H-buffer (20 mM HEPES–KOH, pH 7.5, 1 mM DTT, 3 mM $MgCl_2$, 60 mM NaCl, 0.5% Nonidet P-40), for 30 min at 4 °C. The bead pellet was washed three times with H-buffer and resuspended in 50 μl of Laemmli sample buffer. Half the sample was analyzed by immunoblot using Cdc42p (y-191, Santa Cruz Biotechnology) and Rho1p [9] specific antibodies. Immunoblots were detected with IRDye800 fluorescently-tagged secondary antibodies (Rockland Immunochemicals) using an Odyssey Image analysis system (LiCor).

Vacuole isolation, fusion and GTPase activation reactions. Vacuoles were isolated from yeast spheroplasts by floatation on Ficoll gradients and subjected to *in vitro* membrane fusion reactions as previously described [18]. Standard fusion reactions contained 3.5 μg of vacuoles from each of BJ3505 (pro-ALP fusion reporter strain) and DKY6251 (protease donor strain to cleave pro-ALP upon fusion) in 30 μl of *f*usion *r*eaction *b*uffer (FRB = 20 mM PIPES–KOH, pH 6.8, 125 mM KCl, 5 mM $MgCl_2$, 200 mM sorbitol, 10 μM CoA, 1× PIC), 1× ATPreg and 0.5 mg/ml cytosol. To assay for Rho activation, standard reactions (150 μl) contained 50 μg vacuoles that were pre-incubated at 27 °C in FRB + 1× ATPreg and 40 μM GTPγS to facilitate the detection of activation. About 0.5 mg/ml of cytosol was also included where indicated. Vacuoles were re-isolated (centrifugation at 18,000*g*, 4 °C, 5 min) from pre-incubation reactions after 2-fold dilution in cold PS-buffer and then probed for activated Rho proteins.

Results

Detection of activated yeast Rho proteins

Rho protein activation can be detected with probes derived from and Rho-binding domains of downstream effectors [2,3]. In yeast, Rho1p and Cdc42p binding domains (RBD and CBD, respectively) have been identified on several proteins (Fig. 1A) [19–23]. We cloned these domains as GST-tagged fusion proteins to examine whether they could be used as Rho activation probes. All Cdc42p activation probes worked with similar efficiency (Fig. 1B); however, Rho1p activation probes cloned from yeast were significantly less efficient that the original probe derived from Rhotekin (Fig. 1C). For further biochemical analyses the conventional Rho activation probes were used.

Rho activation probes showed specific association with endogenous Cdc42p and Rho1p (Fig. 2A). Yeast whole-cell lysate and purified vacuoles were subjected to chemical nucleotide exchange to obtain specific nucleotide-bound states, which were then incubated with Rho activation probes. Pre-incubation without nucleotide or with GDP resulted in no probe association. High levels of binding were observed when GTPγS was exchanged, and somewhat less when GTP was exchanged. Therefore, activation probes specifically associated with GTP-bound Cdc42p and Rho1p. However, we were puzzled by the low levels of association in GTP exchanged samples (Fig. 2A, *GTP*). This result suggests that the endogenous GTPase activity of yeast Rho proteins in our samples is sufficiently high and quickly hydrolyze GTP during incubations.

Activation probes were also verified using Rho mutant alleles. Cdc42p and Rho1p proteins from strains expressing constitutively-active mutants (Cdc42p-G12V and Rho1p-G19V) showed high levels of probe association, while the dominant-negative mutants (Cdc42p-T17N and Rho1p-T24N) did not (Fig. 2C, middle panel).

Rho activation during membrane fusion

Since our primary interest is the regulation of membrane fusion, we next examined whether Cdc42p and Rho1p activation could be detected on the vacuole membrane during fusion reactions. Detectable levels of activated Cdc42p and Rho1p were observed when vacuoles, but not whole-cell lysates, were pre-incubated in membrane fusion reaction buffer (Fig. 2B). Activated levels of Cdc42p and Rho1p were significantly enhanced when GTPγS was included in the reaction buffer, with detection levels comparable to chemical nucleotide exchange (Fig. 2B, GTPγS). These results indicate that both Cdc42p and Rho1p undergo activation during vacuole fusion. To avoid underestimation of activation levels due

to GTP hydrolysis, GTPγS was included in all further Rho activation assays.

Examination of vacuoles overexpressing mutant Rho proteins further supports the presence of Rho activation during fusion. Incubation did not trigger the activation of dominant-negative Rho mutants, nor did it increase the levels constitutively-active mutants as detected by probe association (Fig. 2C, bottom panel). However, overexpressed wild-type Rho proteins showed a significant increase in activation levels due to incubation.

We next assayed the kinetics of Cdc42p and Rho1p activation during vacuole fusion. Vacuoles were incubated in fusion reaction buffer and at specific times aliquots were removed and assayed for membrane fusion via content mixing assay. Rho activation via association with GST-probes was examined in parallel. Fusion signals increased linearly for ~60 min (Fig. 3A, hatched bars) and Rho1p activation showed similar activation kinetics, reaching maximum activation at 60 min (Fig. 3A, white bars and C). This suggests that Rho1p regulates a late reaction during membrane fusion at or near the final step of lipid bilayers mixing [13]. The

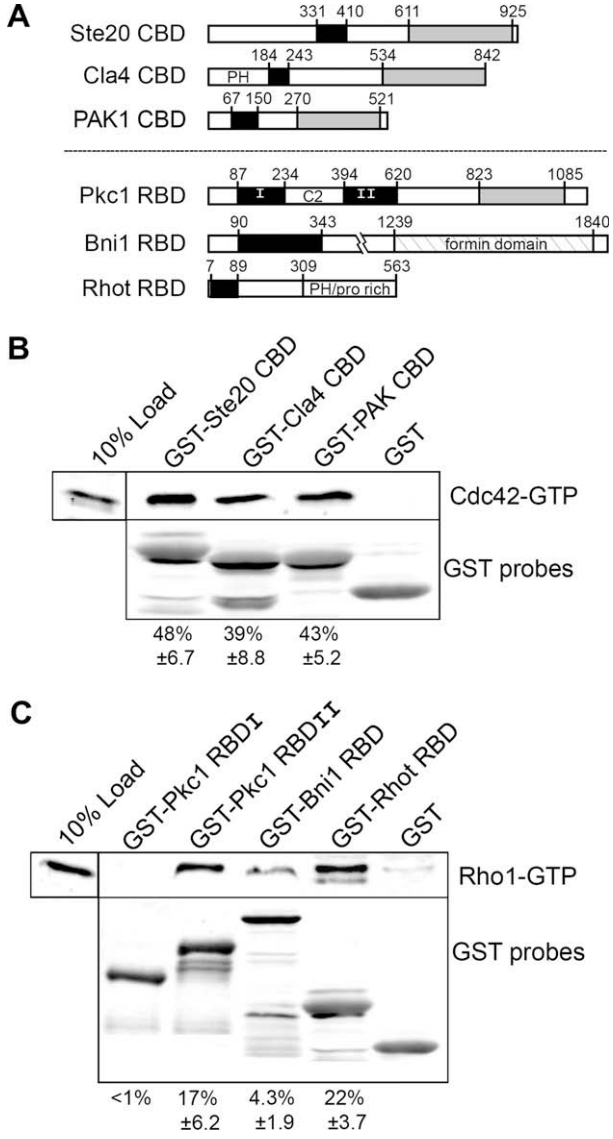

Fig. 1. Yeast specific Rho activation probes. (A) The Cdc42p binding domains (CBD) of yeast Cla4p, Ste20p and human PAK1; the Rho1p binding domains (RBD) of yeast Pkc1p, Bni1p and human Rhotekin (Rhot). These were cloned as GST-fusion proteins and expressed in *E. coli*. (B, C) About 15 μg of each Rho activation probe was immobilized on glutathione beads and incubated with 0.5 μg recombinant Cdc42p (B) or Rho1p (C) pre-loaded with GTPγS. Precipitated amounts were quantified from at least three independent experiments. Quantification of binding showed that all three CBD probes (A) had similar Cdc42-GTP binding efficiency while the Rhotekin RBD probed showed the most efficient Rho1-GTP binding (C, GST-Rhot RBD). 10% Load = 50 ng Cdc42p or Rho1p.

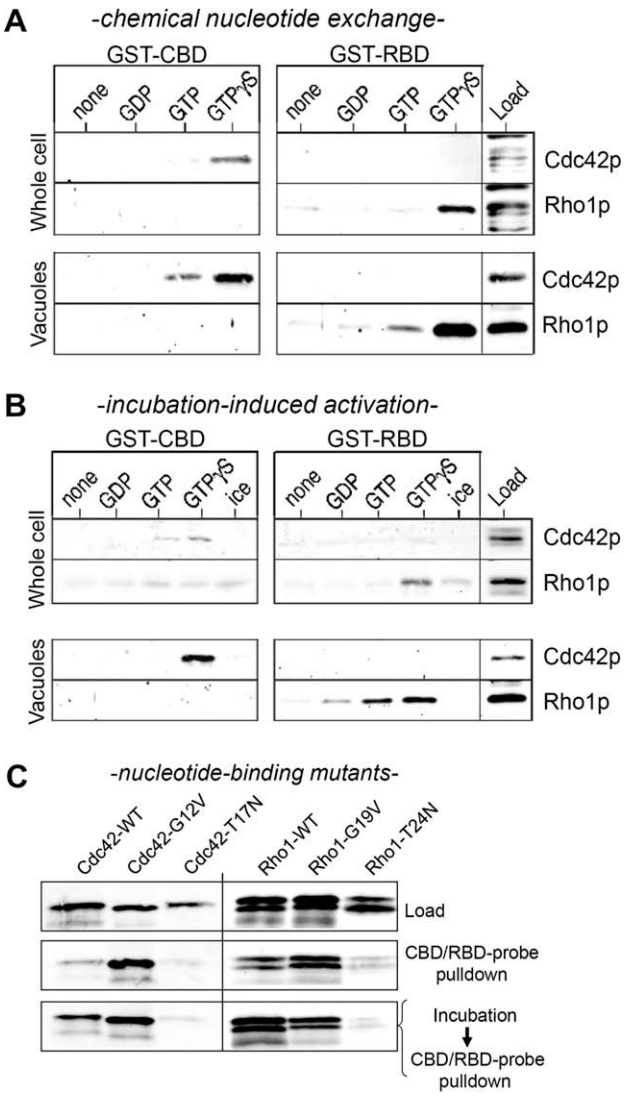

Fig. 2. Specificity and selectivity of GST-CBD and GST-RBD probes. (A) Interaction of Cdc42p and Rho1p with activation probes when specific nucleotide-bound states were induced by chemical nucleotide exchange. About 200 μg of yeast cytosol or 50 μg of purified vacuoles were incubated with 40 μM of the indicated nucleotide in 400 μl H-buffer containing 2.5 mM EDTA and no $MgCl_2$. Samples were incubated for 5 min at 30 °C and then 10 mM $MgCl_2$ was added to quench nucleotide-binding reactions. About 30 μg of GST-CBD or GST-RBD beads were added to affinity isolate activated (GTP-bound) Cdc42p and Rho1p, respectively. (B) Determination of Cdc42p and Rho1p activation by incubation for membrane fusion. About 400 μg of yeast cytosol or 50 μg of purified vacuoles were incubated with 40 μM of the indicated nucleotide in 200 μl FRB + ATPreg. Samples were incubated for 40 min at 27 °C and then 30 μg of GST-CBD or GST-RBD beads were added to affinity isolate activated (GTP-bound) Cdc42p and Rho1p, respectively. (C) Vacuoles were isolated from strains overexpressing wild-type (Cdc42-WT, Rho1-WT), constitutively-active (Cdc42-G12V, Rho1-G19V) or dominant-negative (Cdc42-T19N, Rho1-T24N) forms of Cdc42p and Rho1p. About 50 μg of vacuoles from each strain were solubilized and incubated with 30 μg of GST-CBD probe (left three lanes) or 15 μg of GST-RBD (right three lanes). Only constitutively-active Rho proteins showed significant interaction with probes when vacuoles were not incubated for fusion (middle panel). Incubation of vacuoles for fusion resulted in increased interaction (i.e. activation) of wild-type but not constitutively-active Rho proteins (lower panel). Dominant-negative Rho proteins showed no interaction with Rho activation probes for all conditions. Load = 10%.

kinetics of Cdc42p activation did not mirror the kinetics of fusion, but instead, Cdc42p was rapidly activated, reaching maximum levels by 20 min (Fig. 3A, black bars and B). This kinetically coincides with an early subreaction of membrane fusion, such as priming [13].

Conditions of Rho activation

To further define the conditions that trigger activation of vacuolar Cdc42p and Rho1p, we performed reactions that lacked certain membrane fusion components. Complete reactions, containing physiological salt, ATPreg (ATP regenerating system) and cytosol, were incubated at 27 °C for 40 min followed by incubation with Rho activation probes on ice (Fig. 4A, lane 7). Rho1p activation was reduced when ATPreg was not included in reactions, however, this had no effect on Cdc42p activation (lanes 1 and 5). Cytosol had little effect on Cdc42p activation, but enhanced Rho1p activation (Fig. 4A, compare lanes 1–3 vs. 5–7). This suggests that Cdc42p activation is driven by an ATP-independent mechanism while Rho1p activation requires ATP and is sensitive to cytosolic proteins. Salt-washed vacuoles required cytosol for maximum activation of both Cdc42p and Rho1p (Fig. 4B, compare lanes 7 vs. 3). Therefore, both vacuolar Cdc42p and Rho1p activation is likely driven by peripherally associated cytosolic components that co-purify with vacuoles during isolation. These results also confirm that spontaneous nucleotide exchange does not occur since incubation of samples on ice resulted in no activation (Fig. 4A, compare lane 8 (ice) to lane 7 (27 °C)). Since little active Rho proteins are detected in samples incubated on ice, the major species must be the inactive GDP-bound forms. Incubation in the presence of 5 mM $MgCl_2$ requires an active process to catalyze nucleotide exchange [24].

We also tested a number of previously characterized inhibitors of membrane fusion [13] for their effect on Rho activation (Fig. 4B). Apyrase inhibited Rho1p activation but not Cdc42p, which confirms the ATP requirement for Rho1p activation only. RhoGDI (Rdi1p) inhibited the activation of both Rho1p and Cdc42p as expected, while RabGDI had no effect. This suggests that either Rab functions downstream of Rho, or that Rab and Rho GTPase signaling are not linked. Several antibody inhibitors tested had little effect (i.e. anti-Sec17p shown, anti-Vam3p (SNARE) antibodies not shown). Neomycin, which binds to $PI(4,5)P_2$, and the phosphoinositol-specific binding domain, ENTH [25], inhibited the activation of Rho1p. This suggests that ATP-dependent Rho1p activation is linked to the generation of phosphoinositol lipids during fusion [26]. This was confirmed by enhanced activation when Plc1p was inhibited with U73122 which would reduce $PI(4,5)P_2$ metabolism. As additional controls we examined the effect of blocking calcium flux and diacylglycerol via incubation with the Ca^{2+} chelator BAPTA and the C1b domain, respectively; however, neither of these reagents affected Rho activation.

Discussion

Rho proteins govern the initiation of many spatially defined signaling processes, and hence it is not surprising to find them localized to many intracellular sites [27]. We have previously shown that two Rho GTPases, Cdc42p and Rho1p, are enriched on the vacuole membrane, where they participate in the regulation of membrane fusion [10–12]. This was most conclusively shown using the Rho guanine dissociation inhibitor, Rdi1p, which blocked the fusion of purified vacuoles [10]. Rdi1p theoretically block the activation of Cdc42p and Rho1p, however, previous experiments did not examine this processes. We developed an assay to detect yeast Cdc42p and Rho1p activation in *in vitro* reactions by modification of methods previously developed to affinity isolate activated Rho proteins from mammalian cell lysates [2,3]. We rigorously tested this assay using recombinant Rho proteins, chemical nucleotide exchange experiments and mutant Rho proteins with specific "nucleotide-locking" modifications (Figs. 1 and 2). Although C-terminal lipidation of Ras has been implicated in effector interaction [28], we find that non-lipid modified recombinant Rho proteins interact strongly with activation probes. This assay showed rather weak detection of activated Rho proteins in cell lysates, however, activation of Cdc42p and Rho1p was detected at appreciable levels during vacuole fusion reaction. Pre-incubation of membranes with Rdi1p blocked both Cdc42p and Rho1p activation as did incubation on ice. Hence, the Rho activation mechanism is clearly driven during incubation for membrane fusion.

Vacuolar Cdc42p and Rho1p activation showed significantly different kinetics and conditional requirements (Fig. 3). Cdc42p activation was rapid, while Rho1p activation required much longer incubation time and ATP. Distinct patterns of multi-Rho protein activations are known to be tightly coordinated, and thus drive multi-component mechanisms such as cell movement and phagocytosis [29]; this seems to also occur during vacuole fusion. We have recently shown that an early role for Cdc42p is the regulation of vacuole-associated actin remodeling [12]. The specific function of Rho1p has remained obscure, however judging by the late stage of its activation, we suggest that it may stabilize the fusion complex on docked vacuoles [30].

Rho1p activation is clearly linked to the fusion mechanism. Rho1p activation is sensitive to ligands of $PI(4,5)P_2$, an important signaling lipid which accumulates at membrane fusion domains (i.e. vertex ring; [30]). Inhibition of $PI(4,5)P_2$ metabolism by chemical inactivation of Plc1p enhanced Rho1p activation (Fig. 4C). These result suggest that the ATP requirement for Rho1p activation might be linked to the generation of $PI(4,5)P_2$ which occurs during vacuole fusion [26]. Interestingly, Plc1p has been shown to inhibit membrane fusion [26], but also stimulate membrane fusion in some cases [17]. This is likely due to stage/subreaction-specific requirements for both $PI(4,5)P_2$, and its PLC cleavage products, IP3 and DAG [13]. Our results suggest that $PI(4,5)P_2$ has an initial

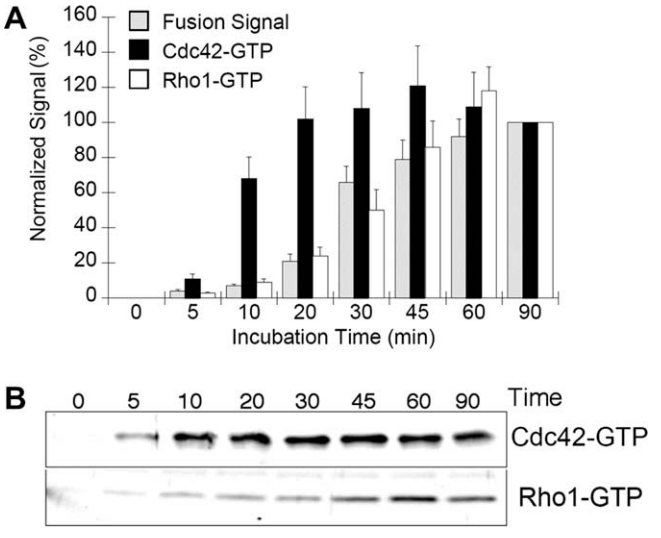

Fig. 3. Kinetics of vacuole fusion and vacuolar Rho GTPase activation. Standard vacuole fusion reactions were performed to assay for levels of fusion and Rho activation. At the indicated times reactions were stopped by placing on ice and assayed for fusion signal via ALP enzyme assay. Cdc42p activation via precipitation with the GST-CBD probe or Rho1p activation via precipitation with the GST-RBD probe was assayed in parallel. (A) Quantification of fusion and Rho activation signals, normalized to 90 min reactions. Shown are the average signals from at least three independent experiments +/− SEM (B, C) Typical immunoblots showing the time course for Cdc42p and Rho1p activation.

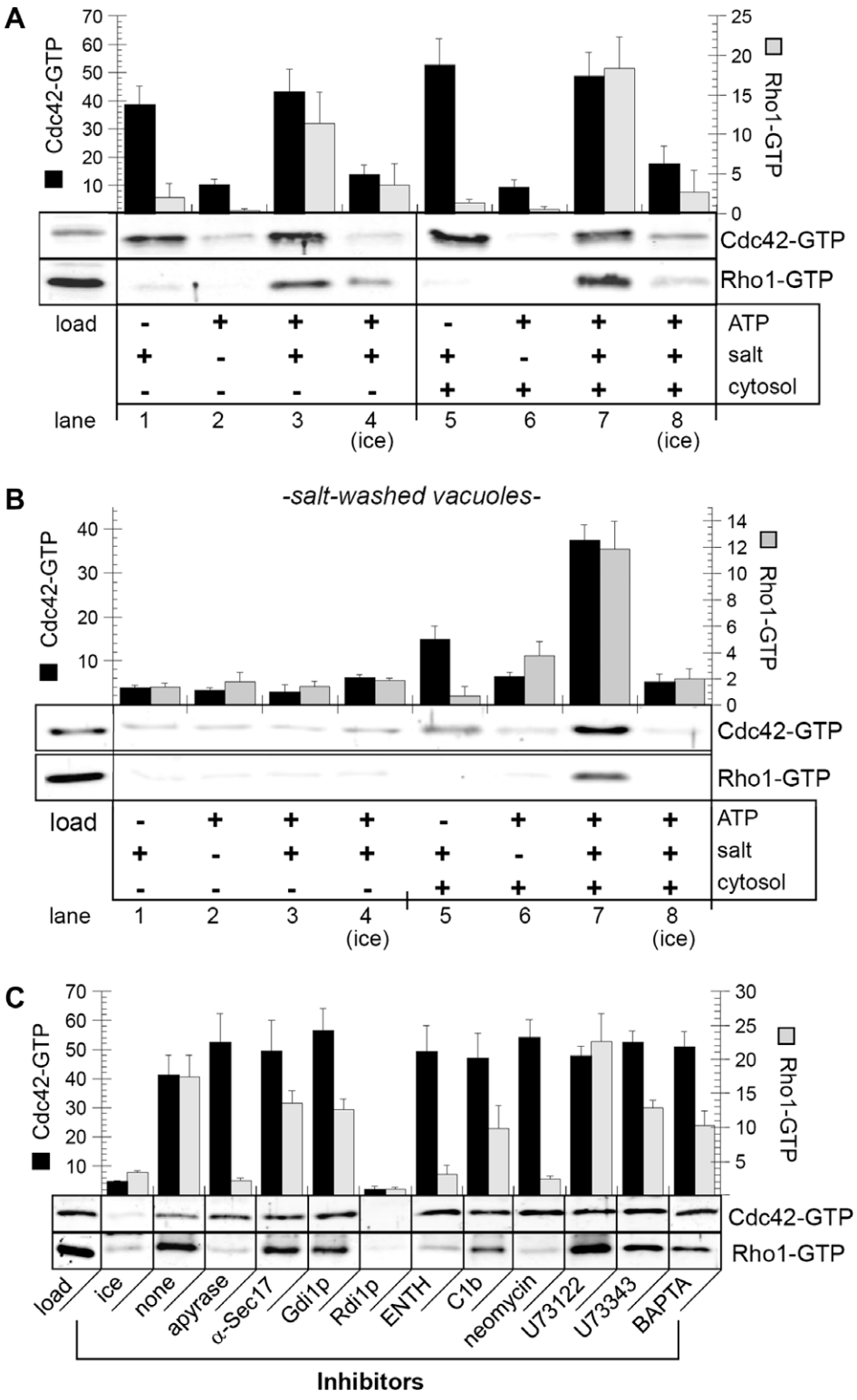

Fig. 4. Conditional requirements for vacuolar Cdc42p and Rho1p activation. (A) Levels of activated Cdc42p and Rho1p were determined from vacuole fusion reactions performed in the absence of specific reaction components. Vacuoles, +/− fusion components as indicated, were incubated for 40 min at 27 °C or on ice prior to assaying for Cdc42p or Rho1p activation. Incubation with 0.5 mg/ml cytosol showed a slight enhancement of activation (compare lanes 1–4 (−cytosol) to lanes 5–8 (+cytosol)). (B) Identical experiment as in (A) except membranes were salt-washed (2× FRB) prior to analysis. Activation of Cdc42p and Rho1p showed a greater dependence on cytosol when vacuoles were salt-washed. (C) Pharmacological inhibition of vacuolar Cdc42p and Rho1p activation. Levels of activated Cdc42p and Rho1p were determined when standard vacuole fusion reactions were incubated in the presence of reagents previously shown to affect vacuole fusion [13]. Reactions were incubated for 40 min at 27 °C with the following reagents prior to assaying for Rho activation: 0.3 U/μl apyrase VII; 0.25 mg/ml α-Sec17 IgG fraction; 0.1 mg/ml Gdi1p; 0.2 mg/ml Rdi1p, ENTH domain or C1b domain; 0.1 mM neomycin, U73122, U73343, or BAPTA. Control reactions without inhibitors were incubated at 27 °C (none) or on ice (ice). Graphs show activation levels (arbitrary units) quantified by comparing immunoblot band intensities of precipitated proteins versus the load. Shown are the average signals obtain from four independent experiments +/− SEM; load = 10% of starting vacuoles.

role in Rho1p activation and is subsequently converted to DAG for final lipid bilayer mixing when membrane negative curvature is required. Similar coordination of events that engage Rho signaling have also been shown to regulate exocytosis [14,15]. Thus, the regulation of Cdc42p and Rho1p activation provides additional layers of regulation for vacuole membrane fusion which might be common to other systems.

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