Direct binding of the Kex2p cytosolic tail to the VHS domain of yeast Gga2p facilitates TGN to prevacuolar compartment transport and is regulated by phosphorylation

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ABSTRACT
Human Golgi-localized, γ-ear-containing, ADP-ribosylation factor–binding proteins (Ggas) bind directly to acidic dileucine sorting motifs in the cytosolic tails (C-tails) of intracellular receptors. Despite evidence for a role in recruiting ubiquitinated cargo, it remains unclear whether yeast Ggas also function by binding peptide-sorting signals directly. Two-hybrid analysis shows that the Gga1p and Gga2p Vps27, Hrs, Stam (VHS) domains both bind a site in the Kex2p C-tail and that the Gga2p VHS domain binds a site in the Vps10p C-tail. Binding requires deletion of an apparently autoinhibitory sequence in the Gga2p hinge. Ser780 in the Kex2p C-tail is crucial for binding: an Ala substitution blocks but an Asp substitution permits binding. Biochemical assays using purified Gga2p VHS–GGA and TOM1 (GAT) and glutathione S-transferase–Kex2p C-tail fusions show that Gga2p binds directly to the Kex2p C-tail, with relative affinities Asp780 > Ser780 > Ala780. Affinity-purified antibody against a peptide containing phospho-Ser780 recognizes wild-type Kex2p but not S780A Kex2p, showing that Ser780 is phosphorylated in vivo; phosphorylation of Ser780 is up-regulated by cell wall–damaging drugs. Finally, mutation of Ser780 alters trafficking of Kex2p both in vivo and in cell-free trans-Golgi network (TGN)–prevacuolar compartment (PVC) transport. Thus yeast Gga adaptors facilitate TGN–PVC transport by direct binding of noncanonical phosphoregulated Gga-binding sites in cargo molecules.

INTRODUCTION
Steady-state localization of singly spanning transmembrane proteins (the processing peptidases Kex2p and Ste13p and the vacuolar protein sorting receptor, Vps10p) to the yeast trans-Golgi network (TGN) depends on cycles of vesicular trafficking between the TGN, the late endosome/prevacuolar compartment (PVC), and maturing late Golgi cisternae (Cooper and Stevens, 1996; Bryant and Stevens, 1997; Brickner and Fuller, 1997). It is believed that these proteins follow a common, retromer-dependent PVC–Golgi retrieval pathway (Seaman et al., 1997; Nothwehr et al., 1999). Signals required for this retrograde transport have been identified in the cytosolic domains (cytosolic tails [C-tails]) of Kex2p, Ste13p, and Vps10p (Wilcox et al., 1992; Nothwehr et al., 1993; Cereghino et al., 1995). In the forward direction, evidence from in vivo and in vitro studies indicates that Vps10p follows a Golgi-localized, γ-ear-containing, ADP-ribosylation factor–binding protein (Gga/GGA)-dependent, AP-1-independent, “direct” pathway from the TGN to the PVC, whereas Kex2p...
The GGA proteins comprise a family of clathrin adaptors that facilitate clathrin-mediated trafficking of cargo between the TGN and endosome by linking cargo sorting and clathrin lattice assembly at vesicle budding sites (Bonifacino, 2004). The modular organization of GGA proteins into Vps27, Hrs, Stam (VHS), GGA and TOM1 (GAT), hinge, and γ-adapten ear (GAE) domains permits the coordination of multivalent contacts with the C-tails of cargo, Arf-GTP, clathrin, and accessory protein involved in vesicle formation. Biochemical and structural studies of binding between the mammalian GGA-VHS domains and the C-tails of TGN receptors sortilin and two mannose-6-phosphate receptors (MPRs) provided the first evidence for a direct interaction between GGAs and cargo (Bonifacino, 2004). The key residues in the C-tails of the cation-dependent MPR (CD-MPR), cation-independent MPR (CI-MPR), sortilin, memapsin 2 (β-secretase [BACE]), and low-density-lipoprotein receptor-related protein 3 for binding to the VHS domain of human GGAs were shown to constitute acidic dileucine motifs with the consensus sequence DXXXL (Nielsen et al., 2001; Puertollano et al., 2001; Takatsu et al., 2001; Zhu et al., 2001; He et al., 2002; Misra et al., 2002). Phosphorylation of a Ser residue within or adjacent to the acidic dileucine motif enhanced the binding of CI-MPR, BACE, sorLA, and sortilin C-tail sequences to human GGAs, suggesting a phospho-regulated mechanism for binding and cargo sorting (Kato et al., 2002; He et al., 2003; Cramer et al., 2010). Comparison of the sequences of the yeast Gga proteins to the structures of the mammalian GGAs, however, suggested that the sorting of the polytopic membrane proteins Gap1p, Fur4p, Ste13p, and Vps10p involves interaction of covalently bound ubiquitin with the GAT domains of the Gga proteins (Bilodeau et al., 2004; Scott et al., 2004; Kim et al., 2007), leading to the notion that yeast GGAs might exclusively mediate sorting of ubiquitin-modified cargo. Recent work, however, suggested that TGN–PVC trafficking of Am1p and Gap1p, albeit Gga dependent, is independent of both ubiquitin modification and the Gga GAT domain, although the mechanism is unknown (Deng et al., 2009; Lauwers et al., 2009). Although the C-tails of both Kex2p and Vps10p are highly acidic, they contain no obvious acidic dileucine motifs. No Gga-binding sites (GBSs) have been identified in the C-tails of these proteins, and the direct interaction of GBSs with the VHS domains of the yeast Gga proteins has not been demonstrated.

To characterize the precise role of the yeast Gga proteins in TGN-to-PVC transport of Kex2p and Vps10p, we used a directed, yeast two-hybrid strategy to detect and map GBSs in the Kex2p and Vps10p C-tails that bind to the VHS domains of yeast Gga proteins.

We used an in vitro binding assay to demonstrate that the Kex2p GBS binds directly to the VHS domain of Gga2p. Efficient binding required Kex2p C-tail residue Ser390, which we found to be phosphorylated in vivo in response to cell wall–damaging agents. Analysis of the effects of Ala and Asp substitutions on the biochemical binding assay, a cell-free TGN–PVC transport assay, and an in vivo assay for Kex2p localization indicated that phosphorylation favored binding to the Gga2p VHS domain and enhanced TGN–PVC sorting by the direct, Gga-dependent pathway.

RESULTS

Two-hybrid interactions between the C-tail of Kex2p and the VHS domains of Gga1 and 2p

On the basis of the demonstrated role of Gga2p in vivo and cell-free TGN-to-PVC transport of transmembrane proteins and on the established mechanism of direct cargo selection by mammalian GGAs in facilitating transport, we predicted that the yeast GGAs would bind to sequence elements in the C-tails of yeast cargo proteins. To probe possible yeast cargo–Gga adaptor interactions, we tested the C-tail of Kex2p and full-length and truncated forms of Gga2p for binding using a directed yeast two-hybrid assay (Figure 1). In the two-hybrid assay, the Kex2p C-tail did not interact with full-length Gga2p but showed a strong interaction with the VHS-GAT domains of Gga2p (Figure 1B), suggesting the presence of an inhibitory element C-terminal to the GAT domain (see later discussion). When the VHS and GAT domains of Gga2p were tested individually for interaction with Kex2p, only the VHS domain bound to the Kex2p C-tail (Figure 1C). Moreover, the VHS domain of Gga1p also bound to the Kex2p C-tail (Figure 1C).

The hinge region of Gga2p contains an autoinhibitory sequence

To locate more precisely the inhibitory region of Gga2p, we tested a set of truncations for interaction by yeast two-hybrid analysis (Figure 2A and Supplemental Figure S1). Whereas full-length Gga2p, Gga2p-VHS-GAT-hinge, and C-terminal truncations that ended at positions 455, 440, 425, 410, and 395 of the Gga2p hinge domain failed to bind to the Kex2p C-tail, truncations that ended at positions 380, 365, and 350 did bind. These results indicate that an end-point of the inhibitory sequence is located between residues 380 and 395. We next determined whether the hinge could interact with the VHS domain in-trans. Figure 2B shows that the Gga2p-hinge domain interacted with the Gga2p-VHS domain. Taken together, these results suggest that an internal inhibitory sequence in the hinge region of Gga2p binds to the VHS domain, thereby preventing it from interacting with cargo. Because the GGAs have been reported to be monomeric in the cytosol, this autoinhibition may result from intramolecular rather than intermolecular binding (Dell’Angelica et al., 2000; Hirst et al., 2000). However, two-hybrid analysis indicated that the VHS domain of Gga2p exhibited both a strong homomeric interaction and a strong heteromeric interaction with the Gga1p VHS domain, whereas the Gga1p VHS domain exhibited a weaker homomeric interaction (Supplemental Figure S2). This raises the possibility that homodimerization or heterodimerization through the VHS domains may play a role in Gga function. Direct biochemical binding experiments would be necessary to confirm these apparent autoinhibitory and homomeric interactions and the exclude involvement of other factors in these binding events.

Identification of the Gga-binding site in the Kex2p C-tail

To identify the Gga-binding site (GBS) in the Kex2p C-tail, we constructed and tested truncations of the Kex2p C-tail for binding to
the Gga2p-VHS-GAT domain in the yeast two-hybrid assay (Figure 3, A and B, and Supplemental Figure S3). C-terminal truncations showed strong binding by C-tail residues 1–90 and a sharp loss on truncation to residue 85 (Figure 3A). N-terminal truncations showed strong binding of C-tail residues 45–115, diminished binding with 50–115, and loss of binding with 60–115. These results suggested that strong binding required C-tail residues 45–90 and that essential binding determinants lay within residues 55–90 (Figure 3B). Consistent with this conclusion, Kex2p C-tail residues 45–90 alone interacted with Gga2p-VHS-GAT as well as the full-length C-tail did (Figure 3C), confirming that this region was sufficient for binding to the Gga2p VHS domain.

To identify individual residues critical for binding, we performed alanine scanning mutagenesis (Figure 3D). Simultaneous mutation of Phe$_{779}$ and Ser$_{780}$ to Ala abrogated binding. Single-Ala substitution for Phe$_{779}$ had only a small effect, whereas the S$_{780}$A mutation nearly eliminated binding (Figure 3E). In contrast, substitution of Asp for Ser$_{780}$ resulted in an interaction comparable to that seen with the wild-type (WT) sequence, suggesting a role for phosphorylation of S$_{780}$ in binding the Gga2p VHS domain.

Two-hybrid interaction between the C-tail of Vps10p and Gga2p

Because yeast two-hybrid analysis showed that the Kex2p C-tail interacted with the Gga2p VHS domain, we asked whether a similar interaction would be observed with the Vps10p C-tail. As shown in Figure 4A and Supplemental Figure S4, full-length Vps10p C-tail did not interact with Gga2p-VHS-GAT. N-terminal truncation of Vps10p C-tail sequences revealed that, whereas a construct containing tail residues 40–164 did not bind, a construct containing tail residues 45–164 did, indicating the N-terminal endpoint of an inhibitory
sequence between tail residues 40 and 45. To identify the minimal requirement for Gga2p binding, we tested additional N- and C-terminal truncation mutants (Figure 4, A and B). Vps10p C-tail residues 55–164 showed full binding, which was diminished in a 55–140 construct and lost in a 55–125 construct. Conversely, residues 100–164 showed full binding, which was diminished in a 110–164 construct and lost in a 125–164 construct. These results suggested that primary determinants for binding lay between 110 and 140, although a slightly extended sequence might be needed for full binding. Consistent with these expectations, Vps10p C-tail residues 100–145 alone interacted with Gga2p-VHS-GAT nearly as well as with residues 55–164 (Figure 4C), indicating that this region is sufficient for binding to Gga2p. Finally, the Vps10p C-tail, like that of Kex2p, interacted specifically with the VHS domain of Gga2p (Figure 4D).

**Kex2p is phosphorylated at Ser780 in vivo**

To determine whether Ser780 in Kex2p is phosphorylated, we raised a rabbit antibody (anti-P-Ser780) against a synthetic peptide corresponding to Kex2p C-tail residues from Leu773 to Phe787 and containing phospho-Ser at the position corresponding to Ser780. Glass bead lysates from kex2Δ strain CBO17 containing plasmids overexpressing either WT Kex2p (pG5-KX22) or the S780A mutant of Kex2p (pG5-KX22-S780A) were analyzed by immunoblotting. When blots were probed with anti–P-Ser780 serum, a band corresponding to Kex2p at 135 kDa (along with numerous background bands) was seen in the lanes containing WT Kex2p or S780A Kex2p (Figure 5A, lanes 3 and 4). Competition by unphosphorylated peptide NPP eliminated the S780A Kex2p band but only reduced the WT Kex2p (Figure 5A, lanes 5 and 6). Competition by the phosphorylated peptide PP2 eliminated both the WT and S780A Kex2p bands (Figure 5A, lanes 7 and 8), implying that a component of the antiserum was specific for P-Ser780. Affinity-purified anti–P-Ser780 antibody (hereafter, anti–P-Ser780 antibody) gave a clearer result. Whereas affinity-purified anti-Kex2p antibody (hereafter, anti-Kex2p antibody) recognized the WT and S780A Kex2p equally well (Figure 5A, lanes 1 and 2), anti–P-Ser780 antibody gave a fourfold stronger signal with WT than with S780A Kex2p (Figure 5A, lanes 9 and 10). On reuse, reactivity of the affinity-purified probe with S780A-Kex2p was completely lost, whereas reactivity with WT Kex2p remained (e.g., compare lanes 1–5 with lanes 9–10).

**FIGURE 3:** Delimiting the Gga-binding site of Kex2p by mutational analysis. (A) C-terminal and (B) N-terminal truncation mutants of Kex2p (C-tail) in the VP16-AD plasmid were tested for interaction with Gga2-VHS-GAT (residues 1–336) in the LexA-BD plasmid. (C) Kex2p C-tail construct expressing residues 45–90 (i.e., Kex2p residues 746–791) in the VP16-AD plasmid was tested for interaction with Gga2-VHS-GAT (residues 1–336) in the LexA-BD plasmid. (D) Ala-scanning mutations in the full-length Kex2p C-tail in the VP16-AD two-hybrid plasmid were tested for interaction with Gga2-VHS-GAT (residues 1–336) in the LexA-BD plasmid. (E) Single-Ala mutations of F779 and S780, double-Ala mutations of F779S780, and the S780D mutation, all in the full-length Kex2p C-tail in the VP16-AD plasmid, were tested for interaction with Gga2-VHS-GAT (residues 1–336) in the LexA-BD plasmid. The regions of Kex2p C-tail within each construct are indicated by C-tail residue number. Strains were spotted as in Figure 1.
A. BD AD Cells: 
| Gga2 VHS-GAT 1-164 | +His 10mM 3AT |
|---------------------|----------------|
| Gga2 VHS-GAT 15-164 |
| Gga2 VHS-GAT 30-164 |
| Gga2 VHS-GAT 45-164 |
| Gga2 VHS-GAT 55-164 |
| Gga2 VHS-GAT 55-140 |
| Gga2 VHS-GAT 55-125 |
| Gga2 VHS-GAT 55-110 |
| Gga2 VHS-GAT 70-164 |
| Gga2 VHS-GAT 85-164 |
| Gga2 VHS-GAT 100-164 |
| Gga2 VHS-GAT 110-164 |
| Gga2 VHS-GAT 125-164 |

B. BD AD Cells: 
| Gga2 VHS-GAT 30-164 | +His 10mM 3AT |
|---------------------|----------------|
| Gga2 VHS-GAT 35-164 |
| Gga2 VHS-GAT 40-164 |
| Gga2 VHS-GAT 45-164 |

C. BD AD Cells: 
| Gga2 VHS-GAT 55-164 | +His 10mM 3AT |
|---------------------|----------------|
| Gga2 VHS-GAT 100-145 |
| Gga2 VHS-GAT VP16 |

D. BD AD Cells: 
| Gga2 VHS VP16 | +His 10mM 3AT |
|---------------|----------------|
| Gga2 VHS Vps10 C-tail 55-164 |

**FIGURE 4:** The C-tail of Vps10p contains a GBS that interacts with the Gga2p VHS domain. Two-hybrid constructs in the LexA-BD plasmid and the VP16-AD plasmid were as indicated. The regions of Vps10p C-tail within each construct are indicated by C-tail residue number. Gga2-VHS-GAT (residues 1–336) and Gga2-VHS (residues 1–169) were used. (A) A GBS in the Vps10p tail is revealed after deletion of an inhibitory sequence. (B) Fine deletion analysis of the N-terminal end of the inhibitory sequence. (C) Minimal GBS in Vps10p C-tail. (D) The Vps10p GBS binds the Gga2p VHS domain. Strains were spotted as in Figure 1.

lanes 6–9 in Figure SC, making the anti-P-Ser780 antibody an effective reagent for detecting P-Ser780 Kex2p. Incubation of extracts containing WT Kex2p with alkaline phosphatase eliminated reactivity with the anti-P-Ser780 antibody but not the anti-Kex2p antibody in immunoblots, confirming the specificity of the anti-P-Ser780 antibody for Kex2p phosphorylated at Ser780 (Supplemental Figure S5).

The experiments in Figure 5A were conducted using a strain overexpressing WT and mutant Kex2p. To ensure that phosphorylation of Ser780 was not an artifact of overexpression, we tested reactivity of affinity-purified anti-P-Ser780 antibody with WT and S780A Kex2p expressed at a range of levels from the WT level to 150 times the WT level (Figure 5B). WT Kex2p was detected by anti-P-Ser780 antibody when expressed at the WT level and overexpressed at 25 and 150 times the WT level (Figure 5B). Judged by the ratio of the anti-C-tail signal to the anti-P-Ser780 signal, the degree of phosphorylation was highest at the WT level of expression (3:4:1) and was diminished with overexpression (5:1 for 25 times; 19:1 for 150 times). This may reflect the fact that overexpression of Kex2p results in mislocalization, with 150-times overexpression resulting in accumulation of Kex2p in aberrant membrane-enclosed structures (Wilcox et al. 1992).

Kex2p function has been tied to the processing of numerous cell wall proteins and enzymes (Mrsa et al., 1997; Moukadiri et al., 1999; Tomishige et al, 2003). To determine whether phosphorylation of Ser780 in the Kex2p C-tail is regulated by cell wall damage–response pathways, we examined the effects of cell wall–damaging agents on phosphorylation in cells expressing WT Kex2p or overexpressing WT or S780A Kex2p. Treatment of cells overexpressing WT Kex2p with calcium white, caffeine, and Congo red for 4 h (Figure 5C, top) or overnight (Supplemental Figure S5) increased phosphorylation of WT Kex2p by twofold to threefold, as measured by immunoblotting with the anti–P-Ser780 antibody, relative to the untreated control. Blotting with anti-Kex2 antibody demonstrated that none of these treatments significantly altered the level of Kex2p itself (shown for 4-h treatment, Figure 5C, bottom). WT Kex2p expressed at the WT level also exhibited enhanced phosphorylation after treatment with caffeine for 4 h (1.9-fold), as well as on growth to high density overnight (3.2-fold), a treatment that has been shown to induce the cell wall–integrity pathways (Figure 5D). Enhancement of phosphorylation under these conditions was reproducible, but the degree of enhancement was variable because of variation of baseline phosphorylation of WT Kex2p (Supplemental Figure S6B). These results suggest that phosphorylation of the Kex2p cytosolic tail reflects physiological regulation of trafficking of the protein.

**Direct binding of the Kex2p C-tail to Gga2p VHS-GAT**

To determine whether the binding of the Kex2p C-tail to the Gga2p VHS domain was direct, we carried out binding assays using glutathione S-transferase (GST)–Kex2p C-tail and C-terminally hexahistidine (His6)-tagged Gga2p-VHS-GAT expressed in and purified from *Escherichia coli* (Figure 6). The VHS-GAT-His6 construct was used because the GST-VHS construct could not be purified in good yield. GST-Kex2p C-tail fusion protein bound to glutathione–agarose retained purified VHS-GAT-His6, with the VHS-GAT-His6–bound fraction increasing as a function of the amount of its input (Figure 6A). This interaction was inhibited by incubation with increasing amounts of purified, untagged VHS-GAT (Figure 6A). Moreover, consistent with yeast two-hybrid data, the binding assay also showed that Kex2p C-tail residues 45–90 alone were capable of retaining Gga2p-VHS-GAT-His6 (Figure 6B). Taken together, these results suggest a direct and specific interaction between the Kex2p C-tail and Gga2p-VHS-GAT-His6. Directed yeast two-hybrid data indicated that substitution of Ala for Ser780 reduced binding of the Kex2p C-tail to the Gga2p VHS domain and that the phosphomimetic substitution of Asp for Ser780 permitted a strong interaction with the Gga2p VHS domain (Figure 3E). To determine how the nature of the residue at position 780 affected direct binding of the Kex2p C-tail to the Gga2p VHS domain, we tested purified WT, S780D, and S780A GST-Kex2p C-tail fusion proteins for binding to purified Gga2p-VHS-GAT-His6. The S780A-Kex2p C-tail exhibited twofold lower binding and the S780D-Kex2p C-tail exhibited twofold higher binding than the WT tail (Figure 6C). Pretreatment of the WT GST-Kex2p C-tail fusion and the S780D GST-Kex2p C-tail fusion with the anti–P-Ser780 antibody reduced binding of purified Gga2p-VHS-GAT-His6 by more...
FIGURE 5: Kex2p Ser<sub>780</sub> is phosphorylated in vivo, and phosphorylation is increased under conditions of cell wall damage. (A) WT Kex2p is phosphorylated at Ser<sub>780</sub> in vivo. After glass bead lysis of CBO17 cells containing pG5-KX22 (WT Kex2p, odd-numbered lanes) or pG5-KX22-S<sub>780</sub>A (S<sub>780</sub>A-Kex2p, even-numbered lanes), cell extracts were analyzed by immunoblotting with anti-Kex2p antibody (top) or anti-Kex2p antibody (bottom). Lane 1 contains extract of CBO17 containing YCpKX22 to provide a marker for the Kex2p band. See Materials and Methods for further details.

Effects of GBS mutations on the cellular trafficking of Kex2p
To determine whether GBS mutations affect trafficking of Kex2p in vivo, we assessed the effects of the mutations on the essential role of Kex2p in processing the yeast mating pheromone precursor pro-α-factor (Figure 7A) and in the rate of vacuolar turnover of Kex2p (Figure 7B; Fuller et al., 1988; Wilcox et al., 1992). The assay in Figure 7A measures the rate of loss of mating competence after shutting off expression of Kex2p. Because the form of Kex2p used contains a Y<sub>113</sub>A mutation, which inactivates TLS1 and blocks retrieval from the late endosome, the assay measures the relative rate of exit of Kex2p from the pro-α-factor processing compartment(s)/TGN in the absence of recycling from the PVC (Wilcox et al., 1992). Strain MAY15S-BB (MATα kex2Δ gaα1Δ), containing WT, Y<sub>113</sub>A; Y<sub>113</sub>A, F5<sub>779,780</sub>AA; or Y<sub>113</sub>A, S<sub>780</sub>D Kex2p under GAL1 promoter control, was grown in galactose media, shifted to glucose media at various times to repress transcription, and then tested for the ability to mate with a MATα tester strain (Figure 7A). As expected (Wilcox et al., 1992), the mating efficiency of cells in which WT Kex2p expression was shut off did not decline substantially over the course of the experiment (Figure 7A), but the mating efficiency of the retrieval-defective Y<sub>113</sub>A Kex2p began to decline by 4 h after shifting to glucose. In the context of the Y<sub>113</sub>A tail, the F5<sub>779,780</sub>AA mutation, which blocks or reduces Gga2p binding, decreased the rate of loss of mating competence, whereas the S<sub>780</sub>D mutation, which enhances Gga binding, accelerated loss of mating competence. These results are consistent with the loss or reduction of Gga-binding reducing the rate of transport of Kex2p from the TGN to the PVC by the direct, Gga-dependent pathway. In contrast, enhancing Gga-binding by the phosphomimetic S<sub>780</sub>D mutation appears to increase the rate of transport of Kex2p from the TGN to the PVC by this pathway. In the context of the Y<sub>113</sub>A mutation, the rate of Kex2p turnover measures the overall rate of transport to the vacuole (Wilcox et al., 1992). Half-lives of WT and mutant forms of Kex2p were measured (Figure 7B) using a GAL1 promoter shut-off protocol similar to that used in Figure 7A. As expected, WT Kex2p was long lived (t<sub>1/2</sub> = 330 min), whereas the Y<sub>113</sub>A mutation resulted in rapid turnover (t<sub>1/2</sub> = 20 min). In the context of the Y<sub>113</sub>A mutation, the F5<sub>779,780</sub>AA reduced (t<sub>1/2</sub> = 26 min), whereas the S<sub>780</sub>D mutation markedly increased (t<sub>1/2</sub> < 7.1 min), the rate of vacuolar turnover. The rate measured for vacuolar delivery of Y<sub>113</sub>A, S<sub>780</sub>D Kex2p
is in fact faster than that of Kex2p with a C-tail deletion (11 min; Wilcox et al., 1992). The likeliest explanation for this is that the S780D mutation accelerates delivery of Kex2p from the TGN to the PVC relative to the C-tailΔ protein, which cannot interact with Gga proteins, and that the subsequent delivery of both proteins to the vacuole occurs at the same rate, as each protein lacks a retrieval signal. The data in Figure 7 establish a strong link between the ability of Kex2p to bind Gga and the ability to exit the TGN.

GBS mutations affect cell-free transport of Kex2p from the TGN to the PVC

We previously established an assay that measures transport of Kex2p from the TGN to the late endosome in a cell-free system. This reaction requires Gga2p, clathrin heavy chain, the dynamin homologue Vps1p, the Vps21p rab GTPase, the Vps45p soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) regulator, and the late endosomal t-SNARE Pep12p and measures the delivery of Kex2p from donor TGN membranes to acceptor PVC membranes, where Kex2p cleaves a chimeric substrate, Pep12Ste13xHA fusion substrate (PSHA; for details of the assay see Materials and Methods and Blanchette et al., 2004; Abazeed et al., 2005; Abazeed and Fuller, 2008). Transport is measured as the fraction of the total PSHA in acceptor membranes that is cleaved (processed) by Kex2p. With Kex2p in donor membranes, the reaction exhibits two phases: an early, rapid phase (30–40% of the total), which is Gga2p and clathrin independent and most likely represents delivery of Kex2p from early endosomes to the PVC; and a second phase (∼60–70% of the total), which is Gga2p and clathrin dependent (Abazeed and Fuller, 2008). This second phase proceeds after a lag of ∼10 min and appears to represent direct TGN–PVC transport (Abazeed and Fuller, 2008).

To assess the effects of mutation of S780 on cell-free TGN–PVC transport of Kex2p, we prepared donor medium-speed supernatant (MSS) membranes from strains expressing wild-type levels of WT, S780D, or FS779,780AA Kex2p. Relative to reactions containing WT Kex2p, reactions containing S780D–Kex2p exhibited an accelerated rate with a reduced lag period, reaching completion at 10 min instead of the more typical 20 min (Figure 8A). Reactions containing...
The FS779,780AA Kex2p, in contrast, exhibited a slower rate and more extended lag period than seen with WT Kex2p. All reactions reached the same final extent (~8% PSHA processed), consistent with the fact that the acceptor PSHA membranes were the same in each case. Because the reduced lag seen with S780D-Kex2p raised the possibility that a larger fraction of the reaction might be occurring through the Gga-independent indirect pathway, the dependence of the reactions on Gga2p was assessed. Previously it was shown that reactions using MSS from MAY17—a strain lacking Gga1p and expressing only a form of Gga2p containing a C-terminal 13-myc epitope tag—could be inhibited by anti-c-myc antibody (Abazeed and Fuller, 2008). MSS was prepared from MAY17 expressing wild-type levels of WT, S780D, or FS779,780AA Kex2p and tested for transport competence. These membranes exhibited time-course profiles (Supplemental Figure S7A) similar to those seen in Figure 8A using MSS from a GGA1 GGA2 WT strain. Preincubation of donor MSS with increasing amounts of anti-c-myc antibody resulted in inhibition of reactions with all three forms of Kex2p and indicating that all of the reactions were Gga dependent (Supplemental Figure S7C).

Unexpectedly, when donor MSS membranes were titrated into the reaction, saturation was seen with the 50-μg donor membrane fraction containing WT Kex2p, whereas reactions with donor membranes containing either S780D or FS779,780AA Kex2p required approximately fourfold higher levels of donor membrane to reach saturation (Figure 8B). The same effect was seen with MSS prepared from the gga1Δ gga2::13myc strain MAY17 (Supplemental Figure S7B). Kex2p specific activity was nearly identical in the three MSS preparations, between 100 and 110 U/mg, and consistent with previously measured WT levels of Kex2 activity (Fuller et al., 1989a), so the difference cannot be accounted for by reduced levels of the S780D mutant enzyme per se. A possible explanation is that the S780A and S780D mutations localized larger fractions of Kex2 to compartments from which Gga2p-dependent transport could not occur. Because of enhanced Gga2p binding and TGN exit, S780D Kex2p might be more enriched in the PVC, whereas decreased Gga2p binding and TGN–PVC transport might result in S780D-Kex2p enrichment in the early endosome. To test the hypothesis that the S780 mutations altered the distribution of Kex2p in TGN/endosomal membranes, we assessed the behavior of donor membranes containing WT and C-tail mutant forms of Kex2p by using the cell-free TGN homotypic fusion reaction (Brickner et al., 2001). This assay depends on direct fusion of TGN membranes containing Kex2p with TGN membranes containing the Ste13α-HA fusion substrate (SHA) fusion protein, which consists of full-length Ste13p fused to C-terminal Kex2-cleavable site and 3x hemagglutinin (HA) tag. Activity in this assay therefore likely reflects Kex2p levels in the TGN (Figure 9). In contrast to the results with the TGN–PVC assay, both membranes containing S780D and S780A Kex2p showed slower kinetics in the TGN homotypic fusion reaction than membranes containing WT Kex2p, with the S780D Kex2p-containing membranes exhibiting the slowest reaction (Figure 9A). This result is consistent with there being less C-tail mutant Kex2p in the TGN donor compartment. Indeed, in donor membrane titration assays, membranes containing S780D Kex2p showed twofold lower activity than membranes containing WT Kex2p, with S780A Kex2p membranes exhibiting intermediate activity (Figure 9B). These results suggest that both S780D and S780A Kex2p are present at reduced levels in TGN membranes and are thus consistent with mutation of Ser780 altering the steady-state distribution of Kex2p. This finding makes it all that much more striking that the S780D mutation renders Kex2p more rapidly recruited into cell-free, Gga-dependent TGN–PVC transport. These results are consistent with the conclusion that phosphorylation of Ser780 regulates Kex2p sorting at the TGN by increasing partitioning of Kex2p into the direct, Gga-dependent pathway.
to block efficient TGN-to-PVC trafficking (Wilcox et al., 1992; Nothwehr et al., 1993; Cereghino et al., 1995). Here we showed that despite the existence of this C-tail–independent TGN–PVC transport mechanism, the C-tail sequences in both Kex2p and Vps10p contain sites that interact with the VHS domains of the yeast Gga proteins, the clathrin adaptors required for direct TGN–PVC trafficking. We showed further, in the case of Kex2p, that binding is direct and ubiquitin independent, that it is enhanced by phosphorylation of a
specific site, and that mutations that affect the binding of the Kex2p GBS to purified Gga2p VHS-GAT also affect TGN–PVC trafficking both in vivo and in a cell-free assay. Thus, although TGN–PVC trafficking of singly spanning proteins such as Kex2p and Vps10p can be achieved by default, we propose that it is ordinarily driven through specific, direct, and regulated binding of C-tail sequences to the Gga adaptors.

This direct interaction between cargo-sorting signals and the Gga1/2p VHS domains stands in contrast to the ubiquitin-dependent sorting of several polytopic membrane proteins, in which covalently bound ubiquitin mediates binding to the GAT domain of the Gga proteins (Lauwers et al., 2010). Recent evidence suggests that the ubiquitin–GAT interaction may be required only for Gga-dependent sorting at the PVC into the luminal vesicles of multivesicular endosomes and that other interactions mediate Gga-dependent sorting of polytopic proteins at the TGN (Deng et al., 2009; Lauwers et al., 2009). Whether the ubiquitin-independent interactions at the TGN involve direct binding to the Gga VHS domains remains to be seen.

**Potential regulation of Gga-GBS binding by apparent autoinhibitory mechanisms**

Detection of the binding of Gga2p to sequences in the Kex2p and Vps10p C-tails required deletion of an internal inhibitory sequence in the Gga2 linker domain that could itself bind to the Gga2p VHS domain. Thus, although recent work called into question the functional significance of autoinhibitory, VHS-binding sequences in the linker regions of mammalian GGA proteins (Cramer et al., 2010), an even more recent study challenged this view (Doray et al., 2012), and our data indicate that this is a property conserved in yeast Gga2p. Furthermore, despite the fact that the hinge regions are poorly conserved between yeast Gga1p and Gga2p (16% identity over 100 residues), the 380–395 deletion interval, which contains the internal Gga2p inhibitory sequence, contains a motif, DLLGD, that is conserved between the two proteins. Although the existence of an autoinhibitory sequence in the Gga1p hinge has not been directly demonstrated, we propose that the DLLGD motif is a reasonable candidate for the internal VHS-binding site. Indeed, Ala scanning indicates that the two Leu residues in this motif are required for autoinhibition (unpublished data). This site resembles neither the typical acidic dileucine motif nor the Kex2p or Vps10p GBS sequences. The only other motif of at least 5 residues conserved between the Gga1p and Gga2p hinge domains, NUDIF (residues 351–355 in Gga2p), has recently been shown to contain overlapping GAE-domain and clathrin-binding motifs. This sequence governs a distinct autoinhibitory mechanism that regulates competing interactions of Gga2p with clathrin and the Ent5p adaptor (Hung et al., 2012). Thus there may be multiple internal mechanisms that regulate the multivalent binding activities of the yeast Gga proteins.

Detection of binding of the Vps10p C-tail to Gga1/2 VHS domains required deletion of membrane-proximal inhibitory sequences in the Vps10p tail. Deletion analysis indicated that the N-terminus of this inhibitory sequence lay between tail residues 40 and 45, which contains a sequence, FYVF, previously shown to regulate Vps10p trafficking (Cereghino et al., 1995; Cooper and Stevens, 1996). Although the mechanism of this inhibition has not been determined, its existence suggests that conformational interactions in the Vps10p tail might be important in regulating exposure of sorting determinants.

**Nature of the GBS in the Kex2p and Vps10p C-tails**

As determined by the two-hybrid assay, the minimal GBS sequence in Kex2p comprised ∼36 residues (C-tail residues 55–90; Supplemental Figure S3) and in Vps10p comprised ∼31 residues (C-tail residues 110–140; Supplemental Figure S4). Alanine scanning mutagenesis of the C-terminal side of the Kex2p GBS identified Ser780 as an important residue for binding and showed that the identity of residues C-terminal to position 780 was unimportant for binding. Further mutagenesis showed that substitution of Asp for Ser780 permitted binding in the two-hybrid assay and suggested that Ser780 might be a phosphorylation site (see later discussion). In direct biochemical binding assays, the S780A substitution decreased and the S780D substitution increased the affinity of Gga2 VHS-GAT for the Kex2p GBS. The Ala and Asp substitutions also had opposite effects on TGN–PVC trafficking both in vivo and in a cell-free assay for TGN–PVC transport. The effects of the mutations were shown in vivo using both a sensitive mating assay that measures the rate of loss of Kex2p activity from the pro-c–α-factor processing compartment, presumably the TGN, and a turnover assay, which measures the overall rate of delivery to the vacuole. In the loss-of-mating-competence assay, the Ala substitution reduced the net rate of exit of Kex2p from the processing compartment, whereas the Asp substitution increased it. In the turnover assay, the S780A substitution decreased and the S780D substitution increased the rate of vacuolar delivery. In the cell-free TGN–PVC assay, the Ala substitution similarly decreased the initial rate of TGN–PVC transport of Kex2p, whereas the Asp substitution increased it. The specificity of the effects of the Asp and Ala substitutions for Ser780 is underlined by the fact that both substitutions reduced Kex2p activity available for TGN homotypic fusion. From this we conclude that Ser780 is an important determinant for binding to the Gga1/2 VHS domain and that Ser780 phosphorylation positively regulates Gga-dependent sorting at the TGN.

However, because the Ala780 substitution only reduced but did not eliminate binding, and because it appeared only to reduce rather than eliminate TGN–PVC trafficking in the in vivo and cell-free assays as well, we conclude that sequences on the N-terminal side of the Kex2p GBS are also likely to be important for binding. Although neither the Kex2p nor the Vps10p GBS contains canonical DXXL Gga-binding motifs, these presumptive VHS-binding sequences contain clusters of acidic and aliphatic residues like the known mammalian VHS-binding sequences. Suggestive alignments can be made both between the Kex2p and Vps10p GBSs and between these GBSs and the Gga-binding sequences of CI-MPR, BACE, sortilin, and SorLA (Supplemental Figure S9). Clustal W alignment of Kex2p and Vps10p C-tails from multiple Saccharomyces species aligns a conserved Thr residue in the Vps10p tails with Ser780 in Kex2p. In each case, these residues are flanked at their N-terminal side by acidic sequences. An alternative manual alignment of the Kex2p and Vps10p sequences with the mammalian GBS motifs aligns Asp residues in the yeast proteins with the conserved Asp of the mammalian GBS motifs and aligns at least one Leu in each yeast sequence with the conserved aliphatic residues of the mammalian GBS motifs. The crystal structures of these motifs bound to the VHS domain of GGA1 (sortilin, BACE, and SorLA) or GGA3 (Cl-M6PR) identify key conserved residues in the mammalian VHS domains that are required for interaction with the Asp and Leu residues (or Asp, Val, and Met residues in the case of SorLA) of the GBS motifs (Misra et al., 2002; Shiba et al., 2002; He et al., 2003; Cramer et al., 2010). As observed by Misra et al. (2002), several of these VHS residues are not conserved in the yeast Gga1/2p VHS domains. This is consistent with the VHS domains of the yeast Gga proteins interacting preferentially with yeast GBS motifs that differ from the mammalian GBS motifs.
Regulation of Gga binding by phosphorylation of Ser\textsubscript{780} in the Kex2p GBS

Phosphorylation of the Kex2p C-tail was reported previously (Brickner, 1998; Johnston et al., 2005). Here, however, we demonstrate the molecular, cellular, and physiological effects of a specific phosphorylation event in the C-tail. Phosphorylation of Ser\textsubscript{780} in the Kex2p C-tail in vivo was confirmed by the fact that an affinity-purified antibody specific for a peptide containing phospho-Ser\textsubscript{780} within the Kex2p GBS preferentially recognized WT versus S\textsubscript{780A} Kex2p in immunoblotting of cell extracts. Biochemical binding assays confirmed that this antibody blocked binding of the Kex2p C-tail to Gga2 VHS-GAT. As indicated earlier, the results of in vivo and in vitro assays indicated that the phosphomimetic substitution of Asp at 780 increased, whereas the Ala substitution decreased, the rate of TGN–PVC transport of Kex2p. As indicated, despite the fact that the Ala substitution completely blocked binding of the Kex2p GBS to the Gga2p VHS domain in the two-hybrid assay, the Ala\textsubscript{780} Kex2p C-tail still bound to the purified Gga2p VHS-GAT protein, although substantially more weakly than did the WT or Asp\textsubscript{780} C-tails. This is consistent with a modulatory role for phosphorylation of Ser\textsubscript{780}, with phosphorylation facilitating Gga-dependent sorting. A modulatory role for phosphorylation would be consistent with the effects of phosphorylation of the C-tails of mammalian proteins on their interactions with mammalian Ggas (Supplemental Figure S9; Cramer et al., 2010).

Unlike Vps10p, which follows only the direct, Gga-dependent pathway to the PVC, Kex2p partitions between this direct pathway and an indirect pathway through the early endosome. We hypothesize that phosphorylation acts as a switch to enhance partitioning into the direct pathway. Evidence supporting this includes the fact that the GBS and Ser\textsubscript{780} phosphorylation site in the Kex2p C-tail overlaps two other targeting signals (Supplemental Figure S10).

First, the motif NPFFS\textsubscript{K}, which includes Ser\textsubscript{780}, is a site that can mediate Sla1p-dependent endocytosis, although Kex2p is not known to travel to the plasma membrane (Tan et al., 1996; Howard et al., 2002). Second, the sequence TNENP, which lies within the minimal GBS, contains the C-terminal domain of TGN-localization signal 2 (TLS2), which operationally functions as a TGN retention signal antagonized by Vps13p function and may represent a signal for TGN–PVC transport of Kex2p through the early endosome, that is, to alter the partitioning of Kex2p through the two pathways. Phosphorylation of the Ste13p N-terminal cytosolic tail at Ser\textsubscript{13} in the context of the fusion protein A-alkaline phosphatase has also been shown to accelerate trafficking from the TGN to the PVC, although it is not known whether this involves enhancement of trafficking via the direct Gga-dependent pathway (Johnston et al., 2005).

It is not known what kinase is responsible for phosphorylation of Ser\textsubscript{780}. Indeed, the site does not resemble known phosphorylation sites in yeast proteins, so that an empirical approach will be required to identify the responsible enzyme or enzymes. Regulation of phosphorylation of Ser\textsubscript{780} by cell cycle–damaging/stressing agents suggests the possibility that alterations in Kex2 trafficking may occur in response to cell wall damage, perhaps to alter the profile of substrates processed by Kex2p. At present, however, there is no known alteration in cleavage of substrates by Kex2 under cell wall damage conditions. However, numerous cell wall proteins undergo processing by Kex2p, and kex2-null mutations result in cell wall defects (Mrsa et al., 1997; Moukadiri et al., 1999; Tomishige et al., 2003). Furthermore, null mutations in KEX2 exhibit synthetic interactions with mutations in the genes encoding yapsins 1 and 2, which have been shown to be required for normal glucan incorporation into the cell wall (Komano and Fuller, 1995; Krysan et al., 2005). Thus it is plausible to hypothesize that alterations in trafficking of Kex2p might be important in adaptation to cell wall stress or damage.

In summary, we have shown that direct interactions occur between peptide-targeting motifs in the C-tails of yeast TGN membrane proteins and the VHS domains of the yeast Gga proteins. These interactions are important for the direct Gga-dependent trafficking between the TGN and PVC in the case of Kex2p. Interaction between the Kex2p C-tail and the Gga proteins can undergo physiological regulation by site-specific phosphorylation that enhances sorting into the direct TGN–PVC pathway.

MATERIALS AND METHODS

Strains and plasmids

Strains used were L40 (MATa his3A200 trp1-901 leu2-3, 112 ade2 lys2::(lexAop)\textsubscript{2}-HIS3 URA3::(lexAop)\textsubscript{2}-lacZ GAL4; Vojtek et al., 1993), DC14 (MATa his1), JBY209 (MATa kex2A2::his Gdap2::kan' pep4::HIS3 ste13::LEU2; Brickner et al., 2001), MAY17 (JBY209 gga1A GGA2::13myc-TRP1; Abazed and Fuller, 2008), CBO18 (MATa KEX2 pep4::HIS3 ppc::HisG ppc::HisG), CBO16 (CBO18 kex2Δ::TRP1), and CBO17 (CBO16 MAT\textsubscript{a}; Wilcox et al., 1992). The diploid strain generated by crossing MAY14 (MATa gga1::TRP1 gga2::HIS3) with KRY24-2A (MATa kex2::LEU2) was sporulated, and tetrads were scored to isolate MAY15-BS (MATa kex2::LEU2 gga1::TRP1). All strains except DC14 were from the W303-1B background and had the following additional genetic markers: ade2-1 can1-100 his3-11,15 leu2-3 trp1-1 ura3-1.

CEN4 URA3 plasmids pCWXX10 and pCWXX20 contain the KEX2 structural gene under control of the KEX2 promoter and the yeast GAL1 promoter, respectively (Wilcox et al., 1992). Overlap-extension PCR was used to generate Nar–Sall fragments containing the substitutions F\textsubscript{5779,780}AA, S\textsubscript{780}D, Y\textsubscript{113}A/F\textsubscript{5779,780}AA, and Y\textsubscript{113}A/S\textsubscript{780}D. These fragments were subcloned into pCWXX10 and pCWXX20, replacing the WT Nar–Sall fragment (Wilcox et al., 1992) to create pCWXX10-F\textsubscript{5779,780}AA, pCWXX10-S\textsubscript{780}D, pCWXX20-F\textsubscript{5779,780}AA, and pCWXX20-S\textsubscript{780}D. Plasmid pPSHA (previously referred to as pPPE12-STE13STMIX3XHA or pPB10) encodes the chimeric protein PSHA (Blanchette et al., 2004; Abazed et al., 2005). Plasmid pPSHA (previously termed pSTE13xha) encodes the chimeric protein Ste13xHA (here termed SHA; Brickner et al., 2001). Yeast two-hybrid plasmids expressing the entire sequences or segments of Gga1p, Gga2p, and the C-tails of Kex2p and Vps10p were made by subcloning PCR fragments encoding the residues indicated in Figures 1–4 and Supplemental Figures S1, S3, and S4 into Lexa-BD and VP16-AD vectors as described (Vojtek et al., 1993). Kex2p C-tail sequences 1–115 correspond to residues 700–814 of the KEX2 open reading frame (Fuller et al., 1989b). Vps10p C-tail sequences 1–164 correspond to residues 1416–1579 of the VPS10 open reading frame (Cereghino et al., 1995). Sequences were amplified by oligonucleotide primers containing BamHI (S' primer) and NotI (3' primer) restriction sites and cloned directionally into the Lexa-BD and VP16-AD vectors. Alamime-scanning mutations were made by overlap-extension PCR in the full-length Kex2p C-tail and cloned into the VP16-AD vector. Plasmids YcPXX22-S\textsubscript{780}A and pG5-KXX2-S\textsubscript{780}A were constructed by mutating the codon for Ser at position 780 to Ala (AGT to GCC) in the KEX2 C-tail in plasmids YcPXX22 and pG5-KXX2, respectively. In YcPXX22 and pG5-KXX2, the full-length KEX2 open reading frame is under the control of the TDI3 promoter in the URA3 CEN4 plasmid YcP50 (YcPXX22) or the
multicyl 2µ URA3 vector pG5 (pG5-KX22; Wilcox et al., 1992). Plasmids YCpKX22 and pG5-KX22, respectively, lead to Kex2p expression elevated ~25-fold and ~150-fold relative to WT cells (Wilcox et al., 1992). E.coli vectors expressing fusions of GST to WT, Ser750Ala, and Ser750Asp-Kex2p C-tails were constructed by inserting sequences encoding residues 700–814 of the Kex2p C-tail into vector pGEX-KG (Guan and Dixon, 1991), generating, respectively, plasmids pGEX-KG-KX, pGEX-KG-KX-S750A, and pGEX-KG-KX-S750D. An E.coli vector expressing a fusion of GST to the VHS-GAT domains of Gga2p was constructed by inserting sequences encoding residues 1–336 of Gga2p into pGEX-KG, generating pGEX-KG-VHS-GAT. In pGEX-KG-VHS-GAT-His6, sequences encoding a hexahistidine tag were appended to the Gga2p C-terminus. Yeast media were as described (Redding et al., 1996a).

Antibodies and reagents

Anti-HA monoclonal antibody (mAb) 12CA5 and anti-c-myc mAb (9E10) were from Roche Applied Science (Indianapolis, IN). Anti-hexahistidine was from Novus Biologicals (Littleton, CO). Anti-GST antibody was from Zymed Laboratories (San Francisco, CA). Anti-yeast phosphoglycerate kinase antibody was from Invitrogen (Carlsbad, CA). Affinity-purified anti-Kex2 C-tail antibody (anti-Kex2p antibody) was as described (Redding et al., 1991). Anti-phosphopeptide-specific antiserum (anti-P-Ser780 serum) and affinity-purified antibody (anti-P-Ser780 antibody) were prepared by 21st Century Biochemicals (Marlboro, MA). Briefly, rabbits were immunized with phosphorylated peptide PP1 (Ac-LeuThrAsnGluAsnProPhe(phospho-Ser)AspProIleLysGlnLysPhe-NH2), corresponding to Kex2p residues 773–787, coupled to keyhole limpet hemocyanin. Affinity purification of serum was conducted by successive chromatography on resins containing nonphosphorylated peptide NPP (Ac-CysLeuThrAsnGluAsnProPheSerAspProIleLysGlnLysPhe-NH2) and phosphorylated peptide PP2 (Ac-CysThrAsnGluAsnProPhe(phospho-Ser)AspProIleLysGlnLysPhe-NH2). Horseradish peroxidase-conjugated donkey anti-rabbit secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Affinity-purified rabbit anti-mouse immunoglobulin G was from Jackson Immuno Research (West Grove, PA). Glutathione–Sepharose 4B and protein A–Sepharose were from Sigma-Aldrich (St. Louis, MO). Antibodies and reagents (GE Healthcare); exposures were made using BioMax MR film (Kodak, Rochester, NY). Competition experiments were performed by preincubating 1 ml of diluted anti-P-Ser780 serum for 30 min at 4°C with 25 μg of phosphorylated peptide PP1 or non-phosphorylated peptide NPP. To measure the effects of cell wall–damaging drugs, log-phase cultures were diluted in yeast extract/peptone/dextrose (YPD) to an OD600 of 0.5, and cell wall–damaging drugs (calcofluor white, 20 mg/ml; Congo red, 200 mg/ml; or caffeine, 6 mM) were added for 4 h or overnight at 30°C (Krysan et al., 2005). For quantification of immunoblots, films of appropriate exposure were scanned to create TIFF files that were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD).

Protein expression

GST-Kex2p C-tail fusions, GST-VHS-GAT, and GST-VHS-GAT-His6 fusion proteins were expressed in E.coli BL21 from their respective plasmids, and the proteins were purified. Four hours prior to harvest, fusion protein expression was induced with 100 mM isopropyl-β-D-thiogalactoside. Extracts prepared from cultures by sonication (three 10-s bursts with intermittent cooling) were cleared by centrifugation (12,000 rpm, 5 min) and then incubated with glutathione–Sepharose 4B beads for 30 min at room temperature. Beads were washed three times with phosphate-buffered saline (PBS; 50 mM sodium phosphate, pH 7.0, 150 mM NaCl) plus 0.05% Tween 20 (10 ml). In the case of GST-Kex2p C-tail fusions and the GST-VHS-GAT fusion protein, glutathione was eluted with PBS containing 20 mM glutathione. Eluted fractions were pooled, concentrated, and dialyzed against PBS. GST-VHS-GAT-His6 beads were washed twice with 1 ml of thrombin cleavage buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2.5 mM CaCl2 and 0.1% 2-mercaptoethanol). Fusion protein bound to beads was cleaved by bovine a-thrombin to release VHS-GAT-His6 (Guan and Dixon, 1991). Thrombin was inactivated with 1 mM phenylmethylsulfonyl fluoride, and the soluble fraction and two 1-ml washes of thrombin-treated beads were pooled, concentrated, and dialyzed against PBS.

Kex2p C-tail–binding assays

Binding reactions containing GST-Kex2p C-tails (30 or 100 μg) and indicated concentrations of VHS-GAT-His6 were conducted in 0.5 ml of binding buffer (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid–KOH, pH 7.5, 150 mM KCl, 1 mM MgCl2, 10% [vol/vol] glycerol, and 0.5 mg/ml bovine serum albumin) for 2 h at 4°C or room temperature (RT), as indicated. Glutathione–Sepharose 4B (25 μl) was added, and incubations were continued for 30 min. Suspensions were transferred to chromatography columns, washed three times with binding buffer, and eluted in SDS–PAGE sample buffer containing 250 μl of lysis buffer (50 mM Tris-HCl, pH 7.0, 6 M urea, 100 mM dithiothreitol, 10% glycerol, 2% [wt/vol] SDS) containing protease inhibitors (complete EDTA-free mini tablet; Roche Applied Science) and phosphatase inhibitors (10 nM okadaic acid, 200 μM sodium vanadate, 50 μM sodium fluoride) and lysed by vortexing with 0.38 g of 0.5-mm glass beads in 13× 100-mm glass tubes for five 20-s intervals alternating with 20 s on ice. Lysates were centrifuged to remove cell debris, and protein was measured in the supernatant fractions by the Bradford assay (Bio-Rad, Hercules, CA). Lysate protein, 100 μg, was heated in SDS–PAGE sample buffer at 98°C for 5 min and subjected to SDS–PAGE (4–20% gradient gels; Invitrogen, Carlsbad, CA). Gels were transferred to nitrocellulose (GE Healthcare, Waukesha, WI), and immunoblots were probed with anti–P-Ser780 (1:1000 dilution), anti–P-Ser780 antibody (3.3 μg/ml), or anti-Kex2p antibody and developed with secondary antibody and ECL reagents (GE Healthcare); exposures were made using BioMax MR film (Kodak, Rochester, NY). Competition experiments were performed by preincubating 1 ml of diluted anti-P-Ser780 serum for 30 min at 4°C with 25 μg of phosphorylated peptide PP1 or non-phosphorylated peptide NPP. To measure the effects of cell wall–damaging drugs, log-phase cultures were diluted in yeast extract/peptone/dextrose (YPD) to an OD600 of 0.5, and cell wall–damaging drugs (calcofluor white, 20 mg/ml; Congo red, 200 mg/ml; or caffeine, 6 mM) were added for 4 h or overnight at 30°C (Krysan et al., 2005). For quantification of immunoblots, films of appropriate exposure were scanned to create TIFF files that were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD).

Two-hybrid analysis

All two-hybrid assays were performed in strain L40 (Vojtek et al., 1993). Transformants containing the indicated LexA-binding domain (LexA-BD) and VP16-activation domain (VP16-AD) plasmid constructs were analyzed by pronging cells onto synthetic dextrose complete (SDC)-Leu-Trp plates and SDC-Leu-Trp-His plates from 96-well microtiter plates. In cases in which nonspecific transactivation of the His reporter was observed, indicate amounts of 3-aminoatrizole were added to -Leu-Trp-His plates.

Analysis of Kex2p Ser780 phosphorylation

 Cultures (25 ml) grown to log phase in SDC-Ura medium (CBO17 containing plasmid pG5-KX22, pG5-KX22-S780A,YCpKX22, or YCpXX22-S780A) or YPAD medium (CBO16 or CBO18) were harvested by centrifugation, and cells were resuspended in 250 μl of lysis buffer (50 mM Tris-HCl, pH 7.0, 6 M urea, 100 mM dithiothreitol, 10% glycerol, 2% [wt/vol] SDS) containing protease inhibitors (complete EDTA-free mini tablet; Roche Applied Science) and phosphatase inhibitors (10 nM okadaic acid, 200 μM sodium vanadate, 50 μM sodium fluoride) and lysed by vortexing with 0.38 g of 0.5-mm glass beads in 13× 100-mm glass tubes for five 20-s intervals alternating with 20 s on ice. Lysates were centrifuged to remove cell debris, and protein was measured in the supernatant fractions by the Bradford assay (Bio-Rad, Hercules, CA). Lysate protein, 100 μg, was heated in SDS–PAGE sample buffer at 98°C for 5 min and subjected to SDS–PAGE (4–20% gradient gels; Invitrogen, Carlsbad, CA). Gels were transferred to nitrocellulose (GE Healthcare, Waukesha, WI), and immunoblots were probed with anti–P-Ser780 (1:1000 dilution), anti–P-Ser780 antibody (3.3 μg/ml), or anti-Kex2p antibody and developed with secondary antibody and ECL reagents (GE Healthcare); exposures were made using BioMax MR film (Kodak, Rochester, NY). Competition experiments were performed by preincubating 1 ml of diluted anti-P-Ser780 serum for 30 min at 4°C with 25 μg of phosphorylated peptide PP1 or non-phosphorylated peptide NPP. To measure the effects of cell wall–damaging drugs, log-phase cultures were diluted in yeast extract/peptone/dextrose (YPD) to an OD600 of 0.5, and cell wall–damaging drugs (calcofluor white, 20 mg/ml; Congo red, 200 mg/ml; or caffeine, 6 mM) were added for 4 h or overnight at 30°C (Krysan et al., 2005). For quantification of immunoblots, films of appropriate exposure were scanned to create TIFF files that were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD).
buffer. Samples were subjected to SDS–PAGE and immunoblotted with anti-His antibody to detect bound VHS-GAT-His6. Competition with untagged VHS-GAT was measured by adding specified concentrations of purified VHS-GAT to the binding reactions. In antibody-blocking assays, Kex2p C-tail and Kex2p C-tail-S780D (30 μg of each) were preincubated with 60 μg of anti–P-Ser780 antibody in 0.5 ml of binding buffer for 30 min at RT before adding VHS-GAT-His6. Competition with phosphorylated and nonphosphorylated peptide was measured by preincubating peptides (15 μg) with VHS-GAT-His6 (50 μg) for 30 min at RT in 0.5 ml of binding buffer before adding GST-Kex2 C-tails (30 μg). To determine relative dissociation rates of complexes of GST-Kex2 C-tails with VHS-GAT-His6, after initial binding incubations at RT, standard reactions were diluted 10-fold with binding buffer and incubated at RT for the indicated times before processing.

Quantitative loss-of-mating-competence assays

Assays for loss of mating competence upon shutting off expression of WT and mutant forms of Kex2p under GAL1 promotor control were conducted as described (Wilcox et al., 1992). Briefly, MAY5-8B cells harboring CEN4 URA3 plasmids encoding WT Kex2p (pCWXX20), Y713A-Kex2p (pCWXX21), Y713A/FS779,780AAA-Kex2p (pCWXX21-FS779,780AA), and Y713A/S780D-Kex2p (pCWXX21-S780D) under the control of the GAL1 promotor were grown in SC-Ura media containing 2% galactose into log phase at 30°C and were switched to media (SC-Ura) containing 2% glucose. After indicated times, ~5 × 10⁶ cells were mixed with ~1 × 10⁶ cells of MATα mating tester strain DC14, and the mixture was collected on 25-mm HAT filters (Millipore, Bedford, MA). The filters were incubated cell side up on YPD plates at 30°C for 4 h, the cells were resuspended in water, and serial dilutions of matings were plated on SC and SDC-Ura. The number of colonies on the SDC-Ura plates represented total the number of diploids plus MATα cells; the number of colonies on SDC plates represented total diploids. Mating efficiency is expressed as the number of diploids divided by the number of diploids plus MATα haploids.

Determination of rates of turnover

MAY15-8B cells containing plasmid pCWXX20, pCWXX21, pCWXX21-FS779,780AA, or pCWXX21-S780D were grown in SC-Ura containing 2% galactose to an OD600 of 0.3 at 30°C, and galactose was added to 2% to repress KEX2 expression. At the indicated time points, cells were harvested by centrifugation, and glass bead lysates were prepared, subjected to SDS–PAGE, and transferred to nitrocellulose, and immunoblots were probed with anti Kex2p antibody, bodies, developed, and quantified as described earlier (see Analysis of Kex2p Ser780 phosphorylation).

Cell-free TGN–PVC transport and TGN homotypic fusion assays

The basic cell-free assays for TGN-to-PVC transport and TGN homotypic fusion were as described (Brickner et al., 2001; Blanchette et al., 2004; Abazeed et al., 2005; Abazeed and Fuller, 2008). The TGN–PVC transport assay measures delivery of Kex2p activity from TGN membranes to PVC membranes containing the chimeric Kex2p substrate PSHA. PSHA consists of the entire sequence of the PVC syntaxin Pep12p, which localizes the protein to the PVC, followed by the catalytic domain of the Ste13p dipeptidyl aminopeptidase A (DPAP), a Kex2p cleavage site from pro–α-factor and a triple-HA epitope tag. The TGN homotypic fusion assay measures fusion of TGN membranes containing Kex2p activity with TGN membranes containing the chimeric Kex2p substrate SHA. The SHA chimera lacks the Pep12p sequences found in PSHA and contains the Ste13p C-tail and TMD and is thus localized to the TGN. In both assays, MS5 was prepared from semi-intact yeast cells by gentle freeze–thaw lysis of yeast spheroplasts, followed by centrifugation at 13,000 × g. MSS prepared under these conditions contains cytosol and late Golgi and endosomal membranes but not endoplasmic reticulum or early Golgi vesicles (Brickner et al., 2001). TGN–PVC transport reactions were performed by combining equal volumes (10 μl) of 1) 3x buffer (containing an ATP-regenerating system), 2) donor MSS, prepared from JBY209 or MAY17 containing pCWXX10 (expressing WT Kex2p), pCWXX10-FS779,780AA, or pCWXX10-S780D, and 3) acceptor MSS prepared from JBY209 or MAY17 containing plasmid pPSHA, which expresses the PSHA chimera (Blanchette et al., 2004; Abazeed and Fuller, 2008). For TGN homotypic fusion assays, MSS was prepared from JBY209 containing plasmid pPSHA, which expresses the SHA chimera (Brickner et al., 2001). Mixtures were incubated at 30°C for 20 min or indicated times to permit transport to occur and terminated by addition of buffer containing 1% Triton X-100. To determine the extent of cleavage of PSHA or SHA by Kex2p, terminated transport reactions were subjected to immunoprecipitation using anti–HA monoclonal antibody, rabbit anti–mouse immunoglobulin G, and Pansorbin (Brickner et al., 2001). The amount of Ste13 dipeptidyl aminopeptidase activity left in the supernatant of the immunoprecipitation (IP) and of a mock-IP (no anti-HA) was determined using the fluorogenic substrate Ala-Pro-AMC. The extent of transport (PSHA) or fusion (SHA) is expressed as the ratio of DPAP activity in the supernatant fractions from the IP and the mock IP (fraction of PSHA or SHA cleaved). For MSS titration experiments, transport reactions were performed by adding donor MSS containing the indicated amount of protein (measured by Bradford assay). Data points represent mean values of triplicates, and error bars represent the SD of the mean. All reactions presented were performed three times with comparable results. At least two independent preparations of MSS were tested.

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