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A Chemical Genetic Screen for Modulators of Exocytic Transport Identifies Inhibitors of a Transport Mechanism Linked to *GTR2* Function[▽]

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Membrane and protein traffic to the cell surface is mediated by partially redundant pathways that are difficult to perturb in ways that yield a strong phenotype. Such robustness is expected in a fine-tuned process, regulated by environmental cues, that is required for controlled cell surface growth and cell proliferation. Synthetic genetic interaction screens are especially valuable for investigating complex processes involving partially redundant pathways or mechanisms. In a previous study, we used a triple-synthetic-lethal yeast mutant screen to identify a novel component of the late exocytic transport machinery, Avl9. In a chemical-genetic version of the successful mutant screen, we have now identified small molecules that cause a rapid (within 15 min) accumulation of secretory cargo and abnormal Golgi compartment-like membranes at low concentration (<2 μM), indicating that the compounds likely target the exocytic transport machinery at the Golgi. We screened for genes that, when overexpressed, suppress the drug effects, and found that the Ras-like small GTPase, Gtr2, but not its homolog and binding partner, Gtr1, efficiently suppresses the toxic effects of the compounds. Furthermore, assays for suppression of the secretory defect caused by the compounds suggest that Gtr proteins can regulate a pathway that is perturbed by the compounds. Because *avl9*Δ and *gtr* mutants share some phenotypes, our results indicate that the small molecules identified by our chemical-genetic strategy are promising tools for understanding Avl9 function and the mechanisms that control late exocytic transport.

Cell growth and proliferation, as well as the regulation of cell surface composition, are achieved by an intracellular transport machinery that delivers proteins and membrane to the cell surface. The transport machinery is regulated by environmental sensing and signaling pathways that are integrated for the fine-tuned control of transport to the cell surface. The mechanisms that regulate cell growth and proliferation are highly robust; therefore, they can function in a wide range of environmental conditions and even when some components of the transport or signaling machinery fail. In eukaryotic cells, this robustness is achieved in part by a complex network of membrane and protein traffic routes to the cell surface (17, 33). Defects in a transport pathway can result in cargo transport by an alternate route, making transport defects difficult to detect in mutant screens (17, 18). Therefore, relatively little is known about the mechanisms by which protein and membrane cargo is transported from late exocytic sorting compartments, the late Golgi compartments and endosomes, and we have yet to identify most of the components that mediate and regulate this process.

Complex processes are more readily understood in relatively simple organisms. For this reason, the budding yeast *Saccharomyces cerevisiae* has become one of the most powerful experimental models for understanding intracellular transport, and most of the conserved components of the exocytic traffic machinery were first discovered by using yeast genetic strategies (27). We used a yeast genetic screen to identify a novel component of the late exocytic transport machinery, Avl9, a member of an ancient eukaryotic protein superfamily (18). Avl9 is essential in a mutant strain lacking Vps1, a dynamin homolog that is thought to function in transport vesicle formation at a late Golgi compartment (26, 34), and also lacking Apl2, a large subunit of the adaptor protein 1 (AP-1) complex, which is required for forming certain classes of clathrin-coated vesicles at late Golgi compartments and endosomes (18, 19, 31, 42). The *apl2*Δ and *vps1*Δ mutants have defects in an exocytic pathway(s), but these mutants, as well as an *apl2*Δ *vps1*Δ double mutant, grow well because cargo is rerouted into a remaining pathway(s) (18). Mutations such as *avl9*Δ, which are lethal in an *apl2*Δ *vps1*Δ strain but not in a wild-type strain, are expected to cause defects in a branch of the exocytic pathway that remains functional in the *apl2*Δ *vps1*Δ strain. Analogous to using mutagenesis to screen for a secretory block in the *apl2*Δ *vps1*Δ mutant, we performed a high-throughput screen of a large library of small molecules to identify compounds that inhibit the growth of the *vps1*Δ *apl2*Δ mutant but which have relatively little effect on wild-type cells. The targets of these compounds are potential components of the secretory machinery, and some of the compounds may interfere with an Avl9-related function. The biochemical function of Avl9 and related proteins is still unknown, and the inhibitors identified by our screen strategy could be valuable tools in understanding the role of Avl9 in both yeast and mammalian cells.

Our high-throughput screen was successful in identifying novel exocytic transport inhibitors, and we describe the phenotypic effects of one structurally similar group of compounds in detail. Furthermore, we show that the toxic effects of this

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group of compounds are inhibited by highly expressing *GTR2*, which encodes a Ras-like small GTPase that plays a role in regulating nutrient-responsive TORC1 (target of rapamycin complex 1) kinase signaling, exocytic cargo sorting at endosomes, and epigenetic control of gene expression (7, 11, 14, 25, 37). Therefore, the small molecules identified by our chemical-genetic approach are promising tools for understanding how signaling pathways that respond to environmental conditions regulate the traffic pathways that mediate cell growth and proliferation.

MATERIALS AND METHODS

Reagents, plasmids, and yeast strains. The minimal medium for growing plasmid-carrying yeast strains was complete synthetic medium (CSM) lacking a nutrient for plasmid selection, with amino acid mixes from Q-Biogene. All other growth media components were from Difco and were prepared following recipes described previously (39). The rich medium was YPD (yeast extract, peptone, 2% glucose) or YPGal (yeast extract, peptone, 2% galactose) unless otherwise stated. Culture growth was monitored by measuring the optical density at 600 nm (OD_{600}) in a Genesys 5 spectrophotometer (Thermo-Fisher). Rapamycin was from Sigma-Aldrich and prepared as described earlier (6). Hit compounds from high-throughput screening were reordered from ChemBridge (KU#1-11) or ChemDiv (KU#12-15).

Our "wild-type" yeast strains were EHY46 and EHY47 (18). EHY361 is a *vps1*Δ mutant strain in the EHY47 background (18). EHY644 is an *apl2*Δ *vps1*Δ mutant strain (obtained from EHY361 crossed to GPY1783-10A [18]). EHY644 was transformed with (i) pEH227, which is pRS316 with *VPS1* (18), to generate EHY658; (ii) pEH331, which is pRS316 with *APL2* (18), to generate EHY1166; or (iii) an "empty" vector, pRS316, which is a *URA3 CEN* plasmid (40), to generate EHY707. EHY807 is an *apl2*Δ strain, generated from EHY47 by integrating a PCR product containing *apl2*Δ::*KanMX4* (obtained from Y12725 by using the primers EH130 and EH131 [18]). EHY1325 is a wild-type diploid generated by crossing EHY47 to EHY46. *GTR2* was deleted in this diploid to generate EHY1326 by integrating a PCR fragment containing *gtr2*Δ::*LEU2* (by using the primers EH212 [GGAAAGGACCGTTTCCGGAC] and EH213 [CG ACCCCCATCGTGAGTGCT]), obtained from strain NBW5Δgtr2 (kindly provided by Takeshi Sekiguchi [25]). EHY1326 was sporulated, and *gtr2*Δ progeny (LZY260) was crossed to EHY644 to obtain the following haploid progeny: *gtr2*Δ *vps1*Δ *apl2*Δ (LZY253), *gtr2*Δ *apl2*Δ (LZY256), and *gtr2*Δ *vps1*Δ (LZY257).

Plasmids containing mutant alleles of *GTR1* and *GTR2* that encode proteins restricted to the GDP- or GTP-bound conformations were a generous gift from Takeshi Sekiguchi (Kyushu University) and are pL146, pL148, pL264, and pL263 (for a description, see references 25 and 43). We switched the *TRP1* auxotrophic marker in these plasmids to *URA3* for use in our strains, using the pTU marker-swapper plasmid (9). Plasmid pLZ43 contains *GTR2* (20 upstream base pairs and 61 downstream base pairs) under the control of the *GAL1* promoter and was isolated from a cDNA library (22). Plasmid pLZ44 contains *GTR1* under the control of the *GAL1* promoter and was generated by cloning a genomic PCR fragment (by using the primers LZP48 [GTAATGTCGTCAAATAATAGGA] and LZP26 [AAACACTCAATTGCCGAATGT]) into pCR-BluntII-TOPO, using a Zero-Blunt TOPO kit (Invitrogen). The *GTR1*-containing insert was then subcloned into pRS316-GAL (22) by using the PstI and SacI restriction enzyme sites.

High-throughput screen. Our high-throughput screen for compounds that selectively inhibit the growth of an *apl2*Δ *vps1*Δ yeast strain was performed at the University of Kansas High Throughput Screening Laboratory, which has a collection of over 100,000 compounds selected from the ChemBridge, ChemDiv, and Prestwick libraries. A total of 101,376 screening compounds were distributed in 384-well plates. Each plate had 352 wells for compounds and 32 wells for positive control (no cells) and negative control (dimethyl sulfoxide [DMSO] without screening compound). The plates were seeded with an overnight (18-h) culture of EHY644 (*apl2*Δ *vps1*Δ) grown at 24°C in YPD to OD_{600} 0.05 (early log phase). An aliquot (80 μl) of culture was mixed with 20 μl of compound dissolved in 2.5% DMSO, for a final concentration of 5 μg of compound/ml and 0.5% DMSO, in 100 μl per well. The plates were then stacked (but not sealed) and incubated at room temperature for 15 to 17 h to a final average OD_{600} of 0.8 (close to late exponential growth under these conditions). Plates were read on an Envision multilabel plate reader (Perkin-Elmer, Wellesley, MA). We defined hits as those compounds that gave an OD_{600} that was 60% lower than that of the negative control. We identified 279 hits from screening with the *apl2*Δ *vps1*Δ mutant. These hits were then tested with both the *apl2*Δ *vps1*Δ mutant (EHY644) and the corresponding wild-type strain (EHY47), in a six-point dose-response assay (0.15 to 5 μg/ml) in 96-well plates. Of the 279 primary screen hits, 15 compounds inhibited the growth of the *apl2*Δ *vps1*Δ mutant strain but were significantly less toxic to the wild-type strain.

Assays for drug effects. All liquid cultures for growth assays and secretion assays were grown at 24°C, with the exception of the pulse-chase assays, which were performed at 30°C. (Growth at 24 and 30°C was compared for the enzymatic invertase assay in selected samples, and no difference in results was observed for the two temperatures.) Yeast on agar plates were incubated at 30°C. For shaking-culture exponential-phase growth assays, the cells were grown overnight to early exponential phase in CSM (minus uracil) medium to maintain plasmids. They were then diluted to an OD_{600} of 0.07, and compounds dissolved in DMSO were added at the indicated concentrations, with final DMSO concentration of 0.25% in each case. Cultures were placed on a rotating platform for aeration, and OD_{600} readings were taken every 2 h at least five times to generate an exponential growth curve. Rates were calculated from an exponential curve fit equation using Kaleidagraph 3.6 (Synergy Software, Reading, PA). The correlation coefficient (Pearson r) was >0.9 in each case.

The invertase, Bgl2, and CPY transport assays were performed as described previously (18). For the invertase assay, we grew cells overnight to exponential phase in either YPD or CSM (to select for CEN plasmids) and then shifted them to fresh YPD with 5% glucose for 2 to 3 h. Compound or DMSO control was then added to this medium at the indicated concentration and cultures were grown for an additional 15 min, followed by shifting cells to YPD with 0.1% glucose (to derepress invertase expression) plus compound or DMSO control for 90 min prior to performing the invertase secretion assay as described previously (18). The results from at least three independent cultures grown on different days were averaged, and variability is indicated as the standard error of the mean (SEM). Statistical significance (using a Student t test) was calculated by using Kaleidagraph 3.6 software. Pulse-chase analysis of transport kinetics was performed as described previously (18). Briefly, exponential-phase cells were inoculated into CSM lacking cysteine and methionine at 4 OD_{600}/ml and shaken at 30°C for 5 min, and compound or DMSO control was added for 20 min prior to a 4-min pulse with 25 μCi of labeling mix/OD_{600} cells. Chase for 2 to 20 min was with excess cold amino acids, and cells were processed for immunoprecipitation and detection of Bgl2, invertase, and CPY as described previously (18). Cells for thin-section electron microscopy were grown at 30°C in YPD and prepared as described previously (18).

Screen for gene overexpression suppressors of drug effects. To screen for genes that, when overexpressed, can suppress the toxic effects of our drugs, we used both a 2μ (high-copy) genomic library (5) and a *GAL*-promoter-driven cDNA library (22). Only the cDNA library yielded a suppressor clone. For that library, we screened for suppressors in strain EHY644 (*apl2*Δ *vps1*Δ). The strain was transformed with library DNA by using the method of Schiestl and Gietz (36), and cells were plated on CSM (lacking uracil), 2% galactose, and compound (1 μM KU#7 or 2 μM KU#4). Plasmids were recovered from colonies that could grow on drug plates and retransformed and retested for suppression in EHY644. Plasmids that retested were sequenced at the insert junctions to identify the suppressing gene.

RESULTS

High-throughput screen for compounds that are selectively toxic to a *vps1*Δ *apl2*Δ mutant strain. We performed a high-throughput screen of a library of drug-like molecules for compounds that inhibit the growth of a yeast strain with *apl2*Δ and *vps1*Δ mutations but which have relatively small effects on a corresponding background strain. Our goal was to identify compounds that generate an AVL phenotype (for *apl2 vps1* lethal), analogous to the phenotype of an *avl9*Δ mutant (18). Of 101,376 compounds screened, 279 significantly inhibited the growth of a *vps1*Δ *apl2*Δ mutant (<40% of growth without drug). These compounds were then screened in dose-response growth assays to eliminate compounds that inhibited the growth of both wild-type and mutant strains. Of our 279 initial hits that inhibited the growth of a *vps1*Δ *apl2*Δ mutant, we identified 15 hit compounds that selectively inhibited the growth of the mutant strain.

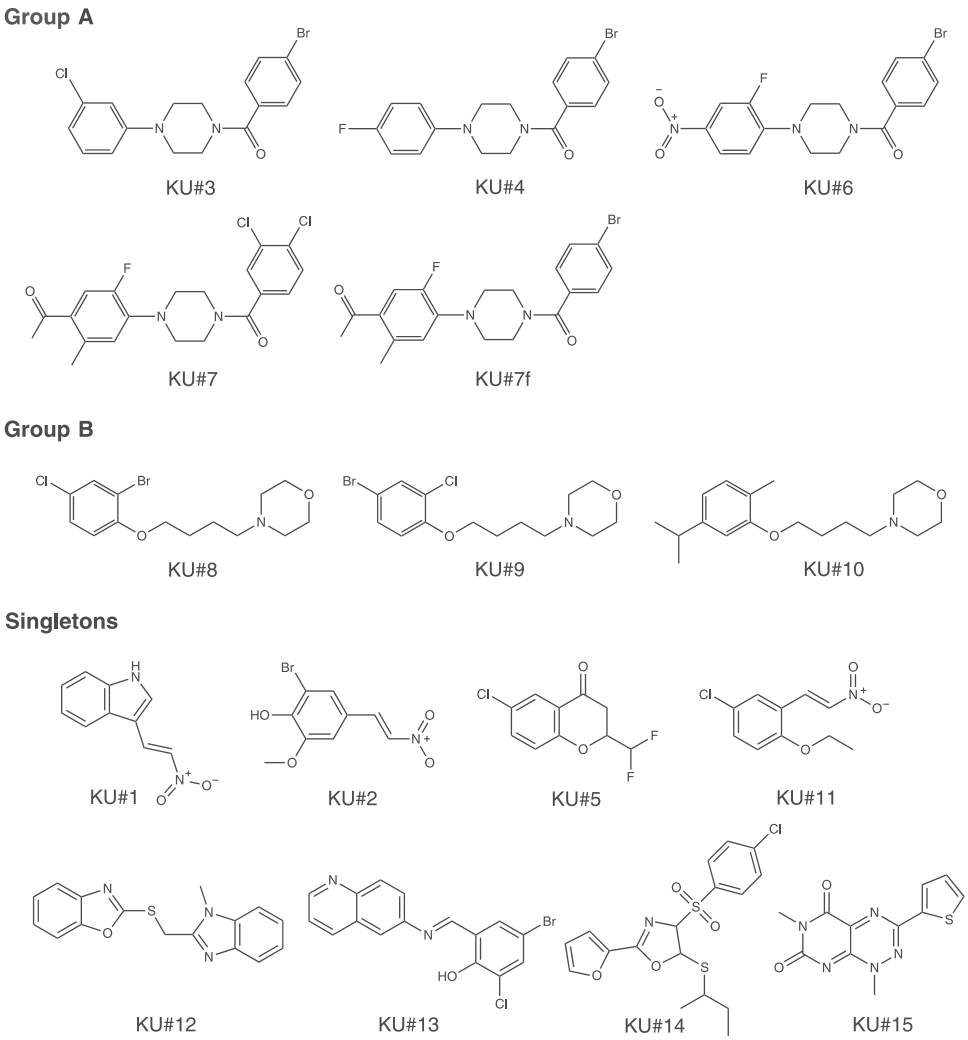

FIG. 1. Structures of all 15 hit compounds identified in a high-throughput phenotypic screen of a 101,376-compound library for small molecules that have an AVL (*apl2*Δ *vps1*Δ lethal) effect. Some of the confirmed hit compounds grouped into two structural groups, group A and group B, whereas other hits had unique structures ("singletons").

Seven of the fifteen hit compounds could be grouped into two groups based on similar structures, whereas the rest of the structures were unique (Fig. 1). We identified four similar compounds that we named group A, and three compounds that we named group B. A fifth group A compound, KU#7f, was not identified in our screen but was purchased as a substitute for KU#7 when that compound was no longer available from the supplier (KU#7f is somewhat more active than KU#7 in our assays). Dose-response growth assays in 96-well plates for representative group A compounds and a group B compound are shown in Fig. 2A. Piperazine rings are common in druglike molecules, but the published bioactive piperazine derivatives that most resembled our group A structures (12, 44) did not have growth-inhibiting activity for our mutant strains, when tested at up to 10 μM. In particular, we found that the *N*-benzoyl substituent was essential for the activity of our group A compounds, because similar piperazine compounds that did not have this substituent (12) had no effect in our assays (our unpublished observations). We did not find published examples of bioactive compounds that resembled our group B compounds.

Group A compounds selectively inhibit the growth of *apl2*Δ and *vps1*Δ mutants. We tested all of our 15 hit compounds in exponentially growing shaking cultures for growth-inhibiting activity and found that the group A compounds had the most rapid (within 30 min), dramatic effect on the growth of either the *apl2*Δ or *vps1*Δ or *apl2*Δ *vps1*Δ mutant strains, with relatively much smaller effects on the growth of our wild-type strain (Fig. 2B). However, when grown on solid medium, the toxic effects of the compounds were specific to the *apl2*Δ *vps1*Δ strain; thus, the group A compounds have an AVL (for *apl2*Δ *vps1*Δ lethal) effect (Fig. 2C). It is possible that rapidly growing cells in shaking cultures, in which membrane trafficking occurs at a greater speed than it does in cells grown on plates, are more sensitive to group A compounds. Alternatively, our results could reflect the time scale of the assays, a few hours in shaking cultures at exponential growth, compared to several days on plates, in which

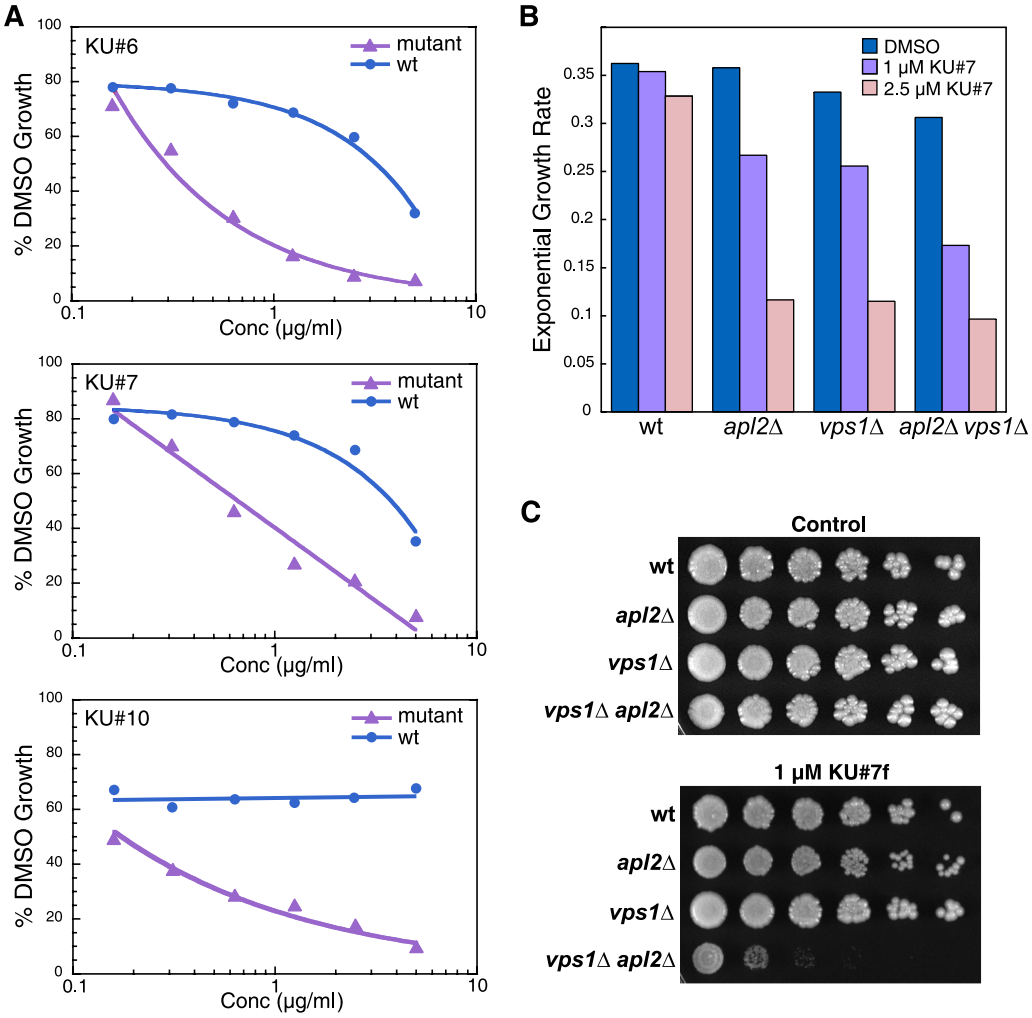

FIG. 2. Group A and group B compounds have mutant-specific effects on growth. (A) Dose-response growth assays for KU#6 (group A), KU#7 (group A), and KU#10 (group B) compounds. Wild-type and *apl2*Δ *vps1*Δ ("mutant") cells were grown in the presence of compound or DMSO control in 96-well plates. Compounds were at the following concentrations, prepared by 2-fold serial dilutions: 5, 2.5, 1.25, 0.625, 0.31, and 0.15 μg/ml. The graphs show percent growth based on the calculation (OD_{600} in compound/OD_{600} in DMSO) × 100, after ~18 h of growth. (B) Growth rates of exponential-phase cells in shaking cultures. Overnight exponential-phase cultures were diluted to an OD_{600} of 0.07, and compounds or DMSO control were added at the indicated concentrations with final DMSO concentrations of 0.25% in each case. Cultures were grown with aeration, and OD_{600} measurements were obtained over ~9 h of growth to generate growth curves. Rates were determined from an exponential curve fit equation (correlation coefficients > 0.9 in each case). (C) Growth on solid medium in the presence of group A compounds. Cultures were grown to an OD_{600} of 1.0 and spotted on a YPD plate containing 1 μM KU#7f and on a control plate, after fourfold serial dilutions. In panels B and C, mutant strains have an EHY47 wild-type background and are identical except for the plasmids they contain: EHY658 (*apl2*Δ), EHY1166 (*vps1*Δ), and EH707 (*apl2*Δ *vps1*Δ).

time the *vps1*Δ and *apl2*Δ single mutants could possibly adapt to the effects of the compounds.

In contrast to group A compounds, most of our hit compounds initially did not appear to inhibit growth of exponentially growing shaking cultures. This included the group B compounds, which consistently strongly inhibited the growth of the *apl2*Δ *vps1*Δ strain but not the wild-type strain when the growth assay was performed in 96-well (nonshaking) liquid cultures (Fig. 2A) or agar plates (not shown). However, we found that after cells were grown for a longer time (>4 h), growth inhibition in shaking liquid cultures became apparent in group B compounds (results not shown). The delay in growth defect was not due to a need for buildup of compound in cells, because we noticed the same time delay in lower and higher concentrations of the drug. It is possible that these compounds affect gene expression or signaling pathways that regulate growth.

Group A compounds inhibit exocytic transport. It is difficult to interpret results with slow-acting growth inhibitors, so we focused on analyzing the effects of group A compounds, which rapidly inhibited the growth of our mutant strains. To determine whether the toxicity of these compounds is due to a transport block, we assayed the effects of the compounds on exocytic cargo transport. In wild-type yeast, two pathways can transport exocytic cargo. One route transports the cell wall protein Bgl2, whereas another route transports the periplasmic enzyme invertase (16). These two cargoes are sorted into one pathway in a *vps1*Δ mutant, in which the normal invertase

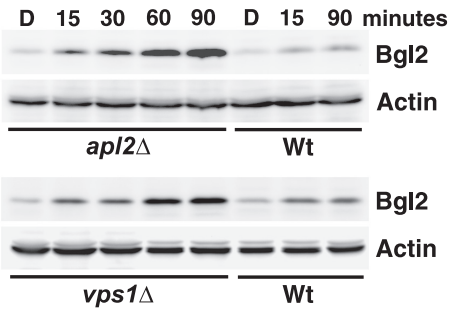

FIG. 3. Group A compounds cause internal accumulation of the cell wall protein, Bgl2, in *apl2*Δ and *vps1*Δ mutant strains. Cells were grown to exponential phase, and compound KU#4 (group A) was added at 10 μM for the indicated times. DMSO controls ("D") represent 60-min time point samples. The cell walls were digested to remove external Bgl2, and internal Bgl2 was assayed by Western blotting. Actin is shown as loading control. Strains are as described in Fig. 2.

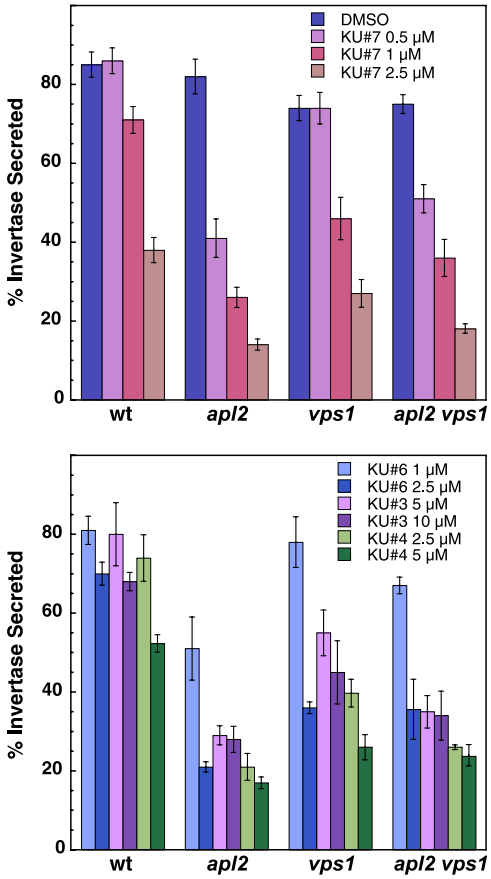

FIG. 4. Group A compounds cause a mutant-specific defect in invertase secretion. Cultures were grown to exponential phase in high-glucose medium, and the compound was added for 15 min before shifting to low glucose with the compound (to derepress invertase expression). Growth in the compound was continued for 90 min. External invertase was compared to total invertase by an enzymatic assay to calculate the percentage of invertase secreted. Strains are as in Fig. 2. The means of three experiments (from three independent cultures) are shown. Error bars, SEM.

pathway appears to be blocked (17). Bgl2 transport can be conveniently assayed, because at steady state most of this protein is in the cell wall, which can be removed by enzymatic digestion (spheroplasting). Wild-type cells have very little Bgl2 after spheroplasting, which represents intracellular Bgl2 in transit to the cell surface. We found that 15 min after the compound was added, intracellular accumulation of Bgl2 was detected in the *vps1*Δ and *apl2*Δ mutants but not in the corresponding wild-type strain (Fig. 3). This accumulation was very dramatic after 90 min, indicating a significant defect in Bgl2 transport.

We also assayed the transport of a periplasmic enzyme, invertase, in the presence of group A compounds. Invertase expression is repressed when cells are grown in 2% glucose, and expression is derepressed when cells are shifted to 0.1% glucose (8). There is no detectable invertase secretion prior to derepressing expression. We added compounds to cells 15 min prior to derepression and then shifted cells into low glucose with compound for 90 min, followed by enzymatic assays for invertase secretion (Fig. 4). We observed a very significant secretory defect for the *apl2*Δ mutant in as low as 0.5 μM compound KU#7, the lowest concentration that we assayed. We found similar effects on secretion for all of our group A compounds, and the *apl2*Δ mutant was consistently the most sensitive to compound in this assay (Fig. 4). The mutant strains were identical except for the plasmids that they contained (double mutant with either empty vector or vector with *VPS1* or *APL2*). It is possible that the double mutant adapts to the transport defect in some way that makes it less sensitive to compound in this assay. Interestingly, the relative invertase secretion defects in the different mutants did not correspond with the relative growth decrease in compounds (Fig. 2B and C).

The secretory assays described above have the advantage of being very sensitive for detecting transport defects. However, the assays show cargo accumulation over time, rather than an immediate effect on transport kinetics. Therefore, we also performed metabolic labeling and pulse-chase analysis of secretory cargo to determine whether the compounds had observable effects on transport kinetics in this assay (Fig. 5). In the absence of inhibitors, about half of the Bgl2 is exported within 5 min, while transport of invertase is faster (Fig. 5A and C shows the results for *apl2*Δ [the results were similar for the wild type]). Both wild-type and *apl2*Δ cells show a defect in Bgl2 transport kinetics in compounds KU#4 and KU#7 (Fig. 5B), and the defect is more substantial in *apl2*Δ cells. The slower transport kinetics was most easily observed at early chase time points, but there was not a complete transport block, and most Bgl2 was secreted by 20 min (not shown). KU#4 and KU#7 also slowed invertase transport in the *apl2*Δ mutant, but not in wild-type cells (Fig. 5D). However, invertase transport is normally very fast, so it is difficult to detect a partial transport block by pulse-chase analysis. Wild-type cells did show a defect in the enzymatic invertase secretion assay (Fig. 4), but internal invertase in that assay represents accumulation over 90 min. Furthermore, the enzymatic assays also show an incomplete transport block for wild-type and even mutants, at the drug concentrations we tested (for comparison, the *sec6-4* conditional secretory mutant secretes <5% invertase at a restrictive temperature in this enzymatic assay [27; our unpublished ob-

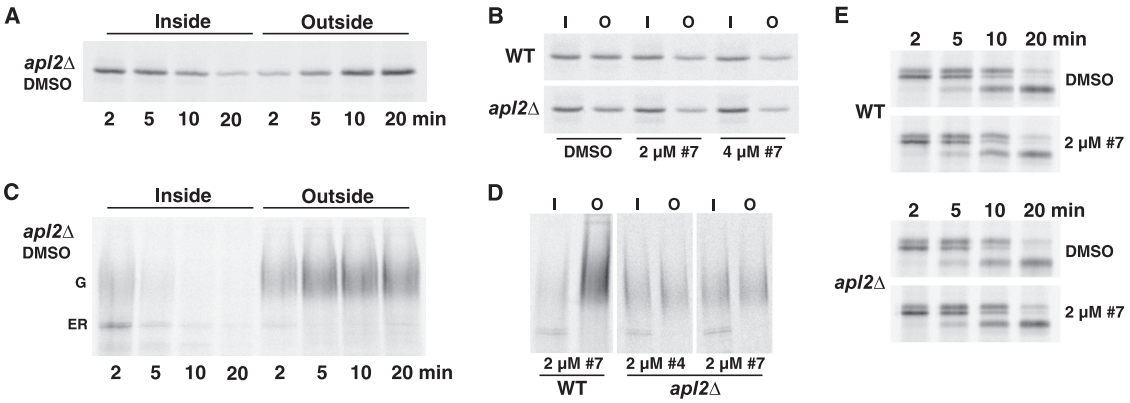

FIG. 5. Pulse-chase analysis shows a kinetic lag of Bgl2 and invertase exocytic transport in the presence of group A compounds. Cells were preincubated with compound or DMSO control for 20 min and metabolically labeled with ^{35}S cysteine and methionine for 4 min, followed by addition of excess unlabeled amino acids for 2- to 20-min chase times (A, C, and E) or for a 5-min chase (B and D). The inside (I) fraction was separated from the cell wall and medium fraction (O), and Bgl2 (A and B), invertase (C and D), and CPY (E) were immunoprecipitated and detected by phosphorimaging. Bgl2 is not visibly modified, whereas invertase has an ER form and a Golgi compartment-modified (G) heterogeneously glycosylated form. CPY has a 67-kDa ER form, a 69-kDa Golgi form, and a 61-kDa vacuole form. Group A compounds do not cause a defect in ER-to-Golgi compartment or Golgi compartment-to-vacuole transport of CPY.

servations]). The transport defects caused by our compounds appear to be specific to the late exocytic pathway, because pulse-chase analysis of transport of a vacuolar protein, CPY, showed no detectable defect in ER-to-Golgi transport or transport from the Golgi to vacuole (Fig. 5E).

Group A compounds cause accumulation of abnormal Golgi compartment-like membranes. To further define the membrane traffic defects caused by our group A compounds, we examined the ultrastructure of cells grown in the presence of KU#7 or KU#3 by thin-section electron microscopy (Fig. 6). The *apl2*Δ strain showed dramatic accumulation of membranes when grown in KU#7 for just 30 min (Fig. 6A and B). Most of the membranes were clusters of vesicles and/or tubules (tubules and fenestrated membranes could appear as clustered vesicles in the plane of a thin section). Another common and striking structure was a ring of discontinuous membranes (enlarged inset in Fig. 6A; also seen in Fig. 6B, C, and E). Similar rings were observed in cells grown in the presence of KU#3 for 60 min (Fig. 6C; KU#3 is our least-effective group A compound). Because the rings were consistently circular, they likely represent fenestrated spheres, structures also observed in temperature-sensitive *sec14* (*sec14-ts*) mutants after a short shift to a restrictive temperature (32) and in an *arf1*Δ mutant (15). Gaynor et al. (15) observed both very large and smaller rings in *arf1*Δ cells and, based on complementing immunolocalization studies by light microscopy, these researchers concluded that the large rings likely represent aberrant endosomes, whereas the smaller rings represent Golgi membranes. In our samples, we did not observe the very large rings seen in the *arf1*Δ mutant. However, the *arf1*Δ and *sec14-ts* mutants have defects in transport from both Golgi and endosomes (15, 23), and the invertase-transporting exocytic pathway likely transits endosomes (17), so our group A compounds could perturb exit from either or both the Golgi and endosomes. Wild-type cells had a similar but less abundant membrane-accumulation phenotype when grown in the presence of KU#7 (Fig. 6E). We did not observe these abnormal membranes in either *apl2*Δ cells or wild-type cells grown in DMSO control (Fig. 6D, F), although the *apl2*Δ cells had occasional small continuous rings which represent cup-shaped structures, similar to but much smaller than Berkeley bodies, thought to represent aberrant Golgi, in *sec7-ts* and *sec14-ts* mutants (27). We also observed small continuous rings in cells grown in KU#7, but these were likewise smaller and rarer than the structures accumulated by *sec7* and *sec14* mutants. Cells grown in KU#7 for 1 h had a similar phenotype (not shown), so the fenestrated spheres did not progress to Berkeley bodies at the drug concentrations we tested. It is possible that the fenestrated spheres and tubules represent an incomplete block in exit from the Golgi (as is the case with the *arf1*Δ mutant described in reference 15). Alternatively, the different abnormal membranes may represent different molecular defects.

The toxicity of group A compounds can be suppressed by overexpressed *GTR2*. A relatively simple strategy for identifying potential drug targets, or genes that are relevant for the function of a drug target, is a screen for gene dosage suppression of the drug effects. We screened both a 2μ (multicopy) genomic library and a *GAL*-promoter-driven cDNA library (22) for genes that, when overexpressed, can suppress the toxicity of group A compounds. We obtained no suppressors from the 2μ library and only one strong suppressor gene from the cDNA library. We identified the suppressor gene as *GTR2* (Fig. 7). Gtr1 and Gtr2 are homologous Ras-like GTP-binding proteins that form hetero- and homodimers (14, 25). They function as nutrient-responsive regulators of the TORC1 signaling pathway (7, 11), exocytic sorting of the Gap1 general amino acid permease at endosomes (14), and epigenetic control of gene expression (37). Although Gtr1 and Gtr2 are thought to function as obligate heterodimers, Gtr1 had no suppressing effect when highly expressed from the *GAL* promoter (Fig. 7). A 2μ (high-copy) plasmid containing *GTR2* under its native promoter could not suppress toxicity either on glucose or galactose (our unpublished observations).

A dominant-negative form of the Gtr complex and decreased TORC1 signaling suppress the secretory defect caused by group A compounds. Our invertase secretion assay is not com-

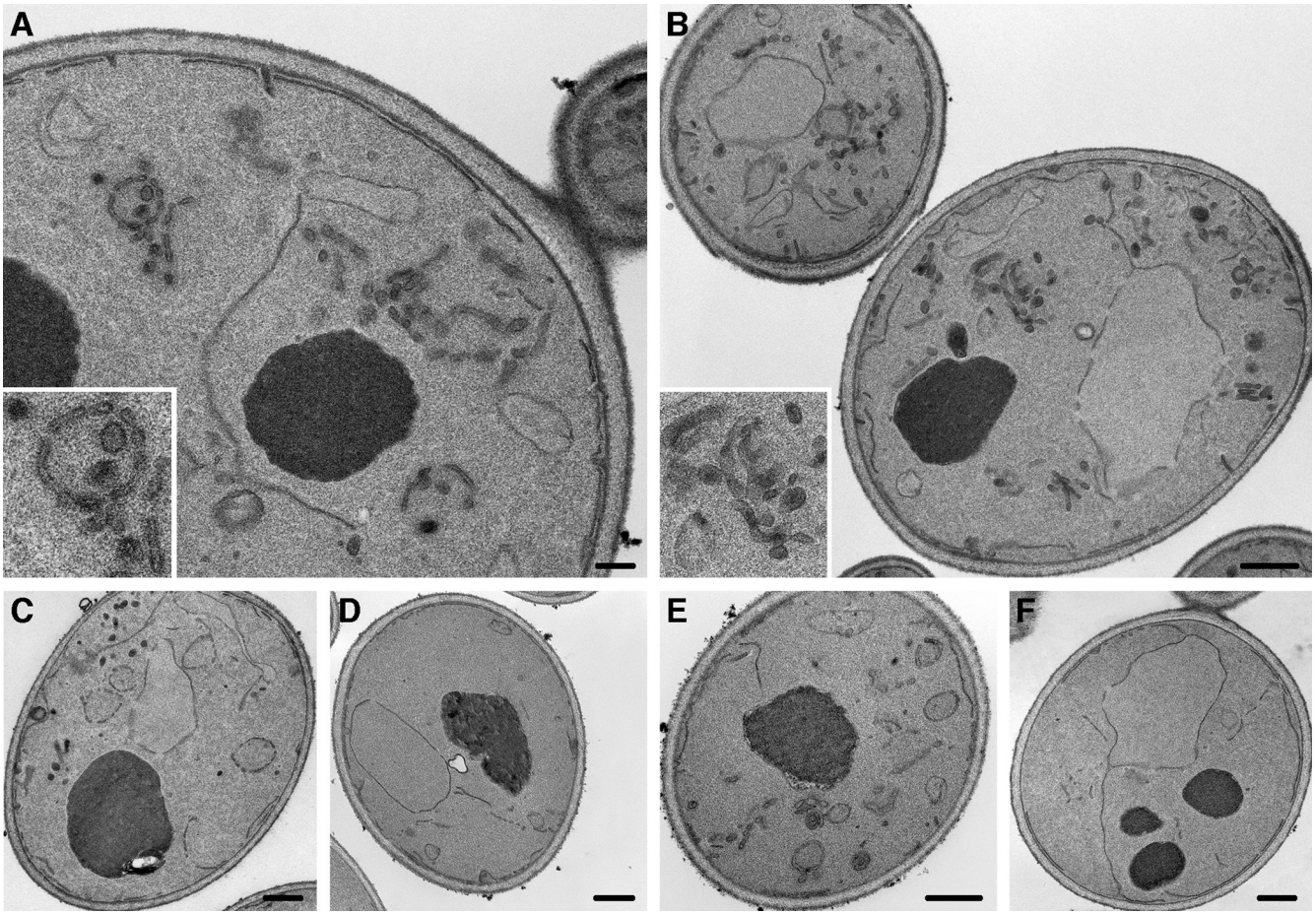

FIG. 6. Group A compounds cause accumulation of Golgi or endosome-like membranes. An *apl2*Δ strain (EHY807 [A and B]) or wild-type (EHY47 [E]) were grown in the presence of 2.5 μM compound KU#7 for 30 min. (C) Similar results were obtained for *apl2*Δ cells grown in 10 μM KU#3 (a less-potent group A compound) for 60 min. DMSO controls without compound are shown in panels D (*apl2*Δ) and F (wild type). Cells were prepared for thin-section electron microscopy as described previously (18). Scale bars: 200 nm (A), 500 nm (B to F).

patible with growth on galactose-containing medium, so we could not use our *GAL-GTR2* construct to test whether overexpression of *GTR2* can suppress the effects of group A compounds on invertase secretion. Instead, we used mutant alleles

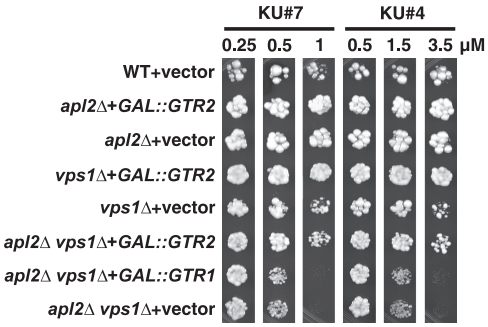

FIG. 7. Overexpression of *GTR2* on galactose-containing medium suppresses the toxic effect of group A compounds. The strains are described in Materials and Methods and are as follows: EHY47 with pRS316, EHY807 with pLZ43; EHY807 with pRS316, EHY361 with pLZ43, EHY361 with pRS316, EHY644 with pLZ43, EHY644 with pLZ44, and EHY644 with pRS316.

of the *GTR* genes expressed from their native promoters in CEN (low-copy) plasmids. The Gtr1/Gtr2 dimer, as well as the metazoan counterpart, RagAB/RagCD, are active in positively regulating TORC1 signaling when Gtr1 (or RagA or RagB) is GTP-bound and Gtr2 (or RagC or RagD) is GDP bound. Furthermore, in the opposite conformations (Gtr1/RagAB in a GDP-bound state and Gtr2/RagCD in a GTP-bound state) have a dominant-negative effect on TORC1 signaling (7, 11, 20, 35). Likewise, the Gap1 permease is sorted from a vacuolar pathway to the exocytic pathway when Gtr1 is GTP-bound and Gtr2 is GDP bound but not when the GTPases are restricted to the opposite conformations (14). We tested *GTR* alleles that express either GTP-restricted or GDP-restricted forms of the proteins for effect on invertase secretion in *apl2*Δ *vps1*Δ cells grown in the presence of KU#7f (Fig. 8A). We found that both Gtr1 and Gtr2 can partially suppress the effects of KU#7f on secretion, and only when the GTPases are restricted to the conformations that are expected to form a dominant-negative complex. Although the suppression of the secretory defect was not dramatic, the Gtr proteins were not overexpressed in these experiments, and the wild-type proteins were also present. Furthermore, the results were consistent in each repeat of the

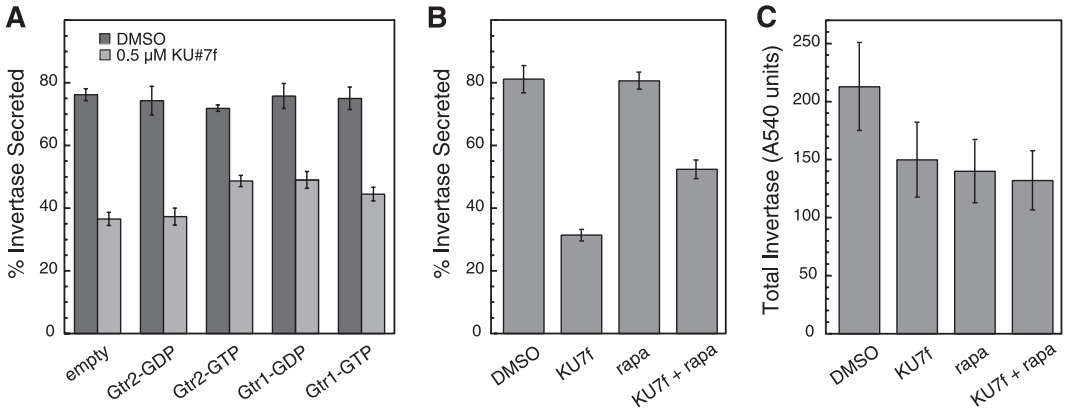

FIG. 8. Dominant-negative Gtr proteins and subinhibitory rapamycin suppress the invertase secretion defect caused by group A compounds. (A) An *apl2*Δ *vps1*Δ mutant strain (EHY644) was transformed with empty vector (pRS316) or pRS-based (CEN) vectors expressing GDP- or GTP-restricted mutant forms of Gtr1 and Gtr2 from their native promoters. The strains were tested for invertase secretion after growth in 0.5 μM KU#7f, as described for Fig. 4. For each strain, the means were calculated from seven repeats (for KU#7f) or three repeats (for DMSO). Each repeat was an independent culture. (B) An *apl2*Δ *vps1*Δ mutant strain (EHY644) culture was divided into four samples, which were treated with either DMSO (control), or 0.5 μM KU#7f, or 10 ng of rapamycin/ml, or 0.5 μM KU7f plus 10 ng of rapamycin/ml together. The means from three independent (predrug) cultures are shown. (C) Total invertase activity (secreted plus internal) from the cultures used in panel B. Invertase is expressed as A_{540} units per OD_{600} cells from the invertase enzymatic assay. Error bars, SEM.

experiment and statistically significant ($P = 0.001$ for secretion with empty vector compared to secretion when Gtr2-GTP is expressed). In contrast, the alleles that are expected to form the TORC1-activating form of the Gtr complex (Gtr2-GDP and Gtr1-GTP) had less or no effect on invertase secretion (Fig. 8A).

Because the "active" Gtr complex positively regulates TORC1 signaling, whereas the "inactive" complex has a dominant-negative effect on TORC1 activity, we sought to determine whether decreasing TORC1 activity also suppresses the secretory defect in the presence of KU#7f. We found that 10 ng of rapamycin/ml (which is the amount typically used as a subinhibitory level, whereas 200 ng/ml is used to inhibit growth [6]) suppressed the secretory defect caused by KU#7f to an extent similar to that of the dominant-negative Gtr proteins (Fig. 8B). A nearly identical result was obtained with 20 ng of rapamycin/ml (data not shown), so our results with both the Gtr proteins and rapamycin may represent the maximum possible suppression. Rapamycin alone, either 10 ng/ml (or 20 ng/ml [data not shown]) had no effect on invertase secretion in the *apl2*Δ *vps1*Δ strain when no group A compound was present (Fig. 8B).

TORC1 positively regulates translation (45), so we speculated that suppression by reducing TORC1 activity may be due to a decrease in cargo flow through the secretory pathway, such that the reduced secretory competence due to effects of our group A compound is less deleterious to the *apl2*Δ *vps1*Δ mutant. Cells are grown in the drugs for 15 min prior to derepression of invertase expression, followed by 90 min more in drug, so some reduction in protein synthesis might eventually occur in the time course of our experiments. However, for the identical samples shown in Fig. 8B, we found that total invertase (secreted plus internal) was essentially the same when just KU#7f was added to cells and when both KU#7f and rapamycin were added (although total invertase level was somewhat lower compared to the DMSO control, whether or not rapamycin and KU#7f were combined or each was present alone). These results suggest that TORC1 activity may regulate membrane traffic in a way that is deleterious when traffic is perturbed in the *apl2*Δ *vps1*Δ mutant by group A compounds. We were unable to detect suppression of the growth-inhibitory effect of group A compounds, by either the mutant alleles of Gtr proteins or the subinhibitory levels of rapamycin. This could be because the invertase assay measures a relatively short-term effect, and the long-term effect on growth is not as readily suppressed.

***GTR2* is not a likely direct target of group A compounds.** Suppression by overexpressed Gtr2 could indicate either that Gtr2 is a target of our group A compounds or that the function of the drug target is relevant for Gtr2 activity. Because group A compounds are toxic to an *apl2*Δ *vps1*Δ mutant, a defect in the drug target could likewise be deleterious to the *apl2*Δ *vps1*Δ strain. We found that crossing a *gtr2*Δ mutation into our *apl2*Δ *vps1*Δ strain did not noticeably reduce the growth of the *gtr2*Δ *apl2*Δ *vps1*Δ triple-mutant progeny (Fig. 9). It is still

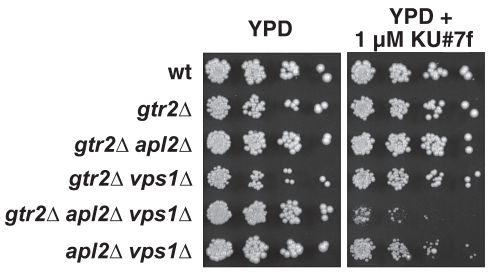

FIG. 9. Gtr2 is not a likely target of our group A compounds. A *gtr2*Δ mutation (in EHY47 background) was crossed into an *apl2*Δ *vps1*Δ strain (EHY644). All double- and triple-mutant progeny were viable; therefore, unlike group A compounds, *gtr2*Δ does not produce an AVL phenotype. The *gtr2*Δ *apl2*Δ *vps1*Δ strain was at least as sensitive to 1 μM KU#7f as was an *apl2*Δ *vps1*Δ strain, indicating that the drug does not interact with Gtr2 for a dominant effect. These results show that group A compounds do not have a direct effect on Gtr2 function.

possible that group A compounds have a dominant effect on Gtr2 or target a regulator of its activity. However, this is unlikely, because in such a case, the deleterious effects of the compounds would require the presence of *GTR2*, and we found that KU#7f is at least as toxic to a *gtr2*Δ *apl2*Δ *vps1*Δ mutant as it is to an *apl2*Δ *vps1*Δ mutant (Fig. 9). Although we have not yet identified the molecular target of our compounds, we can conclude that modulation of TORC1 activity is critical when the effects of group A compounds are combined with the *apl2*Δ and *vps1*Δ mutations, and our results are valuable in suggesting additional target candidates.

DISCUSSION

We screened a 101,376-compound library for small molecules that perturb the late exocytic pathway, with the goal of identifying novel molecular probes to help us understand the mechanisms and regulation of late exocytic transport. We identified 15 compounds that had selective toxicity toward an *apl2*Δ *vps1*Δ mutant, in which exocytic cargo is rerouted from a defective pathway to an alternative exocytic route. Thus, the compounds are expected to perturb a remaining exocytic pathway in this mutant, and cause an AVL phenotype, analogous to the phenotype of the *avl9*Δ mutant identified in a classical yeast mutant screen (18). Some of our hits fell into two groups of similar structures, and one of these groups, which we named group A, we characterized in detail for effects on membrane trafficking.

The group A compounds inhibit the growth of the *apl2*Δ *vps1*Δ strain at a low (<1 μM) concentration and cause an exocytic defect at <0.5 μM both in this double mutant and the *apl2*Δ and *vps1*Δ single mutants. The transport defect appears to be specific to the late exocytic pathway, because the compounds have no detectable effect on transport to the vacuole/lysosome in either *apl2*Δ or wild-type cells. Furthermore, the compounds cause rapid (within 30 min) accumulation of membranes that resemble the aberrant membranes in other mutants with exocytic blocks at late Golgi compartments. With higher concentrations of compound (>2 μM), we detected secretory defects and membrane accumulation in wild-type cells as well. These results indicate that our compounds inhibit exocytic transport from Golgi compartments and possibly endosomes and thus target a molecule(s) that mediates traffic at these steps.

We initiated efforts to identify the molecular target of our group A compounds by screening for genes that suppress the toxicity of the compounds when overexpressed. Although Avl9 is a potential target, it is highly toxic when overexpressed (18), and other strategies are needed to explore it as a potential drug target. Our suppressor screen identified just one gene, *GTR2*, which suppressed the toxic effects of our most potent group A compound, KU7f, when highly expressed from the *GAL* promoter. Gtr2 and its homolog, Gtr1, function as a dimer to regulate numerous nutrient-responsive processes, but the primary function of these GTPases and the corresponding metazoan proteins is thought to be the regulation of TORC1 signaling and thus the regulation of growth (7, 11, 20, 35). Overexpression of *GTR1* could not suppress the toxicity of KU7f, a result that was explained in our subsequent assays with GDP- and GTP-restricted mutant alleles of the GTPases. We found that the dominant-negative alleles of the GTPases, having a GDP-restricted conformation for Gtr1 and GTP-restricted conformation for Gtr2, partially suppressed the invertase secretion defect caused by KU7f in the *apl2*Δ *vps1*Δ mutant. Therefore, the overexpressed wild-type proteins are likely more abundant in a GTP-bound conformation.

The alleles of the GTPases that best suppressed the secretory defect caused by KU7f are dominant-negative regulators of TORC1 signaling and, correspondingly, sublethal concentrations of rapamycin, a TORC1 inhibitor, likewise suppressed the invertase secretory defect caused by KU7f. This result was not due to a reduction in invertase production. Therefore, TORC1 signaling may regulate a pathway that needs modulation in response to secretory transport blocks. Invertase in wild-type cells likely transits endosomes en route to the cell surface (17), and endosomes are the most likely site where the Gtr/Rag complex functions in regulating transport in response to amino acid signals in both yeast and mammalian cells (14, 35). Furthermore, TORC1-mediated signaling functions at endosomes or Golgi compartments for sorting the high-affinity tryptophan and histidine permeases, Tat2 and Hip1, from an endosome-mediated vacuolar pathway to the exocytic pathway (6). It is possible that both group A compounds and TORC1 signaling enhance sorting into an endosome-mediated exocytic transport route that is defective in the *apl2*Δ *vps1*Δ mutant. TORC1 signaling and our compounds may instead, or in addition, downregulate an alternative transport route for invertase that is utilized by the *apl2*Δ *vps1*Δ mutant.

In addition to linking exocytic transport to signaling mechanisms that regulate growth, the identification of *GTR2* as a suppressor of our group A compounds was an exciting result, because it suggests that our chemical-genetic strategy may lead to insights about Avl9 function. The *gtr* and *avl9* mutants were among the top-ranked mutants in a genome-wide screen of the nonessential yeast gene deletion collection for mutants that are hypersensitive to both high hydrostatic pressure and cold temperature (3). The reason for this growth phenotype of *gtr* and *avl9*Δ mutants is not clear, but it could reflect defects in traffic due to reduced membrane fluidity under these conditions. Other mutations in nonessential genes that cause defective transport from the Golgi were also identified in the screen (3). Furthermore, at high pressure and cold temperature, the Tat2 permease is sorted to the vacuole even though TOR signaling seems unaffected (2), suggesting that the TORC1-regulated exocytic route is especially sensitive to conditions that reduce membrane fluidity.

Transcriptional profiles of *S. cerevisiae* subjected either to high hydrostatic pressure or to cold temperature indicate highly upregulated expression of genes involved in remodeling of the cell surface (plasma membrane and cell wall) under anaerobic growth conditions (1, 4, 10, 13, 28). Traffic pathways or mechanisms that involve Avl9 and Gtr function may be involved in cell surface remodeling, and a defect in this process may lead to pressure and cold sensitivity. Alternatively or in addition, the high levels of expression of these same set of genes encoding cell surface components may be toxic to the *avl9*Δ mutant. The *avl9*Δ mutant is SLAM (for synthetic lethality analysis on microarrays) with *mot3*Δ (29), and Mot3 is a repressor of the expression of most of these same cell surface components (38). It is also possible that Avl9 and Gtr proteins

have a role in regulating plasma membrane homeostasis, or an Avl9- and Gtr-dependent sorting or transport process depends on optimized lipid composition. There is much evidence that regulation lipid composition is critical for cargo sorting and transport carrier formation in the late secretory pathway (21, 23, 24, 30, 41).

The signaling pathways that regulate cell growth and proliferation are expected to coordinate with regulation of the traffic pathways that deliver cell surface components and thereby mediate cell growth. Therefore, our result that modulation of a TORC1-mediated process is critical in the presence of our group A compounds suggests that these compounds may serve as powerful probes for understanding the molecular mechanisms by which late exocytic transport is regulated in response to environmental factors. Furthermore, a possible link to Avl9 function via Gtr2-mediated suppression of the drug effects, as well at the AVL phenotype conferred by the compounds, indicate that our new exocytic transport inhibitors may help us to discover the biochemical and biological functions of the Avl9 protein family.

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