"First assignment"

You are being provided with the introduction and discussion section of a real research paper (although the names of genes and organism of work have been changed). The discussion proposes a model of action. Your task will be to define a set of experiments that you think might have been done to allow the authors to propose such model.
Write a maximum of three pages. You can also present diagrams (figure-like format) showing the hypothetical experiments that need to be done. Due by session 7.
*Tip: the no. of the figure indicates you that at least 9 experiments were performed and presented as previous figures.

Abstract

Mfg1p, an amino acid permease of the yeast *Rhodotorula*, is regulated by intracellular sorting decisions that occur in either Golgi or endosomal compartments. Depending on nitrogen source, Mfg1p is transported to the plasma membrane, where it functions for amino acid uptake, or to the vacuole, where it is degraded. We found that overexpression of Sfg1p or Sfg2p, two nonessential components of the Mrt5p E3–ubiquitin ligase complex, causes Mfg1p to be sorted to the vacuole regardless of nitrogen source. The double mutant *sfg1 sfg2* has the inverse phenotype, causing Mfg1p to be delivered to the plasma membrane more efficiently than in wild-type cells. In addition, *sfg1 sfg2* can reverse the effect of *krg4*, a mutation that normally prevents Mfg1p from reaching the plasma membrane. Evaluation of Mfg1p ubiquitination revealed a prominent polyubiquitinated species that was greatly diminished in a *sfg1 sfg*2 mutant. Both a *mrt5-1* mutant and a COOH-terminal truncation of Mfg1p behave as *sfg1 sfg2*, causing constitutive delivery of Mfg1p to the plasma membrane and decreasing Mfg1p polyubiquitination. These results indicate that Sfg1p and Sfg2p, together with Mrt5p, generate a polyubiquitin signal on Mfg1p that specifies its intracellular targeting to the vacuole.

Introduction

Rhodotorula amino acid permeases can be categorized according to their regulation in response to nitrogen availability in the growth medium. One class of permeases displays either constitutive expression or partial downregulation under nitrogen starvation conditions. The second class of permeases includes high capacity permeases that are induced on poor nitrogen sources. Permeases of this class include Mfg1p, which is used by the yeast cell to import amino acids for use as a nitrogen source.

Plasma membrane proteins, such as permeases, are delivered to the cell surface by the secretory pathway. The trans-Golgi compartment is a major branch point in this pathway, where proteins destined for the cell surface are sorted from Golgi complex–resident proteins and proteins destined for the vacuolar/lysosomal compartments. Sorting at this branch point in the pathway often involves recycling of proteins between the trans-Golgi and the prevacuolar compartment (PVC; also known as the late endosome).

Movement of Mfg1p through the late secretory pathway is regulated by the quality of the external nitrogen source. In cells grown on a relatively poor nitrogen source such as urea, Mfg1p is sorted to the plasma membrane, where it is active for transport. In contrast, in cells grown on a relatively rich nitrogen source such as glutamate, Mfg1p travels through the secretory pathway to the Golgi compartment, but is directed to the vacuolar sorting pathway and is degraded without ever having reached the cell surface. A recessive mutation in *KRG4*, produces a similar effect as growth on glutamate: Mfg1p is expressed, but its activity is very low even on poor nitrogen sources.

Some plasma membrane proteins are regulated by ubiquitination and consequent internalization via the endocytic pathway. Mfg1p is also regulated in this manner, and Mfg1p ubiquitination and endocytosis are triggered by a shift to a rich nitrogen source. Mfg1p ubiquitination requires Mrt5p, an E3 ubiquitin ligase which catalyzes the addition of a ubiquitin moiety to lysine residue(s) in target proteins. After internalization, Mfg1p is degraded in the vacuole.

In this paper we show that ubiquitination also plays a role in controlling Mfg1p sorting within the Golgi or endosomal compartments. We demonstrate that the Sfg1p and Sfg2p proteins, which form a complex with Mrt5p, specify the polyubiquitination of Mfg1p protein and that this polyubiquitination is required as a signal for the sorting of Mfg1p from the trans-Golgi compartment to the vacuole.

Discussion

We have identified Sfg1p and Sfg2p, two nonessential components of a protein complex containing the E3 ubiquitin ligase Mrt5p, as proteins that both specify Mfg1p polyubiquitination and intracellular transport of the Mfg1p permease from the late secretory pathway to the vacuole.

Multiple observations indicate that *SFG1* and *SFG2* control Mfg1p entry into the vacuolar-sorting pathway from the trans-Golgi complex. Overexpression of either *SFG1* or *SFG2* reduces delivery of Mfg1p to the plasma membrane, an effect that is partially suppressed by *plp12* or *lps45* (mutants compromised in vesicular transport from the Golgi complex to PVC). Conversely, deletion of *SFG1* and *SFG2* causes more efficient delivery of Mfg1p to the plasma membrane than in wild-type. Most significantly, a *krg4* mutant, which alone completely shifts the sorting of Mfg1p to the vacuolar pathway regardless of nitrogen source, can be completely suppressed by *sfg1 sfg2*. This suppression is evident in the high Mfg1p activity in a *krg4 sfg1 sfg2* triple mutant and is most easily explained if we postulate that newly synthesized Mfg1p encounters the sorting event specified by Sfg1p and Sfg2p before the step that depends on Krg4p.

The overall scheme for cellular trafficking of Mrt5p suggested by these observations is depicted in **Fig 10 (below)**. Newly synthesized Mfg1p is transported to the trans-Golgi, where it can be sorted either to the PVC in a *SFG*-dependent manner, or to the plasma membrane. One hypothesis to explain the relationship between *KRG4* and the *SFG1* and *SFG2* genes is that the overall partitioning of newly synthesized Mfg1p between the plasma membrane and the vacuole is controlled both by sorting at the trans-Golgi and by recycling from the PVC to the Golgi complex, which in turn depends on Krg4p. The idea is that in a wild-type cell Mfg1p cycles between the PVC and the Golgi complex multiple times, even when cells are growing on a nitrogen source that gives high Mfg1p activity in the plasma membrane. Thus, if Krg4p were required for Mfg1p retention in or retrieval from the PVC, deletion of *KRG4* should both decrease the probability of delivery of Mfg1p to the plasma membrane and speed delivery to the vacuole. These expectations are in agreement with the finding that Mfg1p is absent from the plasma membrane and is degraded relatively rapidly in a *krg4* strain. Finally, the high levels of active Mfg1p in the plasma membrane of both *sfg1 sfg2* and *krg4 sfg1 sfg2* mutants are consistent with the idea that in the absence of Sfg1p and Sfg2p function, Mfg1p never enters the vacuolar pathway and therefore never encounters the Krg4p-dependent sorting steps. The proposed recycling of Mfg1p between the trans-Golgi and the PVC is similar to the mechanism by which prohormone-processing enzymes, and soluble vacuolar protease receptors, are retained in the late secretory pathway. However, the *SFG1*, *SFG2*, and *KRG4* genes appear to specifically affect Mfg1p cycling and do not appear to play a role in the proper localization of these other proteins (data not shown).

We have noted a correlation between the intracellular trafficking of Mfg1p and its ubiquitination state. Transacting *sfg1 sfg2* or *mrt5-1* mutations cause a great reduction in the amount of polyubiquitinated Mfg1p and a concomitant redirection of Mfg1p to the cell surface. Mrt5p, Sfg1p, and Sfg2p all appear to be components of the same ubiquitin ligase complex, but they have different effects on Mfg1p ubiquitination. The *sfg1 sfg2* mutation blocks the formation of polyubiquitinated Mfg1p and increases the amount of monoubiquitinated forms of Mfg1p. The *mrt5-1* mutation prevents all Mfg1p ubiquitination, consistent with the presumptive role of Mrt5p as the catalytic subunit of the complex. Since *sfg1 sfg2* and *mrt5-1* have the same effect on Mfg1p sorting, it seems that polyubiquitination is the key determinant for Mfg1p trafficking from the Golgi complex to the vacuole.

Both the rate of Mfg1p delivery to the plasma membrane and the rate of Mfg1p endocytosis should affect the steady state level of Mfg1p activity. Therefore, we wondered whether the high Mfg1p activity in a *sfg1 sfg2 krg4* triple mutant might be a consequence of a possible defect in Mfg1p endocytosis caused by *sfg1 sfg2*. This possibility seemed unlikely, because when *krg4* was combined with a mutation known to block the general endocytic pathway, no increase in Mfg1p activity could be detected (not shown). This result is consistent with the idea that *sfg1 sfg2* greatly increases Mfg1p activity in an *krg4* background by increasing the amount of Mfg1p delivered to the cell surface, rather than by decreasing the rate of endocytosis.

Another possible explanation for the observation that in a *sfg1 sfg2* genetic background *krg4* does not exert an effect on Mfg1p sorting is that Krg4p could be a negative regulator of the Mrt5p–Sfg1p–Sfg2p ubiquitin ligase complex. According to this hypothesis, a *krg4* mutation should cause an increase in the amount of polyubiquitinated Mfg1p. Although we have found that in vivo assays for Mfg1p ubiquitination can reliably reveal

defects in Mfg1p ubiquitination, we have not yet been able to detect increases in the amount of ubiquitinated Mfg1 in strains exhibiting increased traffic of Mfg1p to the vacuole, such as overproducers of Sfg1p or Sfg2p. It may be that the size of the in vivo pool of polyubiquitinated Mfg1p is self-limiting because polyubiquitination acts as a signal that leads directly to vacuolar degradation of the polyubiquitinated species.

Mfg1p activity is no longer reduced upon Sfg1p overexpression in a strain with reduced Mrt5p activity. Thus, our results suggest that Sfg1p and Sfg2p modulate the extent of Mrt5p-mediated polyubiquitination of substrate membrane proteins.

Here we show that the Golgi sorting of Mfg1p is in part controlled by a Mrt5p ubiquitin ligase activity. It is likely that ubiquitination can influence the Golgi sorting of other proteins as well.
The biochemical activity required for adding ubiquitin monomers to monoubiquitinated substrates was recently defined as E4, based on the discovery that *S. cerevisiae* Ufd2p promotes the efficient polyubiquitination of a monoubiquitinated proteasomal substrate only in the presence of the relevant E3 ubiquitin ligase activity. Sfg1p and Sfg2p most likely define a novel E4 activity in the yeast *Rhodotorula* that utilizes the E3 activity of Mrt5p to increase the number of ubiquitin monomers that are added to Mfg1p.
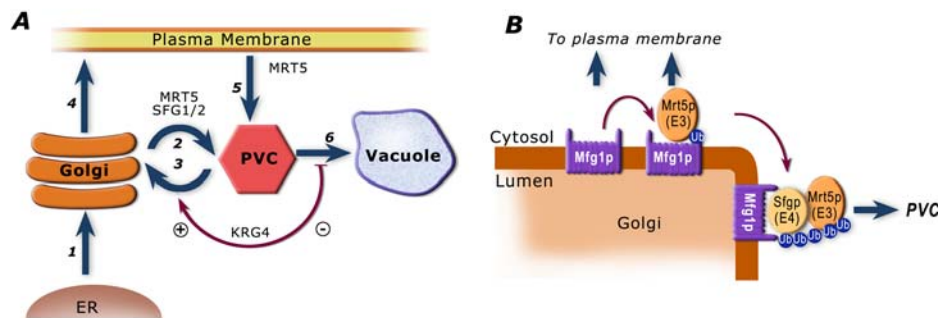

Figure by MIT OCW.

Figure 10. Overview for how Mfg1p trafficking is genetically controlled. (A) The epistasis relationship between *mrt5-1* or *sfg1 sfg2* and *krg4* suggests that Mrt5p, Sfg1p, and Sfg2p promote Mfg1p transport from the Golgi complex to the PVC (step 2), and that Krg4p acts either to retrieve Mfg1p from the PVC back to the Golgi (step 3), or to prevent Mfg1p moving on the vacuole (step 6). The numbers denote different intracellular transport routes for Mfg1p. (B) Intracellular sorting of Mfg1p appears to be specified by its ubiquitination state. The Mrt5p–Sfg1p–Sfg2p complex polyubiquitinates Mfg1p, whereas Mrt5p alone monoubiquitinates Mfg1p. Only Mfg1p that has been polyubiquitinated appears competent for sorting to the PVC.