**Mini-Review**

**A selective autophagy pathway that degrades gluconeogenic enzymes during catabolite inactivation**

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In *Saccharomyces cerevisiae*, glucose starvation induces key gluconeogenic enzymes such as fructose-1,6-bisphosphatase (FBPase), malate dehydrogenase (MDH2) and phosphoenolpyruvate carboxykinase, while glucose addition inactivates these enzymes. Significant progress has been made in identifying mechanisms that mediate the "catabolite inactivation" of FBPase and MDH2. For example, the site of their degradation has been shown to change, depending on the duration of starvation. When glucose is added to short-term starved cells, these proteins are degraded in the proteasome. However, when glucose is added to long-term starved cells, they are degraded in the vacuole by a selective autophagy pathway. For the vacuole pathway, these proteins are first imported into novel vesicles called Vid (vacuole import and degradation) vesicles. Following import, Vid vesicles merge with the endocytic pathway. Future experiments will be directed at understanding the molecular mechanisms that regulate the switch from proteasomal to vacuolar degradation and determining the site of Vid vesicle biogenesis.

**Introduction**

Protein levels in a cell reflect a balance between the processes of protein synthesis and protein degradation. For protein degradation, two major pathways have been identified. These are the proteasome dependent and the lysosomal dependent degradation pathways.1-9 The proteasomal system requires the ubiquitination of protein substrates using E1, E2 and E3 enzymes.1-4 These ubiquitinated proteins are then targeted to the proteasome and destroyed.1-4 The other major degradation pathway in cells occurs in the lysosome.5-9 Lysosomes are acidic organelles that contain a variety of hydrolases. In general, lysosomes are responsible for degrading most of the long-lived proteins and organelles.5-9 This is in contrast to the proteasome, which primarily degrades short-lived cytosolic proteins.1-4 Lysosomal protein degradation can be regulated by nutrients and hormones. For example, both amino acid starvation and glucagon addition increase lysosomal degradation, while the presence of insulin inhibits this process.8,9 Proteins are targeted for degradation via multiple pathways. One example is macroautophagy, a non-selective degradation process. This pathway, which has been studied extensively, plays an important role in the survival of cells during periods of nutrient deprivation. In addition to its role in starvation, autophagy is required for the normal development of worms, flies, mice and humans.10-14 As such, defects in this process can lead to diseases.10-14

In contrast to the non-discriminate nature of macroautophagy, there are also selective autophagy pathways. One type of lysosomal degradation selects proteins with a specific pentapeptide sequence. Proteins with KFERQ in their sequence are recognized by the heat shock protein Hsc73.15-17 These proteins are then bound by the lysosomal receptor protein LAMP2.18 and transported into the lumen of lysosomes. Chaperone mediated autophagy also requires the assistance of several luminal chaperones,19 in conjunction with the function of cytosolic chaperones. Since, a relatively high percentage of proteins contain the KFERQ sequence, this form of autophagy plays an important role in the degradation of bulk cytosolic proteins during starvation. However, other proteins such as the mutant forms of synuclein can be degraded via chaperone mediated autophagy, along with oxidized proteins and other damaged proteins.20-24 Interestingly, this pathway is impaired during aging25 and it is stimulated by ketone bodies.19

**Multiple Protein Targeting Pathways to the Yeast Vacuole**

In yeast, the vacuole is the homolog of mammalian cell lysosomes.26-28 Multiple trafficking pathways carry proteins to the vacuole, where they are either degraded or processed. For example, proteins can be endocytosed from the plasma membrane and targeted for degradation in the vacuole.26-28 In contrast, most vacuole resident proteins are synthesized and travel to the vacuole via the Vps pathway.29-31 However, one vacuolar resident, aminopeptidase I, is delivered directly from the cytosol to the vacuole for processing via the Cvt pathway.10,12,32-35 The Cvt and macroautophagy pathway shares many genes. Furthermore, both pathways can be divided into distinct steps, i.e., the nucleation, expansion and sequestration steps. These steps are, in turn, regulated by a number of ATG genes.10-12,32-35 Finally, unique ubiquitin-like conjugation36 and lipidation processes37 play a role in regulating autophagy.
The vacuole is also the site for organelle degradation. For example, peroxisomes can be degraded in the vacuole via two pexophagy pathways that can be distinguished morphologically. When methylotrophic yeast are grown in methanol and shifted to ethanol, peroxisomes are degraded by macropexophagy. By contrast, when these cells are shifted from methanol to glucose, peroxisomes are degraded by micropexophagy.

**Catabolite Inactivation in Yeast**

In *Saccharomyces cerevisiae*, a number of gluconeogenic enzymes are induced by glucose starvation. Typically, these enzymes are inactivated when cells are replenished with fresh glucose. The most studied example of this “Catabolite Inactivation” is the key gluconeogenic enzyme fructose-1,6-bisphosphatase (FBPase). In addition to FBPase, other gluconeogenic enzymes including malate dehydrogenase (MDH2) and phosphoenolpyruvate carboxykinase (PEPCK) are inactivated by glucose. Likewise, glucose inactivates sugar transporters such as the high affinity glucose transporter, the galactose transporter and the maltose transporter. Peroxisomes are involved in fatty acid metabolism and they are also inactivated by glucose. Thus, catabolite inactivation applies, not only to enzymes involved in sugar transport and gluconeogenesis, but also to peroxisomes involved in fatty acid metabolism. Catabolite inactivation of the maltose transporter and the galactose transporter is mediated by endocytosis of these proteins from the plasma membrane followed by degradation in the vacuole.

Catabolite inactivation of FBPase has been examined extensively. During catabolite inactivation, FBPase is phosphorylated and inactivated via a cAMP mediated process. In wild-type cells, cAMP levels increase when glucose is added following a period of starvation. However, in the *nar2* mutant, cAMP levels do not increase following glucose stimulation, and FBPase is not phosphorylated. Phosphorylation of FBPase most likely occurs at multiple sites. Phosphorylation at serine 11 has been demonstrated following a glucose shift. Interestingly, mutation of serine 11 has little or no effect on FBPase degradation. FBPase degradation is blocked in the *nar2* mutant, suggesting that phosphorylation events are important for FBPase degradation. At present, it is unknown whether the phosphorylation of FBPase plays a required role in the degradation of FBPase in the vacuole. Alternatively, the phosphorylation of other molecules may be required for this degradation event.

**The Site of FBPase Degradation**

The site of FBPase degradation was a matter of debate for several years, although this has been resolved recently. Previously, the Wolf lab showed that FBPase is degraded in the proteasome following glucose addition. In contrast, our lab demonstrated that FBPase was not degraded in the proteasome following glucose shift. However, this protein was detected in the vacuole following a glucose shift, providing the most direct evidence that FBPase is targeted to the vacuole for degradation when prolonged starved cells are shifted to glucose. Thus, our results indicated that the site of FBPase degradation varies, at least in part, due to the duration of glucose starvation. When glucose is added to cells that are starved for a short time (1d), FBPase is degraded in the proteasome. By contrast, when glucose is added to cells that are starved for longer periods of time (3d), FBPase is degraded in the vacuole. These results have been confirmed via a variety of methods. Re-distribution of FBPase from the cytosol to the vacuole has been observed using indirect fluorescence. Moreover, FBPase targeting to the vacuole has been reconstituted in vitro using semi-intact permeabilized cells lacking the endogenous FBPase. Under the in vitro condition, a fraction of exogenously added FBPase was protected from protease K digestion, suggesting that FBPase was sequestered inside organelles. Furthermore, sequestered FBPase was detected in the vacuole in semi-intact cells using indirect immunofluorescence.

MDH2 Shares the Same Degradation Characteristics as FBPase

The cytosolic protein malate dehydrogenase (MDH2) is inactivated and degraded in response to glucose. In order to test whether MDH2 was also degraded in the vacuole in prolonged starved cells, we followed the degradation of MDH2 in short term starved and long term starved Δ*pep4Δaprb1Δaprc1* cells using the same strategy mentioned above. In the Δ*pep4Δaprb1Δaprc1* mutant, MDH2 was degraded normally following 1d of starvation. However, when this mutant was starved for 3d and shifted to glucose, MDH2 degradation was inhibited. Thus, vacuole proteinases are required for MDH2 degradation in 3d starved cells, but not for 1d starved cells. To determine whether MDH2 was indeed targeted to the vacuole for degradation, the Δ*pep4* vacuole mutant was transformed to express MDH2-GFP. In 3d starved Δ*pep4* cells, MDH2-GFP was detected in the cytosol before a glucose shift. However, this protein was not detected in the vacuole following a glucose shift, providing direct evidence that this protein is also targeted to the vacuole when long term starved cells are replenished with fresh glucose.

The degradation of MDH2 shares many characteristics with FBPase. For example, both are degraded in the proteasome when glucose is added to short term starved cells. Moreover, both are degraded in the vacuole when glucose is added to long term starved cells. The degradation of both proteins also requires the N-terminal sequence. Proline is the first amino acid of MDH2 and FBPase and it is required for degradation under either the...
proteasome or the vacuolar specific conditions. When the proline residue was changed to a serine residue, degradation of FBPase and MDH2 was blocked in both 1d and 3d starved cells.\textsuperscript{63} This suggests that the N-terminal proline is required for both proteasomal and vacuole dependent degradation. This observation raises an interesting question regarding how the same residue is utilized for two distinct pathways. One possible explanation is that this residue interacts with the same protein molecules, and these, in turn can interact with distinct binding partners, one for the vacuole and another for the proteasomal pathway. Alternatively, this residue may interact with the same protein molecules or complexes, but these molecules are modified differently depending on the length of starvation.

The Vacuole Import and Degradation Pathway

FBPase is targeted to the vacuole via a selective autophagy pathway. This process requires several genes, collectively called VID (Vacuole Import and Degradation) genes. Mutants defective in FBPase degradation have been isolated from an isopropenol library, a deletion library, and by screening cells generated by random mutagenesis.\textsuperscript{68-70} Characterization of these mutants based on FBPase distribution within cells indicated that they can be classified into at least two categories.\textsuperscript{68} Following a glucose shift, most of the mutants displayed cytosolic staining of FBPase, while a subset of mutants showed FBPase distribution in distinct punctate structures.\textsuperscript{68} When lysates were fractionated using a size column, at least two peaks were found to contain FBPase. The first peak also contained the plasma membrane protein Pma1p, while the second peak contained Vid vesicles. Kinetic studies indicate that Vid vesicles are intermediate carriers in the FBPase degradation pathway.\textsuperscript{71} As such, FBPase appears to be associated with these vesicles prior to targeting to the vacuole.

Purified Vid vesicles retain the ability to transport exogenously added FBPase in vitro.\textsuperscript{72} For the in vitro system, FBPase was purified from wild-type cells that were starved for prolonged periods of time and shifted to glucose for 20 min. Vid vesicles were purified from FBPase deletion strains that were also shifted to glucose for 20 min. In this in vitro system, FBPase sequestration into purified Vid vesicles was dependent on an ATP regenerating system and cytosol.\textsuperscript{72} Furthermore, the cytosolic heat shock proteins Ssa1p was found to interact with purified FBPase. The first peak also contained the plasma membrane protein Pma1p, while the second peak contained Vid vesicles. Kinetic studies indicate that Vid vesicles are intermediate carriers in the FBPase degradation pathway.\textsuperscript{71} As such, FBPase appears to be associated with these vesicles prior to targeting to the vacuole.

The biogenesis of Vid vesicles is regulated by the UBC1 (ubiquitin conjugating enzyme 1) gene.\textsuperscript{74} Thus, in the absence of this gene, the formation of Vid vesicles is substantially reduced. At least one of the VID gene products, Vid24p, localizes to Vid vesicles.\textsuperscript{75} Vid24p is present at low levels in cells prior to a glucose shift. However, following a shift to glucose containing media, levels of this protein increase, Vid24p is associated with Vid vesicles as a peripheral protein. Although the exact role of Vid24p is unknown, in cells lacking this gene, significant amounts of FBPase were still found in Vid vesicle enriched fractions.\textsuperscript{75,76} This suggests that Vid24p plays a role in a trafficking step subsequent to the import of FBPase into Vid vesicles. Because of its unique distribution, Vid24p is now routinely used as a specific marker for Vid vesicles.

COP I Coatomer Subunits are Present on Vid Vesicles

Although Vid vesicles can be purified from glucose shifted cells, it is not known whether these vesicles are present prior to a glucose shift, or whether they are subsequently derived from precursor membranes. As stated above, Vid24p is a specific marker for Vid vesicles. Unfortunately, this protein has not been useful in identifying the site of Vid vesicle biogenesis. This is because Vid24p levels are low in glucose-starved cells, and as such, it is difficult to detect structures that may associate with this protein prior to their stimulation with glucose. In order to address this issue, we attempted to identify other Vid vesicle proteins, some of which might be constitutively expressed. For these experiments, Vid vesicles were purified and subjected to MALDI analysis. A number of proteins were identified via this approach. Most notably, components of COPI vesicles such as Sec28p, Ret1p, Ret2p and Sec21p were all found on purified Vid vesicles.\textsuperscript{70} COPI vesicles have a well-known role in the trafficking of vesicles from the Golgi to the ER.\textsuperscript{77-80} Likewise, they are important in intra Golgi trafficking.\textsuperscript{77-80} Interestingly, a subset of COPI proteins have been found in endocytic compartments in mammalian cells\textsuperscript{81-85} and in yeast.\textsuperscript{86} These proteins appear to actively participate in endosomal trafficking, in mammalian cells\textsuperscript{81-85} and in multivesicular body sorting in yeast.\textsuperscript{86}

The association of COPI coatomer proteins with Vid vesicles has been demonstrated experimentally.\textsuperscript{70} Coatomer proteins were observed to co-localize with Vid24p and FBPase. Coatomer proteins were present at low levels during glucose starvation. Following a glucose shift, however, coatomer proteins in Vid vesicle fractions first increased, and then decreased. Thus, there is a transient increase of Vid vesicle coatomer proteins in response to glucose. Interestingly, coatomer proteins were found to form a large protein complex with Vid24p. Interaction of coatomer proteins with Vid24p increased following a glucose shift. Furthermore, they play an important role in recruiting Vid24p to Vid vesicles. For example, in mutants defective in coatomer subunits, Vid24p association with Vid vesicles was reduced.

Coatomer Protein Sec28p Traffics to Endocytic Compartments and to the Vacuole

Since Sec28p is a component of Vid vesicles we have followed the distribution of this protein as a means to track Vid vesicle trafficking.\textsuperscript{70} When glucose was added to prolonged starved wild-type cells for 15–30 min, Sec28p-GFP was detected in punctuate structures. These structures also contained FM4-64, a lipophilic dye that is internalized from the plasma membrane to early endosomes, late endosomes, and the vacuole membrane.\textsuperscript{87} When we examined FM distribution in mutants that block our degradation pathway, its distribution varied. Some mutants such as Δpp1 and Δvam3 showed small circles, while others such as Δpha1 showed large FM-labeled circles. In wild-type cells expressing Sec28p-GFP, FM co-localized with Sec28p after a brief glucose shift, suggesting that Sec28p resides on or traffics to endosomes. To identify genes that control the trafficking of Sec28p to endosomes, we screened mutants that failed to show co-localization of Sec28p with FM following a glucose shift. We found that, Sec28p did not co-localize with FM containing endosomes in the Δvam3 mutants, suggesting that Sec28p distribution on endosomes is controlled by VAM3.

The trafficking of Sec28p was also studied using a Δubc1 mutant. Previously, we demonstrated that Vid vesicle formation is regulated by the UBC1 gene.\textsuperscript{74} As such, in the absence of this gene, Vid vesicle formation is significantly reduced. Because of
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for extended periods of time. Thus, our results suggest that Sec28p vesicles can form from the vacuole membrane.

A Model for the Vid Vesicle Trafficking Pathway

Based on our Sec28p localization studies, we proposed the following model for the Vid trafficking pathway (Fig. 1). Sec28p resides on Vid vesicles as a peripheral protein, where it interacts with other coatomer proteins. The Sec28p complex acts to recruit Vid24p to Vid vesicles. Accordingly, when coatomer genes are mutated, Vid24p association with Vid vesicles is reduced. Following the recruitment of Vid24p, the Vid vesicles converge with the endocytic pathway. This process is regulated by \textit{VAM3}, since Vid vesicles failed to merge with endosomes in the absence of this gene. Sec28p then moves from endosomes to the vacuole. After fusion, Sec28p is distributed on the vacuole membrane. Sec28p then concentrates in areas of the membrane that later bud off from the vacuole membrane.

Coatomer proteins play an important role in the budding of COPI vesicles. During the formation of COPI vesicle, coat proteins are assembled at the budding site. Therefore, if Sec28p plays a similar role in Vid vesicles formation, then this protein should localize to areas that form these buds. Localization to the budding site may be more stable in mutants with defective coatomer subunits. Therefore, we used a \textit{ret2-1} mutant that encodes a temperature sensitive product of the 60 kD subunit of the coatomer complex. In the \textit{ret2-1} mutant, Sec28p initially co-localized with FM, indicating that Sec28p traffics to endosomes. However, at later time points, FM was on the vacuole membrane and Sec28p was in dots adjacent to the vacuole membrane. This can be explained in two ways. Sec28p is either in vesicles that are ready to fuse with the vacuole membrane, or Sec28p is in vesicles that are budding from the vacuole membrane. To address this issue, we pre-labeled the vacuole membrane with FM in the \textit{ret2-1} strain and examined the distribution of Sec28p-GFP. Following a shift to glucose, Sec28p localized to small buds on the vacuole membrane for extended periods of time. Thus, our results suggest that Sec28p vesicles can form from the vacuole membrane.

In support of our model, FBPase was observed to follow the same distribution pattern as Sec28p, most notably in mutants that block various steps of the pathway. As mentioned above, the \textit{VAM3} gene controls the merger of Vid vesicles with the endocytic pathway. In cells lacking this gene, FBPase was in Vid vesicles and failed to co-localize with FM vesicles. In the \textit{Δvph1} mutant, FBPase was found inside large FM-labeled circles. Thus, this suggests that
FBPase enters these endocytic compartments as a luminal protein. Along these same lines, we have shown that FBPase is sequestered in the lumen of Vid vesicles. Therefore, this protein should also be found in the lumen of endosomes after Vid vesicles fuse with these structures. Sec28p, on the other hand, remains on the outside of the membrane as a peripheral protein. The same topology can be seen after the fusion of endosomes with vacuoles. FBPase is in the lumen, while Sec28p is on the outside of the membrane. Thus, our results suggest that Vid vesicles undergo a series of fusion events leading to the degradation of FBPase in the lumen of the vacuole.

**Prospectives**

Based on our studies with Sec28p, we have shown that the Vid vesicle pathway merges with the endocytic pathway. However, a number of questions remain to be answered. For example, are Vid vesicles derived from retrograde vesicles that bud from the vacuole membrane? Alternatively, do retrograde vesicles cycle to the plasma membrane and then form Vid vesicles at this location? If this is the case, are they a distinct type of early endosome? To address the latter possibility, it will be useful to utilize endocytic mutants that block the internalization process. If Sec28p or Vid24p are localized to the plasma membrane when internalization is blocked, it may suggest that Vid vesicles are derived from the plasma membrane. A number of endocytic mutants that block the plasma membrane internalization process are also defective in FBPase degradation. However, the exact mechanisms regarding why internalization is required for FBPase degradation is not clear.

The actin cytoskeleton may play an important role in Vid vesicle formation. Many endocytic mutants affect actin organization at the plasma membrane.88-93 Furthermore, actin patches are the sites of plasma membrane internalization, since these patches co-localize with receptor proteins and the FM dye. In addition, various actin-binding proteins associate with actin patches, albeit with different mobility. Some are relatively immobile, while others move rapidly toward the center of cells.93 If Vid vesicles are derived from the plasma membrane, we may see Sec28p, Vid24p or FBPase targeted to this location. In some mutants, they may be distributed evenly on the plasma membrane, while in others, they may show localized distribution. If this is the case, further experiments will be conducted to determine whether they co-localize with actin patches. If Vid vesicles are indeed derived from the plasma membrane, then the next question is how does FBPase get to the lumen of Vid vesicles. Is it possible that FBPase is translocated or exocytosed to the media and then internalized into Vid vesicles? Is it possible that Vid vesicles are formed first and FBPase is then translocated into Vid vesicles from the cytosol? If this is the case, Vid vesicles may be incompetent for import initially, but become competent at some time point.

It is interesting that the same set of gluconeogenic enzymes including FBPase, and MDH2 are degraded in the proteasome under one condition, but are degraded in the vacuole under different conditions. How do cells decide whether they should be degraded in the proteasome or the vacuole? Differential modification may account for the site of their degradation. As stated above, FBPase is ubiquitinated prior to degradation in the proteasome, while it is phosphorylated before targeting to the vacuole.56,58,59 This may be caused by the activation of particular signaling pathways under certain conditions. For the vacuole dependent pathway, FBPase phosphorylation requires the RAS2 gene and protein kinase A.4,55 When the first proline residue was mutated to serine, FBPase degradation was inhibited in both 1d and 3d cells, suggesting that this residue is important for both proteasome and vacuole degradation pathways. However, these may be other domains or sequences utilized for either the proteasome or the vacuole pathway. In future studies, it would be desirable to screen for FBPase mutants that are defective in 1d versus 3d cells. Mutations that are defective in 1d, but not 3d may be useful to study the proteasomal pathway. Likewise, mutations that show normal FBPase degradation in 1d cells, but defective degradation in 3d cells may uncover sequences required for the vacuole pathway.

It is also interesting that a subset of VID or GID genes are involved in both the proteasomal and vacuolar pathways. In our hands, Vid24p is a structural protein on Vid vesicles.76 This protein is required for FBPase trafficking after FBPase is sequestered inside Vid vesicles. However, recent evidence from the Wolf group suggests that Vid24p is also part of the ubiquitin ligase complex. As such, it is involved in the ubiquitination of FBPase that occurs prior to its proteasomal dependent degradation.94 Thus, Vid24p appears to be involved in two very different cellular functions depending on whether FBPase is targeted to the proteasome or the vacuole. Given that Vid24p plays dual roles in the proteasomal versus vacuolar degradation pathways, it is reasonable to assume that other VID or GID genes may code for multifunctional proteins. Thus, a major focus of our future research will be to sort out the roles that these proteins play in the proteasomal versus vacuole dependent degradation of FBPase.

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