The key gluconeogenic enzyme fructose-1,6-bisphosphatase is secreted during prolonged glucose starvation and is internalized following glucose re-feeding via the non-classical secretory and internalizing pathways in *Saccharomyces cerevisiae*

**Bennett J. Giardina and Hui-Ling Chiang***

Department of Cellular and Molecular Physiology; Penn State College of Medicine; Hershey, PA USA

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**Abbreviations:** FBPase, fructose-1,6-bisphosphatase; Icl1p, isocitrate lyase; Pck1p, phosphoenolpyruvate carboxykinase; MDH2, malate dehydrogenase

Gluconeogenic enzymes are induced when *Saccharomyces cerevisiae* are grown in media containing low glucose. However, when glucose is added to glucose-starved cells, these enzymes are inactivated and degraded. These enzymes include fructose-1,6-bisphosphatase (FBPase), isocitrate lyase (Icl1p), phosphoenolpyruvate carboxykinase (Pck1p) and malate dehydrogenase (MDH2). Inactivation and degradation of gluconeogenic enzymes during glucose re-feeding prevents energy futile cycles that are harmful to cells. FBPase is degraded either in the vacuole or in the proteasome depending on the length of glucose starvation. For the vacuole-dependent pathway, several intermediate compartments are utilized. Vid (vacuole import and degradation) vesicles are small vesicles, whereas Vid/endosomes contain clusters of Vid vesicles. Recent evidence indicates that FBPase is secreted into the periplasm during glucose starvation. Following glucose re-feeding, FBPase is internalized into Vid/endosomes in the cytoplasm. FBPase internalization is dependent on the ARC18 (Arp2/3 complex subunit) and SLA1 (Synthetic Lethal with ABP1) genes involved in actin polymerization/endocytosis. FBPase internalization also requires the VPS34 gene encoding PI3K. Using these unconventional pathways, secreted FBPase is retrieved into the cytoplasm and subsequently degraded in the vacuole.

**Autophagy and Human Diseases**

Autophagy is a process by which proteins or organelles are degraded in the lysosome/vacuole. Multiple autophagic pathways have been identified. The best example is the non-selective macroautophagic pathway, which is induced when cells are starved of nutrients. This pathway recycles amino acids for reuse and is important for survival during starvation. In addition, autophagy is critical for a number of biological processes such as extension of life span, developmental regulation and defense against the invasion of pathogens. Altered autophagy is associated with many pathological conditions including aging, cancer, neuromuscular degeneration and neurodegeneration. In animal models of neurodegeneration, rapamycin which induces autophagy reduced large protein aggregates and improved the performance of affected animals. Therefore, induced autophagy has the
potential to treat patients with aggregates-prone diseases such as Parkinson disease, Huntington's disease, or Alzheimer disease.

Catabolite Inactivation

A novel autophagic pathway that degrades gluconeogenic enzymes during glucose re-feeding has been studied in *Saccharomyces cerevisiae*. Gluconeogenic enzymes fructose-1,6-bisphosphatase (FBPase), isocitrate lyase (Icl1p), phosphoenolpyruvate carboxykinase (Pck1p) and malate dehydrogenase (MDH2) are induced when cells are grown in media containing low glucose.16–18 However, following a transfer of glucose-starved cells to media containing high glucose, these enzymes are inactivated and degraded.17,24 This inactivation was called “catabolite inactivation” by Dr. H. Holzer more than 20 years ago.18 However, the mechanisms responsible for catabolite inactivation have not been completely elucidated. In addition to gluconeogenic enzymes, catabolite inactivation has also been described for mitochondrial enzymes such as the F1 subunit of the ATPase,25 Ach1p involved in acetate metabolism,26 the galactose,27,28 the maltose,29,30 and the high-affinity glucose transporters.18 Thus, catabolite inactivation applies to multiple enzymes involving different metabolic pathways.

The Site of Degradation

Fructose-1,6-bisphosphatase is the key gluconeogenic enzyme and has been used extensively to study the mechanisms for glucose-induced inactivation and degradation.20,21,31–36 This protein has been reported to be degraded either in the vacuole20,21,37–45 or in the proteasome.31,36 Interestingly, the site of degradation of FBPase varies depending on the length of glucose starvation.32 FBPase is degraded in the proteasome when glucose is added to cells that are starved for 1 d. In contrast, FBPase is degraded in the vacuole when glucose is added to cells that are starved for 3 d. Malate dehydrogenase (MDH2) is another gluconeogenic enzyme that is degraded in the proteasome upon addition of glucose to 1 d-starved cells. This protein is degraded in the vacuole following a transfer of 3 d-starved cells to glucose.32 Likewise, two other gluconeogenic enzymes Pck1p and Icl1p are targeted and then degraded in the vacuole when prolonged-starved cells are replenished with glucose.40 Trafficking of gluconeogenic enzymes to the vacuole in response to glucose has been demonstrated using indirect immunofluorescence microscopy, immuno-transmission electron microscopy (immuno-TEM) and fluorescence microscopy with GFP targeted cargo proteins.20,21,32,40 Targeting of FBPase to the vacuole has also been reconstituted in vitro using semi-permeabilized cells.34

The Vacuole Import and Degradation Pathway

A number of *VID* (vacuole import and degradation) genes have been identified as being required for the degradation of FBPase in the vacuole.55 Homologs of these *VID* genes are also found in mice and human, suggesting that *VID* genes are evolutionarily conserved. The degradation of FBPase, MDH2, Pck1p and Icl1p was retarded in cells lacking the *VID24* gene,40 indicating that the Vid pathway mediates the degradation of these proteins in the vacuole. The fact that multiple gluconeogenic enzymes are degraded in the vacuole via the Vid pathway highlights the importance of this pathway. Furthermore, the Vid pathway is a selective degradation pathway. Cargo proteins are degraded when they are no longer needed in new environments. This is different from the starvation-induced autophagic pathway that degrades proteins non-selectively. *GID* (glucose induced degradation) genes were isolated as being required for the degradation of FBPase in the proteasome.56 Interestingly, many of these *GID* genes are also involved in vacuole-dependent degradation of FBPase in response to glucose addition.32

For the Vid pathway, FBPase is associated with intermediate compartments prior to being delivered to the vacuole. Vid vesicles are small vesicles and have smooth surfaces.56 These vesicles were detected in glucose-starved cells, suggesting that they are formed prior to the addition of glucose. Vid24p is a peripheral protein that resides on Vid vesicles.44,47 COPI coatomer proteins are also present on Vid vesicles and are required to recruit Vid24p to these vesicles.44 COPI coatomer proteins are involved in multiple trafficking pathways in mammalian cells and in yeast. For example, COPI proteins are critical for retrograde transport from the Golgi to the ER. Furthermore, these proteins are localized to endosomes and play important roles in endosomal sorting.48–52 In the absence of the *UBCI* (ubiquitin conjugating enzyme 1) gene, levels of Vid24p were reduced in the Vid vesicle enriched fraction, suggesting that the *UBCI* gene is involved in the formation of Vid vesicles.53 The import of FBPase into Vid vesicles has been reconstituted in vitro. The sequestration of FBPase requires the heat shock protein Ssa2p, cyclophilin A and Vid22p.36,42,43 Recent evidence indicates that Vid30p is also distributed to Vid vesicles and forms a large protein complex with Vid24p and Sec28p.55 Moreover, the type I phosphatase Reg1p-Glc1p54 and the vacuole ATPase55 play important roles in the Vid pathway.

**FBPase is Localized to Endosomes Following Glucose Addition**

Vid vesicles exist in at least two forms. Individual Vid vesicles are 30–50 nm in diameter.40 Vid vesicles can also aggregate to form Vid/endosomes that contain the endosomal protein Pep12p, the Vid-vesicle protein Vid24p and the cargo protein FBPase.39 Vid/endosomes have been purified and examined at the ultra-structural level.39 FBPase and Vid24p were in small compartments inside Vid/endosomes. Vid24p was also present at multiple locations on the surface of Vid/endosomes.39 It is difficult to assess whether or not a layer of common membranes surrounds Vid/endosomes, as such membranes may not be preserved during fixation and processing for negative staining and TEM. The *VPH1* gene is required for the Vid pathway at a late step. Therefore, FBPase accumulated in late endosomes in cells lacking this gene. In the Δ*pch1* mutant, FBPase-GFP was inside endosomes that were surrounded by a thin layer of FM4–64, suggesting that common membranes are present in these structures.39 FM 4–64 (FM) is a red fluorescence dye that is internalized and subsequently
targeted to endosomes and then to the vacuole.\textsuperscript{60} As such, this dye has been used to label compartments in the endocytic pathway. In addition to FBPase, Vid vesicle-proteins Vid24p and Sec28p also co-localize with FM-containing endosomes upon a transfer of glucose-starved cells to glucose.\textsuperscript{44}

When the distribution of FBPase was examined at the ultra-structural level, FBPase was in areas near the plasma membrane following glucose re-feeding for 15 min.\textsuperscript{39} In yeast, actin polymerization is needed for scission of endocytic vesicles and generation of force to propel endocytic vesicles.\textsuperscript{57,60} Interestingly, FBPase and MDH2 associated with actin patches transiently and they dissociated later.\textsuperscript{39} Vid-vesicle proteins such as Vid24p and Sec28p also associated with actin patches initially. However, less co-localization was observed at later time points.\textsuperscript{39} The utilization of the endocytic pathway enables cells to remove molecules from the extracellular and intracellular spaces simultaneously. This may provide an efficient way for cells to clear unwanted proteins and to adapt quickly to the new environments.

Anterograde and Retrograde Trafficking to and from the Vacuole

Co-localization of cargo proteins and Vid vesicle proteins such as Vid24p, Sec28p and Vid30p with endosomes suggests that these proteins are delivered to endosomes and then to the vacuole via the anterograde trafficking pathway. However, cargo proteins are degraded in the vacuole, whereas Vid vesicle proteins are not degraded in the vacuole. Thus, a retrograde transport pathway should be used to retrieve Vid vesicle proteins from the vacuole. Without retrograde transport, these molecules would be trapped in the vacuole.

To study retrograde transport, the vacuole was pre-labeled with FM4-64 for 16 h in glucose-starved wild-type cells that expressed GFP tagged proteins. Cells were then re-fed with glucose and examined for the distribution of GFP proteins in retrograde transport vesicles that budded from the vacuole. In these studies, Sec28p was detected in vesicles that formed from the vacuolar membrane.\textsuperscript{40,44} Co-localization of Sec28p in retrograde vesicles requires the \textit{UBCI} and the \textit{RET2} genes (encodes the δ subunit of COPI coatomers).\textsuperscript{73} The Tor1 complex (TORC1) plays an essential role in the Vid pathway. Interestingly, the TORC1 subunits Torlp and Tco89p were also found in retrograde transport vesicles.\textsuperscript{40} In addition to having a role in retrieving proteins from the vacuole, retrograde transport is also critical to maintain the size of the vacuole. Without retrograde transport, the vacuole membrane would expand. As such, retrograde transport is as important as anterograde transport.

\textbf{VID30 Plays a Critical Role in the Association of Vid Vesicles with Actin Patches}

\textit{VID30} was originally identified via a transposon library screening for mutants defective in the Vid pathway.\textsuperscript{53} Vid30p is constitutively expressed and localized to Vid vesicles.\textsuperscript{53} This protein is associated with actin patches during glucose starvation and less association was observed at later time points. In the absence of the \textit{VID30} gene, Vid24p and FBPase did not co-localize with actin patches, suggesting that \textit{VID30} is required for the association of Vid vesicles with these patches.

Vid30p contains a lissencephaly type 1-like homology domain (LisH). Mutations in the \textit{LIS1} gene cause Miller-Dieker lissencephaly disorder and early death. Vid30p also contains a C-terminal to the LisH domain (CTLH) involved in microtubule dynamics.\textsuperscript{61-63} These domains are important for Vid30p interaction with Vid24p and Sec28p. In the absence of these domains, FBPase accumulated in punctate structures. In contrast, in the complete absence of \textit{VID30}, FBPase showed diffused distribution. Because FBPase displayed different distribution patterns, the LisH and CTLH domains are likely to be involved in a late step in the FBPase degradation pathway.\textsuperscript{53}

\textbf{FBPase is Secreting in Glucose-Starved Cells and Internalized in Glucose Re-fed Cells}

The finding that FBPase was distributed to the FM-containing endosomes in glucose re-fed cells raised the possibility that FBPase itself is secreted into the periplasm prior to glucose addition. To investigate this, FBPase distribution was examined at the ultra-structural level.\textsuperscript{44} When wild-type cells were grown in media containing high glucose, FBPase levels were low. In cells that were starved for 3 d, FBPase was induced and a high percentage of FBPase was in the periplasm. Thus, FBPase is secreted into the periplasm during prolonged starvation. Following the addition of glucose to 3 d-starved cells for 15 min, FBPase appeared in Vid/endosomes in the cytoplasm. In cells that were starved for 3 d and then re-fed with glucose for 120 min, total amounts of FBPase decreased, indicating that most of the FBPase is degraded by this time point.

The appearance of FBPase in the extracellular fraction was further demonstrated using a protocol that extracts extracellular proteins from whole cells. In these studies, wild-type cells were starved, re-fed with glucose and extracellular proteins were extracted. Proteins were then separated into the extracellular and intracellular fractions and the distribution of proteins in these fractions was determined. This protocol was used to detect the secretion of mammalian galectin-1 expressed in \textit{Saccharomyces cerevisiae}.\textsuperscript{55} Similar protocols have been employed to study cell-wall associated proteins in \textit{C. albicans}.\textsuperscript{66}

This protocol was utilized to demonstrate that molecules involved in the Vid pathway were distributed mostly in the intracellular fraction.\textsuperscript{64} Lst8p and Torlp are subunits of the Torlp complex and were mainly in the intracellular fraction. Likewise, the majority of the Sec28p, Vid24p, Vid30p and Vps34p were in the intracellular fraction. By contrast, FBPase was in both intracellular and extracellular fractions. Interestingly, the appearance of FBPase in the extracellular fraction depends on the availability of glucose in the media. When wild-type cells were grown in media containing high glucose, FBPase was not expressed and was not detected in the extracellular fraction. When cells were grown in media containing low glucose for 1 d, FBPase was induced. However, this protein was not detectable in the extracellular fraction. In 2 d-starved cells, low levels of FBPase
The glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is on the cell surface of *Saccharomyces cerevisiae* grown in medium containing high glucose. As such, substantial amounts of GAPDH were in the extracellular fraction in cells grown in high glucose media. However, levels of extracellular GAPDH decreased in cells grown in low glucose media for 1 and 2 d. Amounts of extracellular GAPDH increased following growth in low glucose medium for 3 d. Thus, the distribution of FBPase and GAPDH in the extracellular fraction changes depending on the availability of glucose in the media.

**Vps34p is Critical for the Internalization of Extracellular FBPase in Response to Glucose Addition**

Phospholipids and sterols play critical roles in many protein trafficking pathways. The *VPS34* gene encodes a class III phosphatidylinositol (PtdIns) 3-kinase (PI3K) which phosphorylates phosphatidylinositol at the 3’ hydroxyl position to produce PtdIns 3-phosphate (PtdIns3P). The yeast Vps34p is involved in multiple protein trafficking events including endocytosis, sorting of vacuolar proteins, vacuole segregation, and protein trafficking pathways. The yeast Vps34p is involved in the internalization of EBPsase requires the Vps34p associates with actin patches constitutively. The association of Vid vesicles with actin patches persists in the absence of the *VPS34* gene.

**VPS34** has an important role in the reduction of extracellular FBPase in response to glucose re-feeding. In 3 d-starved Δ*vps34* mutant, substantial amounts of FBPase were in the periplasm as shown by immuno-TEM. However, following the addition of glucose, most of the FBPase remained in the periplasm. These results were further confirmed using the extraction procedure. In 3 d-starved Δ*vps34* mutant, a high percentage of FBPase was in the extracellular fraction. Following a transfer of the Δ*vps34* mutant to media containing high glucose, significant amounts of FBPase remained in the extracellular fraction.

**The N736 Residue and the C-terminal 11 Amino Acids of Vps34p are Critical for the Reduction of Extracellular FBPase Following Glucose Addition**

The N736 residue of Vps34p is critical for PI3K activity and plays important roles in many vacuole pathways including the Vid pathway. FBPase degradation was retarded in cells harboring the N736K mutation. In addition, the N736K mutant protein did not co-localize with actin patches. Moreover, the N736 mutant delayed the clearance of extracellular FBPase in response to glucose re-feeding. The C-terminal 11 amino acids of Vps34p (amino acids 864–875) is implicated in the association of Vps34p on the membrane. FBPase degradation was inhibited in cells in which the C-terminal 11 amino acids were deleted. Moreover, the ΔC11 mutant protein and actin patches did not co-localize. In addition, the absence of the C-terminal 11 amino acids retarded the clearance of extracellular FBPase in response to glucose. These results indicate that the N736 residue and the C-terminal 11 amino acids are critical for Vps34p association with actin patches and the reduction of extracellular FBPase following glucose re-feeding.

**The Vid Pathway Model**

Based on the current knowledge about the Vid pathway, the following model has been proposed (Fig. 1). When cells are grown in low glucose for 3 d, FBPase is secreted into the periplasm. The secretion of FBPase into the extracellular fraction/periplasm increases as cells are starved longer. Interestingly, levels of the glycolytic enzyme GAPDH in the extracellular fraction also depend on the growth conditions. Because FBPase and GAPDH do not colocalize with actin patches, these proteins are secreted via the non-classical pathway. FBPase internalization enables cells to retrieve most of the extracellular FBPase into the cytoplasm, allowing this protein to be targeted to the vacuole for degradation.

![Figure 1](image_url)

**Figure 1.** A model for the Vid pathway. When wild-type cells are starved of glucose for a prolonged period of time, significant amounts of FBPase are secreted into the periplasm. Following glucose re-feeding, FBPase is internalized into Vid/endosomes. The internalization of FBPase requires the *SLA1*, *ARC18* and *VPS34* genes. Under the same conditions, most of the Vid24p, Sec28p, Vid30p and Vps34p are in the intracellular fraction. Vid24p, Sec28p and Vid30p associate with actin patches initially and dissociate later, whereas Vps34p associates with actin patches constitutively. Following internalization, FBPase is targeted to the vacuole and then degraded in the lumen.
is dependent on the VPS34 gene encoding PI3K. Vps34p association with actin patches is linked to the decline of extracellular FBPase. When the C-terminal 11 amino acids were deleted or when the N736 residue was mutated, Vps34p association with actin patches was impaired and the clearance of extracellular FBPase was retarded. Interestingly, most of the extracellular FBPase was cleared in the first 30 min of glucose addition, suggesting that this is a rapid process. FBPase is unlikely to be internalized via the receptor-mediated endocytic pathway due to its lack of a signal sequence. Under the same conditions, molecules involved in the Vid pathway such as Vid24p, Sec28p, Vid30p and Vps34p are retained in the intracellular fraction. In the future, it will be important to elucidate the mechanisms responsible for the secretion and internalization of FBPase via the non-classical pathways.

**Prospectives**

Why is FBPase secreted in glucose-starved cells and internalized in glucose re-fed cells? One possibility is that FBPase is secreted when the need for this enzyme inside the cells is decreased. FBPase may be in greater demand in the intracellular fraction for 1 d-starved cells. However, the need for FBPase in the intracellular fraction may decrease in 3 d-starved cells. As such, more FBPase may be in greater demand in the intracellular fraction for when the need for this enzyme inside the cells is decreased. In glucose re-fed cells? One possibility is that FBPase is secreted because the plasma membrane or enter through protein- or vesicle-conducting channels? Are Vid/endosomes similar to the multi-vesicular bodies implicated in endocytosis in mammalian cells? Why are proteins secreted in vesicles? Since both membrane proteins and luminal proteins can be packaged in vesicles, the number of proteins secreted into the extracellular space may increase substantially. Furthermore, vesicles may protect proteins from sudden changes in the environments. For instance, when cells are challenged with toxic materials, surface proteins may be damaged, but luminal proteins may be spared. Given that exosomes contain mRNA and microRNA, they may be involved in the transfer of genetic material to recipient cells. As such, they have the potential for gene therapy. Therefore, secreted proteins may have multiple roles, to protect cells from toxic materials, to adjust metabolic needs in response to changing environments, to participate in cell-to-cell communication and to transfer genetic materials to recipient cells.

Bacteria, viruses, fungi and parasites secrete a large number of signal-less proteins during infection. Cancer cells also secrete many signal-less proteins during growth and invasion. Defects in endocytosis have also been linked to various neurological disorders. For instance, Vps34p is involved in endocytosis in mammalian cells and the deletion of VPS34 in sensory neurons causes neurodegeneration by disrupting the endosomal pathway. Thus, understanding the molecular mechanisms responsible for the unconventional secretory and internalizing pathways should have far-reaching impacts on many biological processes such as metabolic regulation, protein trafficking, pathogen infection and cancer growth and invasion.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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Plant Signaling & Behavior volume 8 issue 8

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