When *Saccharomyces cerevisiae* are grown in media containing low glucose, gluconeogenic enzymes are induced. However, when glucose is added to prolonged-starved cells, these enzymes are inactivated and degraded in the vacuole via the Vid (vacuole import and degradation) pathway. These enzymes include fructose-1,6-bisphosphatase (FBPase), malate dehydrogenase (MDH2), isocitrate lyase (Icl1p), and phosphoenolpyruvate carboxykinase (Pck1p). Recent evidence indicates that the key gluconeogenic enzyme FBPase is secreted when cells were starved of glucose for three days. Since other gluconeogenic enzymes MDH2, Icl1p, and Pck1p are also degraded in the vacuole via the same Vid pathway, they should show distribution patterns similar to those of FBPase. As such, they should also be secreted in glucose-starved cells. To test this, we used immuno-TEM techniques to determine the distribution of these enzymes at the ultra-structural level. To confirm our immuno-TEM studies, we used an extraction procedure that extracts extracellular proteins from whole cells. Our results indicated that approximately 33.7–76.7% of these enzymes were secreted in glucose-starved cells. Given that these proteins do not contain typical ER signal sequence, they are secreted via the non-classical pathway. Thus, gluconeogenic as well as non-gluconeogenic enzymes utilize the unconventional pathway to be secreted during prolonged starvation.

Fructose-1,6-bisphosphatase, Malate Dehydrogenase, Isocitrate Lyase, Phosphoenolpyruvate Carboxykinase, Glycerdehyde-3-phosphate Dehydrogenase, and Cyclophilin A are secreted in *Saccharomyces cerevisiae* grown in low glucose
Coatomer subunit Sec28p is distributed mainly in the intracellular fraction as shown previously\textsuperscript{22,24} and was used as a negative control. Because antibodies against Pck1p and Icl1p were not available, we produced a wild-type strain expressing Icl1p tagged with HA and Pck1p-tagged with Myc. We then used HA and Myc antibodies to detect these tagged proteins. For these experiments, wild-type cells co-expressing Icl1p-HA and Pck1p-Myc were grown in YPKG media containing low glucose for three days and processed for immuno-TEM studies. First, we used western blotting to examine whether or not these antibodies recognized proteins at the expected molecular weights in total lysates. As is shown in Figure 1A, polyclonal antibodies against Sec28p, FBPase, MDH2, HA, Myc, GAPDH, and Cpr1p all reacted with a single protein band at the predicted molecular weights.

We next examined the distribution of these proteins by incubating thin sections of glucose-starved wild-type cells in the absence or presence of purified primary antibodies followed by secondary antibodies conjugated with 10 nm gold particles (Fig. 1B). When thin sections of cells were incubated in the absence of primary antibodies, there were very few gold particles observed between the cell wall and the plasma membrane. In a similar manner, low levels of Sec28p were detected in the extracellular space. In contrast, substantial amounts of the positive control protein FBPase were present in areas between the cell wall and the plasma membrane. Likewise, significant amounts of MDH2, Icl1p-HA, Pck1p-Myc, GAPDH, and Cpr1p were also found in the extracellular space in cells that were grown in low glucose for a prolonged period of time. Quantification of immunogold particles indicated that approximately 76.7% of FBPase, 36.9% of MDH2, 46.9% of Icl1p, 57.6% of Pck1p, 33.7% of GAPDH, and 42.7% of Cpr1p were in the extracellular space in glucose-starved cells (Table 1).
Table 1. Quantification of the number of gold particles per cell in the I and E fractions

| Protein          | I          | E          |
|------------------|------------|------------|
| No primary antibodies | 4.1 ± 2.1  | 3.2 ± 1.3  |
| Sec28p           | 60.3 ± 10.2| 3.5 ± 2.3  |
| FBPase           | 49.7 ± 13.1| 165.8 ± 23.5|
| MDH2             | 121.1 ± 15.3| 70.6 ± 9.9 |
| Icl1p-HA         | 88.3 ± 10.1| 78.2 ± 5.9 |
| Pck1p-Myc        | 130.8 ± 18.3| 178.3 ± 21.2|
| GAPDH            | 117.9 ± 33.9| 59.8 ± 15.2|
| Cpr1p            | 94.5 ± 1.9 | 70.3 ± 14.2|

Thin sections of wild-type cells expressing Icl1p-HA and Pck1p-Myc were incubated in the absence or presence of primary antibodies directed against Sec28p, FBPase, MDH2p, HA, Myc, GAPDH, and Cpr1p followed by secondary antibodies conjugated with 10 nm gold particles. The number of gold particles per cell in the intracellular (I) and extracellular/periplasmic fraction (E) was counted. Mean and Standard Error of the Mean (SEM) were obtained by counting the number of gold particles in the intracellular and extracellular fractions from 3 micrographs.

We next used the extraction procedure to confirm the presence of these proteins in the extracellular fraction. Wild-type cells expressing Icl1p-HA and Pck1p-Myc were grown in low-glucose media for three days and subjected to the extraction procedure. Following the extraction protocol, proteins were separated into the intracellular and extracellular fractions. The distribution of these proteins in these fractions was then examined by western blotting (Fig. 2). FBPase is the soluble cell wall protein and was used as a control for proteins that reside in the extracellular fraction. Not surprisingly, the majority of FBPase was in the extracellular fraction. In contrast, most of the Sec28p was distributed in the intracellular fraction. As expected, significant amounts of FBPase were in the extracellular fraction. In a similar manner, MDH2, Icl1p-HA, Pck1p-Myc, GAPDH, and Cpr1p were all found in the extracellular fraction. Taken together, our results indicate that FBPase, MDH2, Icl1p-HA, Pck1p-Myc, GAPDH, and Cpr1p are secreted when cells were grown in media containing low glucose. Because these enzymes do not contain typical ER signal sequence, they are secreted by the non-conventional pathway.

Secretion of signal-less proteins in extracellular vesicles has been observed in different fungi including Cryptococcus neoformans, Candida parapsilosis, Candida albicans, Histoplasma capsulatum, Paracoccidioides brasiliensis, Sporothrix schenckii, Saccharomyces cerevisiae, and Malassezia sympodialis. Extracellular vesicles isolated from different species showed differences in their sizes. For example, 20–400 nm vesicles were observed in C. neoformans, while 10–350 nm vesicles and 20–200 nm vesicles were found in H. capsulatum and in P. brasiliensis respectively. Extracellular vesicles of 50–250 nm were also detected in culture media from S. cerevisiae. Interestingly, FBPase, MDH2, Icl1p, Pck1p, GAPDH, and Cpr1p were identified in extracellular vesicles isolated from Histoplasma capsulatum. We have shown previously that FBPase is associated with small vesicles (Vid vesicles) in the cytoplasm in S. cerevisiae. These vesicles exist in at least two forms. Free vesicles are 30–50 nm in diameter. Vid vesicles can also aggregate to form large clusters in the cytoplasm. Given that substantial amounts of gluconeogenic enzymes are secreted in glucose-starved cells, these proteins may also be secreted in vesicles. Future experiments will be needed to elucidate the molecular mechanisms responsible for the secretion of signal-less proteins and vesicles across the plasma membrane and the cell wall.

Methods

Cell Culture, Media and Antibodies

Yeast strains used in this study included wild-type (BY4742, MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0), wild-type tagged with Icl1p-HA and Pck1p-Myc (MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 trp1–1 Icl1p-HA-TRP1 Pck1p-Myc-HIS3), and wild-type expressing Scw4p-GFP (MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 Scw4p-GFP::HIS3). Cells were first grown in YPKG (1% yeast extract, 2% peptone, 1% potassium acetate and 0.5% glucose) for three days and processed. Antigen affinity purified anti-GAPDH polyclonal antibodies were purchased from Protein Tech Group. Affinity purified anti-HA polyclonal antibodies were purchased from Immunology Consultant Laboratory. Affinity purified polyclonal anti-Myc antibodies were obtained from Santa Cruz Biotechnology. Wild-type cells expressing Scw4p-GFP were purchased from Invitrogen. Anti-GFP polyclonal antibodies were purchased from Abcam.

Immuo-TEM

Immuo-TEM was performed as described. After three days in YPKG media, cells were fixed with 3% paraformaldehyde and 0.2% glutaraldehyde overnight at room temperature. Cells were serially dehydrated and embedded in LR White. Embedded samples were sectioned and placed onto Formvar-carbon-coated...
nickel grids. Grids containing cell sections were incubated on a droplet containing 0.1% BSA/PBS for 30 min. Next, the grids were incubated with or without primary antibody (Sec28p:10 x, FBPase: 50 x, MDH2: 20 x, pHA: 20 x, pMyc: 20 x, GAPDH: 10 x, and Cpr1p: 20 x) diluted in 1% BSA/PBS for 2 h. Grids were then washed and incubated in 50 x dilution of goat anti-rabbit IgG conjugated with 10 nm immunogold particles (Ted Pella). After washing, grids were stained with 2% uranyl acetate for 1 min and viewed on a JEOL JEM-1400 electron microscope with an Orius SCI1000 CCD camera.

**Extraction and Western Blotting**

Extraction was performed as described.2-24 Cells were grown in 2 ml YPKG for three days and harvested. Cells (OD600 = 10/ ml) were incubated with 100 μl of extraction buffer containing 0.1 M Tris, pH 9.4 and 10 mM β-mercaptoethanol in a 37°C shaker for 15 min. Following incubation, cells were pelleted at 3,000 g for 5 min. The supernatant fraction was precipitated with 15% TCA, washed and resuspended in SDS-PAGE buffer. The distribution of proteins in the extracellular and intracellular fractions was examined using polyclonal antibodies directed against Sec28p, FBPs, MDH2, HA, Myc, GAPDH, and Cpr1p followed by peroxidase-conjugated donkey anti-rabbit IgG diluted 1:10,000 (GE Healthcare). The distribution of Sec24p-GFP was examined using anti-GFP antibodies. Bands were developed using Western Lightning Plus ECL (Perkin Elmer).

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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