Intracellular vesicle clusters are organelles that synthesize extracellular vesicle–associated cargo proteins in yeast

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Extracellular vesicles (EVs) play important roles in cell-cell communication. In budding yeast (Saccharomyces cerevisiae), EVs function as carriers to transport cargo proteins into the periplasm for storage during glucose starvation. However, intracellular organelles that synthesize these EV-associated cargo proteins have not been identified. Here, we investigated whether cytoplasmic organelles—called intracellular vesicle clusters (IVCs)—serve as sites for the synthesis of proteins targeted for secretion as EV-associated proteins. Using proteomics, we identified 377 IVC-associated proteins in yeast cells grown under steady-state low-glucose conditions, with the largest group being involved in protein translation. Isolated IVCs exhibited protein synthesis activities that required initiation and elongation factors. We have also identified 431 newly synthesized proteins on isolated IVCs. Expression of 103Q-GFP, a foreign protein with a long polyglutamine extension, resulted in distribution of this protein as large puncta that co-localized with IVC markers, including fructose-1,6-bisphosphatase (FBPase) and the vacuole import and degradation protein Vid24p. We did not observe this pattern in cycloheximide-treated cells or in cells lacking VID genes, required for IVC formation. The induction of I03Q-GFP on IVCs adversely affected total protein synthesis in intact cells and on isolated IVCs. This expression also decreased levels of EV-associated cargo proteins in the extracellular fraction without affecting the number of secreted EVs. Our results provide important insights into the functions of IVCs as sites for the synthesis of EV-associated proteins targeted for secretion to the periplasm.

Extracellular vesicles (EVs)² contain proteins, lipids, DNAs, and RNAs that are secreted from various cell types and organisms (1–4). These vesicles are found in extracellular fluids, including blood, urine, breast milk, and cerebrospinal fluid. They function as intercellular carriers and participate in cell-cell communication (1–8). Under certain pathological conditions such as cancer, the secretion of EVs increases, and this can be used to detect growth and metastasis of cancer cells (1, 9, 10, 12, 13). Secreted proteins include metabolic enzymes, molecular chaperones, and oxidative stress proteins (1–8). Toxic proteins such as prions, α-amylloid peptide, superoxide dismutase, and α-synuclein are also secreted in EVs (14–20). Prion is responsible for the Creutzfeldt-Jakob disease in humans and bovine spongiform encephalopathy in cattle (15). The amyloid precursor protein and cleaved products are involved in Alzheimer’s disease (18, 19). Additionally, α-synuclein and superoxide dismutase I are associated with Parkinson’s disease and myotrophic lateral sclerosis, respectively (18–20).

EVs have been found in fungi such as Cryptococcus neoformans (21–23), Paracoccidioides brasiliensis (24–26), Histoplasma capsulatum (27), and Saccharomyces cerevisiae (28–31). Published data from our laboratory have identified more than 300 EV-associated proteins in S. cerevisiae that are classified into various groups based on their functions (31–33). They include proteins involved in oxidative stress, as well as molecular chaperones that participate in the folding of newly synthesized proteins. Enzymes that play important roles in the metabolisms of carbohydrates, lipids, amino acids, and nucleotides have also been detected (31–33). These include gluconeogenic/glycolytic enzymes fructose-1,6-bisphosphatase (FBPase), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and phosphoglycerate kinase (PGK1) (31–33).

We have shown that cells grown in low glucose media (0.5%) for 3 days release EVs carrying gluconeogenic enzymes into the periplasm (31–33). When these cells are transferred to media containing high glucose (2%), EVs are internalized and gluconeogenic enzymes are degraded in the vacuole via the vacuole import and degradation (Vid) pathway (31–33). This degradation provides amino acids that can be used to synthesize new proteins. These gluconeogenic enzymes serve as convenient markers to study the trafficking of EVs across the plasma membrane.

The Vid pathway is divided into multiple steps, including 1) the secretion of vesicles to the periplasm, 2) the internalization of vesicles into the cytoplasm, and 3) targeting of vesicles to the vacuole for degradation (34–40). Work from our laboratory

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indicates that the degradation of gluconeogenic enzymes requires the presence of Vid proteins (Vid21p, Vid24p, Vid27p, Vid28p, Vid30p, and COPI coater proteins) (35–40). These proteins are peripheral proteins on vesicles, and their interactions are critical for vesicles to form aggregates for their retention in the cytoplasm. For instance, Vid24p interacts with Vid21p, Vid27p, Vid28p, and Vid30p to form a large protein complex (36–38). When this interaction is disrupted, clusters fail to form and vesicles are released into the periplasm (36–38).

Vesicles carrying gluconeogenic enzymes are distributed in multiple locations including the cytoplasm and the periplasm (31, 34, 40). In the cytoplasm, the majority of these vesicles form aggregates (intracellular vesicle clusters, IVCs), although a small percentage exists as free vesicles (30–50 nm in diameter) (31, 34, 40). Given that cargo proteins are found on IVCs during steady-state growth of cells in low glucose media, we hypothesize that IVCs are the intracellular organelles involved in the synthesis of EV-associated cargo proteins. Accordingly, more than 300 EV-associated proteins have been identified using proteomics analysis (31). In contrast, most of the proteins on IVCs have not been identified.

The goals of this study were to identify proteins present on IVCs and to investigate the potential roles of IVCs in the synthesis of EV-associated proteins. We have identified 377 proteins that were present on IVCs using proteomics-based analysis. A high percentage of IVC-associated proteins were components of translational machinery involved in protein synthesis. Isolated IVCs exhibited protein synthesis activities, and 431 nascent proteins were synthesized on IVCs. A newly synthesized foreign protein containing a long polyglutamine extension (103Q-GFP) was also distributed on IVCs and co-localized with the IVC markers FBPase and Vid24p. This foreign protein reduced levels of EV-associated proteins in the extracellular fraction/pace without affecting the number of EVs secreted to the periplasm. Our results indicate that IVCs contain protein synthesis activities and play important roles in the synthesis of EV-associated cargo proteins.

Results

Identification of proteins associated with IVCs

Previously, we showed that FBPase and Vid24p are associated with IVCs (34). However, most of the proteins present on IVCs have not been identified. To find additional IVC-associated proteins, IVCs from WT cells grown in low glucose media were isolated as described in the “Experimental procedures.” Examination of the 100,000-g pellet fraction (IVC1) using transmission EM (TEM) revealed highly enriched vesicle clusters (Fig. 1A). When the 100,000-g pellet fraction was further separated by centrifugation on sucrose density gradients (IVC2), vesicle clusters containing FBPase were enriched in fractions 8 and 9 (Fig. 1F) and were the most abundant organelles found in these fractions (Fig. 1B).

To identify IVC-associated proteins in cells grown during steady-state conditions in low-glucose media, IVCs were isolated from 100,000-g pellet fraction (IVC1) or from the sucrose density gradient following differential centrifugation (IVC2). IVCs were solubilized with Triton X-100, digested with trypsin, and the resulting peptides were analyzed by mass spectrometry (MS). Table S1 lists 377 IVC-associated proteins (213 in IVC1 and 337 in IVC2) that were found. As shown in Fig. 1, C and D, the largest group consisted of proteins involved in translation (49.8% in IVC1 and 38.0% in IVC2, compared with 2.8% in the yeast genome); the second largest group contained enzymes in the metabolism of carbohydrates (19.2% in IVC1 and 19.0% in IVC2, compared with 3.1% of the total yeast genome). These studies also identified proteins involved in the metabolism of amino acids, lipids, and nucleotides, as well as molecular chaperones that participate in the folding of newly synthesized proteins.

Given that proteins involved in translation were identified on IVCs, we determined whether components of translational machinery were present on IVCs. WT cells expressing the GFP-tagged translation initiation factor Tif2p (eIF4A), as well as elongation factors Tef2p (eEF-1α) and Eft2p (eEF2), were grown in low-glucose media. Lysates were subjected to differential centrifugation followed by sucrose density gradients. The distribution of FBPase and GFP-tagged proteins was determined by Western blotting using FBPase and GFP antibodies. FBPase, Tif2p, Tef2p, and Eft2p were all distributed in fractions 8 and 9 containing IVCs (Fig. 1E), suggesting that these initiation and elongation factors are present on IVCs.

Newly synthesized foreign protein containing a long polyglutamine extension is distributed on IVCs

A polypeptide containing a long expanded glutamine (103Q) domain fused with exon 1 of the human Huntington gene formed aggregates upon induction in yeast cells when examined by fluorescence microscopy (41). In contrast, no aggregates were observed in 25Q cells containing a short extension of the polyglutamine domain (41). Previous studies in our lab indicate that the IVC marker Vid24p appears as intracellular puncta using fluorescence microscopy (34, 39). Because a large number of proteins associated with IVCs are involved in translation (Table S1), we explored the possibility that newly synthesized 103Q-GFP is distributed on IVCs. WT cells containing either a short (25Q-GFP) or long polyglutamine extension (103Q-GFP) were grown in YNB containing 2% raffinose overnight. These cells were then induced with 2% galactose in the presence or absence of cycloheximide for 2, 4, and 16 h. Fig. 2A shows that the induction of 103Q-GFP with galactose resulted in increased amounts of 103Q-GFP in large puncta. This was observed at 2 h following treatment and persisted for up to 16 h, relative to cells suppressed in raffinose media at t = 0. Large 103Q-GFP containing puncta was not found in cells pre-treated with the protein synthesis inhibitor cycloheximide followed by galactose induction (Fig. 2B). In contrast to the large puncta observed in 103Q-GFP cells, 25Q-GFP appeared to be diffused in the cytoplasm following galactose induction (Fig. 2C). As expected, the addition of cycloheximide blocked the expression of 25Q-GFP (Fig. 2D). Given that cells treated with galactose for 16 h were viable and showed similar distribution patterns as those in earlier time points, all subsequent experiments concerning induction of these proteins by galactose were performed at 16 h. This incubation time was also required to detect cargo proteins such as FBPase.
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A  IVC1

B  IVC2

C  IVC1

D  IVC2

E  -FBPase

- Tif2p-GFP

- Tef2p-GFP

- Eft2p-GFP

Fraction 1 2 3 4 5 6 7 8 9 10 11 12
The distribution of 103Q-GFP was visualized using confocal microscopy. The expression of Vid proteins, the 103Q-GFP signals were weak and presented as small dots treated with galactose for 16 h. In cells lacking Vid proteins, the 103Q-GFP signals were weak and presented as small dots in the cytoplasm (Fig. 4A). This result indicates that the presence of Vid proteins is required for the formation of IVCs and, hence, the distribution of newly synthesized 103Q-GFP on these organelles.

Previous reports indicated that Vid24p and FBPase are associated with IVCs (34). To further confirm that newly synthesized 103Q-GFP is distributed on IVCs, WT cells co-expressing 103Q-GFP and FBPase-RFP were incubated with galactose for 16 h to induce both FBPase and 103Q. As illustrated in Fig. 4B, 103Q-GFP co-localized with FBPase-RFP in large puncta on IVCs. Likewise, WT cells that contained both 103Q-RFP and GFP-Vid24p also showed co-localization of 103Q with Vid24p following galactose treatment (Fig. 4C). Taken together, these results further support the notion that newly synthesized 103Q-GFP is localized to IVCs.

To determine whether IVCs are related to the secretory pathway, the distribution of 103Q-GFP on IVCs was examined in sec mutant strains that block different steps of the secretory pathway at the nonpermissive temperature (37 °C). 103Q-GFP was expressed in sec62–1 (endoplasmic reticulum), sec7–1 (Golgi), or sec1–1 (exocytosis) following galactose induction at either the permissive (25 °C) or nonpermissive (37 °C) temperature. Fig. 4D shows that 103Q-GFP was found as large puncta in sec mutants at either temperature after galactose induction, indicating that the distribution of 103Q-GFP on IVCs is not affected in these sec mutants.
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**A**

<table>
<thead>
<tr>
<th>Raffinose</th>
<th>103Q-GFP</th>
<th>Galactose</th>
<th>Galactose + CHX</th>
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**B**

<table>
<thead>
<tr>
<th>Raffinose</th>
<th>25Q-GFP</th>
<th>Galactose</th>
<th>Galactose + CHX</th>
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**Figure 3. Newly synthesized 103Q-GFP is distributed on IVCs.** A, WT cells expressing GFP-tagged 103Q with a long expanded polyglutamine were grown in YNB media containing 2% raffinose overnight. Cells were induced by an addition of 2% galactose for 16 h in the absence or presence of cycloheximide (10 μg/ml). The distribution of 103Q-GFP on isolated IVCs was visualized using immuno-TEM. Scale bar = 100 nm. Arrow indicates the presence of 103Q-GFP on IVCs. B, WT cells containing 25Q-GFP with a short glutamine domain were treated as above in the absence or presence of cycloheximide for 16 h and examined by immuno-TEM. Scale bar = 100 nm. Results are representative data from three independent experiments.

**Isolated IVCs contain protein synthesis activities in vitro**

The distribution of newly synthesized 103Q-GFP on IVCs raises the possibility that IVCs exhibit protein synthesis activities. Additionally, our proteomic studies have identified components of the translational machinery as IVC-associated proteins. To address this connection, we investigated whether isolated IVCs contain protein synthesis activities and incorporate radioactive amino acids into proteins. Increasing amounts of isolated IVCs were incubated with 35S-methionine/cysteine (35S-met/cys) in the presence of an ATP regenerating system for 60 min. The incorporation of 35S-met/cys into trichloroacetic acid (TCA) precipitable proteins was determined. The highest specific activity was observed in the presence of 10–20 μg of IVCs (Fig. 5A). To investigate whether intact vesicles on IVCs are required for the incorporation of 35S-met/cys into proteins, equal amounts of IVCs (20 μg) were pre-treated with or without 2% Triton X-100 and examined for protein synthesis activities. The disruption of vesicles on IVCs by Triton X-100 decreased protein synthesis activity as compared with those incubated in the absence of this detergent (Fig. 5B).

To determine whether cytosol is needed for protein synthesis activities on IVCs, increasing amounts of cytosol were added to the reaction mixture. Protein synthesis activity was the highest in the absence of cytosol and decreased with increasing amounts of cytosol (Fig. 5C), suggesting that cytosol adversely affects the in vitro reactions. We also investigated the effects of various incubation times, temperatures, and concentrations of ATP in the regenerating system on in vitro protein synthesis activities. Incubation of IVCs for 60 min (Fig. 5D) at 30 °C (Fig. 5E) in the presence of 4 mM ATP (Fig. 5F) resulted in high protein synthesis activities. Hence, all subsequent in vitro labeling experiments were performed at 30 °C for 60 min using 20 μg of IVCs and 4 mM ATP in the regenerating system.

We next examined whether the protein synthesis activity associated with IVCs is affected by the growth of cells under different glucose concentrations. IVCs were isolated from WT cells grown in either YPD media (2% glucose) or YPKG media (0.5% glucose) for 1 or 3 days and examined for protein synthesis activities. IVCs isolated from cells grown in high or low glucose for either 1 or 3 days exhibited similar protein synthesis activities (Fig. 6A). Thus, protein synthesis activity on IVCs is independent of durations of growth under different glucose concentrations.

If the incorporation of 35S-met/cys into proteins reflects the synthesis of new proteins, in vitro reactions should be blocked by protein synthesis inhibitors. As illustrated in Fig. 6B, pre-treatment of IVCs with increasing concentrations of cycloheximide decreased protein synthesis activities as compared with IVCs incubated in the absence of this drug. Thus, this indicates that the incorporation of 35S-met/cys into proteins represents the synthesis of new proteins.

To study whether the presence of IVC-associated proteins and RNAs is important for protein synthesis, IVCs were pre-treated with proteinase K or RNase A to degrade proteins and RNAs, respectively. Incubation of IVCs with increasing amount of proteinase K (Fig. 6C) or RNase A (Fig. 6D) decreased in vitro protein synthesis activities as compared with control IVCs in the absence of these inhibitors.

We determined whether components of the protein translational machinery play important roles in this process. IVCs isolated from mutant cells lacking either initiation factors or elongation factors were examined for protein synthesis activities.
Reduced protein synthesis activities were observed when IVCs were isolated from cells lacking these factors, as compared with those isolated from WT cells (Fig. 6E). Hence, these factors are required for protein synthesis activities on IVCs. Our proteomic studies indicated that IVCs contained enzymes involved in metabolism (FBPase) and oxidative stress (thioredoxin peroxidase, Tsa1p). However, protein synthesis activities of IVCs isolated from mutant cells lacking FBPase or Tsa1p were similar to those isolated from WT cells (Fig. 6E).

**Identification of nascent proteins synthesized on IVCs**

Given that IVCs exhibited protein synthesis activities (Figs. 5 and 6), we used an *in vitro* system and CLICK chemistry to identify nascent proteins synthesized on IVCs. Isolated IVCs from WT cells were incubated with a methionine analog L-homopropargyl glycine (HPG) in the presence of an ATP regeneration system at 30 °C for 60 min. Nascent proteins containing incorporated HPG were conjugated with desthiobiotin-PEG₃-azide (DSB), affinity purified on streptavidin resins, and eluted with biotin. Purified proteins were digested with trypsin and the resulting peptides were subjected to MS-based analysis. We identified 431 newly synthesized proteins (277 from SYN1 and 335 from SYN2) that were classified into several functional groups (Table S2). As shown in Fig. 7, A and B, the largest group contained proteins involved in translation (40.4% in SYN1 and 37.6% in SYN2, compared with 2.8% in the total genome). The second largest group consisted of enzymes involved in the metabolism of carbohydrates (18.1% in SYN1 and 18.2% in SYN2, compared with 3.1% of the total genome). These proteins included well-characterized cargo proteins (FBPase, GAPDH, and PGK1). Enzymes involved in the metabolisms of amino acids, lipids, and nucleotides were also identified. Of the newly synthesized proteins, 76.1% (328 out of 431) overlapped with 87.0% (328 out of 377) of proteins associated with IVCs isolated from WT cells grown under steady-state conditions in low-glucose media (Fig. 7C).
Figure 5. Isolated IVCs exhibit protein synthesis activities in cell-free systems. A, increasing amounts of IVCs isolated from WT cells grown in YPKG media (0.5% glucose) were incubated with 2 μCi of 35S-met/cys for 1 h in the presence of an ATP regenerating system. B, isolated IVCs (20 μg) were pre-treated with or without 2% Triton X-100 (TX) for 15 min followed by incubation with 35S-met/cys for 1 h. C-F, isolated IVCs (20 μg) were incubated in the presence of 35S-met/cys and an ATP regenerating system containing varying concentrations of cytosol (C), incubation durations (D), temperatures (E), and ATP concentrations (F). Each error bar represents the mean ± S.D. of three independent experiments. Results were normalized to total proteins used in each reaction and expressed as counts per minute (cpm). **, p < 0.01; ***, p < 0.001; ****, p < 0.0001 versus control values.

103Q-GFP expression inhibits total protein synthesis in vitro and in vivo

The induction of 103Q-GFP inhibits endocytosis and contributes to cytotoxicity (41). Because the above results indicated that 431 nascent proteins as well as newly synthesized 103Q-GFP were distributed on IVCs, we explored the possibility that the expression of 103Q-GFP affects the synthesis of other proteins on IVCs. 103Q-GFP cells grown in YNB media containing 2% raffinose were treated with galactose for 16 h. IVCs were isolated from these cells and examined for their abilities to synthesize nascent proteins in vitro. Fig. 8A shows that induction of 103Q-GFP resulted in an approximate 90% decrease in the incorporation of 35S-met/cys into total proteins compared with IVCs isolated from cells maintained in raffinose media. In contrast, the amounts of labeled proteins in 25Q-GFP cells were not affected by either raffinose or galactose treatment (Fig. 8B).

The above in vitro studies (Fig. 8, A and B) indicated that the majority of the protein synthesis activity on IVCs was suppressed by the expression of 103Q-GFP. We investigated whether the induction of 103Q-GFP affects total protein synthesis under in vivo circumstances. Cells containing 25Q-GFP or 103Q-GFP were incubated with galactose or raffinose for 16 h and then labeled with 35S-met/cys in the presence of galactose or raffinose for 4 h. Induction of 103Q-GFP by galactose decreased (~50%) the incorporation of 35S-met/cys into proteins as compared with control 103Q-GFP cells in raffinose media (Fig. 8C). Protein synthesis was not affected whether 25Q-GFP cells were treated with raffinose or galactose (Fig. 8D). Taken together, the expression of 103Q-GFP inhibits protein synthesis on isolated IVCs and in intact cells.

103Q expression decreases levels of newly synthesized proteins and EV-associated proteins in the extracellular fraction

If IVCs have important roles in the synthesis of EV-associated cargo proteins for their secretion to the periplasm, the expression of 103Q-GFP may decrease levels of newly synthesized proteins or EV-associated proteins in the extracellular fraction/space. We investigated whether 103Q-GFP induction affects the amounts of newly synthesized proteins in vivo. Cells transformed with 25Q-GFP or 103Q-GFP were incubated with raffinose or galactose for 16 h and then labeled with 35S-met/cys for 4 h. Proteins were separated into intracellular (I) and extracellular (E) fractions for the determination of total labeled proteins in each fraction. Fig. 9A shows that induction of...
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A

SYN1

- Protein Translation: 40.4%
- Carbohydrate Metabolism: 18.1%
- Amino Acid Metabolism: 10.5%
- Lipid Metabolism: 7.2%
- Nucleobase Metabolism: 6.5%
- Transport: 7.6%
- Proteolysis: 2.9%
- Chaperones: 2.5%
- Other: 4.3%

B

SYN2

- Protein Translation: 37.6%
- Carbohydrate Metabolism: 18.2%
- Amino Acid Metabolism: 17.0%
- Lipid Metabolism: 9.9%
- Nucleobase Metabolism: 3.8%
- Transport: 2.4%
- Proteolysis: 1.8%
- Chaperones: 5.4%
- Other: 3.9%

C

SYN 103 328 49 IVC
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103Q-GFP with galactose decreased (~50%) the incorporation of 35S-met/cys into proteins in the I fraction as compared with cells maintained in raffinose. The amounts of labeled proteins in the I fraction were similar in 25Q-GFP cells incubated in either raffinose or galactose media (Fig. 9B). Likewise, a decrease (~60%) in the amount of labeled proteins in the E fraction of 103Q-GFP cells was detected in the presence of galactose relative to the E fraction of cells maintained in raffinose (Fig. 9C). Levels of labeled proteins in the E fraction of 25Q-GFP cells were unaffected by raffinose or galactose treatment (Fig. 9D). Thus, the expression of 103Q decreases amounts of newly synthesized proteins in the E fraction.

Finally, we determined whether 103Q expression affects the levels of EV-associated cargo proteins in the E fraction. Cells containing 25Q-GFP or 103Q-GFP were treated with galactose for 16 h and amounts of EV-associated proteins in this fraction were analyzed. Levels of cargo proteins such as FBPase were below detection in the presence of raffinose (data not shown). In 103Q-GFP, expressing cells following galactose induction, amounts of EV-associated proteins including FBPase, GAPDH, and PGK1 in the E fraction decreased several-fold relative to those in 25Q-GFP cells (Fig. 10, A and B).

To examine whether decreased levels of cargo proteins in the E fraction of 103Q-GFP cells are because of a reduction in the number of EVs, EVs were isolated from these cells following galactose induction. As shown in Fig. 10, C and D, the number of EVs was similar in 25Q-GFP or 103Q-GFP cells.

Discussion

In the present study, 377 IVC-associated proteins were identified during steady-state growth of cells in low-glucose media, with the largest group playing established roles in translation. Using yeast cells expressing the foreign protein 103Q-GFP, we found evidence indicating that this protein was synthesized and localized to IVCs. Furthermore, the distribution of newly synthesized 103Q-GFP on IVCs was blocked in the presence of cycloheximide. Several lines of evidence support the notion that the observed cytoplasmic puncta represent IVCs. For instance, newly synthesized 103Q-GFP co-localized with known IVC markers such as FBPAse and Vid24p. This protein was also detected on purified IVCs using immuno-TEM after induction with galactose. Finally, the distribution of 103Q-GFP on IVCs was disrupted and presented as small dots in cells lacking Vid proteins. This suggests that the formation of IVCs is important for the distribution of 103Q on these organelles.

Interactions of peripheral proteins on Vid vesicles are critical for the formation of IVCs (36–38). These types of interactions have been noted for numerous Vid proteins such as Vid21p, Vid24p, Vid27p, Vid28p, or Vid30p, with the binding of Vid proteins on vesicles to one another underlying the formation of
IVCs (36–38). In the current study, we did not observe a direct interaction of 103Q-GFP with either Vid24p or FBPase. However, we found 149 IVC-associated proteins that interacted with 103Q-GFP following galactose induction, relative to only four proteins observed in raffinose (Table S3). The majority of these 103Q-interacting proteins were involved in translation; a small percentage were metabolic enzymes, chaperones, and transporters. It is possible that these interacting proteins play roles in the formation of aggregates. Future experiments will be necessary to test this idea.
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The distribution of 103Q-GFP on IVCs inhibited protein synthesis in vivo and in vitro. When compared with 25Q-GFP cells, there was a significant decrease in protein synthesis, both in cells expressing 103Q-GFP and on isolated IVCs in response to galactose. These results suggest that the ability of IVCs to synthesize proteins is negatively influenced by the presence of 103Q-GFP. Adverse effects on cell functions have also been reported by others, where aggregation of 103Q-GFP led to defects in endocytosis as well as contributing to cellular toxicity (41). It is possible that the induction of 103Q-GFP causes changes on IVCs leading to a reduced ability of these organelles to synthesize proteins. Given that the majority of 103Q-interacting proteins on IVCs are components of translational machinery, the expression of 103Q-GFP may cause the sequestration of molecules available for active synthesis of proteins. This, in turn, leads to decreased total protein synthesis activities. Along this line, we observed that a high percentage of nascent proteins synthesized on IVCs are designated for secretion to the periplasm as EV-associated cargo proteins. For instance, we found that levels of EV-associated proteins such as FBPane, GAPDH, and PGK1 in the E fraction were much lower in 103Q-GFP cells than those detected in the 25Q-GFP cells. This decline appears because of decreased synthesis of cargo proteins on IVCs without affecting the number of EVs secreted to the periplasm (Fig. 10, C and D).

Previously we have shown that endocytosis of EVs in yeast is mediated via a cup-shaped channel located on the plasma membrane (11, 38). However, the mechanism by which EVs are released as intact vesicles is unknown. We propose that cargo proteins are synthesized on IVCs and remain associated with these organelles. Individual vesicles carrying cargo proteins are then released from IVCs in the cytoplasm prior to their export across the plasma membrane by a process similar to that involved in endocytosis. This model is different from the reports in mammalian cells in which multivesicular bodies fuse with the plasma membrane and release their internal vesicles to the extracellular space (1–6).

Taken together, the results from this study provide important insights into the functions of IVCs as the sites for the synthesis of 103Q-GFP and EV-associated cargo proteins. The expression of 103Q-GFP decreased the amounts of EV-associated cargo proteins in the E fraction without affecting the number of secreted EVs. We conclude that IVCs are novel intracellular organelles involved in the synthesis of EV-associated proteins targeted for secretion to the periplasm.

Experimental procedures

Strains, plasmids, and reagents

Plasmids containing either short (25Q-GFP) or long (103Q-GFP) expanded polyglutamine domains under the GAL1 promoter were gifts from Dr. Sherman (Boston University School of Medicine, Boston, MA). The 103Q-RFP plasmid was generated in two steps. The 103Q-TOPO plasmid (B599) was produced by PCR using the 103Q-GFP template, a forward primer (P215) containing GAC GTC CTA GCA GCT GTA ATA CGA CTC ACT ATA GGG AAT ATT, and a reverse primer (P216) containing GTT AAT TAA CAC CAG GGA TCC CCC GGG CTG CAG TTG TTG CTG. The PCR products were purified and cloned into a pYES2.1 vector. The 103Q-TOPO plasmid was digested with PacI and SacI and cloned into PacI-Sacl sites on a plasmid containing RFPLYS2 (B594) to produce 103Q-RFP (B601). Plasmid containing FBP-RFP-LEU2 (B502) was produced by digesting FBP-RFP-HIS3 (B430) with PacI and SacI and ligated into RFP-LEU2 (B455) that was also digested with PacI and SacI. WT and mutant yeast strains used in this study are listed in Table S4. Deletion strains were obtained from Euroscarf. WT cells containing GFP-tagged proteins were purchased from Invitrogen. GFP-Vid24p was produced as described (34).

Rabbit polyclonal anti-FBPane antibody was generated using purified FBPane as an antigen. Anti-GFP antibody was purchased from Abcam. 35S-met/cys was obtained from PerkinElmer (Waltham, MA). Goat anti-rabbit antibodies conjugated with 10 nm gold particles for immuno-TEM was from Electron Microscopy Sciences. Secondary antibodies used for immunoblotting were polyclonal IRDye goat anti-rabbit 680 RD or monoclonal IRDye goat anti-mouse 800 CW from LI-COR Odyssey (Lincoln, NE). Click chemistry desthiobiotinylation and pulldown kit (product 1132), desthiobiotin-PEG3-azide (1107), and L-homopropargylglycine (1067) were obtained from Click Chemistry Tools LLC. (Scottsdale, AZ).

Yeast growth and GAL induction

WT and mutant strains were grown in either high glucose YPD media (1% yeast extracts, 2% peptone, 2% glucose) or low glucose YPKG media (1% yeast extracts, 2% peptone, 1% potassium acetate, and 0.5% glucose). Cells transformed with 25Q-GFP or 103Q-GFP were grown in yeast nitrogen base (YNB) containing ammonium sulfate, all standard amino acids except uracil and 2% raffinose (as a control to suppress the expression of the GAL1 promoter). The expression of 25Q-GFP or 103Q-GFP was induced by adding 2% galactose to YNB media for the indicated time points. For protein synthesis inhibitor experiments, cells were incubated with cycloheximide (10 μg/ml) for 15 min prior to the addition of 2% galactose. At each time point, NaN3 was added to stop the reactions.

Isolation of IVCs

IVCs were isolated from 1-liter culture of WT yeast strains grown in YPD or YPKG media for 1 or 3 days. To obtain IVCs from WT cells transformed with either 25Q-GFP or 103Q-GFP, cells were grown in 1 liter of 2% raffinose YNB media and then treated with 2% galactose in the absence or presence of cycloheximide (10 μg/ml). Cells were spun down at 3000 × g and pellets were re-suspended in 10 ml FBPase buffer (50 mM HEPES, pH 7.2, 5 mM MgSO4, 40 mM (NH4)2SO4, 0.1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride). Cells were disrupted by vortexing 4–5 times (30 s/time) with 425–600 micros microns acid-washed glass beads (Sigma) at 4 °C. Total lysates were subjected to sequential centrifugation at 3000 × g for 10 min, 13,000 × g for 20 min, and then 100,000 × g for 2 h at 4 °C. The resultant 100,000 × g pellets containing IVCs were processed for TEM or used for the in vitro labeling experiments. Alternatively, the 100,000 × g pellets were loaded on a 10-ml 40–80% (2 ml 40%, 2 ml 50%, 2 ml 60%, 2 ml 70%, and 2 ml 80%)
Sucrose density gradients and centrifuged at 100,000 × g for 16 h at 4 °C. Proteins from each fraction (0.8 ml) were collected from the top and precipitated with 20% TCA. Proteins were solubilized with 2× Laemmli sample buffer (LSB) and determined by Western blotting. For immuno-TEM, fractions 8 and 9 that contained IVCs were pooled and re-centrifuged at 100,000 × g for 2 h. The pellet was processed for immuno-TEM studies.

Transmission electron microscopy

The above isolated IVCs were fixed in 3% paraformaldehyde with 0.2% glutaraldehyde for 2 h at 4 °C. Fixed IVCs were incubated for 1 h on poly-lysine–coated nickel grids. The samples containing fixed IVCs on grids were washed once with PBS, twice with distilled water for 5 min, dried with Whatman filter papers, and stained with 2% uranyl acetate for 5 min. Samples were visualized using a JEOL JEM-1400 electron microscope with an Oriou SC 1000 CCD camera. For immuno-TEM, fixed IVCs on coated grids were washed with PBS and PBS containing 0.1% BSA/PBS, PBS and water, the grids were stained with 2% uranyl acetate and viewed on electron microscope.

2D-LC separation and mass spectrometry

Proteomic analyses was performed at the Mass Spec and Proteomics Core Facility (Penn State College of Medicine). Isolated IVCs (100 μg) were solubilized with 2% Triton X-100, precipitated with TCA, and digested in 50 mM ammonium bicarbonate, pH 8.0, with trypsin at 48 °C for 16 h. Digestions were done with Promega Sequencing Grade Trypsin, which cuts on the C-terminal side of lysine and arginine residues, unless they are followed by a proline. The resulting tryptic peptides were separated by an inline NanoFlow column (75 μm × 150 mm Waters 1.7 μm BEH 130 C18) run at 300 nl per min, and sprayed directly into a Sciex 5600+ TripleTOF mass spectrometer for MS and MS/MS analyses of the top 50 peaks over 2.75 s of each elution fraction.

The database searched was the NCBI RefSeq S. cerevisiae database of Jan. 3 2016, containing 5837 proteins plus a list of 536 common lab contaminants, for a total of 6373 protein sequences. A reversed database of these 6373 proteins was also created and searched simultaneously to calculate estimated false discovery rates (FDR) from database hits (IDs) of nonexistent protein sequences from that reversed Decoy database, such that a total of 2 × 6373 = 12,746 sequences were searched by the Paragon Algorithm contained in the ProteinPilot 5.01 analysis suite (Sciex). To minimize the potential number of false positive proteins, the Paragon Algorithm search results were further narrowed by using a Local FDR estimation. This FDR was calculated according to the Proteomics System Performance Evaluation Pipeline (PSPEP). Search parameters were as follows: cysteine alkylation: iodoacetamide, ID focus: biological modifications, search effort: thorough. Protein IDs with a local FDR estimate of <5% were accepted as valid IDs, and the number of protein IDs meeting these criteria were 213 (IVC1), 337 (IVC2), 277 (SYN1), 335 (SYN2), 4 (Raf), and 149 (Gal). The function of each protein was characterized using the Gene Ontology Slim Mapper program in the Saccharomyces Genome Database (Stanford University).

Synthesis of extracellular vesicle proteins

Confocal fluorescence microscopy

WT cells expressing GFP- or RFP-tagged proteins were grown in 2 ml YNB without uracil in the presence of 2% raffinose overnight. Cells were inducted with 2% galactose in the presence or absence of cycloheximide for the indicated time points. The fluorescence of GFP and RFP was visualized on glass slides using a Leica SP8 confocal microscope with software: Leica Application Suite X (Leica, Germany) and a 63× objective (NA 1.4).

Protein synthesis in vitro

Isolated IVCs (20 μg) were incubated with PBS buffer, 4 mM ATP regenerating system (320 mM creatine phosphate and 1.6 mg/ml creatine phosphate kinase) and 2 μCi 35S-met/cys for 60 min at 30 °C. Thereafter, reaction mixtures were spotted on Whatman 450 filter papers (24 mm), air dried, and washed several times with TCA (1 time with 10% cold TCA, 1 time with 5% cold TCA, 1 time with boiled TCA, and final wash with 5% cold TCA). Filters containing proteins with incorporated 35S-met/cys were soaked with 1 ml Solvable for 10 min prior to 10 ml LSC mixture (Opti-Fluor) and counted using a Beckman scintillation counter (PerkinElmer, Waltham, MA). Protein concentration was determined using Bio-Rad DC protein kit (Bio-Rad). Specific activities were defined as total counts per min (cpm) divided by the amounts of total proteins (μg) used in each reaction.

Synthesis of nascent proteins using the CLICK Chemistry Kit (1132)

IVCs isolated from WT cells grown in low-glucose YPKG media were incubated with an alkyne-tagged methionine analog HPG in the presence of an ATP regenerating system for 60 min at 30 °C. The modified nascent proteins containing incorporated HPG were conjugated with DSB using a copper-catalyzed CLICK reaction. Newly synthesized proteins were captured with streptavidin agarose resins and eluted with biotin according to the manufacturer’s protocol. Proteins were digested with trypsin and peptides were analyzed by MS.

Separation of intracellular and extracellular proteins

WT cells expressing 25Q-GFP or 103Q-GFP grown in 2% raffinose YNB media were induced with 2% galactose for 16 h. Equal amounts of cells (A600 = 10/ml) were pelleted and extracted with 200 μl extraction buffer (0.1 m Tris, pH 9.4, and 10 mm β-mercaptoethanol) in a 37 °C shaker for 15 min. Following incubation, cells were centrifuged at 3000 × g for 10 min. Proteins from the supernatant were precipitated with 20% TCA to obtain the E proteins. Cell pellets containing I proteins were lysed in 200 μl of FBPase buffer (50 mM HEPES, pH 7.2, 5 mM MgSO4, 40 mM (NH4)2SO4, 0.1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride). Protein from both fractions of lysates
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were denatured in 2× LSB and analyzed by Western blotting. The amounts of FBPase, GAPDH, and PGK1 in the I and E fractions were quantified using ImageJ software and expressed as relative distribution (%) in the E fraction.

Western blotting and co-immunoprecipitation

For immunoblotting experiments, proteins were electrophoresed on denaturing polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were blotted with 5% nonfat dry milk in TBS and incubated with antibodies of interest (1:1000). Unbound primary antibodies were removed by washing with TBS containing 0.1% Tween-20 (ICI Americas, Inc., Wilmington, DE). Blots were incubated with secondary goat anti-rabbit 680 RD or goat anti-mouse 800 CW antibodies (1:10,000) in the dark for 1 h. The blots were visualized using a LI-COR Odyssey CLX Imaging system and quantified with the image studio software. For co-IP experiments, IVCs isolated as described above were solubilized with 2% Triton X-100 for 1 h at room temperature and pre-cleared with 50% slurry streptavidin agarose CL4B beads (Sigma). Primary antibody (1:200) was added to equal amounts of proteins and rotated overnight at 4 °C. A total of 50 μl of 50% slurry protein A Sepharose beads were added and rotated for additional 1 h. Immunoprecipitates were washed three times with PBS, denatured with 2× LSB, and boiled for 5 min. The eluted proteins were precipitated with 20% TCA overnight at 4 °C, washed three times with water, and subjected to MS analysis.

Statistical analysis

An unpaired Student’s t test was performed. Data are presented as mean ± S.D. of three repeated individual experiments. A value of p < 0.05 was considered to be statistically significant.


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References


