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Biogenesis of extracellular vesicles in yeast

Many questions with few answers

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The cellular events required for unconventional protein secretion in eukaryotic pathogens are beginning to be revealed. In fungi, extracellular release of proteins involves passage through the cell wall by mechanisms that are poorly understood. In recent years, several studies demonstrated that yeast cells produce vesicles that traverse the cell wall to release a wide range of cellular components into the extracellular space. These studies suggested that extracellular vesicle release involves components of both conventional and unconventional secretory pathways, although the precise mechanisms required for this process are still unknown. We discuss here cellular events that are candidates for regulating this interesting but elusive event in the biology of yeast cells.

Key words: secretion, extracellular vesicles, exosome, trans-cell wall transport, yeast box

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Protein secretion is a widely studied cellular phenomenon. To reach the extracellular milieu, intracellularly synthesized proteins are targeted to the cell surface for release to the extracellular space.[1] In mammalian cells, the plasma membrane is the final barrier to be crossed during secretion. Such processes, which involve both conventional and unconventional mechanisms, have been studied in detail and a number of excellent reviews are available in the literature.[1-6]

Secretory systems in microbes and mammalian cells show points of convergence and divergence.[7] Fungi and prokaryotes are surrounded by thick cell walls, a key difference in comparison with mammalian and other eukaryotic cells (e.g., protozoa) that adds significant complexity to secretion systems in these organisms. A number of mechanisms have been proposed for the trans-cell wall molecular transport in prokaryotes.[8] In fungi, however, the mechanisms required for passage of molecules across the cell wall are poorly understood. Recently, extracellular vesicle release has been described as a mechanism used by yeast cells to secrete many molecules across the cell wall.[9-12]

Extracellular vesicles produced by fungal cells share morphological and biochemical similarities with mammalian exosomes,[13,14] including an ability to modulate the function of immune cells.[15] Plant cells also produce exosome-like vesicles,[16] supporting the notion that vesicular release is a mechanism of trans-cell wall passage shared by cell-wall containing eukaryotes. In contrast to what is observed for mammalian exosomes,[17] the pathways required for extracellular vesicle biogenesis and release in both plant and fungal cells remain virtually unknown. One remarkable feature of mammalian exosomes and fungal extracellular vesicles is the abundance of cytoplasmic proteins lacking a signal peptide that directs proteins to the endoplasmic reticulum in conventional secretory processes.[13,14,18-20]

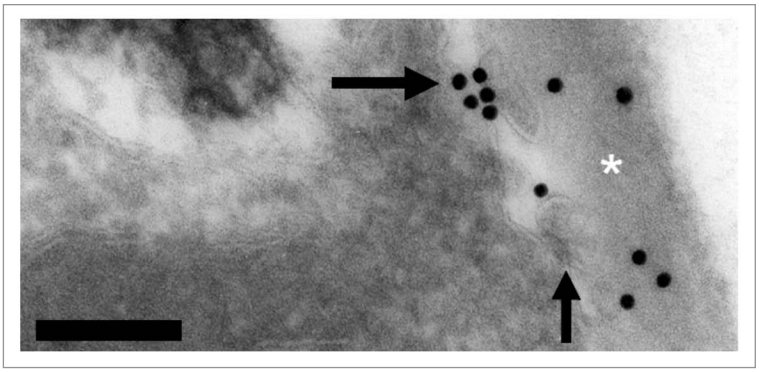

Figure 1. *Cryptococcus neoformans*, a yeast pathogen, produce vesicle-like structures (arrows) that apparently bud from the plasma membrane to be deposited at the cell wall, as evidenced from transmission electron microscopy. Gold labeling represents reactivity of fungal glucosylceramide with human antibodies. Scale bar, 0.1 μm. Asterisk denotes the cell wall. For experimental details, see Rodrigues and colleagues.[26] Modified from Barreto-Bergter et al.[25] courtesy of Dr. Kildare Miranda.

In a recent study, we evaluated the contribution of both conventional and unconventional pathways of secretion in the formation of extracellular vesicles in the model yeast *Saccharomyces cerevisiae*.[21] Our approach was based on the study of mutants with defects in two major secretion pathways: conventional post-Golgi secretion[2] and exosome formation, a mechanism of unconventional secretion.[17] The use of this model was based on the facts that: (1) genes required for conventional, post-Golgi secretion were implicated in the formation of extracellular vesicles in fungi;[22] and (2) exosomes and fungal vesicles share many similarities.[18-20,23,24]

Defects in the formation of multivesicular bodies (MVB) are expected to directly affect the formation of exosomes.[17] Surprisingly, yeast mutants with defects in MVB formation produced similar amounts of extracellular vesicles in comparison to WT cells.[21] The protein composition of vesicles from WT and mutant cells was essentially equivalent, but approximately 50% of these vesicular proteins had their abundance modified in mutant cells. Remarkably, most of the proteins (75%) found in vesicular fractions lacked signal peptides. These puzzling results indicate that, although MVB-related mutations apparently do not affect vesicle release, MVB formation is somehow related to the formation of extracellular vesicles in yeast.

Post-Golgi secretory vesicles usually fuse with the plasma membrane to release their cargo, so they are not expected to interfere with formation of vesicular structures outside the cell.[6] In our analyses, however, yeast mutants lacking Sec4p, a secretory vesicle-associated Rab GTPase essential for Golgi-derived exocytosis,[6] had reduced kinetics of vesicle release to the extracellular milieu.[21] The fact that cells with defects in a post-Golgi event of secretion, but not with disturbed MVB formation, affected vesicle release raised an obvious and still unanswered question: how is a double layered vesicle secreted from yeast cells?

The simplest and more tangible explanation for the release of any extracellular vesicle is the fusion of MVB with the plasma membrane. However, studies by our group[25,26] clearly show that double-layered vesicles can bud from the plasma membrane of yeast cells (**Fig. 1**). Therefore, one could speculate that proteins required for post-Golgi conventional secretion could be required for addressing vesicle components to the plasma membrane. Vesicles would then be formed by membrane budding and sequential transfer to the cell wall and extracellular space. That would be consistent with previous hypotheses raising the possibility that formation of extracellular vesicles can involve membrane budding.[4] Budding from the plasma membrane would also be in line with the complex vesicle composition including cytoplasmic elements, as observed in our analyses.[13,14,21] It remains unknown how these vesicles traverse the cell wall, but many cell wall degrading enzymes were observed in extracellular vesicle samples obtained from *S. cerevisiae* cultures.[21] We hypothesize that these enzymes could hydrolyze cell wall components to facilitate vesicle passage through this cellular barrier.

The methods currently used for vesicle purification do not discriminate between vesicles of different origins. This implies that heterogeneous preparations are obtained during vesicle isolation. In this context, the possibility that the collection of mutations analyzed in our recent study[21] is affecting different types of vesicles cannot be ruled out. The current knowledge on how fungal extracellular vesicles are formed, in fact, strongly suggests the involvement of multiple—and perhaps still unknown—pathways of secretion.[9,11,13] As recently described in independent studies, unconventional protein secretion can also involve autophagosomes,[27,28] which are intracellular structures whose functions were initially attributed to many catabolic steps.[29]

In autophagy, cytosolic material is sequestered by an expanding membrane compartment, the phagophore, resulting in the formation of a double-membrane vesicle, the autophagosome.[29] Autophagosomes then fuse with the lysosome/vacuole where, as initially supposed, the sequestered material is degraded.[29] Independent studies, however, have shown that yeast cells can also direct the autophagic content for secretion, in a process called exophagy.[27-29] In fact, the autophagic machinery participates in the packaging and delivery of the soluble yeast protein acyl-Coenzyme A-binding protein Acb1 to the cell surface. Therefore, these studies suggest the existence of a vesicular mechanism that utilizes the same machinery for both secretion and degradation of cellular components. It is interesting to note that secretion of Acb1 from yeast as well as secretion of the *Dictyostelium discoideum* Acb1 homologue, AcbA, depends on the Golgi associated protein GRASP,[27,28,30] which is apparently required for extracellular vesicle release in yeast cells.[21] These observations add to an already long list of candidates that can regulate vesicle formation in yeast cells.

After our initial description of fungal extracellular vesicles in 2007,[12] eight different studies showing their functions in fungal physiology or pathogenesis have been reported in the literature.[13-15,21,22,31-33] Vesicle

release has been associated to protein and polysaccharide secretion,[12-14,22,32] surface architecture,[31] virulence,[10,12,13] pigmentation[33] and modulation of macrophage function.[15] Despite their apparent multiple functions in yeast, the cellular components controlling their biogenesis and release remain elusive. We emphasize the supposition that the methods currently used for preparation of extracellular fractions containing vesicles may co-isolate vesicular compartments of different cellular origins, which limit the application of studies based on the generation of punctual mutations. Post-Golgi components required for conventional secretion, proteins involved in MVB formation, GRASP and even autophagy-related events may be involved in the formation of extracellular vesicles. Although much progress has been made in the last three years, the route to understand how fungal extracellular vesicles are formed still seems long and laborious.

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