MINIREVIEW

Chitosomes: past, present and future

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Abstract

José Ruiz-Herrera's discovery that chitin microfibrils could be made by a fungal extract paved the way for elucidating the intracellular location of chitin synthetase. In collaboration with Charles Bracker, chitosomes were identified as the major reservoir of chitin synthetase in fungi. Unique in size, buoyant density, and membrane thickness, chitosomes were found in a wide range of fungi. Their reversible dissociation into 16S subunits is another unique property of chitosomes. These 16S subunits are the smallest molecular entities known to retain chitin synthetase activity. Further dissociation leads to complete loss of activity. From studies with secretory mutants, yeast researchers concluded that chitosomes were components of the endocytosis pathway. However, key structural and enzymatic characteristics argue in favor of the chitosome being poised for exocytotic delivery rather than endocytotic recycling. The chitosome represents the main vehicle for delivering chitin synthetase to the cell surface. An immediate challenge is to elucidate chitosome ontogeny and the role of proteins encoded by the reported chitin synthetase genes in the structure or function of chitosomes. The ultimate challenge would be to understand how the chitosome integrates with the cell surface to construct the organized microfibrillar skeleton of the fungal cell wall.

Introduction

The story of chitosomes can be traced back to Ruiz-Herrera's *in vitro* synthesis of chitin microfibrils reported in 1974. It was also the auspicious start of a close and long collaboration (Ruiz-Herrera & Bartnicki-Garcia, 1974). The discovery that visible quantities of chitin microfibrils could be made from UDP–*N*-acetyl-D-glucosamine (UDP-GlcNAc) by a solubilized extract from a fungus had important implications: (1) it validated Leloir's sugar nucleotide pathway for chitin synthesis, for which only trace (radioactive) evidence had existed (Glaser & Brown, 1957); (2) it demonstrated that not just the carbohydrate polymer but an entire microfibril – the structural unit of the fungal cell wall – could be assembled outside the cell; (3) it paved the way for elucidating the intracellular location of the chitin-synthesizing enzyme and the subsequent discovery and characterization of chitosomes. Besides chitin and chitosomes, Ruiz-Herrera had multiple research and academic interests evident in his prolific career (Bartnicki-Garcia, 2006).

Synthesis of chitin microfibrils *in vitro*

Ruiz-Herrera found a clever way to extract the chitin-synthesizing activity from a crude membrane preparation of the fungus *Mucor rouxii*. The resulting, seemingly soluble, extract was capable of producing visible quantities of chitin microfibrils from the substrate UDP-GlcNAc. Amazingly, under the electron microscope, the resultant product was a pure meshwork of microfibrils resembling fragments of the fibrillar skeleton of isolated cell walls (Fig. 1). Clearly, the synthesis of cell wall microfibrils did not require the integrity of the living cell.

Search for the location of chitin synthetase in the cell

Having an enzymatic system to make chitin *in vitro* provided a golden opportunity to trace the location of the polymerizing enzyme in the fungal cell. It was soon realized that, despite the initial impression, the enzyme was not truly

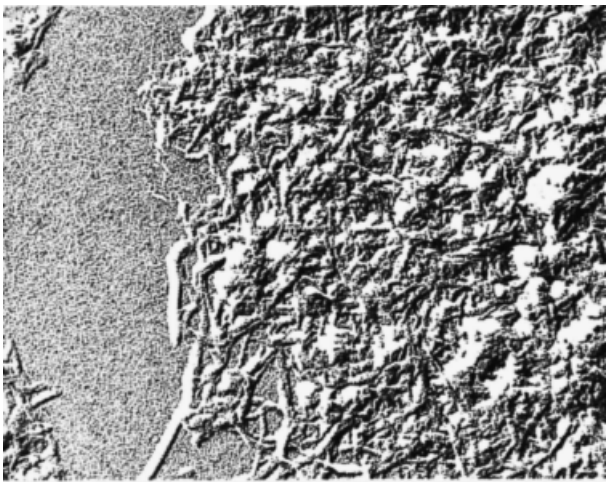


Fig. 1. Electron micrograph of chitin microfibrils synthesized *in vitro*, shadow cast with Pd (Ruiz-Herrera & Bartnicki-Garcia, 1974).

soluble but consisted of minute particles that could be sedimented by centrifugation at high speed. Electron microscopy of the sediment led to the notion that the chitin synthetase was in the form of granules (Ruiz-Herrera *et al*., 1975), but any further characterization was temporarily hampered by technical shortcomings in both electron microscopy and cell fractionation procedures. From his laboratory at Purdue University, aware of our past findings and intrigued by current difficulties in characterizing chitin synthetase particles, Charles Bracker offered to spend a sabbatical year in my laboratory at the University of California, Riverside, coincident with José Ruiz-Herrera's stay. That was a most fortuitous reunion, combining the rigorous standards of electron microscopy that had earned Bracker a worldwide reputation with the biochemical dexterity of Ruiz-Herrera.

Discovery of chitosomes

As this tripartite collaboration unfolded, it became evident that a major obstacle in the characterization of the chitin-synthesizing particles was the presence of an overwhelming number of ribosomes in the cell-free extracts. Despite the fact that initial sedimentations eliminated the vast majority of ribosomes, the sheer abundance of ribosomes in the cell-free extracts and the scarcity of chitin synthetase particles posed a serious problem. Ruiz-Herrera devised an intricate procedure (Fig. 2) to isolate the chitin-synthesizing particles by relying on the destruction of contaminating ribosomes by a massive dose of ribonuclease. The final step of the purification procedure was density gradient centrifugation. Painstakingly, Bracker examined each of the fractions under the electron microscope by a simple but powerful method: negative staining. It thus became unmistakably clear that the peak of chitin-synthesizing activity coincided with a population of microvesicles (Fig. 3). Instead of solid granules, the chitin synthetase particles turned out to be hollow microvesicles, to which the name chitosomes was ceremoniously attached (Bracker *et al*., 1976).

Chitosomes and chitin synthesis

Chitosomes were described as small spheroidal vesicles mostly 40–70 nm in diameter (Fig. 4). In thin sections, isolated chitosomes appear as microvesicular structures with a tripartite membrane 6.5–7.0 nm thick (Fig. 4). Under the electron microscope, similar structures can be seen in sections of whole cells (Bracker *et al*., 1976). Because of their unique size and low buoyant density, chitosomes could be detected without much difficulty in cell-free extracts of a wide range of fungi, including yeasts (Bartnicki-Garcia *et al*., 1978). The totality and reproducibility of the evidence collected prompted the conclusion that chitosomes were the cytoplasmic containers and conveyors of chitin synthetase zymogen en route to the cell surface (Bracker *et al*., 1976; Bartnicki-Garcia, 1990). The only fungus in which no chitosomes have been found is *Saprolegnia monoica* (Leal-Morales *et al*., 1997). This should not be too surprising, given the deep phylogenetic divergence between the *Oomycetes* and other fungi. Since chitin is not essential for the growth of *Saprolegnia monoica* (Leal-Morales *et al*., 1997), there seems to have been no need to develop a microvesicular vehicle suitable for microfibril assembly.

Chitosomes and fibrillogenesis *in vitro*

Apart from their significance in cell wall biogenesis, chitosomes provide an exquisite system for integrated ultrastructural–biochemical studies of microfibril biogenesis *in vitro*. Upon addition of substrate and activators, isolated chitosomes undergo a seemingly irreversible series of transformations (Bracker *et al*., 1976). The internal structure of the chitosomes changes, and a coiled microfibril (fibroid) appears in the chitosome. The shell of the chitosome is opened or shed, and an extended microfibril arises from the fibroid particle (Fig. 5). During prolonged incubation, the fibroid coils become less common and extended microfibrils appear thicker. This process raises intriguing questions as to how exactly the fibrils are made *in vivo* and how the chitosomes are integrated into the cell surface; that is, the topology of fibril formation in the living fungal cell is still unexplored territory.

Chitosome unique properties

Buoyant density

Low buoyant density is a distinguishing feature of fungal chitosomes (Bartnicki-Garcia *et al*., 1984; Ruiz-Herrera

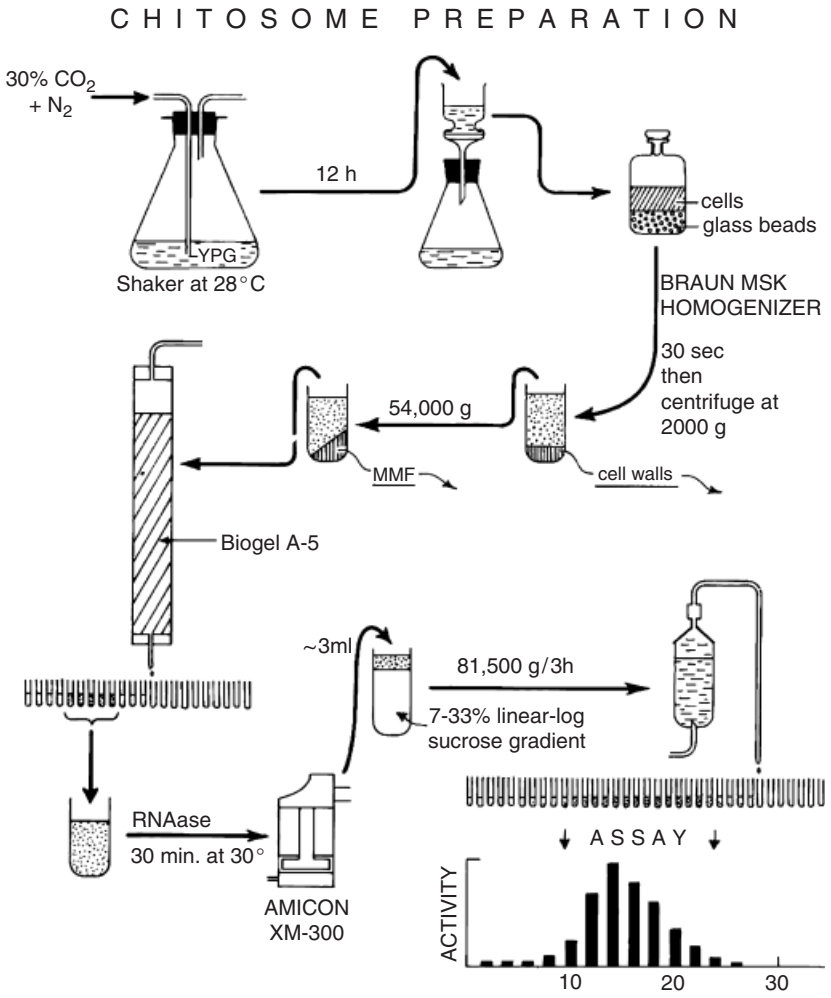


Fig. 2. Procedure for the isolation of chitosomes from fungal cell-free extracts (Bracker *et al.*, 1976; Ruiz-Herrera *et al.*, 1977).

et al., 1984; Leal-Morales *et al.*, 1988, 1994; Martinez *et al.*, 1989; Lending *et al.*, 1990; Kamada *et al.*, 1991a). Because of this and their small size, chitosomes can be effectively separated from other subcellular particles by centrifugation. Initially, rate-zonal sedimentation was suitable for isolating chitosomes as long as ribosomes had been eliminated by enzymatic digestion. By isopycnic centrifugation, it became possible to separate chitosomes directly from crude cell-free extracts without requiring RNase digestion; chitosomes from yeast cells of *M. rouxii* sedimented as a sharp symmetrical peak of chitin synthetase at a buoyant density of $d = 1.14–1.15\ g\ cm^{-3}$; the only significant contaminants were particles of fatty acid synthetase complex (Ruiz-Herrera *et al.*, 1984). With the use of high-performance rotors (angle or vertical) capable of generating much higher ***g*** forces, the isopycnic separation of chitosomes became more practical (Leal-Morales *et al.*, 1988; Lending *et al.*, 1990). The exact buoyant density of chitosomes varies according to the fungal species and also to the developmental state of the organism (Leal-Morales *et al.*, 1988; Martinez *et al.*, 1989; Kamada *et al.*, 1991a). Lipids are estimated to comprise one-third of the chitosome weight in *M. rouxii* (Hernandez *et al.*, 1981), thus raising the possibility that the variability in buoyant density of chitosomes results from differences in lipid composition.

Membrane thickness

One important distinction between chitosomes and other membranous organelles is the thickness of the membrane. The plasma membrane, the vacuole membrane and the membranes of secretory vesicles are considerably thicker (8–9 nm) than the chitosome shells (6.5–7 nm) (Fig. 4b; Bracker *et al.*, 1976).

Zymogenicity

Nearly all of the chitosomal chitin synthetase in cell-free extracts of *M. rouxii* occurred in a zymogenic form that required proteolytic activation (Ruiz-Herrera *et al.*, 1977).

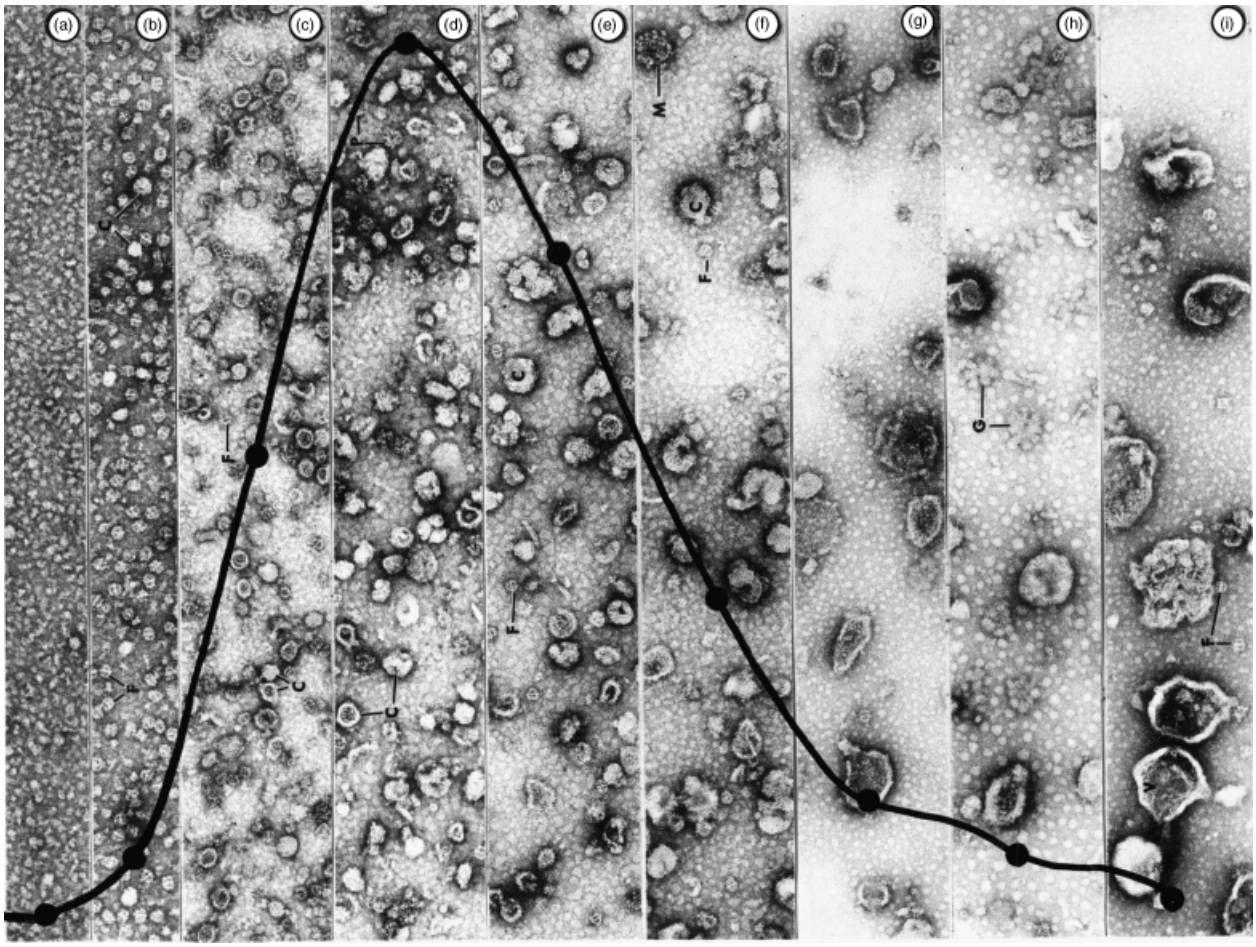


Fig. 3. Separation of chitosomes by velocity sedimentation on a 7–33% sucrose gradient (81 500 ***g*** for 3 h). Chitosomes are the predominant structure in the peak fraction of chitin synthetase (black line) (Ruiz-Herrera *et al.*, 1984).

Evidence of zymogenicity of chitin synthetase, first reported by Cabib & Farkas (1971), was found in crude preparations from all five fungal genera examined (Bartnicki-Garcia *et al.*, 1978). There were, however, specific differences among the fungi in the response of the zymogen to acid or neutral proteases and in the retention of zymogenicity during chitosome isolation. In assessing the degree or apparent lack of zymogenicity of a chitin synthetase preparation, it is important to consider that the cell-free extracts contain powerful proteases that not only activate the zymogen spontaneously but could also irreversibly destroy all chitin-synthesizing activity (Kamada *et al.*, 1991b).

Chitosome dissociation into 16S subunits

One of the most important but often overlooked discoveries was the reversible dissociation of chitosomes into 16S subunits (Ruiz-Herrera *et al.*, 1980). Digitonin causes disintegration of the chitosome and the release of a homogeneous population of chitosome subunits (7–12 nm) with a sedimentation coefficient of 16S (Fig. 6). Zymogenicity is retained with only a minor overall loss in chitin synthetase activity. To date, the 16S subunit, a ~500-kDa particle, is the smallest molecular entity known to retain chitin synthetase activity. The product synthesized by chitosome subunits was characterized by X-ray diffractometry as α-chitin and was by this criterion indistinguishable from α-chitin made by preparations of undissociated chitosomes. However, in the electron microscope, the chitin microfibrils made from chitosome subunits were in general much shorter than those produced by undissociated chitosomes and exhibited a needle-like appearance (Ruiz-Herrera *et al.*, 1980).

Self-assembly

One remarkable property of the 16S subunits is their intrinsic capacity for self-assembly into vesiculoid membranous structures upon removal of the digitonin

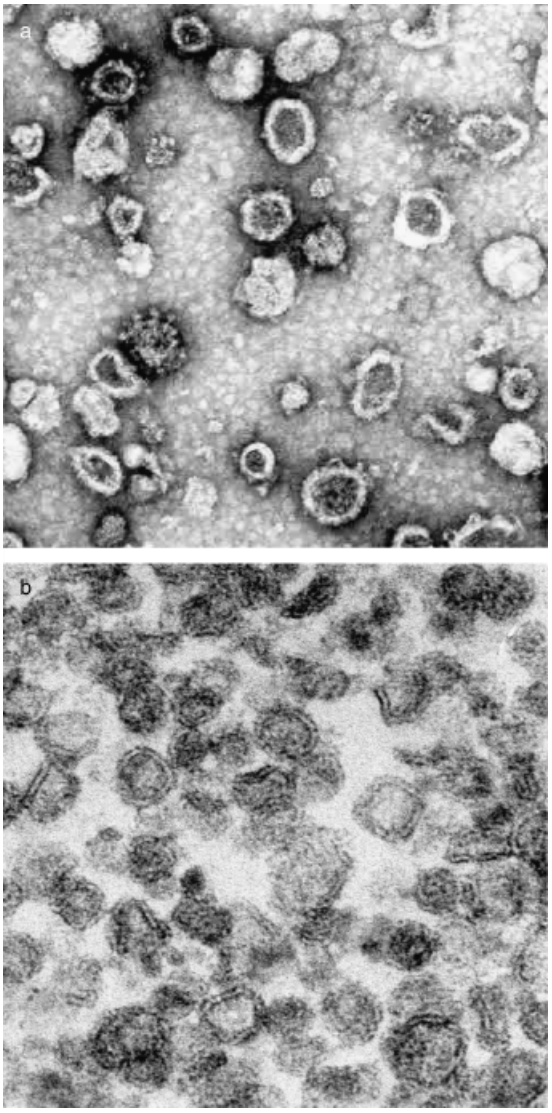


Fig. 4. Electron micrographs of chitosomes: (a) negative staining; (b) thin section of an embedded pellet of sedimented chitosomes (Bracker *et al.*, 1976).

(Fig. 6) (Bartnicki-Garcia *et al.*, 1979; Bartnicki-Garcia & Bracker, 1984). This ability to reconstitute membranes has been demonstrated for 16S subunits from *M. rouxii* and *Agaricus bisporus* (Hanseler *et al.*, 1983a). The self-assembling property poses the intriguing suggestion that chitosomes may not be generated within the conventional secretory path from endoplasmic reticulum to Golgi to vesicle, but instead result mainly from the self-assembly of membranes from 16S subunits synthesized either in the cytosol or inside vacuoles, creating the multivesicular bodies often seen in electron micrographs of fungi (Bracker *et al.*, 1976).

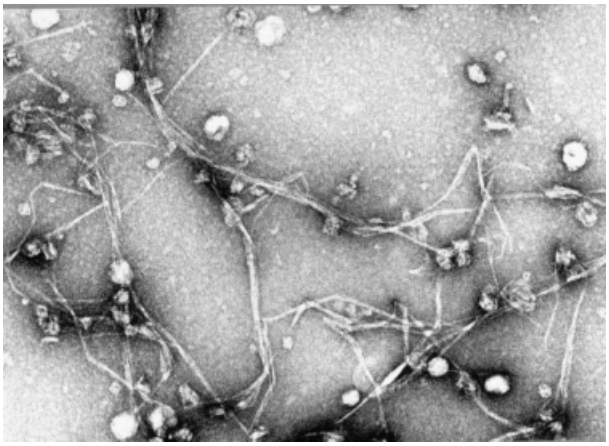


Fig. 5. Synthesis of chitin microfibrils by a chitosome preparation from *Mucor rouxii* incubated with substrate (UDP–*N*-acetyl-D-glucosamine) and activators (*N*-acetyl-D-glucosamine and acid protease) (Bracker *et al.*, 1976).

The purest form of active chitin synthetase – so far

Multiple efforts to further dissociate the 16S subunit, and identify the catalytic polypeptide(s), invariably led to complete loss of enzymatic activity. The highly purified 16S subunits yielded seven polypeptide bands, four of which (21, 23, 33 and 39 kDa) correlated tightly with chitin synthetase activity (Lending *et al.*, 1991). Significantly, there were no polypeptides in the dissociated 16S particles compatible with the larger size predicted by the sequences of the various chitin synthetase genes from fungi (Roncero, 2002; Ruiz-Herrera *et al.*, 2002).

Validity of the chitosome concept

The evidence obtained in various laboratories examining a wide diversity of fungi, notably *M. rouxii* (Bracker *et al.*, 1976), *Neurospora crassa* (Bartnicki-Garcia *et al.*, 1978, 1984), *Blastocladiella emersonii* (Mills & Cantino, 1981), *Phycomyces blakesleeanus* (Herrera-Estrella *et al.*, 1982), *A. bisporus* (Hanseler *et al.*, 1983b), *Candida albicans* (Gozalbo *et al.*, 1987) and *Saccharomyces cerevisiae* (Leal-Morales *et al.*, 1988), provided evidence for the existence of chitosomes throughout the fungi. Accordingly, these microvesicles constitute a separate secretory route distinct from the typical secretory pathway; chitosomes are an effective vehicle for delivering a protected cargo of latent chitin synthetase to the cell surface in an organized manner. However, other researchers challenged this concept by regarding chitosomes as either disruption artefacts or endocytosis products.

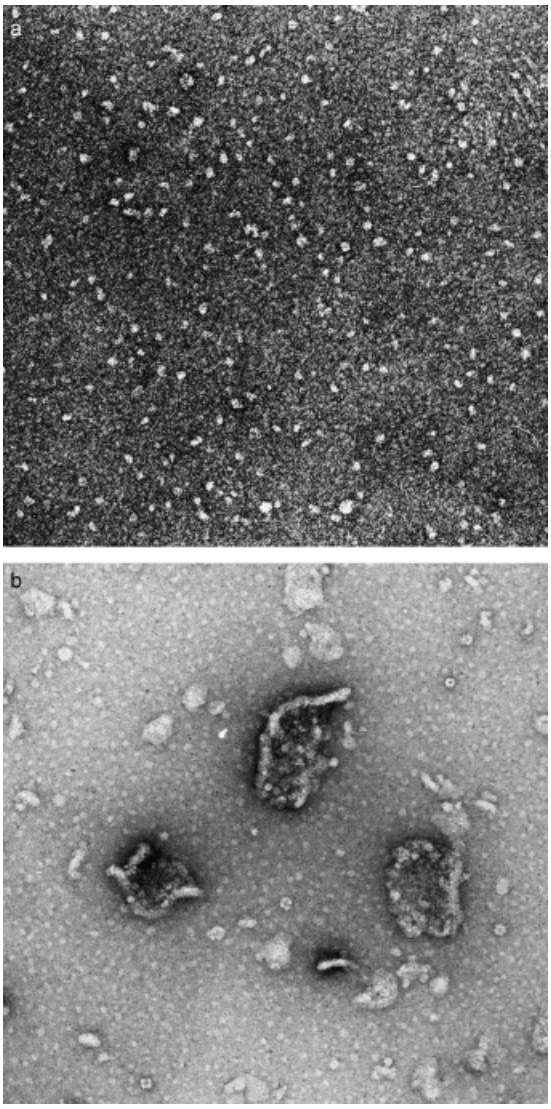


Fig. 6. Dissociation and reassembly of chitosomal membranes: (a) 16S particles purified after dissociation of chitosomes with digitonin (Ruiz-Herrera *et al.*, 1980); (b) reassembled vesiculoid membranes obtained after removal of digitonin (Bartnicki-Garcia *et al.*, 1979).

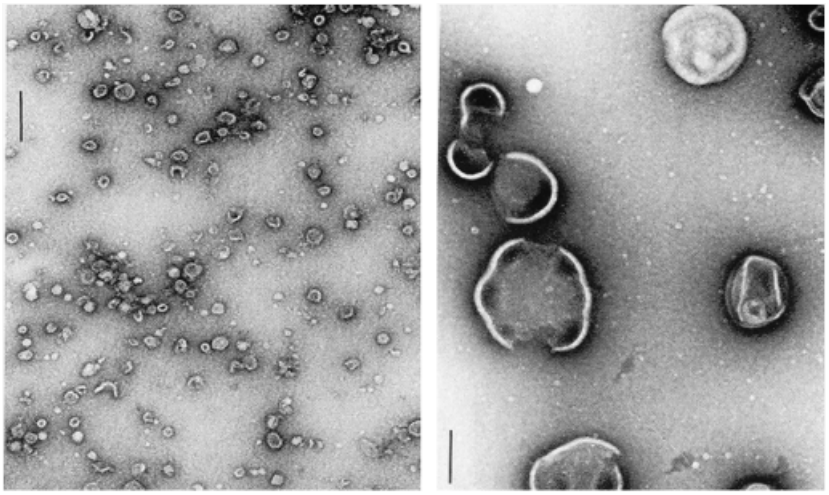
Disruption artefacts?

Initially, the chitosome concept led to considerable skepticism, particularly from Cabib and his coworkers (Duran *et al.*, 1979; Farkas, 1979; Cabib, 1981). Convinced that chitin synthetase was a plasma membrane-bound enzyme, they regarded chitosomes as artefacts of cell disruption. This initial rejection of the chitosome concept seemed strange, particularly as a microvesicle-bound enzyme provided a much-needed vehicle for delivering chitin synthetase to the plasma membrane/cell surface.

Much later, with the advent of more powerful centrifugation procedures, the main reason behind the controversial claims for the intracellular location of chitin synthetase was discovered (Leal-Morales *et al.*, 1988; Flores Martinez & Schwencke, 1988). With the moderately high speeds and short times utilized in earlier work (velocity sedimentation), the chitin synthetase from *Saccharomyces cerevisiae* sedimented as a single broad peak. However, using isopycnic sedimentation with high-perfomance rotors generating much greater forces (265 000 ***g***) and by spinning for a much longer time (21 h), it was possible to separate neatly two populations of chitin synthetase particles from cell-free extracts of exponentially growing cells of *Saccharomyces cerevisiae*. The two populations were of nearly equal abundance, but with markedly different buoyant densities and particle diameters. One population contained 45–65% of the total chitin synthetase and was identified as chitosomes because of microvesicular size (median diameter = 61 nm) and characteristic low buoyant density (1.15 g cm^{-3}); it also lacked β-1,3-glucan synthetase activity. The second population (35–55%) was identified as plasma membrane because of its high buoyant density (1.22 g cm^{-3}), large vesicle size (median diameter = 252 nm), and the presence of vanadate-sensitive ATPase. This fraction cosedimented with the main peak of β-1,3-glucan synthetase, a bona fide plasma membrane component (Fig. 7).

Endocytosis vesicles?

On the basis of studies with secretory mutants of *Saccharomyces cerevisiae*, Schekman and coworkers (Chuang & Schekman, 1996; Ziman *et al.*, 1996) confirmed the existence of chitosomes, but they regarded chitosomes as a product of endocytosis, instead of having the exocytotic role described above. However, this view overlooks the published body of experimental evidence and related physiologic conclusions that argue against chitosomes being primarily products of endocytosis. A major endocytic derivation of chitosomes from the plasma membrane runs contrary to the following observations. The substantial difference in membrane thickness between the chitosome membrane and the plasma membrane or the membranes of typical secretory vesicles makes an endocytic ontogeny unlikely. The almost full zymogenicity of the chitin synthetase in chitosomes makes improbable an endocytic derivation from the plasma membrane, where exposure to periplasmic or plasma membrane-bound proteases would have activated the enzyme irreversibly. β-1,3-Glucan synthetase, a marker of the yeast plasma membrane, is absent from the chitosome fraction (Leal-Morales *et al.*, 1988). There is also the growing realization that despite many common features between yeast and hyphal morphogenesis, there are some key cellular and molecular differences between these two modes of development (Harris & Momany, 2004; Harris *et al.*, 2005). The growing hyphal tip requires a continuous source of fresh chitin synthetase (McMurrough *et al.*, 1971). Chitin



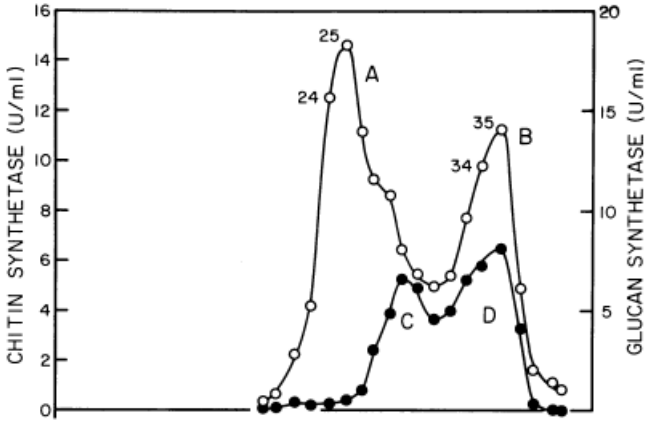


Fig. 7. Isopycnic separation of two chitin synthetase populations from a crude homogenate of *Saccharomyces cerevisiae*. The sample was applied to a 10–65% sucrose gradient and centrifuged in a 70 Ti rotor at 265 000 ***g*** for 21 h. Open circles indicate the peaks of chitin synthetase activity. Peak A contains chitosomes shown in the electron micrograph on the top left. Peak B contains plasma membrane fragments shown in the electron micrograph on the top right. For reference, the distribution of β-1,3-glucan synthetase activity (solid circles) was determined; most of this cosediments with the plasma membrane peak, and essentially none with the chitosome peak.

synthetase recycling does not solve the supply problem. An endocytic origin and recycling to the plasma membrane (Valdivia & Schekman, 2003) suggested by yeast mutant studies may represent a secondary route that could involve a separate subpopulation of microvesicles. In either case, the physiologic significance of the recycling of chitin synthetase zymogen remains an open question.

Conclusion

The extensive structural and biochemical evidence gathered on the existence and uniqueness of chitosomes supports the role of chitosomes as major containers of chitin synthetase in the cell. The prodigious growth rate of the hyphae of many fungi depends on a highly efficient and sharply polarized secretory apparatus; the conspicuous accumulation of vesicles at the tip (i.e. the Spitzenkörper) is testimony to this activity. The presence of at least two distinct populations of vesicles, macrovesicles and microvesicles, became evident in the earlier electron-microscopic studies of hyphal tip cytology (Girbardt, 1969; Grove & Bracker, 1970). The identification of a chitin-synthesizing role for the microvesicles suggests a clear division of labor. Whereas the polymers and enzymes that comprise the amorphous phase of the wall are secreted in macrovesicles, i.e. typical secretory vesicles, chitin synthetase zymogen is delivered separately in microvesicles. This division of labor makes physiologic sense. The chitosomes are safe vehicles to supply the growing tip with latent enzyme. Given that the substrate UDP-GlcNAc is present throughout the cytosol (Martinez *et al.*, 1987), zymogenicity is essential to prevent the premature synthesis of chitin inside the cell. Only after the zymogen is delivered at the surface will the enzyme become activated and microfibrils be formed external to the cell.

To complement existing autoradiographic (Sentandreu *et al.*, 1984) and immunologic (Sietsma *et al.*, 1996) evidence for the intracellular presence of chitosomes, the immediate challenge is to demonstrate the operation of chitosomes *in vivo* by constructing green fluorescent protein fusions to trace the path of the various chitin synthetase gene products in the cell (Riquelme *et al.*, 2006). The ultimate challenge will be to elucidate how the chitosome integrates or interacts with the plasma membrane to deliver,

deploy and activate the chitin synthetase zymogen, and thus begin to unravel the secrets of the final steps in the assembly of a fungal cell wall. Equally important will be to determine the role of the various chitin synthetase genes in the structure and function of the chitosome and resolve the contradiction that exists between the low molecular size of the polypeptides dissociated from purified 16S particles and the much larger size of the polypeptides encoded by the various chitin synthetase genes (Roncero, 2002; Ruiz-Herrera *et al*., 2002).

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