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EVIDENCE THAT *BLASTOCLADIELLA EMERSONII* ZOOSPORE CHITIN SYNTHETASE IS LOCATED AT THE PLASMA MEMBRANE

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***Blastocladiella emersonii* zoospores are not encased by a cell wall and do not detectably synthesize or contain chitin; accompanying de novo cell wall formation during zoospore encystment, chitin rapidly accumulates and is incorporated into the cell wall. Essential for understanding this abrupt change in chitin synthesis is the location of zoospore chitin synthetase. The enzyme has previously been reported to be sequestered with distinctive cytoplasmic organelles (gamma particles) characteristic for the zoospore cell type. Using similar differential and equilibrium density centrifugation procedures to those reported previously, we have observed the vast majority of zoospore homogenate chitin synthetase activity in fractions distinct from the gamma particle-enriched fractions. Over 90% of the homogenate enzyme activity could be recovered in a sucrose buoyant density region (1.14–1.18 g/ml) containing membranous elements and well separated from the region enriched for gamma particles (1.30–1.34 g/ml). When zoospores were surface-labelled with [^{3}H]concanavalin A prior to homogenization, the buoyant density regions of radioactivity and of chitin synthetase activity exhibited nearly complete coincidence. At least the bulk of zoospore chitin synthetase appears to be located at the plasma membrane, rather than in gamma particles.**

Introduction

Several fungi have been used in recent years to investigate cell wall biogenesis at the cellular level (see Refs. 1 and 2 for recent reviews). Aquatic fungi continue to furnish exceptional material for certain aspects of this study in that a cell wall appears de novo during an abrupt transition in the life cycle (encystment). Chitin, a homopolymer of *N*-acetylglucosamine (GlcNAc), is the major macromolecular component of the cell wall of the aquatic fungus, *Blastocladiella emersonii* [3]. Accompanying the transition from the free-swimming, wall-less zoospore to the sessile, wall-encased cyst, chitin accumulates and is incorporated into the cell wall [4].

B. emersonii zoospores do not contain enough substrate (UDP-GlcNAc), by over an order of magnitude, to account for the amount of chitin appearing during zoospore encystment [4,5]. However, all four hexosamine pathway-specific enzymes, as well as chitin synthetase (UDP-2-acetamido-2-deoxy-D-glucose: chitin 4-β-acetamidodeoxy-D-glucosyltransferase, EC 2.4.1.16), have been observed at high specific enzyme activities in zoospore extracts [5–9]. In addition, zoospores have been reported to contain an unusual glycolipid primer for chitin synthesis [10,11]. Chitin accumulation and cell wall biogenesis occur on schedule when zoospores encyst in the presence of protein synthesis-inhibiting doses of cycloheximide [4,12]. Thus, post-translational controls presumably regulate the absence of the cell wall and of chitin synthesis during the zoospore phase of the

life cycle, as well as the abrupt activation of chitin synthesis and of cell wall biogenesis during encystment.

A model has been proposed [13,14] whereby zoospore chitin synthetase is localized in characteristic cytoplasmic organelles, the gamma particles; according to this model, the enzyme is delivered to the cell surface during de novo cell wall biogenesis via release of vesicles from the gamma particles and fusion of these vesicles with the plasma membrane. The distribution of enzyme activity in zoospore homogenates has previously been presented in terms of chitin synthetase specific activities (activities/protein) in certain homogenate fractions [13]. In this paper, we follow the proportions of total enzyme activity present in different homogenate fractions, with and without prelabelling of the zoospore cell surface with [^{3}H]concanavalin A. Fractions are also viewed by transmission electron microscopy. The bulk of catalytically active chitin synthetase appears to be located at the zoospore plasma membrane.

Materials and Methods

Chemicals. UDP-[^{14}C]GlcNAc was obtained from New England Nuclear Co. and Amersham Corp. [^{3}H]Concanavalin A was obtained from New England Nuclear Co. Nonradioactive chemicals were obtained from Sigma Chemical Co. except where otherwise noted. Concanavalin A was purchased from Miles Co., Ficoll from Pharmacia and Renografin-60 from Squibb.

Cell culture. Stock cultures were initiated from clonal plates monthly and were maintained as mass cultures on standard Cantino peptone-yeast extract-glucose agar by subculturing on a 24 h, 20°C life cycle schedule [15]. Freshly released zoospores were harvested after flooding the agar cultures with sterile distilled water. Zoospore suspensions were immediately filtered through Whatman 541 filter paper to remove cellular material other than zoospores and the filtrates were washed with cold distilled water by centrifugation at 4°C (5–10 min at $500 \times g$ in an International Centrifuge swinging bucket head No. 269). Growth cultures (plant cells) were prepared using the medium and suspension culture conditions previously described [16] except that one liter water-jacketed spinner flasks (Bellco) were used. The cultures were harvested by filtration after 6 h at 27°C and the plant cells collected on the filters were washed by centrifugation as above.

Cell homogenate preparation. The method for osmotic lysis was adapted from that described by Myers and Cantino [13]. Washed zoospore pellets were resuspended at final concentrations of 10^8 cells/ml in cold 1 M sucrose containing 25 mM potassium phosphate buffer, 1 mM KCl and 0.5 mM EGTA (phosphate /KCl/EGTA) at pH 6.8 and were incubated in an ice-water bath for 5 min. After centrifugation at $500 \times g$ for 4 min at 4°C (using the same centrifuge head as above, with 15 ml adapters in the 50 ml tube holders), the pellets were resuspended to concentrations of $5 \cdot 10^8$ cells/ml in phosphate/KCl/EGTA containing 0.4 M sucrose and incubated in an ice-water bath for 10 min to permit cell lysis. Lysates were centrifuged at $500 \times g$ for 5 min at 4°C and the supernatants were centrifuged at $5000 \times g$ for 5 min at 4°C in a Sorvall HB-4 swinging bucket head. The resulting supernatants were harvested and used as the starting material for analyses of chitin synthetase activity. In certain experiments, zoospores were stained with neutral red prior to osmotic shock [13]; washed zoospore pellets were resuspended in 50 μg/ml neutral red (Allied Chemical Corp.), held for 15 min in an ice-water bath and centrifuged at $500 \times g$ for 5 min at 4°C. The pellets were then carried through the osmotic lysis procedure, as above.

To prepare homogenates by sonication, washed cell pellets were resuspended in cold 25 mM Tris-HCl, pH 8.5 at concentrations of $5 \cdot 10^8$ cells/ml for zoospores and of $5 \cdot 10^7$ cells/ml for plants. The suspensions were held on ice and subjected to 10-s bursts with a Branson Sonifier set at 40 watts output, with one min between bursts to allow cooling. Two or three bursts were sufficient to disrupt zoospores while five to ten bursts were necessary to break over 90% of the plants. Homogenates were centrifuged at $500 \times g$ for 4 min at 4°C to remove any remaining intact cells or plant cell walls.

To prepared zoospore homogenates by Dounce homogenization, conditions were identical to those used for sonication except that a glass homogenizer with a tightly fitting plunger (Kontes) was

used to disrupt cells. Approximately 75 strokes of the plunger were required.

[^3H]Concanavalin A labelling of zoospores. Washed zoospore pellets were resuspended in 25 mM Tris-HCl containing 5 mM $MgCl_2$, pH 8.5 (Tris-HCl/$MgCl_2$) and pelleted at $500 \times g$ for 10 min. These pellets were resuspended at 10^9 cells/ml in Tris-HCl/$MgCl_2$ containing 0.50 μCi [^{3}H]concanavalin A (0.25 mg/ml) and held for 10 min at 23°C. The cells were then washed several times with Tris-HCl/$MgCl_2$ by centrifugation ($500 \times g$, 5 min, 4°C) to remove unbound label. Final pellets were resuspended in Tris-HCl/$MgCl_2$ containing 1 mg/ml DNAase, homogenized as noted in Results, incubated for 5 min at 23°C and centrifuged at $500 \times g$ for 5 min. Supernatants were then used as described in Results. When using the osmotic lysis procedure, cell pellets were resuspended, incubated and washed in phosphate/KCl/EGTA rather than Tris-HCl/$MgCl_2$ and the DNAase treatment was omitted. [^{3}H]Concanavalin A radioactivity was quantitated by counting samples, in Aquasol, with a Beckman liquid scintillation spectrometer.

In control experiments, the concanavalin A treatment was found not to alter total chitin synthetase activity recovered, regardless of the method of homogenization employed. The concanavalin A procedure employed appeared to 'stabilize' the plasma membrane, as in similar procedures used with *Neurospora crassa* [17] and *Saccharomyces cerevisae* [18]. Zoospore preparations were always examined under phase contrast optics after concanavalin A labelling as well as after the washing procedures. The cells appeared intact and in any case, the debris from any lysed cells would have been removed during the extensive washing procedure following the concanavalin A-labelling treatment. The cells also appeared to be stabilized as judged by the increased difficulty in breaking lectin-treated zoospores by Dounce homogenization relative to control cells.

Gradient centrifugation. Sucrose, Ficoll and Renografin gradients were preformed with an ISCO model 570 gradient former and were centrifuged in SW41Ti or SW39L rotors in a Beckman L2-65B ultracentrifuge as specified in the figure legends. Gradient fractions were collected by pumping with a fluorinert chase solution (ISCO) using an ISCO model 180 pump, a model 184 gradient fractionator and a Gilson drop-counter fraction collector, usually set to deliver 0.25 ml fractions. The positions of neutral red and other visible (turbid white) bands of homogenate material in the gradients were indexed prior to collecting fractions. Sucrose and Ficoll concentrations in gradient fractions were determined using an Abbe refractometer. Renografin concentrations were determined by weighing known volumes of each fraction.

Chitin synthetase assay. 15 μl of sample was incubated with 15 μl of reaction mixture containing (final concentrations) 12 mM $MgCl_2$, 20–50 mM Tris-HCl, pH 8.5, 20 mM GlcNAc and 2–10 mM UDP-[^{14}C]GlcNAc (0.09–0.45 Ci/mol). Incubation was at 23°C for 15–30 min and the reaction was stopped by addition of 5 μl glacial acetic acid or by immersion in a boiling water bath for 3 min. The lower buffer concentration was used in more recent experiments since it was observed to yield higher enzyme activity. The substrate concentration and incubation time were constant within each experiment, but varied between experiments according to the enzyme activity expected. Reaction mixtures containing samples from the osmotic lysis procedure contained halfstrength phosphate/KCl/EGTA. Enzyme activity did not differ ($\pm$ 10%) when phosphate buffer was used in place of Tris-HCl buffer, or over the range pH 7–9. Assays were routinely performed in duplicate, except for gradient fractions, which were assayed singly.

Reaction mixtures were spotted on Whatman No. 1 filter paper and developed by descending chromatography for 18 h using ethanol/1 M ammonium acetate, pH 7.0 (7:3, v/v) or isobutyric acid/1 M ammonium hydroxide (5:3, v/v). Chitin remained at the origin while substrate did not [19]. The origins were cut out and counted in PPO/toluene (4 g/liter) in a Beckman liquid scintillation spectrometer.

Electron microscopy. Prefixation and then fixation were with 0.02% and 0.1% osmium tetroxide, respectively, in buffered 1.25% poly(vinyl alcohol), as previously described [20]. Pelleted samples were then washed, embedded in plastic, sectioned, mounted and stained as previously described [20]. Several randomly selected regions of pelleted samples were viewed.

Results

Differential and gradient centrifugation analyses of chitin synthetase activity

Previous work on the cellular location of zoospore chitin synthetase (Ref. 13; see also Refs. 10 and 14) utilized homogenates prepared by osmotic lysis; chitin synthetase activities were monitored in fractions enriched for gamma particles after differential centrifugation (20000 × *g* pellets) followed by sucrose density centrifugation (peak density of gamma particles at 1.32 g/ml). Here, homogenates have been prepared by three methods, including osmotic lysis, and the distributions of enzyme activity have been analyzed after differential centrifugation and after density gradient centrifugation in sucrose, Ficoll or Renografin.

Only a minor fraction of homogenate enzyme activity sedimented at 20000 × *g* (less than 20% in six experiments using homogenates prepared by osmotic lysis; see experiment of Fig. 1, below) and the remainder was recovered in the corresponding 20000 × *g* supernatants. The data of Table I indicate that chitin synthetase activity in zoospore homogenates resides in physically heterogeneous particulate material, with increasing enzyme activities recovered in pelleted material with increasing centrifugal forces. Virtually complete recovery of enzyme activity was observed in 100000 × *g* pellets of homogenates prepared by osmotic lysis, sonication (96%, Table I) or Dounce homogenization.

TABLE I

DIFFERENTIAL CENTRIFUGATION OF ZOOSPORE CHITIN SYNTHETASE ACTIVITY

Zoospore homogenates were prepared by sonication as described in Materials and Methods, except that the suspending solution was 50 mM Tris-HCl, pH 8.5 containing 5 mM $MgCl_2$. Homogenates were centrifuged at 5000 × *g* for 15 min and, in separate experiments, the supernatants were recentrifuged at the indicated centrifugal forces for 30 min. The 'fraction activity sedimented' refers to the ratio of chitin synthetase activity in the final pellet to the sum of the activity in the final pellet plus final supernatant. In each experiment, the latter sums corresponded closely with the enzyme activities measured in the respective 5000 × *g* supernatants used as starting materials for the centrifugations.

$\times g_{av}$	Fraction activity sedimented
20000	0.15
32000	0.70
51000	0.78
73000	0.85
100000	0.96

Both crude homogenates and 20000 × *g* pellets were analyzed by density gradient centrifugation. Representative sucrose gradient analyses of enzyme activity are shown in Fig. 1. This experiment utilized an osmotic lysis homogenate in which the 20000 × *g* pellet contained 17% of the crude homogenate enzyme activity. With both the crude homogenate (Fig. 1A) and the 20000 × *g* pellet (Fig. 1B), the main buoyant density region of enzyme activity occurred over the range 1.14–1.18 g/ml. The same buoyant density region, containing ⩾ 90% of the homogenate activity, has been observed in additional experiments using crude homogenates prepared by osmotic lysis (three experiments), sonication (three experiments) or Dounce homogenization (two experiments). Centrifugation from one to nineteen hours did not alter the peak density of enzyme activity (average for all experiments, 1.165 g/ml). Very small percentages of enzyme activity (less than 10%, usually less than 5%) were also observed at the bottom of sucrose gradients (see Fig. 1) at a density range (1.30–1.34 g/ml) characteristic for gamma particles (Ref. 13 and below). One main region of enzyme activity was also observed with equilibrium Ficoll or Renografin gradients with average peak densities of 1.18 g/ml and 1.19 g/ml respectively, again clearly separated from the characteristic gamma particle buoyant density region.

In the experiment of Fig. 1, the zoospores were prestained with neutral red, a putative vital stain for gamma particles (see Ref. 13). Observations by light microscopy of both intact cells and homogenates did reveal that very small (less than 1 μm diameter) particles were stained by the dye. The only visibly colored region observed with sucrose gradients was a discrete pink band (band B, Figs. 1A and B) at a buoyant density of 1.14 g/ml. A visually dense, white band (band C) did coincide with the peak position of chitin synthetase activity with both crude homogenates (Fig. 1A) and 20000 × *g* pellets (Fig. 1B). This correlation was not altered when cells were not prestained with neutral

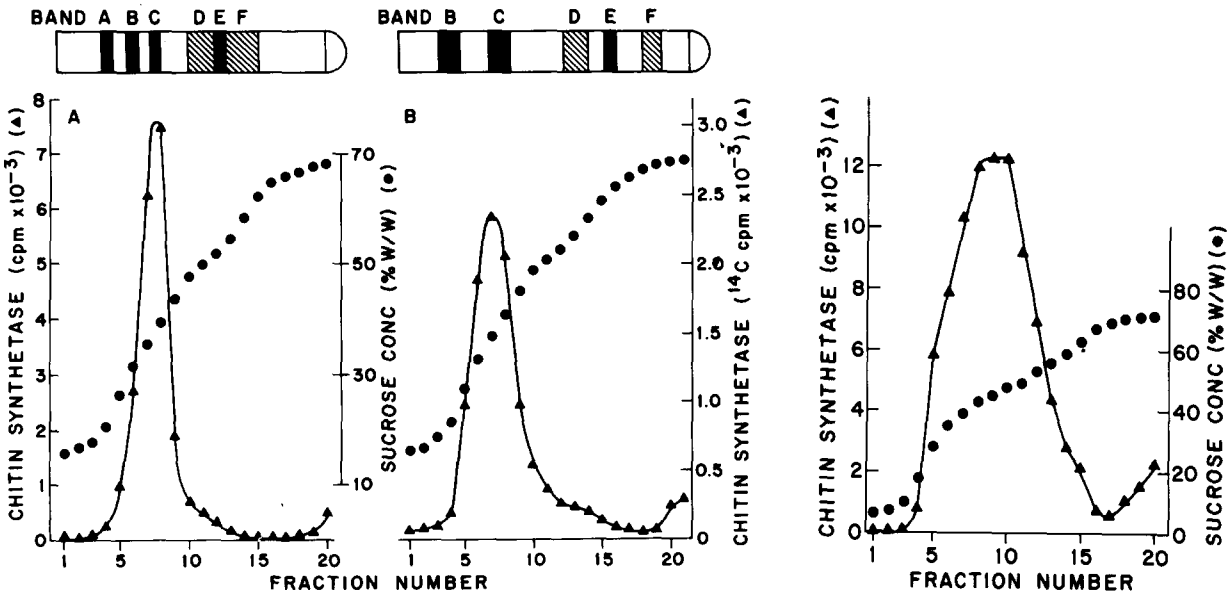

Fig. 1. Equilibrium sucrose gradient centrifugation of zoospore chitin synthetase activity. Zoospores were prestained with neutral red, lysed by osmotic shock and processed through sucrose gradient centrifugation as described in Materials and Methods. The respective samples were layered over a 4 ml 0.4–2.7 M sucrose gradient in phosphate/KCl/EGTA and centrifuged at 130000×*g* for 2 h at 2°C in a SW39L rotor. The positions of visible bands in the gradients were as recorded at the top of each graph. Hatched bands were diffuse and dark bands were dense. Only band B was pink-colored; the remainder were turbid white. Equal volume gradient fractions were assayed for chitin synthetase activity (▲) and for sucrose concentration (●). (A) One ml of a 5000×*g* supernatant was layered over the gradient. Enzyme specific activities (nmol GlcNAc incorporated per min per mg protein) were as follows in this experiment: 5000×*g* supernatant, 17.7; combined chitin synthetase peak fractions 7 and 8, 84; combined gamma particle-enriched fractions 19 and 20, 55 (values for this experiment were representative with respect to four independent experiments). (B) The 5000×*g* supernatant was first centrifuged at 20000×*g* (Sorvall HB-4 rotor) for 20 min at 4°C and the pellet was resuspended in 0.4 M sucrose in phosphate/KCl/EGTA before layering one ml over the gradient. Enzyme specific acivities, in the same terms as above, were: 5000×*g* supernatant, 12; 20000×*g* supernatant, 11.3; 20000×*g* pellet, 70 (values for this experiment were representative with respect to six independent experiments). The enzyme specific activities for chitin synthetase peak fraction 7 and the combined gamma particle-enriched fractions 20 and 21 were 173 and 279, respectively. The values for gamma particle-enriched fractions in A and B were subject to imprecision because the protein concentrations were near the lower limit of assay.

Fig. 2. Equilibrium centrifugation of chitin synthetase activity from vegetative cells. Plant cell homogenates were prepared by sonication in phosphate/KCl/EGTA buffer, as described in Materials and Methods, and were centrifuged at 5000×*g* for 5 min at 4°C. One ml of the 5000×*g* supernatant was layered on 4 ml of a 0.4 M–2.7 M sucrose gradient in phosphate/KCl/EGTA and centrifuged exactly as in Fig. 1. Equal volume gradient fractions were assayed for chitin synthetase activity (▲) and sucrose concentration (●).

red and a dense white band at the same buoyant density position was also observed after the two other methods of homogenate preparation.

For comparative purposes, homogenates were prepared by sonication from growing plants, lacking gamma particles. One main region of enzyme activity was observed, again clearly separated from a minor region at the bottom of the gradient (Fig. 2). In different experiments, the main region of enzyme activity exhibited either a narrower distribution over the density range characteristic for zoospore homogenates or a somewhat broader distribution with a peak density at about 1.20 g/ml (Fig. 2). It is likely that the latter distribution resulted from the variable retention of attached, small cell wall fragments during sonication (the larger fragments would have been removed during the preliminary low speed centrifugation; zoospore homogenates are not subject to this source of variation since zoospores lack a cell wall).

Electron microscopic examinations of centrifugal fractions

Zoospore extracts prepared by the osmotic lysis procedure were separated into 20000 × *g* pellet supernatant fractions and the two fractions were separately examined by transmission electron microscopy. In the experiment from which the micro-

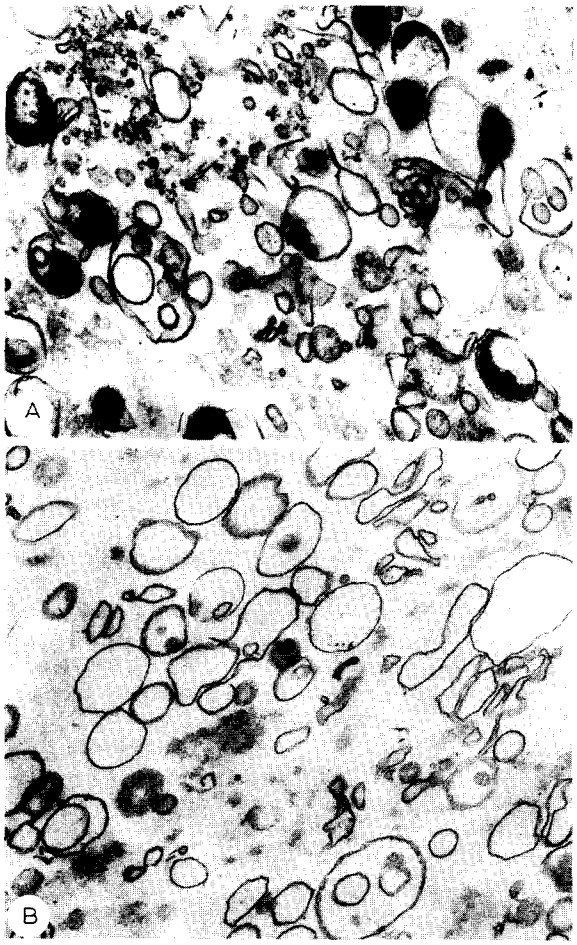

Fig. 3. Electron micrographs of fractions prepared by differential centrifugation (×17850; 1 μm=17.85 mm). A 5000×*g* supernatant from a zoospore homogenate prepared by the osmotic lysis procedure was centrifuged at 20000×*g* for 20 min at 2°C. (A) Thin section micrograph of the 20000×*g* pellet fraction, which contained 19% of the chitin synthetase activity. The objects with electron dense interiors are sections through gamma particles. (B) Thin section micrograph of the 20000×*g* supernatant fraction, which contained 81% of the chitin synthetase activity. Note the absence of sections through gamma particles (occasionally such sections were seen, but at a frequency certainly less than one per field at ×8800 microscope magnification).

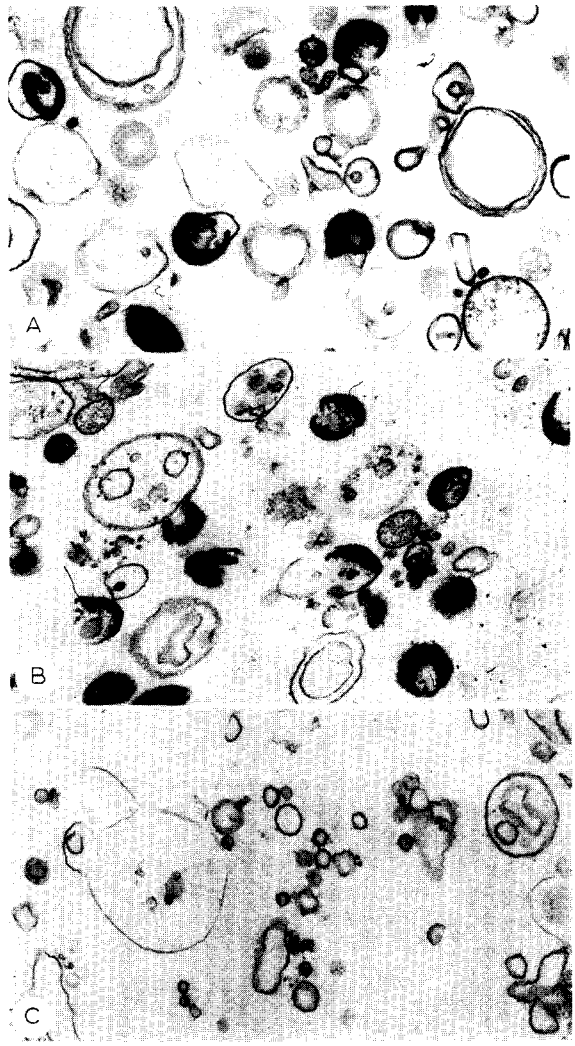

Fig. 4. Electron micrographs of sucrose density gradient fractions. The fractions were from an experiment performed exactly as in Fig. 1A; the positions of visible bands and the distribution of chitin synthetase activity in the gradient were as depicted in Fig. 1A. All micrographs are ×17850 final magnification, as in Fig. 3. (A) Thin section micrograph of the 5000×*g* supernatant material layered on the gradient. (B) Representative thin section micrograph of material observed in the most dense (1.30–1.34 g/ml) region of the gradient. (C) Representative micrograph of material observed in the peak chitin synthetase activity fractions (as fractions 7 and 8, Fig. 1A at 1.16–1.17 g/ml buoyant density). Compare the presence of sections through gamma particles in A and B vs. their absence in C (such sections were exceptionally observed in viewing many thin sections from peak enzyme activity fractions, but certainly much less frequently than once per microscopic field at 8800×magnification).

graphs shown were taken, 19% of the chitin synthetase activity resided in the 20000 × *g* pellet fraction whereas the remainder (81%) resided in the 20000 × *g* supernatant fraction. A striking difference was observed; micrographs of the 20000 × *g* pellet exhibited many typical sections through gamma particles (Fig. 3A; particles with characteristic electron dense interiors), confirming data previously reported [13], whereas gamma particles were virtually absent in the 20000 × *g* supernatant (Fig. 3B). In addition to gamma particles, sections through various other membranous elements were routinely observed in 20000 × *g* pellets (Fig. 3A). These observations were confirmed in an independent experiment.

Sucrose gradient fractions from two independent experiments performed exactly as in Fig. 1A were also examined by electron microscopy. In each experiment, as in Fig. 1A, over 90% of the chitin synthetase activity was recovered in a symmetrical region with peak density at 1.16–1.17 g/ml and the dense white band C (Fig. 1A) was observed at this position. The material layered onto such gradients, the 5000 × *g* supernatant of zoospore osmotic shock lysates, displayed a variety of membranous elements in thin section, including gamma particles (Fig. 4A). With respect to the gradient fractions, sections through gamma particles were predominantly observed in the bottommost, 1.30–1.34 g/ml fractions (Fig. 4B), confirming previous results [13]. Micrographs of these fractions also displayed other membranous elements, as in Fig. 4B. Micrographs of the peak enzyme fractions were notable for the scarcity of gamma particle sections (Fig. 4C). Several of the membranous elements in the latter micrographs measured 1.5–2 μm in diameter, or one-fourth the diameter of the zoospore, supporting the interpretation that these elements were fragments of the plasma membrane. However, since cross-sectional size heterogeneities of membranous elements were observed in several other gradient fractions, firmer evidence was sought for the plasma membrane location of chitin synthetase activity.

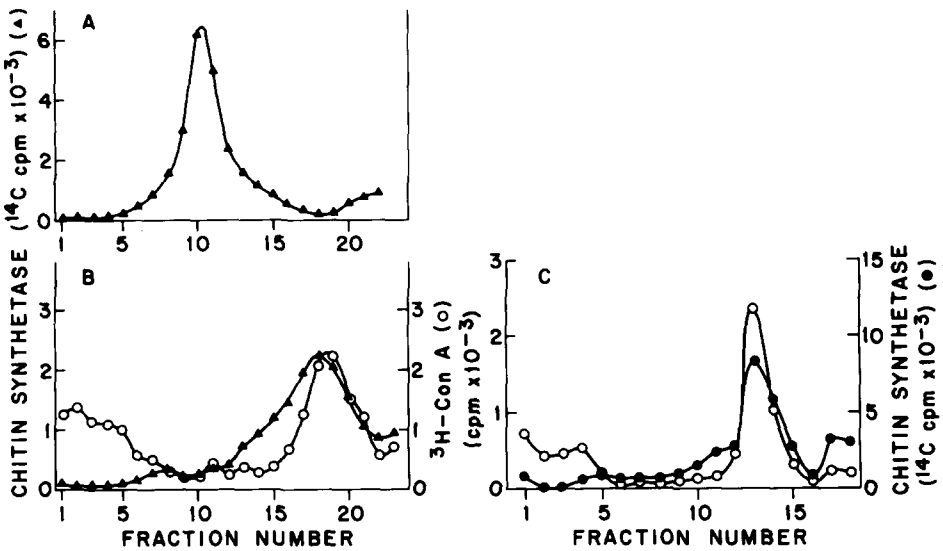

Fig. 5. Sucrose gradient centrifugation of chitin synthetase activity and [^{3}H]concanavalin A. Zoospores were prelabelled with [^{3}H]concanavalin A, washed, Dounce homogenized, incubated with DNAase and then centrifuged at 500 × *g* as described in Materials and Methods. Concanavalin A was either omitted (A) or included (B and C) during otherwise identical procedures. The analyses in A and B were from the same experiment, using the same original zoospore population, whereas the sample analyzed in C was from a separate experiment. In each graph, one ml of a 500 × *g* supernatant was layered on a 4 ml 0.15–2.1 M sucrose gradient (vs. the 0.4–2.7 M gradients of Fig. 1) in Tris-HCl/$MgCl_2$ and each gradient was centrifuged at 130000 × *g* for 1 h at 2°C in an SW39L rotor. Gradient fractions were assayed for chitin synthetase activity (▲, in A and B; ●, in C), [^{3}H]concanavalin A radioactivity (○, in B and C) and sucrose concentration (not shown). Peak enzyme activities occurred at buoyant densities of 1.16–1.17 g/ml in A, 1.19 g/ml in B and 1.20 g/ml in C.

Gradient analyses of [^{3}H]concanavalin A-labelled cells

Zoospores were labelled with [^{3}H]concanavalin A under conditions where the cells remained intact (see Materials and Methods). After extensive washing to remove unbound label, zoospore pellets were homogenized and low speed ($500 \times g$) supernatants were subjected to sucrose equilibrium centrifugation, as usual. In such experiments, another portion of the original zoospore population was carried through the same procedure, but treatment with the lectin was omitted (control cells). Representative analyses of gradient fractions are presented in Fig. 5. The buoyant density distributions of bound [^{3}H]concanavalin A and of chitin synthetase activity were nearly coincident (Figs. 5B and C). The main region of enzyme activity was shifted by the lectin treatment from a narrow zone with peak density at 1.16–1.17 g/ml in homogenates from control cells (Fig. 5A; Fig. 1A) to a usually broader region with peak density at 1.19–1.20 g/ml (Figs. 5B and C), still clearly separate from the 1.30–1.34 g/ml region characteristic for gamma particles. The latter two figures were chosen to depict the extremes of the relative distributions observed of enzyme activity vs. bound [^{3}H]concanavalin A. In the experiment of Fig. 5B, the two distributions were not completely coincident (on the lighter density side) whereas in Fig. 5C, the distributions were almost completely coincident. In control experiments, α-methylmannoside was added after [^{3}H]concanavalin A treatment and cell homogenization in attempts to release previously bound [^{3}H]concanavalin A. Substantial, but incomplete, increases were observed in the proportion of radioactivity in the region of the applied sample (first 4–5 fractions); this release of bound [^{3}H]concanavalin A did not alter the buoyant density distributions of chitin synthetase activity or of the remaining bound label.

A further point of interest has to do with the previously noted white band coincident in buoyant density position with the peak of chitin synthetase activity in homogenates prepared by osmotic lysis (Fig. 1A, band C). An identically located white band was observed in the gradient of the control sample of Fig. 5A, prepared by Dounce homogenization. This band was not visible in the gradients of the lectin-treated samples (Figs. 5B and C), but a more diffuse white band was then observed within the broader, increased density region of chitin synthetase activity.

The nearly complete coincidence of the buoyant density regions of bound [^{3}H]concanavalin A and of chitin synthetase activity has been confirmed in repeat experiments using homogenates prepared by Dounce homogenization (five additional experiments), osmotic lysis (one experiment) and sonication (one experiment) and using sucrose gradients of various density ranges. Nearly complete coincidence has also been observed using a 5.7–57% Renografin gradient. It must be stressed that in all cases, the main buoyant density region of chitin synthetase activity from homogenates of lectin-pretreated cells was clearly distinct from the buoyant density of the bottom-most, gamma particle-enriched gradient fractions.

Discussion

Previous information concerning the distribution of chitin synthetase activity in *B. emersonii* zoospore homogenates has been presented in terms of specific enzyme activities (enzyme activity per protein in crude homogenates vs. enzyme activity per protein in gamma particle-enriched fractions; see Tables IX and X in Ref. 13). Enrichments for chitin synthetase specific activities in gamma particle-enriched fractions were indeed observed. We have observed comparable enrichments (see Fig. 1, legend), which suggests that the differing conclusions about zoospore chitin synthetase localization have not resulted from differences in experimental techniques. In addition, where comparisons can be made, the enzyme specific activities reported here are roughly an order of magnitude greater than the values previously reported (compare values for $5000 \times g$ supernatant, $20000 \times g$ pellet and gamma particle-enriched sucrose density region from $20000 \times g$ pellet in Fig. 1 legend with Table X, second column, in Ref. 13). This suggests that our results, in comparison to the previous results, are not attributable to excessive or differential enzyme inactivation during preparative procedures.

A critical point of this paper is that, in our hands, enzyme specific activities do not reflect the relative proportions of total enzyme activity present in different homogenate fractions. When the

distribution of enzyme activity is represented in the latter fashion, the bulk of homogenate enzyme activity is observed in fractions other than those enriched for gamma particles ($20000 \times g$ supernatant vs. $20000 \times g$ pellet, Table 1, Fig. 1 and text; sucrose buoyant density regions 1.14–1.18 g/ml vs. 1.30–1.34 g/ml, Fig. 1 and text). These distributional patterns have been observed here after each of three different methods of homogenate preparation, including an osmotic shock method similar to the one previously used [13].

We discuss the evidence supporting a plasma membrane location for at least the bulk of zoospore chitin synthetase catalytically active in zoospore homogenates, before evaluating some issues relevant to the situation in intact zoospores. The partial sedimentation of homogenate enzyme activity over a range of intermediate centrifugal forces and the virtually complete sedimentation at $100000 \times g$ are behaviors consistent with many fragmented plasma membrane preparations. The buoyant density distribution of $\geqslant 90\%$ of the enzyme activity in equilibrium sucrose gradients, over the range 1.14–1.18 g/ml with peak density at 1.16–1.17 g/ml, is characteristic for vesicularized plasma membranes from several sources (see Refs. 21, 22) and is clearly distinct from the density distribution of gamma particles, over the sucrose density 1.30–1.34 g/ml with peak density at 1.32 g/ml (Ref. 13 and above). Plasma membrane fractions frequently display size heterogeneity when viewed in thin sections by transmission electron microscopy; such heterogeneity was observed with peak enzyme activity fractions, though many of the membrane-bound elements were large enough that they were unlikely to have been derived from a source other than the plasma membrane. A particularly troublesome source of confusion on these ultrastructural grounds using other fungal preparations has been the endoplasmic reticulum (see Refs. 1, 2 for summary and references). This is evidently not a problem here, since many published thin sections of *B. emersonii* zoospores have uniformly failed to display typical long segments of endoplasmic reticulum (such segments have been observed only following zoospore encystment, accompanying the breakdown of the membrane-bound nuclear cap and the release of its ribosome into the cytoplasm; see Ref. 15). Another previously troublesome factor with other fungal preparations, the cell wall, is not of concern here, since zoospores lack cell walls.

We attach particular importance to the results using [^{3}H]concanavalin A-prelabelled zoospores. Cell-bound [^{3}H]concanavalin A was recovered in one main sucrose density region after each of the three methods of cell homogenization and this region overlapped nearly completely with the discrete main region of chitin synthetase activity recovered in the same preparations. The lectin-prelabelling procedure differed sharply from a concanavalin A procedure previously used to induce zoospore lysis [23], but rather was similar to procedures previously used to localize chitin synthetase to the plasma membrane of protoplasts derived from *Saccharomyces cerevisiae* [18], *Schizophyllum commune* [24] and both the yeast and hyphal phases of the dimorphic fungus, *Candida albicans* [25]. The zoospore plasma membrane was evidently 'stabilized' [17,18] by the concanavalin A -prelabelling procedure (see Materials and Methods). While possibilities for redistribution of some surface-bound [^{3}H]concanavalin A during homogenization have not been eliminated, at least the bulk of the label would nevertheless be expected to remain bound to cell surface material (see an additional argument about this point, below).

An alternative interpretation of our results, based on evidence that gamma particles form and then release vesicles as they decay morphologically [13,14,26], can be envisaged. To entertain the notion that these vesicles contain the bulk of catalytically active homogenate chitin synthetase, additional assumptions would be required to account for data presented here—namely, that most (some 90%) of the gamma particles decayed during sample preparation and/or centrifugation and that the released vesicles displayed the same buoyant density as plasma membrane-derived material. The recovery of gamma particle-enriched fractions in a much greater buoyant density region, just as previously reported [13] and well separated from the main buoyant density region of chitin synthetase activity, furnishes evidence against this interpretation. The interpretation can also be challenged by additional, apparently more critical, evidence. The main buoyant density region of chitin synthetase activity from [^{3}H]concanavalin A-prelabelled zoo-

spores displayed a density shift (peak density, 1.19–1.20 g/ml) relative to zoospores that were not pretreated with the lectin (peak density, 1.16–1.17 g/ml), and this shifted density region of enzyme activity overlapped nearly completely with the main buoyant density region of cell bound [^{3}H]concanavalin A (Fig. 5). It seems unwarranted to assume that the buoyant density of the internal gamma particle-derived vesicles was altered by the concanavalin A pretreatment in exactly the same fashion as the bulk of the [^{3}H]concanavalin A-prelabelled, plasma membrane-derived material. Nevertheless, in our judgement, it remains of value to test the alternative interpretation by other, independent techniques; we are continuing to explore alternative methods of differentially labelling the intact zoospore plasma membrane, chitin synthetase and gamma particles.

While gamma particle-enriched fractions were not pure with respect to gamma particles in our hands (see Fig. 3B), the issue must nevertheless be raised as to whether the minor fraction ($\leq$ 10%) of chitin synthetase activity observed in the appropriate sucrose density region has physiological relevance. This, in turn, raises the subject of chitin synthetase zymogenicity. Protease-mediated increases in in vitro chitin synthetase activity have been reported with several different fungal enzyme preparations whether the enzyme source has been the plasma membrane (see Ref. 27 for review) or small (40–70 nm diameter) particles, thought to be cytoplasmic in origin, called chitosomes (see ref. 28 for review). The in vivo importance of this particular form of chitin synthetase activation has been challenged [29–31]. With certain other fungal preparations, chitin synthetase has been recovered in an 'active' as well as 'zymogenic' form; with such preparations, enzyme activity is relatively high without protease treatment and protease treatments result in only small increases in enzyme activity at best [24,25,32–35], often followed by rapid declines in enzyme activity. *B. emersonii* zoospore homogenates display this latter pattern (Dalley, N.E. and Sonneborn, D.R., in preparation). The usual interpretation of this pattern has been that the zymogenic form of the enzyme has been activated by cellular proteases during extract preparation. However, with *B. emersonii* zoospores, inclusions of common protease inhibitors throughout extract preparation have not as yet yielded chitin synthetase preparations with significantly lower catalytic activities (Dalley, N.E. and Sonneborn, D.R., unpublished data). Moreover, a biologically interesting correlation appears to exist with respect to other preparations where plasma membrane-localized, largely active rather than zymogenic, forms of chitin synthetase have been reported [24,25,33,34]. Each of the preparations was obtained from protoplasts that can deposit chitin broadly over the cell periphery during wall regeneration, as opposed to the local deposits that occur naturally during germ tube emergence, hyphal tip growth or yeast bud formation. The zymogen model has been particularly useful in attempts to account for the latter local wall formation situations. By comparison, the *B. emersonii* zoospore is effectively a natural protoplast, lacking a cell wall,; the chitinous wall abruptly constructed during zoospore encystment encompasses the entire cell (By analogy with published evidence using the related organism, *Allomyces macrogynus* [36], the local surface region where the germ tube emerges may be deficient in chitin).

While we conclude that our evidence supports a plasma membrane, rather than gamma particle, location for the bulk of zoospore chitin synthetase activity, we also recognize that gamma particle decay via vesicularization followed by vesicle-plasma membrane fusions remains an extremely attractive morphogenetic model (see Refs. 13 and 14) for developmentally regulated cellular export of some molecule(s) involved in zoospore germination. Similar ultrastructural evidence has recently been provided with respect to *Allomyces macrogynus* zoospore germination [36], though the association of zoospore chitin synthetase with gamma particles has also been questioned in this organism [37]. Other macromolecular products besides chitin are presumably deposited extracellularly during *B. emersonii* encystment. The cell wall itself contains other components, particularly polypeptides and neutral sugar carbohydrates; presumably, only the inner, microfibrillar layer of the wall contains chitin [3,38]. As cells encyst, they adhere to solid surfaces, or to each other in dense suspension culture; adherence is presumably not mediated directly by chitin per se. In addition, encysted cells still contain at least some apparently intact gamma

particles [38,40], which evidently decay by the succeeding phase of germ tube emergence [39,41]. Characteristic 80–90 nm vesicles accumulate at the emerging germ tube, though these vesicles have previously been interpreted not to arise from gamma particles [41].

Finally, the available evidence does not yet provide a rigorous explanation for either the absence of chitin in the zoospore or the rapid accumulation of chitin during zoospore encystment. Indirect evidence exists that the substrate, UDP-GlcNAc [4,5], and a glycolipid acceptor [10,11] may not be sufficiently available to the zoospore plasma membrane chitin synthetase, but may then be brought to the cell surface during encystment. It has been proposed that the above acceptor is delivered to the surface via gamma particle-derived vesicles [10,11]. Alternatively, chitin synthetase may be oriented in the zoospore plasma membrane in such a way that membrane rearrangements early during encystment (see Refs. 42–44), or during zoospore homogenate preparation, permit accessibility of substrate and acceptor to the enzyme.

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