The Prespore Vesicles of Dictyostelium discoideum

PURIFICATION, CHARACTERIZATION, AND DEVELOPMENTAL REGULATION*

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The coordinate fusion of the prespore vesicles (PSVs) with the plasma membrane at the terminal stage of spore differentiation in Dictyostelium discoideum is an important example of developmentally regulated protein secretion. However, little is known about the composition of the vesicles, the molecular signals regulating secretion, or the mechanics of the membrane fusion. Taking a biochemical approach, we purified PSVs from different developmental stages. These preparations are highly enriched for their specific cargo of spore coat proteins while devoid of markers for other cellular compartments. Electron microscopic observations show that the PSV preparations are homogenous, with the soluble spore coat protein PsB/SP85 distributed throughout the lumen and the acid mucopolysaccharide localized in the central core. During development the PSVs increase in size and density concomitant with an increase in their protein cargo. The PSVs contain approximately 80 proteins, and we have identified a PSV-specific GTP-binding protein that may be involved in regulating vesicle fusion. The PSVs are not clathrin-coated and do not contain the SpiA spore coat protein. The PSV preparations are ideal for a global proteome analysis to identify proteins involved in signal reception, vesicle movement, docking, and fusion in this developmentally regulated organelle.

Virtually all cells secrete specific proteins that function in a variety of cellular processes, including cell-substrate interactions and intercellular signaling. In certain cell types protein secretion is constitutive, as in the case of pancreatic beta cells where secretion of insulin is the main cellular event, and the mechanics of the process are now quite well understood (1). In other cell types, such as neurons, protein secretion is regulated in a spatial and temporal pattern (2). Certainly protein secretion also plays important roles in specific events in multicellular development, although this has received much less attention. This is primarily due to the difficulty in identifying and characterizing these processes within the tissues of complex embryos. A dramatic case of developmentally regulated secretion occurs at the terminal stage of spore cell differentiation in the cellular slime mold Dictyostelium discoideum (3), and the relative simplicity of morphogenesis in this organism allows for the in depth analysis of the mechanisms that underlie this cellular event.

As long as there is a source of food, Dictyostelium amoebae remain as single cells and divide by mitosis. When the food source is exhausted or removed, the cells form multicellular aggregates of approximately 10^5 cells. These multicellular aggregates then undergo a program of cytodifferentiation and morphogenesis to produce an intermediate slug and ultimately a mature fruiting body with a cellular stalk that supports about 80,000 mature spores. Proper cell differentiation results in environmentally resistant spores that are an evolutionary advantage to the organism, as they allow cells to remain viable for extended periods of time. A complex and well studied program of gene expression underlies the process of cell differentiation (4, 5).

The slug stage of development shows a clear spatial distribution of cells. The cells that are destined to differentiate into spores are called prespore cells and comprise the rear 80% of the multicellular slug. The prestalk cells, destined to become stalk cells, reside in the anterior end of the slug. Prespore cells in the slug are characterized by the presence of specialized, cell-type-specific secretory vesicles, called the prespore vesicles (PSVs) (6–8). The PSVs contain components that are essential for the formation of the future spore coat. Several structural spore coat proteins have been identified and studied, such as SP96, PsB (SP85), SP75, SP70, and SP60 (9–13). Four of these spore coat proteins, SP96, PsB, SP70, and SP60, are O-glycosylated (14–16) and are pre-assembled in the prespore vesicles in a multiprotein complex, the PsB complex, that has endogenous cellulose binding activity (17–20, 70). Upon secretion from the PSVs, the PsB complex and other unassembled spore coat proteins are incorporated into a rigid, trilaminar spore coat by an unknown mechanism. The spore coat is composed of approximately 50% cellulose and 50% glycoprotein (21), and the cellulose binding property of the PsB complex is required for the maintenance of the structural integrity of the spore coat and viability of the spore (19, 20, 22).

The pivotal event for spore cell differentiation is the developmentally regulated fusion of the PSVs with the plasma membrane and the subsequent release of their contents into the extracellular matrix. When development is arrested at the migratory slug stage, the PSVs accumulate spore coat proteins, but they do not fuse with the plasma membrane. Hence the regulated secretion of the PSV contents is a true signal-mediated event that underlies cell-type differentiation of prespore cells to spores.

Many significant questions remain unanswered about the

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1. The abbreviations used are: PSV, prespore vesicle; ELISA, enzyme-linked immunosorbent assay; GPS, galactose/N-acetylgalactosamine-containing polysaccharide; mAb, monoclonal antibody; BCA-I, Ricinus communis agglutinin I; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin.

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genesis of the spore coat and the role of PSVs in this process. 1) Do the PSVs undergo changes in protein content and/or morphology as development proceeds? 2) What is the nature of the developmental signal that initiates the synchronous events resulting in exocytosis? 3) What are the molecular mechanisms by which the PSVs move to, and fuse with, the plasma membrane? 4) What are the membrane proteins that must be involved in these processes? In addition, we know nothing of the content of the PSVs beyond the most abundant structural spore coat proteins. It is reasonable to assume that other proteins reside in the lumen of these vesicles and that they may have catalytic roles in the assembly of the spore coat.

The most direct approach to these biochemical questions is to develop a procedure that results in a high level of purification of the vesicles. PSVs can then be isolated and characterized at different stages of morphogenesis. Moreover, the purified material can be effectively analyzed in the proteome project that is under way for Dictyostelium (25). The unique ability to produce large quantities of synchronously developing Dictyostelium cells has made it possible to undertake this analysis. Previous reports have presented approaches to the purification of PSVs but, the reports did not include the requisite analyses using the appropriate organelle-specific markers to establish the purity of the preparations (24–27), and we found that these methods did not result in an adequate level of purification needed to address the questions outlined above.

In this report we demonstrate the purification of the PSVs from four different developmental stages of D. discoideum. Using the now abundant assays available to us, including electron microscopy, we establish the purity of the PSV preparations and the absence of contaminating organelles. We demonstrate that newly synthesized spore coat proteins continue to accumulate specifically in the PSVs until the cells receive the signal to begin the processes that ultimately lead to fusion and exocytosis and that this signal is temporally associated with the slug to fruit transition. During development the PSVs undergo maturation, including changes in structure. A 21-kDa GTP-binding protein becomes specifically associated with the membrane fraction from the PSVs and is a good candidate for a regulatory protein that coordinates the rate of PSV/plasma membrane fusion (28). In addition, it is clear that the protein content of the PSVs is complex, containing many more proteins than just the structural spore coat proteins. Proteome analysis of both the membrane and luminal proteins will allow us to identify many of these proteins and determine their functions in this highly regulated developmental process.

**EXPERIMENTAL PROCEDURES**

**Strains and Conditions for Growth and Development—**Wild-type WS380B was grown in association with Klebsiella aerogenes on SM agar plates at 22 °C (29). Cells were harvested when they reached mid-log phase (2.4 × 10^8 cells/100-mm plate). Residual bacteria were washed away by differential centrifugation in LPS buffer (20 mM KCl, 2.5 mM MgCl_2, 0.5 g/liter streptomycin sulfate, 40 mM potassium phosphate buffer, pH 6.5), and the cells were resuspended in the same buffer. To obtain slugs, 5 × 10^8 washed cells were plated on 100-mm plates containing 1.5% agar in distilled water. LPS agar was used to obtain preculminants (30). Development is synchronous, and the slug stage is reached at 10 h in this rapidly developing wild type strain (31).

**Disaggregation of Slugs—**Slugs were harvested into cold LPS and pelleted. The pellet of slugs was resuspended in 3 volumes of homogenizing medium (50 mM Tris-HCl, pH 7.6, 250 mM sucrose, 25 mM KCl, 5 mM MgCl_2, and 1 mM EDTA) at 4 °C. A mixture of protease inhibitors was added from a 100 × stock (peptatin A, 100 μg/ml; leupeptin, 10 μg/ml; phenylmethylsulfonyl fluoride, 50 mM). The cells were then broken with 30 strokes of a tight-fitting Wheaton Dounce homogenizer. The homogenate was spun at 700 × g for 10 min to pellet the nuclei and unbroken cells. The 700 × g supernatant was set aside while the pellet was resuspended in 3 volumes of homogenizing medium. The suspension was homogenized again, followed by centrifugation at 700 × g. This step was repeated in order to increase the yield of PSVs, because we found that the first homogenization step left 30% of the cells unbroken. The 700 × g supernatants were pooled and spun at 4000 × g for 20 min. The 4000 × g pellet containing the PSVs was resuspended in homogenizing medium, placed on top of a 25% Percoll gradient (Sigma), and spun at 25,000 × g for 1 h using a type 80 Ti rotor in a Beckman LE-80K ultracentrifuge. 1-mL fractions from the Percoll gradient were resuspended to 12 ml in homogenizing medium and spun at 100,000 × g for 1 h in an SW 41 Ti rotor with no brake deceleration. For biochemical experiments, the vesicle pellets were recovered and resuspended to 200 μl in 10 mM sodium phosphate, pH 6.8, 1% Triton X-100, and the protease inhibitors. All samples were stored at −20 °C.

**Protein Quantitation, Electrophoresis, and Western Blotting—**Protein quantitation was performed by the colorimetric BCA assay (Pierce), using BSA as a standard. Protein gel electrophoresis and Western blotting were performed as described previously (33, 34). Antibody detection of proteins on the blots was done by standard procedures (18, 31). The mAb MUD102 was used to detect PsB (14), and the mAb MUD62 was used to detect SP96 and SP75 (15). Discoidin I was detected using the rabbit antiserum 4290 (35), and clathrin was detected using the rabbit antiserum 4290 (35). Discoidin I was detected using the rabbit antiserum 4290 (35), and clathrin was detected using the rabbit antiserum V498-III (36), a gift from T. O’Halloran. HL351 is a SpiA null strain made by homologous recombination (37, 38); gifts from D. Fuller and W. Loomis. Genomic DNA was prepared using DNAzol (Molecular Research Center, Inc.) and detected on a 0.8% agarose gel stained with ethidium bromide.

**ELISAs—**ELISAs were performed as described previously (39). Titers were determined as the end point dilution of each titration curve.

**Enzyme Assays—**Enzyme assays for α-mannosidase (40), alkaline phosphatase (41), and α-glucosidase-2 (42) were performed as described previously.

**Phase Partitioning with Triton X-114—**Phase Partitioning experiments using Triton X-114 were done as described previously (31, 43). GTP Binding Assay—The GTP binding assay was adapted from Vater et al. (44). Equal protein samples (10–20 μg) were separated by 10% SDS-PAGE with 4 μ urea and blotted onto nitrocellulose. The blots...
were incubated for 15 min in 100 ml of buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20 and blocked for 1 h in blocking buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5% BSA, 0.1% Tween 20) at room temperature. The blots were incubated for 30 min in 50 ml of hybridization buffer (20 mM Tris-HCl, pH 7.5, 2 mM MgCl₂, 2 mM dithiothreitol, 0.3% BSA, 0.5% Tween 20, 120 µg/ml tRNA) containing 100 mCi of [α-³²P]GTP (3000 mCi/mmol, ICN Biochemicals) and washed three times for 20 min in blocking buffer. The blots were then air-dried and autoradiographed.

Electron Microscopy—Vesicle preparations removed from the Percoll gradient were fixed in homogenizing medium containing 0.5% glutaraldehyde for 15 min, then spun at 100,000 × g for 1 h to pellet the vesicles. The vesicle pellet was washed three times in homogenizing medium, then fixed in 1% osmium tetroxide for 30 min. The samples were embedded in 2% agar before serial dehydrations in ethanol, followed by infiltration and final embedding in Spurr’s resin (Electron Microscopy Sciences). Disaggregated D. discoides cells from different developmental stages were fixed in 2% glutaraldehyde for 30 min and 2% osmium tetroxide for 1 h. Fixed cells were dehydrated and embedded in Spurr’s resin. For immunolabeling, samples were fixed in glutaraldehyde alone, and embedded in LR White resin (Electron Microscopy Sciences). Thin sections were blocked in 1% BSA for 30 min and stained with MUD102 for 1 h. Serial washes in sterile water were followed by incubation with 2 nm gold-conjugated secondary antibody for 1 h. RCA-1 labeling was performed as described previously (30). 20 nm gold-conjugated RCA-1 (EY Laboratories) was used at a dilution of 1:250. All thin sections were stained with 4% uranyl acetate in 70% ethanol (18 min) and Reynold’s lead citrate (12 min) and viewed in a JEOL 1200EX transmission electron microscope at 80 kV.

RESULTS

Primary Subcellular Fractionation of Prespore Cells—Early attempts at purification of the prespore vesicles did not include the appropriate assays necessary to establish the purity of the PSVs (24–27). In numerous attempts to repeat these protocols, we found that the PSVs were not fractionated as a discrete peak on the gradients used and that all the fractions containing PSVs also contained a complex mixture of markers for other organelles (data not shown). Therefore, using the biochemical markers and the corresponding antibodies for the definition of specific organelles, which are now well characterized in Dictyostelium, we have developed a protocol to yield a highly enriched PSV fraction that is devoid of markers for other organelles.

Our initial aim was to test whether the nuclei, PSVs, and lysosomes were separable by low speed centrifugation. In a typical experiment, we grew 10¹⁰ cells of wild-type WS380B, which were allowed to undergo development to yield 10⁵ slugs. The homogenate was then divided into aliquots and subjected to fractionation at forces up to 18,000 × g, resulting in 100 mCi of [α-³²P]GTP (3000 mCi/mmol, ICN Biochemicals) and a quantitative enrichment of the PSV marker protein PsB at 1.12 g/ml tRNA. The post-nuclear supernatant from the 700 × g sediment of the nuclei, PsB, SP96, and SP75 were used as markers for PSVs (8, 17, 18) and detected by Western blots. Additionally, ELISAs were done using the anti-PsB antibody to obtain a quantitative measurement of the specific enrichment of PsB during different stages of the purification procedure. The PSVs were found almost exclusively in the most dense band (band 3).

Earlier work has shown that a force of 12,000 × g is required for mitochondria to sediment (46).

Organelle Distribution and Assessment of Purity of PSV Fractions from Percoll Gradients—We chose Percoll gradient centrifugation for further purification due to its successful use in the fractionation of post-Golgi vesicles in other organisms (47). The 25% Percoll gradient fractionation of the 4000 × g pellet consistently produced three bands at different densities: band 1 at 1.07 g/ml, band 2 at 1.10 g/ml, and band 3 at 1.12 g/ml. (Note: we show later that the density of band 3 changes with developmental stage.) Each band was tested for the enrichment of PSV markers, and the absence of markers from other organelles, as is shown in Fig. 3. The spore coat proteins PsB, SP96, and SP75 were used as markers for PSVs (8, 17, 18) and detected by Western blots. Additionally, ELISAs were done using the anti-PsB antibody to obtain a quantitative measurement of the specific enrichment of PsB during different stages of the purification procedure. The PSVs were found almost exclusively in the most dense band (band 3). Table I presents a quantitative enrichment of the PSV marker protein PsB at different stages of the purification procedure and shows that the PSVs were enriched approximately 90-fold over the starting cell homogenate, with an overall yield of the PsB protein marker of 60%. This, coupled with an overall protein recovery of 0.7% in band 3 shows that the PSVs represent approximately 1% of the total cellular protein in prespore cells at this stage of development. Further purification of band 3 from the Percoll gradient using either continuous sucrose gradients or S-1000 gel filtration did not appreciably increase the degree of enrichment.

Each fraction was then assayed for other organelle-specific
markers, including α-mannosidase for lysosomes (48, 49), alkaline phosphatase for plasma membrane (50, 51), α-glucosidase-2 for endoplasmic reticulum (42), and discoidin I for cytosol (52, 53). In each case, the Percoll gradient fraction containing PSVs did not contain markers for any other organelle (Fig. 3). These results have been consistently obtained in multiple purifications. The levels of all enzymes tested in homogenates were consistent with previous published reports (40–42). Based on these data, we concluded that band 3 from the Percoll gradient is the PSV marker, PsB, was present in band 3 from the Percoll gradient (lane 8), as shown by Western blotting and quantified by ELISA. The spore coat proteins SP96 and SP75 are also highly enriched in PSV fractions (lane 8). The lysosomal marker, α-mannosidase, is found predominantly in band 2 from the Percoll gradient (lane 7), but is entirely absent from the fraction containing PSVs (lane 8). Enzyme assays for the plasma membrane marker, alkaline phosphatase and endoplasmic reticulum marker, α-glucosidase-2, show that they are also absent from band 3. Discoidin I is known to be in the cytosol, and a Western blot with an anti-discoidin antibody (4290) also showed that it was absent from band 3. P, pellet; S, supernatant.

Electron Microscopy of Purified Prespore Vesicle Preparations—The purity of the PSV preparations was examined by electron microscopy. Thin sections of the purified PSVs show that the vesicle preparation appears quite homogeneous, and the vesicles have diameters of approximately 200–250 nm (Fig. 4A). As described previously in intact cells (6), the PSVs contain an electron-dense region that is surrounded by a electron-lucent gap and enclosed by a typical membrane bilayer (Fig. 4A). It is clear that the PSVs are discrete vesicles and not a tubular network as suggested previously (21). We then tested the purified PSVs for the presence of specific markers. The purified PSVs stain uniformly with MUD102 (Fig. 4B), indicating that the PsB protein is soluble and present throughout the compartment (17). In contrast, the lectin RCA-I used to detect the galactose/N-acetylgalactosamine-containing acid mucopolysaccharide (GPS) of the spore coat (10, 21) selectively stained the electron dense PSV inner core (Fig. 4C). The inclusion of the haptin inhibitor galactose (300 mM) blocked all labeling with RCA-I, showing that the staining was specific for GPS (not shown). Thus the inner electron dense region of the PSVs is made up of GPS, and other soluble proteins are present throughout the organelle.

Not All Spore Coat Proteins Are Located in the PSVs Prior to Secretion—We have demonstrated that the purified PSVs contain the spore coat proteins PsB, SP96, and SP75. We assume that the coat proteins SP70 and SP60 are also localized in the PSVs, because they are stoichiometrically associated with PsB in the PsB multiprotein complex (17). All of these proteins are coordinately synthesized during a period starting at the early aggregate stage and ending at culmination (11, 17, 18). Another spore coat protein, SpiA, is synthesized late in development, just prior to culmination, and is localized to the inner wall of the spore coat (37, 38). The difference in timing of its synthesis compared with other spore coat proteins suggested that it may not be routed to, and accumulate in, the PSVs. Therefore we tested whether PSVs purified from the preculminant stage of development contained SpiA. Fig. 5 (lane 1) shows the presence of the 30-kDa SpiA protein in wild type whole cell extracts from preculminants. Extracts from cells in which the spiA gene (Dd31) has been disrupted lack the protein (Fig. 5, lane 2), as expected. If SpiA was present in the PSVs, we would expect it to be enriched in the purified vesicles. However, the purified PSVs completely lack SpiA (Fig. 5, lane 3). The data indicate that SpiA is transported out of the cell by a second pathway, independent of the PSVs.

Developmental Changes Associated with the Prespore Vesicles—Our characterization of PSVs isolated from slugs demonstrated the presence of all three of the spore coat protein markers assayed. Earlier analyses using Western blotting and immunoprecipitation from this and other laboratories have shown a dramatic increase in the rate of synthesis of all spore coat proteins as development proceeds (9, 13, 17). Using ELISA, we extend this observation showing that there is an approximately 10-fold increase in the PsB concentration in prespore cells at each stage as they develop from aggregates to preculminants (Fig. 6A). Analysis of purified PSVs from cells at four different developmental time points shows that the newly synthesized PsB protein accumulates in the PSVs. At each developmental stage, PsB specific activity in the purified PSVs is increased approximately 60–80-fold over the homogenate. A very different situation exists for the lysosomal enzyme α-mannosidase. It also increases steadily during the same period of development (40), and this is confirmed in Fig. 6B. However, there was a complete exclusion of this lysosomal enzyme from the purified PSVs at all developmental stages. Thus there is a selective increase in the spore coat protein content in the PSVs as development proceeds.

The increase in storage cargo shown above is accompanied by an increase in density of the purified PSV fraction (band 3) from the Percoll gradient. The density of PSVs from preculminants is 1.15 g/ml, compared with 1.12 g/ml seen at earlier stages of development. We demonstrate that this increase in PSV density correlates with morphological changes in the PSVs, as observed by electron microscopy in thin sections of prespore cells. PSVs from the aggregate stage of development are approximately 140 nm in diameter (Fig. 7A) and are located in the interior of the cell. PSVs from slug prespore cells are larger, approximately 200–250 nm in size (Fig. 7B). Dramatic changes in PSV structure and location can be seen in prespore cells from preculminants. The electron-lucent gap seen at earlier developmental stages appears to diminish (Fig. 7C, arrowheads). At this developmental stage, the PSVs range from 400...
to 500 nm in size. Moreover, while PSVs from prespore cells from early developmental stages are located in the interior of the cell, the PSVs in preculminants are located close to the cell periphery. The micrographs shown in Fig. 7 are representative of more than 20 sections examined for each developmental stage. These observations show that there are changes in size and structure as development progresses and that in preculminants the PSVs are found in close proximity to the plasma membrane in preparation for docking and fusion.

Analysis of Proteins in Purified Prespore Vesicles—The purified PSVs provided the material necessary to begin analyzing their protein components. We first performed phase partitioning studies in order to assess the soluble and membrane protein content of the PSVs. We used Triton X-114 to selectively extract membrane-bound proteins in the detergent phase, while soluble proteins remain in the aqueous phase. The results are shown in Fig. 8. Proteins from the soluble and membrane-bound fractions were separated by SDS-PAGE and stained with Coomassie blue or silver nitrate.

TABLE I

| Purification stage | Units/ml ($\times 10^3$) | Protein concentration mg/ml | Total protein mg | Specific activity ($\times 10^6$) | Fold purification of PsB protein | Recovery of PsB protein % |
|-------------------|--------------------------|------------------------------|-----------------|---------------------------------|-------------------------------|---------------------------|
| Homogenate        | 3.13                     | 32.07                        | 1731.78         | 97.2                            | 1.0                           | 100                       |
| 700 × g supernatant | 3.13                     | 18.99                        | 852.30          | 164.0                           | 1.7                           | 83.3                      |
| 4000 × g pellet    | 25.0                     | 25.34                        | 65.88           | 986.5                           | 10.1                          | 36.8                      |
| Percoll band 3: PSVs | 200                      | 23.42                        | 11.7            | 8540.0                          | 87.9                          | 59.4                      |

a Units are measured as the reciprocal of the antibody dilution at the endpoint of the titration curve.

b Specific activity is defined as units/mg protein.

FIG. 5. The spore coat protein, SpiA, is absent from the PSVs. Blots containing equal protein amounts were tested for the presence of the SpiA protein using the antiserum 506-3. The 30-kDa SpiA protein is detected in the homogenate from preculminants in wild type strain WS380B (lane 1) and is absent from the SpiA null strain, HL351 (lane 2). Purified PSVs do not contain the SpiA protein (lane 3). The developmental stage shown in the figure is the same as in Fig. 6.

FIG. 6. Accumulation of the spore coat protein PsB in PSVs during development. PSVs were purified from four sequential D. discoideum developmental stages. A, PsB levels in the homogenates and in the PSVs were measured by ELISA. B, α-mannosidase activity in homogenates and PSVs was assayed. The specific activity is recorded as nmol/mg/min. Open bars, homogenate; solid bars, PSVs.

FIG. 4. Electron microscopy of the purified PSV fraction. A, a thin section of purified PSVs shows that the purified PSVs are approximately 200–250 nm in size. Bar, 0.14 μm. B, MUD102 immunostaining of purified PSVs. 2 nm gold-conjugated secondary antibody was used to detect MUD102. The PSVs stain uniformly, indicating that PsB is present throughout the organelle. Bar, 0.08 μm. C, RCA-I (20 nm gold conjugate) staining of purified PSVs. The inner electron-dense core of the PSVs stains specifically with RCA-I (arrowheads), showing that it contains GPS. Bar, 0.07 μm.
with Coomassie Brilliant Blue. Fig. 8A (lane 1) shows the protein composition of the purified PSVs. Several aqueous and membrane-bound proteins are enriched (Fig. 8A, lanes 2 and 3), none of which have been identified previously. The soluble marker PsB was found exclusively in the aqueous phase in the 4000 × g pellet as well as in the PSVs (Fig. 8B, lanes 1 and 3), demonstrating that the fractionation worked as expected.

We were specifically interested in proteins that may be mechanistically involved in the fusion of the PSVs with the plasma membrane at the onset of exocytosis. Clathrin is known to be involved both in endocytosis and exocytosis (54), and in *Dictyostelium*, it has been shown that clathrin heavy chain is specifically required for spore cell differentiation (36). The defective phenotype of the clathrin null cells could be due to the inability of the PSVs to fuse with the plasma membrane. Thus, we wished to determine whether PSV membranes from wild type cells were indeed clathrin-coated. An immunoblot of PSV proteins with an anti-clathrin antibody (V498-III) showed no staining for clathrin (Fig. 9A, lane 3), although it is present in cell homogenates (Fig. 9A, lane 1). The PSVs are therefore not clathrin-coated organelles, and their fusion with the plasma membrane is not clathrin-mediated.

We then proceeded to look for other molecules that are known to regulate membrane fusion in eukaryotes. Small (20–25 kDa) GTP-binding proteins of the Ras superfamily, called Rab GTPases, are known to regulate membrane fusion in all eukaryotic cells (reviewed in Ref. 55). Rab GTPases are found on the surface of donor vesicle membranes, and their absolute levels control the rate and timing of fusion with their cognate target membranes (56, 57). To determine whether we could detect a protein with GTP binding activity in the purified PSV fraction, a GTP binding assay was performed using [α-32P]GTP. Samples were separated by SDS-PAGE and transferred to nitrocellulose, and the membrane was then incubated with [α-32P]GTP. A single strong band at 21 kDa that binds GTP was associated with the PSVs (Fig. 9B, lane 1), while at least three bands can be detected in whole cell extracts (lane 1). Because Rabs are known to be present on vesicle membranes, we examined whether the GTP-binding protein in the PSVs was membrane-bound. Phase partitioning using TX114 was done on the PSVs to separate the membrane-bound proteins from the soluble proteins. Fig. 9B (lane 6) shows that the GTP-binding protein from the PSVs is entirely associated with the membrane fraction. Thus the PSVs contain a 21-kDa membrane-bound GTP-binding protein that may potentially be a regulatory molecule that mediates membrane fusion.

It has been shown that the absolute levels of Rab GTPases, rather than the rate of nucleotide hydrolysis, determine the rate of membrane fusion (56, 57). The PSVs are developmentally regulated organelles. Their biogenesis begins at the late aggregate stage (5–8 h) into development, but they do not fuse with the plasma membrane until culmination, which begins at 16–18 h of development. If the developmentally regulated fusion of the PSVs is modulated by the GTP-binding protein, we should see an increase in the level of this protein during development. To test this prediction, prespore vesicles were purified from four different developmental stages. Equal protein amounts were subjected to the GTP binding assay. The results are shown in Fig. 9C. During the aggregate stage of development (6–8 h), the PSVs do not contain the 21-kDa GTP-binding protein (Fig. 9C, lane 4), although a low level of reactivity is detectable in the 4000 × g pellet (Fig. 9C, lane 1). PSVs from the slug stage (10 h) contain the protein, as noted previously (Fig. 9B, lane 3). Migrating slugs (12–14 h) show an increase in

**Fig. 7.** PSVs undergo structural changes during development. A, electron micrograph showing a prespore cell from the aggregate stage. The PSVs (arrows) are 140 nm in diameter. Bar, 0.28 μm. B, PSVs from slug prespore cells (arrows) are approximately 250 nm in diameter. Bar, 0.40 μm. C, in preculminants, PSVs are 400–500 nm across and are observed to be closer to the plasma membrane (arrowheads). Bar, 0.33 μm. M, mitochondria; N, nucleus; PM, plasma membrane.

**Fig. 8.** Triton X-114 fractionation of aqueous versus membrane-bound proteins from purified PSVs. A, PSV proteins from band 3 of the Percoll gradient were fractionated into aqueous and detergent phases, separated on a 7–15% SDS-polyacrylamide gel, and stained with Coomassie Brilliant Blue. B, Triton X-114-extracted proteins were separated on 10% SDS-PAGE minigels (Bio-Rad) and transferred to nitrocellulose. The blot was probed with the mAb MUD102, which recognizes the soluble protein, PsB. T, total; A, aqueous phase; D, detergent phase.
the amount of the protein associated with the PSVs (Fig. 9C, lane 5), and precullinants (15 h) show the highest level of accumulation of the GTP-binding protein associated with the prespore vesicles (Fig. 9C, lane 6). Thus as development progresses, we observe an increase in the amount of the GTP-binding protein associated with the prespore vesicles, with maximal levels just prior to fusion and terminal spore cell differentiation. These data are consistent with the hypothesis that the 21-kDa GTP-binding protein may be a regulatory GTPase that controls the rate of fusion by virtue of its levels in the PSVs during the course of development.

**DISCUSSION**

The prespore vesicles appear in the posterior prespore cells during the latter half of morphogenesis in *D. discoideum*. They contain spore coat proteins and other polysaccharide components that accumulate throughout development. At culmination, the PSVs move to, and synchronously fuse with, the plasma membrane. Their contents are deposited into the extracellular space and then incorporated into an environmentally resistant spore coat that surrounds the mature spore. Thus, the developmentally regulated secretion of PSV contents is the pivotal event involved in terminal spore differentiation in this organism. Despite its importance, the developmental signal that coordinates PSV fusion precisely with fruiting body construction and morphogenesis is unknown.

Genetic studies have defined a number of genes that are required for the progression of spore differentiation. Protein kinase A is known to be essential for continuous spore coat protein expression (58). Two genes, tagB and tagC, are expressed at the prestalk/prespore cell boundary in culminants and are thought to transmit a peptide signal for encapsulation to prespore cells (59). A histidine kinase, dhkA, may act as a receptor for the TagB/C signal in prespore cells (60). Deletion of *regA*, a bipartite cAMP phosphodiesterase and a response regulator, results in bypassing the need for the TagB/C signal (61), presumably because cAMP levels remain high in the absence of the phosphodiesterase. cAMP-dependent protein kinase A therefore remains active, and the block to spore cell differentiation is removed.

While these genetic studies have revealed upstream components in the signal transduction pathway leading to spore formation (5), little is known about the downstream events and changes in the PSVs that result in the signal-mediated secretion of their contents at a specific point in development. A biochemical approach to study this phenomenon is essential to understand this process at the molecular level. The initial step in this analysis is to be able to isolate and purify the PSVs, in order to characterize their component proteins.

We developed a procedure that resulted in a highly enriched PSV population free of nuclei, lysosomes, plasma membrane, endoplasmic reticulum, mitochondria, and cytosol. We obtained an overall purification of approximately 80-fold over the starting cell homogenate from four different stages of development. The purified PSVs do not contain appreciable levels of other organelles as judged by the assays of marker proteins, as well as electron microscopy. The purified material is ideal for analysis in the large proteome project that is under way for *Dictyostelium* (23), in order to identify and characterize the structural and enzymatic components in the PSVs. We have already begun to analyze these preparations by two-dimensional gel electrophoresis. The results show that these vesicles have a protein complexity that is greater than may have been initially expected. There are approximately 80 proteins associated with this single organelle.3 One-dimensional gel electrophoresis and Edman sequencing have been used to identify some of the major proteins found in purified endocytic vesicles in *D. discoideum* (62).

Secretory granules from differentiated cells such as adrenal chromaffin cells are known to undergo a maturation process before fusion with the plasma membrane (63). PSVs purified from different developmental stages showed a dramatic increase in the accumulation of the spore coat protein, PsB. Interestingly, they also show an increase in the amount of the GTP-binding protein associated with them. This is consistent with the putative role of this protein in regulating PSV fusion, because its level is highest at a time when the PSVs are about to fuse with the plasma membrane. Concomitant with the biochemical changes, we observed that the PSVs appear to increase in size from 140 to 500 nm in diameter as development progresses. As there does not seem to be a dramatic decrease in the number of PSVs throughout development, it is likely that this increase in size is due to addition of new cargo, from as yet unidentified intermediate post-Golgi vesicles, rather than PSV-PSV fusion. The PSVs, like many other secretory granules, also undergo maturation and selectively acquire the contents required for regulated exocytosis at the proper time in development.

3 S. Srinivasan, M. Molloy, K. Williams, H. Alexander, and S. Alexander, unpublished data.
It has been suggested that the PSVs may have originated from lysosomes, based on co-fractionation of α-mannosidase and acid phosphatase on 35% Percoll gradients at a density of 1.13 g/ml (26). We believe that the reason for these observations was due to two bands of similar densities co-fractionating on the 35% gradient. However, our data clearly show that the lysosomes and PSVs are completely separable on shallower 25% Percoll gradients, with densities of 1.10 and 1.12–1.15 g/ml (depending on the developmental stage), respectively, and the PSV preparations do not contain α-mannosidase at any of the developmental stages tested (Figs. 3 and 6B).

Clearly, many of the major spore coat proteins are targeted to the PSVs. A notable exception is the SpiA protein, which ultimately resides in the inner wall of the spore coat (37). Unlike the proteins in the PsB multiprotein complex which are synthesized coordinately at aggregation, SpiA is expressed much later in development, at the onset of culmination. Based on our data, we suggest that a different, PSV-independent pathway is used to route this protein out of the cell.

In preparation for fusion, the PSVs move from the interior of the cell in aggregates, to the cell periphery in preculinants. However, we still do not know how the PSVs are transported to the cell surface or how they fuse once they get there. In pancreatic cells from rats, it has been shown that zymogen granules move toward the plasma membrane selectively along microtubule tracks (64). Indeed, we have demonstrated that nocodazole inhibits terminal spore differentiation in dissociated prespore cells exposed to 8-bromo-cAMP, while control cells differentiate into spores (4). An analysis of the membrane bound PSV proteins will allow us to search for components that associate with the cytoskeleton, such as kinesin (65), that could mediate this movement.

In our attempts to identify molecules that mediate PSV fusion, we first tested for presence of clathrin. Clathrin has been shown to be an important molecule in regulated exocytosis as well as in endocytosis (66). Recently, a mutant strain lacking clathrin heavy chain has been isolated and has specific defects in the transition from prespore to spore cell differentiation (36). It was possible that the inability of clathrin null cells to differentiate into mature spores was because clathrin is normally involved in PSV docking and fusion. It is clear from our experiments that PSVs are not clathrin coated, and PSV fusion is therefore not clathrin-mediated.

Small GTP-binding proteins (20–25 kDa) are known to be associated with post-Golgi vesicles (55). The Rab family of GTPases is thought to regulate the rate and timing of membrane fusion (28, 56), while the Rac family is known to coordinate vesicular transport along the cytoskeleton (67). We wished to differentiate into mature spores was because clathrin is normally involved in PSV docking and fusion. It is clear from our experiments that PSVs are not clathrin coated, and PSV fusion is therefore not clathrin-mediated.

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Prespore Vesicles of Dictyostelium

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