Proteomics opens doors to the mechanisms of developmentally regulated secretion

Stephen Alexander¹,³, Supriya Srinivasan² and Hannah Alexander¹

¹Division of Biological Sciences
University of Missouri
Columbia, MO 65211-7400

²Gladstone Institute of Cardiovascular Disease
University of California-San Francisco
San Francisco, CA 94141

³ Address correspondence to Stephen Alexander
573-882-6670 TEL
573-882-0123 FAX
alexanderst@missouri.edu

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Abstract.

The program of multicellular development in Dictyostelium discoideum culminates with the assembly of a rugged, environmentally resistant spore coat around each spore cell. After synthesis, the proteins that will constitute the coat are stored in prespore vesicles (PSVs) until an unknown developmental signal triggers the PSVs to move to the cell surface where they fuse with the plasma membrane and secrete their cargo by exocytosis. These events occur synchronously in 80% of the cells in each developing multicellular aggregate, and thus the system offers a unique opportunity to study the developmental regulation of protein secretion in situ. Proteomic analysis of purified PSVs identified many of the constituent proteins, which in turn has lead to novel hypotheses and new experimental avenues regarding the molecular mechanisms regulating secretion from the PSVs.
Protein secretion is a central element of multicellular development.

The organized movement of proteins through cellular compartments is one of the major physiological activities of eucaryotic cells. The secretion of proteins by exocytosis to the outside of cells is an important subset of protein trafficking events. Secretion can be either constitutive - occurring continuously - or regulated - occurring on demand, as a result of an extracellular signal. Both genetic and biochemical approaches have combined to produce our current understanding of eucaryotic protein secretion, although there are clearly many questions that remain unanswered (1). These studies generally are done in yeast or cultured animal cells, each of which has its own experimental advantages. Taken to the next level, one would like to understand the regulation of protein secretion in cells within a developing multicellular organism. Numerous unknown temporal and spatial regulatory mechanisms must control secretion during the course of development. However, these mechanisms take place in cells that are often part of more complex tissues, making their isolation, or study in situ, difficult. For example, migrating neural crest cells are guided by many extracellular matrix molecules, including tenascin (2) and thrombospondin-I (3), which are secreted by the neural crest cells and surrounding mesenchymal cells of the sclerotome, respectively. Similarly, optic nerve growth cone guidance involves an interplay between the extracellular netrin-I and laminin-I proteins that provide positive and negative cues (4). At least part of the problem in studying "developmentally regulated secretion" is finding a system that is amenable to biochemical analysis.
*Dictyostelium discoideum* cells synchronously secrete proteins from unique vesicles during development.

The eucaryotic cellular slime mold *D. discoideum* provides a unique opportunity to study the mechanisms regulating a specific secretory event that is triggered by the developmental program (5). *D. discoideum* cells divide mitotically as long as there is a source of nutrients. The cells resemble mammalian cells, having a simple plasma membrane and typical organelles. When the cells are depleted of nutrients, starvation initiates the onset of a complex and now well described developmental program leading to the generation of environmentally resistant spores (Figure 1) (6-8). The starving cells stochastically begin to secrete cAMP and the surrounding cells respond by migrating up the chemical gradient and relaying the signal to the cells behind them. This process divides up the cells into aggregation territories containing approximately $10^5$ cells each. Thus, $10^9$ cells on a 100 mm dish will produce $10^4$ identical developing aggregates. The newly formed aggregates are true multicellular tissues in which the cells adhere to each other via cell-cell and cell-ECM interactions, communicate and differentiate. The multicellular aggregates continue through development and the cells differentiate into two determined cell populations - prestalk and prespore cells in a ratio of 1:4. The prestalk cells occupy the anterior of the aggregate and ultimately differentiate into vacuolated stalk cells. The prespore cells occupy the rear, and differentiate into the spores. At maturity each fruiting body has an apical mass of 80,000 spores supported by a slender stalk, composed of 20,000 stalk cells (9). The entire process takes 24 hrs and morphogenesis and cell differentiation are synchronous. This allows consistent biochemical studies on an essentially unlimited number of identically staged multicellular "embryos."
The *Dictyostelium discoideum* spore coat is the end product of regulated secretion. Terminally differentiated spores are surrounded by a rugged polarized tri-laminar spore coat which is composed of approximately equal parts of cellulose and glycoproteins (10). The outer layer is electron dense and comprised of loosely associated proteins that can be removed by cold sodium dodecyl sulfate. The middle layer is cellulose, and the inner layer is made up of proteins that are covalently coupled and require heat and denaturing agents for extraction (11, 12). A galactose/N-acetyl-galactosamine-containing polysaccharide (GPS) is also part of the spore coat, residing proximal to the plasma membrane of the cell. Biochemical studies of isolated spore coats have identified about 10 abundant proteins and several minor proteins (13, 14). (A complete list of spore coat proteins, corresponding antibodies and references can be found at [http://www.biosci.missouri.edu/alexander/](http://www.biosci.missouri.edu/alexander/)).

Four of the most abundant spore coat glycoproteins - SP90, PsB/SP85, SP70 and SP60 - exist as a preassembled multiprotein complex (PsB complex) that is held together by a combination of covalent and noncovalent bonds (15, 16). These spore coat proteins are synthesized during aggregation. They are localized exclusively to the prespore cells during the slug stage. During terminal differentiation and spore formation, these proteins, along with other less well-characterized proteins, are deposited into the spore coat by a process of regulated secretion. A series of spore coat protein deletion mutants was used to define the order of assembly of these proteins into the complex (17). Immunostaining has shown that SP96 resides only in the outer layer of the coat, while PsB/SP85 is located only in the inner layer. Thus, the PsB complex is incorporated into the coat with a specific polarity and spans the central cellulose layer. Although it is not known how this polarity is achieved, it is important to note that the PsB
complex has an endogenous cellulose binding activity, which is necessary for proper spore coat assembly (17-20). The spores represent an important evolutionary advantage for this organism allowing the cells to remain viable under extreme environmental conditions including drought, heat and radiation.

The prespore vesicles and regulated secretion of the spore coat proteins.

The prespore cells are defined by the presence of unique secretory vesicles - the prespore vesicles (PSVs) - which appear de novo at the time of aggregation (21). They were originally characterized as carrying a cargo of GPS, which was known to become part of the spore coat. It was later shown that some of the major spore coat proteins, including the PsB multiprotein complex, were also cargo within the PSVs. The origin of the PSVs is a mystery. They appear concomitantly with the appearance of GPS and the spore coat proteins, which are coordinately expressed (15, 16, 22, 23). The proteins are packaged into the PSVs soon after their synthesis (Figure 2). At terminal differentiation, an undefined developmental signal induces the PSVs to migrate to the cell periphery, where they fuse with the plasma membrane and secrete their cargo to the extracellular space where it assembles into the spore coat (Figure 2). When aggregates of cells containing PSVs are made to enter an alternative developmental sequence, where the aggregates become elongated motile "slugs", they do not complete spore differentiation and morphogenesis (24). The PSVs in these cells never secrete unless the aggregates are induced to re-enter the normal developmental sequence. When this happens, secretion from the PSVs is rapidly and coordinately initiated, coinciding with spore formation. Thus, the induction of the secretory process is a true signal-mediated event. Essentially nothing is known about the
molecular mechanisms that underlie this precise spatial and temporal fusion of the PSVs with the plasma membrane, and the coordination of secretion with terminal differentiation.

The questions and a proteomic strategy.

Several significant questions regarding secretion from the PSVs needed answering: 1) What is the genesis of the PSVs?; 2) What is the routing signal(s) used by PSV cargo proteins?; 3) What is the developmental signal that activates the secretion process?; 4) What is the mechanism of fusion between the PSVs and the plasma membrane? and 5) What are the biochemical mechanisms of spore coat protein assembly? Answering these questions required a biochemical approach because there was no reasonably convenient way to screen or select for mutations that affect secretion late in development. An axiom of biochemistry has been that it was necessary to define the components of a reaction or system in order to begin to understand how it functioned. Proteomics is merely a larger version of this strategy. Thus, we set out to purify the PSVs in order to identify and study their constituent proteins.

Purified PSVs begin to provide the answers.

We developed a purification scheme aimed at providing purified PSVs for biochemical analysis. (25, 26). We used enzymatic and immunological assays to show that the PSVs were highly purified and devoid of markers for other organelles. Immuno-electron microscopic analysis showed that purified vesicles had the same morphology and contents of PSVs observed in cells. Moreover, examination of the PSVs purified from cells at different times throughout development showed that they increased in size, but not number, while continuing to take up more of the spore coat protein cargo.
One dimensional SDS-PAGE separation of the proteins in the purified PSVs showed that the PSVs contained many more proteins than just the contents of the future spore coat. The purified PSV preparations were immediately useful for testing some ideas. Based on work on regulated secretion in yeast, we hypothesized that the PSVs would contain small GTP-binding proteins that regulate fusion. \( \gamma^{32} \)GTP-binding assays showed that PSVs indeed contained a PSV-specific developmentally regulated GTP-binding protein, although its identity was still unknown. In addition, we asked if PSVs contained clatharin, which is often associated with endo- and exocytotic vesicles. We showed that the PSVs did not contain clatharin although the cells contained it in abundance. This "one protein at a time" approach was severely limited by our preconceived ideas regarding mechanism and by the availability of assays and antibody reagents (25). Clearly, we needed a more global approach to identify all the proteins in the PSVs so that we could begin to understand the process of developmentally regulated secretion in this system.

The ability to obtain biochemical quantities of pure PSVs allowed us to use a proteomic approach.

The proteomic solution.

The purified PSV preparations were solubilized and separated by two-dimensional polyacrylamide gel electrophoresis (2D PAGE). Approximately 100 proteins were separated. Multiple PSV preparations produced reproducible separations, further confirming the specificity of the purification scheme. Eighty of the most abundant protein spots were excised from the 2D gel, digested with trypsin and the peptides were analyzed by MALDI-TOF spectrometry. These analyses provided identification of more than 50% of the proteins in the PSVs (27; includes a full
list and accession numbers). The *D. discoideum* genome is not yet completely sequenced, and we anticipate the identification of the rest of the PSV proteins upon completion of the sequencing project. Except for the spore coat proteins none were known previously to be associated with the PSVs. The identified proteins fell into several functional groups including:

1. Spore coat proteins - SP96, SP87, SP60. These are the known cargo proteins of the prespore vesicle which ultimately assemble into the spore coat following secretion. Although these proteins are heavily O-glycosylated (28), they were identifiable.

2. Pumps/ATPases - F1 ATPase, P-type ATPase. Proton pumps have been shown to be associated with other secretory vesicles. The acidification of the lumen of these vesicles results in the concentration of their cargo. A similar role is expected for the PSV associated pumps.

3. Calcium binding proteins - Calreticulin and calfumirin. Calcium has previously been associated with the regulation of vesicle traffic protein secretion (29), but assigning functions to specific Ca^{2+} binding proteins has been difficult because of the large number of such proteins and because they often have multiple functions. Specifically associating these proteins with the PSVs allows targeted studies regarding their role in this organelle. Interestingly, calreticulin has been previously associated with the endoplasmic reticulum and recently shown by proteomics to function in the phagosome as well (30, 31).

4. Protein disulfide isomerases - Thioredoxin 2 and Thioredoxin 3. It had been hypothesized that disulfide isomerases were involved in cross-linking the proteins during spore coat maturation and that they might be delivered to the extracellular space via the PSVs (10, 32). The role of these enzymes in cross-linking spore coat proteins can now be examined in strains lacking these enzymes. The genes encoding these proteins are also expressed early in development, and it is not currently known whether the thioredoxins in PSVs are synthesized *de*
novo later in development. In yeast, it was shown that thioredoxin associates with SNARE complexes forming the LMA1 protein complex (33, 34). Thus, the thioredoxins found in the PSVs may play a role in vesicle docking at the plasma membrane as well.

5. cAMP regulated prespore specific proteins - PB74, DG17, and D7. These proteins are expressed exclusively in prespore cells and their cognate genes are regulated by cAMP (35-37). They have frequently been used as markers for cell differentiation, but there has not been any previous suggestion as to their function in spore cell differentiation.

6. Cytoskeletal elements - actin, coactosin, profilin and BiP. The identification of these proteins suggests an actin based mechanism for transporting the PSVs to the plasma membrane for fusion.

7. Esterases - crystal protein and D2. Both proteins have been shown to be associated in a complex with esterosomes, and eventually localize to the spore coat (38, 39). Esterase activity has also been shown to be involved in the lysis of the spore coat during germination (40). We predict that these enzymes are assembled into the spore coat after secretion. The crystal protein has also been shown to associate with the actin cytoskeleton (41), and may therefore function in anchoring the PSVs to the cytoskeleton for transport to the cell surface.

8. Regulatory proteins - PI3 kinase, NDP kinase, and Rab7. Rab GTPases are known to regulate the rate and timing of vesicle traffic (42). The absolute level of GTP-bound Rab associated with secretory vesicles, rather than the rate of GTP hydrolysis per se, dictates fusion competence (43, 44). The Rab7 GTPase was previously shown to function in endosomal traffic in mitotically dividing D. discoideum cells (45). NDP kinase is the GTP-binding protein that was shown earlier to be associated with the purified PSVs (25). It uses ATP to generate other nucleoside triphosphates including GTP, is localized at sites of increased GTP concentration and it thought
to be the major source of GTP in the cell (46). Interestingly, NDP kinase is also expressed early in development, and a mechanism must exist for importing it to the PSVs. The localization and activity of NDP kinase may control the GTP-bound state of Rab7, which in turn may regulate the fusion of the PSVs with the plasma membrane.

9. Other proteins. An important outcome of the PSV proteome analysis was the identification of proteins for which there are corresponding ESTs, but no known function. Although we do not know the specific biochemical functions of these proteins, we do know that they are an integral part of the PSVs and therefore function in some step of regulated secretion. The homology of these gene products with those of other organisms suggests a similar function in those species as well. It will be especially exciting to elucidate the function of these novel proteins.

Lessons learned.

The proteomic analysis of the PSVs resulted in a number of important observations that bear on any study of an isolated organelle. 1) The PSV proteome was specific. A limited and reproducible set of proteins was identified in the analysis, indicating that these proteins are intimately associated with this organelle. 2) Interestingly, several of the PSV proteins previously had been implicated in other cellular/developmental functions, such as those described for Rab7 and NDP kinase. This suggests that proteins can have multiple roles in cells at different times in development, and that function can be dictated by specific subcellular localization (although how this changes during development remains to be elucidated). This observation also has been made in other systems. Analysis of phagosomes revealed the presence of endoplasmic reticulum proteins and lead to the novel discovery that the ER is a source of phagosome membrane (30, 31). 3) This study has suggested functional roles for cell-type
specific proteins of previously unknown function. Many genes in *D. discoideum* are activated only in the developing prespore cell population, and have long been used as markers for differentiation. Although much is known about the regulation of these genes, their function in prespore cells has remained unknown. The proteomics study shows that some of the cognate proteins have a direct role in PSV structure and/or function.  4) One of the PSV proteins was homologous to a protein of unknown function in Arabidopsis. Thus we suggest that the Arabidopsis protein will have a function in protein secretion. This demonstrates that a proteomic analysis in one organism can help understand a protein's function in another organism.  5) Perhaps most importantly, the identification of specific PSV associated proteins readily suggests hypotheses that can be tested to determine the precise mechanism of developmental control of PSV biogenesis, signal perception, vesicle movement and fusion with the plasma membrane.

The next step - back to molecular genetics.

The most obvious initial approach is to use homologous recombination to make gene disruptions in each of the identified PSV proteins and monitor the effect of these mutation on spore differentiation. Presumably, at least some of these would block spore maturation but it would still be unclear what mechanistic event was affected.

A novel strategy is to make a general tester strain in which to systematically interrogate the effects of various null mutations. This strain would express one of the spore coat genes (e.g., the gene encoding SP60) fused with the gene encoding green fluorescent protein (GFP) under the control of its own promoter. (The loss of SP60 has no discernable effect on the growth and development of *D. discoideum.*) The GFP-SP60 would localize to - and thus define - the PSVs.
Single and double null mutations of the genes of interest can then be produced in this genetic background, and fluorescent localization of the GFP-SP60 can be used to assess in living cells if the biogenesis, maturation and fusion of the PSVs are disrupted in the mutants. This will allow the association of each of the cognate proteins with a specific step of the pathway of developmentally regulated secretion from the PSVs (Figure 3). The molecular genetic tools available for use with *D. discoideum* make this approach eminently feasible.

An interesting example of going from protein identification to function is the case of NDP kinase and Rab7. NDP kinase is the major source for GTP in the cell. Its biochemistry has been extensively studied in *D. discoideum* as well as other systems (47-51). Since it is associated with the PSVs in a developmentally regulated manner, its continuous expression and localization may be required to generate GTP to drive vesicle fusion. The role of NDP-kinase can be studied by replacing the wild-type copy of the gene in the GFP-SP60 tester strain with a tetracycline (tetR) inducible copy (52). This allows the expression of NDP kinase to be turned on and off at different times during differentiation, and to follow its role in controlling PSV secretion by examining GFP-SP60 localization.

The interaction of NDP-kinase with Rab7 can be probed further using crosslinking and co-immunoprecipitation approaches that have been used successfully in other proteomic applications as well (53, 54). We propose that NDP kinase may be the proximal source of GTP for the Rab7, which suggests that it plays a major role in regulating the onset of the secretory event. As such we believe that the two proteins may be physically associated.
Lastly, we can test directly whether NDP kinase supplies GTP to Rab7, by incubating prespore cells with $\gamma^{32}$P-ATP, and sampling throughout late development. The GTP generated by NDP kinase will be labeled with the $\gamma^{32}$P from ATP. The association of this $\gamma^{32}$P-GTP with Rab7 will be detectable by immunoprecipitating Rab7. No $\gamma^{32}$P-GTP should be associated with Rab7 if the NDP kinase is switched off. This will provide direct evidence that NDP kinase generates GTP for Rab7 activity.

The foregoing discussion shows that the global discovery-based nature of proteomics is having a major impact on the progress of elucidating fundamental mechanisms in cell biology (Figure 4). Similar proteomic successes have come from studies of the molecular basis of cancer, resistance to chemotherapeutic drugs and the discovery of drug targets, where large scale studies of changes in the proteome have identified new protein targets for drug design and therapy (55-57). We anticipate that probing the specific functions of the PSV associated proteins identified in the proteomic studies will provide molecular details of the developmental regulation of secretion of the spore coat proteins. The mechanistic information we gain from the studies in this accessible model system can then be applied to other examples of protein secretion in development. This work clearly shows that proteomics can rapidly lead to numerous new experimental avenues in diverse experimental systems.

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References

1. Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., and Walter, P. (2002) Molecular biology of the cell, 4 ed, Pages. Garland, New York.
2. Tucker, R. P., and McKay, S. E. (1991) The expression of tenascin by neural crest cells and glia. Development 112, 1031-1039
3. Tucker, R. P., Hagios, C., Chiquet-Ehrismann, R., Lawler, J., Hall, R. J., and Erickson, C. A. (1999) Thrombospondin-I and neural crest cell migration. Dev. Dyn. 214, 321-322
4. Hopker, V. H., Shewan, D., Tessier-Lavigne, M., Poo, M., and Holt, C. (1999) Growth-cone attraction to netrin-1 is converted to repulsion by laminin-1. Nature 401, 69-73
5. Srinivasan, S., Alexander, H., and Alexander, S. (2000) Crossing the finish line of development: regulated secretion of Dictyostelium proteins. Trends Cell Biol. 10, 215-219
6. Kessin, R. H. (2001) Dictyostelium - Evolution, cell biology, and the development of multicellularity., Pages. Cambridge Univ. Press, Cambridge.
7. Alexander, S., and Rossomando, E. F. 1992. Regulation of morphogenesis in Dictyostelium discoideum, p. 29-61. In Rossomando, E. F. and Alexander, S. (ed.), Morphogenesis: An analysis of the development of biological form. Dekker, New York.
8. Loomis, W. F. (1982) The Development of Dictyostelium discoideum, Pages. Academic Press, New York.
9. Srinivasan, S., Alexander, H., and Alexander, S. (2000) The Dictyostelium fruiting body - A structure of cells and cellulose. Trends Cell Biol. 10, 315
10. West, C. M., and Erdos, G. W. (1990) Formation of the Dictyostelium spore coat. Dev. Genet. 11, 492-506
11. Erdos, G. W., and West, C. M. (1989) Formation and organization of the spore coat of Dictyostelium discoideum. Exp. Mycology 13, 169-182
12. Hemmes, D. E., Kojima-Buddenhagen, E. S., and Hohl, H. R. (1972) Structural and enzymatic analysis of the spore wall layers in Dictyostelium discoideum. J. Ultrastr. Res. 41, 406-417
13. Wilkinson, D., Wilson, J., and Hames, B. (1983) Synthesis of spore proteins during development of Dictyostelium discoideum. Biochem. J. 216, 567
14. Orlowski, M., and Loomis, W. F. (1979) Plasma membrane proteins of Dictyostelium: the spore coat proteins. Dev. Biol. 71, 297-307
15. Watson, N., McGuire, V., and Alexander, S. (1994) The PsB glycoprotein complex is secreted as a preassembled precursor of the spore coat in Dictyostelium discoideum. J. Cell. Sci. 107, 2567-2579
16. Watson, N., Williams, K. L., and Alexander, S. (1993) A developmentally regulated glycoprotein complex from Dictyostelium discoideum. J. Biol. Chem. 268, 22634-22641
17. McGuire, V., and Alexander, S. (1996) PsB multiprotein complex of Dictyostelium discoideum. Demonstration of cellulose binding activity and order of protein subunit assembly. J. Biol. Chem. 271, 14596-14603
18. Srinivasan, S., Griffiths, K. R., McGuire, V., Champion, A., Williams, K. L., and Alexander, S. (2000) The cellulose-binding activity of the PsB multiprotein complex is required for proper assembly of the spore coat and spore viability in Dictyostelium discoideum. Microbiol. 146, 1829-1839
19. Zhang, P., McGlynn, A., Loomis, W. F., Blanton, R. L., and West, C. M. (2001) Spore coat formation and timely sporulation depend on cellulose in *Dictyostelium*. *Differentiation* 67, 72-79

20. Zhang, Y. Y., Zhang, P., and West, C. M. (1999) A linking function for the cellulose-binding protein SP85 in the spore coat of *Dictyostelium discoideum*. *J. Cell Sci.* 112, 4367-4377

21. Hohl, H., and Hamamoto, S. (1969) Ultrastructure of spore differentiation in *Dictyostelium*: the prespore vacuole. *J. Ultrastruct. Res.* 26, 442-453

22. Devine, K. M., Bergmann, J. E., and Loomis, W. F. (1983) Spore coat proteins of *Dictyostelium discoideum* are packaged in prespore vesicles. *Dev. Biol.* 99, 437-446

23. Fosnaugh, K. L., and Loomis, W. F. (1991) Coordinate regulation of the spore coat genes in *Dictyostelium discoideum*. *Dev. Genet.* 12, 123-132

24. Newell, P. C., Telser, A., and Sussman, M. (1969) Alternative developmental pathways determined by environmental conditions in the cellular slime mold *Dictyostelium discoideum*. *J. Bact.* 100, 763-768

25. Srinivasan, S., Alexander, H., and Alexander, S. (1999) The prespore vesicles of *Dictyostelium discoideum* - Purification, characterization, and developmental regulation. *J. Biol. Chem.* 274, 35823-35831

26. Maeda, Y., and Takeuchi, I. (1969) Cell differentiation and fine structures in the development of the cellular slime molds. *Dev. Growth Diff.* 11, 232-245

27. Srinivasan, S., Traini, M., Herbert, B., Sexton, D., Harry, J., Alexander, H., Williams, K. L., and Alexander, S. (2001) Proteomic analysis of a developmentally regulated secretory vesicle. *Proteomics* 1, 1119-1127

28. Mreyen, M., Champion, A., Srinivasan, S., Karuso, P., Williams, K. L., and Packer, N. H. (2000) Multiple O-glycoforms on the spore coat protein SP96 in *Dictyostelium discoideum* - Fuc(alpha1-3)GlcNAc-alpha-1-P-Ser is the major modification. *J. Biol. Chem.* 275, 12164-12174

29. Brunger, A. T. (2000) Structural insights into the molecular mechanism of Ca(2+)-dependent exocytosis. *Curr. Opin. Neurobiol.* 10, 293-302

30. Gagnon E, Duclos S, Rondeau C, Chevet E, Cameron PH, Steele-Mortimer O, Paiment J, Bergeron JJ, and M., D. (2002) Endoplasmic reticulum-mediated phagocytosis is a mechanism of entry into macrophages. *Cell* 110, 119-131

31. Garin J, Diez R, Kieffer S, Dermine JF, Duclos S, Gagnon E, Sadoul R, Rondeau C, and M., D. (2001) The phagosome proteome: Insight into phagosome functions. *J. Cell Biol.* 152, 165-180

32. Alexander, S. 1997. Developmental regulation and function of glycoproteins in *Dictyostelium discoideum*, p. 349-362. In Maeda, Y., Inouye, K., and Takeuchi, I. (ed.), *Dictyostelium*: a model system for cell and developmental biology. Universal Academy Press, Tokyo.

33. Xu, Z., and Wickner, W. (1996) Thioredoxin is required for vacuole inheritance in *Saccharomyces cerevisiae*. *J. Cell Biol.* 132, 787-94

34. Xu, Z., Mayer, A., Muller, E., and Wickner, W. (1997) A heterodimer of thioredoxin and I(B)2 cooperates with Sec18p (NSF) to promote yeast vacuole inheritance. *J. Cell Biol.* 136, 299-306
35. Driscoll, D. M., and Williams, J. G. (1987) Two divergently transcribed genes of 
Dictyostelium discoideum are cyclic AMP-inducible and coregulated during 
development. Mol. Cell. Biol. 7, 4482-4489
36. Agarwal, A., Sloger, M. S., Oyama, M., and Blumberg, D. D. (1994) Analysis of a novel 
cyclic AMP inducible prespore gene in Dictyostelium discoideum: Evidence for different 
patterns of cAMP regulation. Differentiation 57, 151-162
37. Hopkinson, S. B., Pollenz, R. S., Drummond, I., and Chisholm, R. L. (1989) Expression 
and organization of BP74, a cyclic AMP-regulated gene expressed during Dictyostelium 
discoideum development. Mol. Cell. Biol. 9, 4170-4178
38. Bomblies, L., Biegelmann, E., Döring, V., Gerisch, G., Krafft, C. H., Noegel, A. A., 
Schleicher, M., and Hummel, B. M. (1990) Membrane-enclosed crystals in Dictyostelium 
discoideum cells, consisting of developmentally regulated proteins with sequence 
similarities to known esterases. J. Cell Biol. 110, 669-679
39. Rubino, S., Mann, S., Hori, R., Pinko, C., and Firtel, R. (1989) Molecular analysis of a 
developmentally regulated gene required for Dictyostelium aggregation. Dev. Biol. 131, 
27-36
40. Cotter, D., Miura-Santo, L., and Hohl, H. (1969) Ultrastructural changes during 
germination of Dictyostelium discoideum. J. Bact. 100, 1020-1026
41. Chia, C. P., Bomblies, L., and Taylor, E. K. (1998) Cytoskeletal association of an 
esterase In Dictyostelium discoideum. Exp. Cell Res. 244, 340-348
42. Novick, P., and Zerial, M. (1997) The diversity of Rab proteins in vesicle transport. Curr. 
Opin. Cell Biol. 9, 496-504
43. Lupashin, V. V., and Waters, M. G. (1997) t-SNARE activation through transient 
interaction with a Rab-like guanosine triphosphatase. Science 276, 1255-1258
44. Rothman, J. E. (1994) Mechanisms of intracellular protein transport. Nature 125, 55-63
45. Buczynski, G., Bush, J., Zhang, L., Rodriguez-Paris, J., and Cardelli, J. (1997) Evidence 
for a recycling role for Rab7 in regulating a late step in endocytosis and in retention of 
lysosomal enzymes in Dictyostelium discoideum. Mol Biol Cell 8, 1343-60
46. Bominaar, A. A., Molijn, A. C., Pestel, M., Veron, M., and Van, H. P. (1993) Activation 
of G-proteins by receptor-stimulated nucleoside diphosphate kinase in Dictyostelium. 
EMBO J. 12, 2275-2279
47. Aguado-Velasco, C., Veron, M., Rambow, J. A., and Kuczmański, E. R. (1996) NDP 
kine can modify contraction of Dictyostelium cytoskeletons. Cell Motil. Cytoskel. 34, 
194-205
48. Dumas, C., Lascu, I., Moréra, S., Glaser, P., Fourme, R., Wallet, V., Lacombe, M. L., 
Véron, M., and Janin, J. (1992) X-ray structure of nucleoside diphosphate kinase. EMBO 
J. 11, 3203-3208
49. Lacombe, M. L., Wallet, V., Troll, H., and Veron, M. (1990) Functional cloning of a 
nucleoside diphosphate kinase from Dictyostelium discoideum. J. Biol. Chem. 265, 
10012-10018
50. Moréra, S., Chiadmi, M., LeBras, G., Lascu, I., and Janin, J. (1995) Mechanism of 
phosphate transfer by nucleoside diphosphate kinase: X-ray structures of the 
phosphohistidine intermediate of the enzymes from Drosophila and Dictyostelium. 
Biochemistry 34, 11062-11070
51. Wallet, V., Mutzel, R., Troll, H., Barzu, O., Wurster, B., Veron, M., and Lacombe, M. L. 
(1990) Dictyostelium nucleoside diphosphate kinase highly homologous to Nm23 and
Awd proteins involved in mammalian tumor metastasis and *Drosophila* development. *J. Natl. Cancer Inst.* **82**, 1199-202

52. Blaauw, M., Linskens, M. H. K., and van Haastert, P. J. M. (2000) Efficient control of gene expression by a tetracycline-dependent transactivator in single *Dictyostelium discoideum* cells. *Gene* **252**, 71-82

53. Gavin, A. C., Bosche, M., Kräus, R., Grandi, P., Marzioch, M., Bauer, A., Schultz, J. E., Rick, J. M., Michon, A. M., Cruciat, C. M., Remor, M., Hofert, C., Schelder, M., Brajenovic, M., Ruffner, H., Merino, A., Klein, K., Hudak, M., Dickson, D., Rudi, T., Gnau, V., Bauch, A., Bastuck, S., Huhse, B., Leutwein, C., Heurtier, M. A., Copley, R. R., Edelmann, A., Querfurth, E., Rybin, V., Drewes, G., Raida, M., Bouwmeester, T., Bork, P., Seraphin, B., Kuster, B., Neubauer, G., and Superti-Furga, G. (2002) Functional organization of the yeast proteome by systematic analysis of protein complexes. *Nature* **415**, 123-124

54. Ho, Y., Gruhler, A., Heilbut, A., Bader, G., Moore, L., Adams, S., Millar, A., Taylor, P., Bennett, K., Boutiller, K., Yang, L., Wolting, C., Donaldson, I., Schandorff, S., Shewnarane, J., Vo, M., Taggart, J., Goudreault, M., Muskat, B., Alfarano, C., Dewar, D., Lin, Z., Michalickova, K., Willems, A., Sassi, H., Neilsen, P., Rasmussen, K., Andersen, J., Johansen, L., Hansen, L., Jespersen, H., Podtelejnikov, A., Neilsen, E., Crawford, J., Poulsen, V., Sorensen, B., Matthiesen, J., Hendrickson, R., Gleson, F., Pawson, T., Moran, M., Durocher, D., Mann, M., Hogue, C., Figeys, D., and Tyers, M. (2002) Systematic identification of protein complexes in *Saccharomyces cerevisiae* by mass spectrometry. *Nature* **415**, 180-183

55. Rohlf, C., and Hollis, K. (2003) Modern proteomic strategies in the study of complex neuropsychiatric disorders. *Biol. Psychiatry* **53**, 847-853

56. Verrills, N. M., and Kavallaris, M. (2003) Drug resistance mechanisms in cancer cells: a proteomics perspective. *Curr. Opin. Mol. Ther.* **5**, 258-265

57. Wulfkuhle, J. D., Pawelz, C. P., Steeg, P. S., Petricoin, E. F., and Liotta, L. (2003) Proteomic approaches to the diagnosis, treatment, and monitoring of cancer. *Adv. Exp. Med. Biol.* **532**, 59-68
Figure Legends.

Figure 1. Morphogenesis and cell differentiation in *Dictyostelium discoideum*. When nutrients are exhausted, cells stop dividing and aggregate into multicellular structures each containing $10^5$ cells. As development proceeds, the anterior cells (yellow) differentiate into prestalk cells while the posterior cells (orange) differentiate into prespore cells. Morphogenetic movements ultimately give rise to mature fruiting bodies which have a mass of 80,000 spores resting on top of a slender multicellular stalk composed of 20,000 stalk cells. Under nutrient conditions, the spores germinate and begin mitotic cell division. For a more complete description of the *D. discoideum* developmental cycle see Kessin (6).

Figure 2. The prespore vesicles (PSVs) contain proteins destined for assembly into the spore coat. Each prespore cell contains prespore vesicles (PSVs) in which the spore coat proteins exist as specific preassembled multiprotein complexes (PsB/SP85 multiprotein complex). The PSVs continue to accumulate the PsB/SP85 complex until an unknown developmental signal initiates the vesicles to synchronously move to, and fuse with, the plasma membrane resulting in the exocytosis of the PsB/SP85 complex. The complexes, which have endogenous cellulose binding activity, assemble with newly synthesized cellulose and GPS to form the environmentally resistant coat of the spores.

Figure 3. Predicted phenotypes of mutants lacking individual prespore vesicle proteins. Null mutants (knockouts) will be produced by homologous recombination in a standard tester strain expressing a GFP-SP60 fusion. This will allow assessment of the secretory process by monitoring the fate of the fluorescent PSVs throughout development. The predicted classes of
phenotypes include A) PSV genesis is blocked; B) PSVs are made but do not move to plasma membrane during development; C) PSVs move to plasma membrane but do not fuse; and D) Fusion and exocytosis occur but spore coat proteins cannot assemble into a mature spore coat.

**Figure 4. Overview of the proteomic approach to understanding the regulation of developmental secretion.** Flow of investigation from the identification of the prespore vesicles in *D. discoideum* prespore cells (21), to the purification of the PSVs (25), to proteomic analysis of the PSV proteins and development of a model predicting their functions in controlling secretion during development (27).
Figure 1

1. Dividing cells
2. Aggregation
3. Early Aggregate
4. Late Aggregate
5. Fruiting Body

- Spores
- Prestalk cells
- Prespore cells
- Stalk cells
PsB multiprotein complex

Prespore cell containing PSVs

Secreting prespore cells

PsB complex assembled in spore coat

Mature spore
Figure 3

A. No genesis of PSVs

B. No movement of PSVs to plasma membrane

C. No exocytosis from PSVs

D. No assembly of PsB multiprotein complex into spore coat
