The PsB glycoprotein complex is secreted as a preassembled precursor of the spore coat in *Dictyostelium discoideum*

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**SUMMARY**

The PsB glycoprotein in *Dictyostelium discoideum* is one of a diverse group of developmentally regulated, prespore-cell-specific proteins, that contain a common O-linked oligosaccharide. This post-translational modification is dependent on the wild-type modB allele. The PsB protein exists as part of a multiprotein complex of six different proteins, which have different post-translational modifications and are held together by both covalent and non-covalent interactions (Watson et al. (1993). *J. Biol. Chem.* 268, 22634-22641). In this study we have used microscopic and biochemical analyses to examine the cellular localization and function of the PsB complex during development. We found that the PsB complex first accumulates in prespore vesicles in slug cells and is secreted later during culmination and becomes localized to both the extracellular matrix of the apical spore mass of mature fruiting bodies and to the inner layer of the spore coat. The PsB associated with the spore coat is covalently bound by disulfide bridges. The PsB protein always exists in a multiprotein complex, but the composition of the PsB complex changes during secretion and spore maturation. Some of the PsB complex proteins have been identified as spore coat proteins. These data demonstrate that some of the proteins that form the spore coat exist as a preassembled precursor complex. The PsB complex is secreted in a developmentally regulated manner during the process of spore differentiation, at which time proteins of the complex, as well as additional spore coat proteins, become covalently associated in at least two forms of extracellular matrix: the inter-sporo matrix and the spore coat. These and other studies show that proteins with modB dependent O-linked oligosaccharides are involved in a wide variety of processes underlying morphogenesis in this organism. These developmental processes are the direct result of cellular mechanisms regulating protein targeting, assembly and secretion, and the assembly of specific extracellular matrices.

**Key words:** secretion, exocytosis, glycoprotein complex, O-glycosylation, extracellular matrix, phosphorylation

**INTRODUCTION**

*Dictyostelium discoideum* is a widely studied organism in developmental and cell biology. When their bacterial food source becomes depleted, the vegetative amoebae aggregate and undergo a complex program of morphogenesis and cyto-differentiation, which ultimately gives rise to three specialized tissues (basal disk cells, stalk cells and spores) in the terminal fruiting body. The multicellular development of *D. discoideum* has many features in common with the morphogenesis of higher organisms, including intercellular cohesion and signalling, cell movement and patterning (Loomis, 1982; Alexander and Rossomando, 1992). There has been much interest in the role of glycoproteins in the morphogenesis of this organism (Freeze, 1992). Monoclonal antibodies (mAbs) have been produced as probes for glycoprotein structure and function (Grant and Williams, 1983; West and Erdos, 1990). The mAb MUD50 defines a family of largely prespore-specific glycoproteins (Alexander et al., 1988). MUD50 is specific for an O-linked oligosaccharide determinant that is missing in glycosylation-defective modB mutant strains (Alexander et al., 1988; Gooley, 1992; Gooley et al., 1992). Two of the MUD50-reactive prespore cell-specific proteins, PsA and PsB, are also recognized by mAbs MUD1 and MUD102, respectively, which are directed to the polypeptide backbones of these glycoproteins (Grant et al., 1985; Smith et al., 1989).

The MUD50-reactive family of glycoproteins is a diverse group of proteins, which have different temporal patterns of developmental expression (Alexander et al., 1988; Smith et al., 1989). Some, such as PsA, are integral membrane proteins, while others, such as PsB, are soluble. The specific intracellular localization of most of the soluble glycoproteins is not known, although some become part of the extracellular matrix sheath of the slug (Zhou-Chou, 1993). Some MUD50-reactive O-linked glycoproteins are also N-glycosylated, and some differ in their degree of phosphorylation.

The availability of specific antibodies to glycoproteins such as PsA and PsB provides an opportunity to examine the biosynthesis, processing and intracellular sorting of O-glycosylated proteins, which have been studied less thoroughly than the N-
linked glycoproteins (Hart et al., 1988, 1989). Our recent studies on the PsB O-linked glycoprotein have shown that PsB is part of a specific complex of soluble proteins (Watson et al., 1993). Newly synthesized PsB appears in a complex containing six different proteins (p112, PsB (95 kDa), p78, p70, p63 and p58) held together by both covalent and non-covalent bonds. The PsB complex is developmentally regulated with synthesis and assembly beginning before the slug stage, and with maximum synthesis at early culmination. Interestingly, only the PsB protein in the complex has the MUD50 O-linked oligosaccharide determinant. None of the proteins in the complex is N-glycosylated as assessed by digestion with peptide N-glycosidase F (Alexander and Elder, 1989) and one of the proteins is heavily phosphorylated. Our goal is to understand the differential processing and assembly of the structurally distinct proteins in the PsB multi-protein complex, as well as the function of the complex in Dictyostelium development in order to gain understanding of the basic cell biological mechanisms that underlie development.

Our approach was to exploit specific monoclonal antibodies to determine the cellular localization and function of the PsB complex, using microscopy and biochemical fractionation. In this report we demonstrate that the PsB complex is secreted in a developmentally regulated manner during culmination and spore formation. Biochemical localization experiments show that the PsB protein is secreted during culmination and becomes localized in two extracellular matrices: the matrix around the spores and the spore coats. Immunostaining revealed that the PsB antigen first accumulates in prespore vesicles (PSVs) in slug cells and is later secreted during culmination. In terminal fruiting bodies, PsB is not detected in basal disk cells, but is present in tracks on stalks and in the interspore matrix. Mature spores do not stain with the PsB-specific antibody, but spore coats from germinated spores stain well, indicating that PsB becomes associated with the inner layer of the spore coat during spore formation. These observations indicate that the PsB complex plays a role in spore morphogenesis. Although its cellular location changes during development, PsB always remains associated in a complex, while the overall composition of the complex changes during spore maturation. In addition, we demonstrate that two of the PsB complex proteins are previously identified spore coat proteins. Thus, the PsB multiprotein complex is a preassembled precursor subunit of the spore coat. This work has direct significance to a number of central problems in cell biology, including regulated protein sorting and secretion, processing of multiprotein complexes, and assembly of extracellular matrices.

### MATERIALS AND METHODS

#### Strains and culture conditions

Strains NP73, W3S80B and W576 each contain a different polymorphic form of the PsA and PsB proteins (Grant et al., 1985; Smith et al., 1989). Cells were grown in association with *Klebsiella aerogenes* on SM agar plates, and developed on black paper filters (10^6 cells/filter) saturated with LPS buffer (20 mM KCl, 2.5 mM MgCl₂, 40 mM potassium phosphate, pH 6.5, containing 0.5 mg/ml of streptomycin sulfate) (Sussman, 1987).

#### Cell labeling and lysis

Cells were radiolabeled with [35S]methionine (ICN, 1180 Ci/mmol) or [32P] (ICN, 8835 Ci/mmol) as described (Watson et al., 1993). Cells were lysed in 1% (v/v) Nonidet P-40 detergent in 10 mM Tris-HCl, pH 8.0, 30 µg/ml phenylmethylsulfonyl fluoride (PMSF), 30 µg/ml 4-(2-aminoethyl)benzenesulfonyl fluoride-HCl (AEBSF; Sigma) (Alexander et al., 1988).

#### Electrophoresis and western blotting

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described (Laemmli, 1970). All gels were reducing 7% to 15% polyacrylamide gradient gels, 19 cm in length, which allowed for separation of the proteins of the complex (Watson et al., 1993). Western immunoblotting was performed using a semidy blotting apparatus (Towbin et al., 1979; Kyhse-Andersen, 1984). The mAbs used are shown in Table 1. The IgG class mAbs were detected with affinity-purified rabbit anti-mouse IgG (whole molecule) peroxidase-conjugated antibody. The MUD102 mAb (IgM class) was detected with affinity-purified goat anti-mouse IgM (mu chain-specific)-peroxidase-conjugated antibody.

#### Immunoprecipitation

Immunoprecipitation of radioactively labeled extracts was performed as described (Alexander et al., 1984; Watson et al., 1993). For immunoprecipitations with IgG class mAbs, rabbit anti-mouse IgG (whole molecule) was used as a secondary antibody to increase the signal. For immunoprecipitations with MUD102 (an IgM mAb), goat anti-mouse IgM (mu chain-specific) was used as a secondary antibody. Pansorbin (Calbiochem) was used to bind the antibody-antigen complexes.

#### Immunofluorescent staining

All manipulations were carried out at room temperature. Vegetative cells were harvested from clearing bacterial plates and washed four times in distilled water. The final cell pellet was suspended to 1×10^9 cells/ml and 50 µl samples were dispensed in a 60 mm line at one end of 100 mm diameter Petri dishes containing 1.5% agar in distilled water. The Petri dishes were placed in dark boxes with the lines of cells opposite and parallel to an open slit for light. Ethanol-washed 12 mm diameter coverslips were placed on the agar surface of the plates about 10 mm from each line of cells on the side closest to the slit for light. The boxes were incubated in an illuminated 22°C incubator. After 12 hours the cells had aggregated and developed into slugs, which had begun moving towards the light. After the slugs moved onto the coverslips, they were fixed for microscopy or were exposed to overhead light to induce culmination (Newell et al., 1969) and allowed to develop into mature fruiting bodies before fixing for microscopy.

To prepare coverslips for staining (Alexander et al., 1992), they were removed from the agar surface, flooded with 3.7% formaldehyde in LPS, incubated for 3.5 minutes and washed for 10 minutes in LPS. To permeabilize cells, the coverslips were incubated with 0.5% NP-40 in

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**Table 1. Summary of mAbs used in this study**

<table>
<thead>
<tr>
<th>mAb</th>
<th>Cognate antigen proteins</th>
<th>Epitope structure recognized</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>MUD3</td>
<td>SP96</td>
<td>Protein</td>
<td>Browne et al. (1989)</td>
</tr>
<tr>
<td>MUD50</td>
<td>modB-dependent O-linked glycoproteins</td>
<td>O-linked oligosaccharide</td>
<td>Grant et al. (1985)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Alexander et al. (1988)</td>
</tr>
<tr>
<td>MUD62</td>
<td>SP96</td>
<td>O-linked fucose oligosaccharide</td>
<td>Grant and Williams (1983)</td>
</tr>
<tr>
<td></td>
<td>SP75</td>
<td></td>
<td>Grant et al. (1985)</td>
</tr>
<tr>
<td>MUD102</td>
<td>PsB</td>
<td>Protein</td>
<td>Smith et al. (1989)</td>
</tr>
<tr>
<td></td>
<td>mAb16.2*</td>
<td></td>
<td>Watson et al. (1993)</td>
</tr>
<tr>
<td></td>
<td>SP85</td>
<td></td>
<td>West et al. (1986)</td>
</tr>
<tr>
<td></td>
<td>(IgG)</td>
<td></td>
<td>West and Erdos (1988)</td>
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*This mAb has the same specificity as mAb16.1 in the references cited.*

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were lysed in 1% (v/v) Nonidet P-40 (NP-40) detergent in 10 mM Tris-HCl, pH 8.0, 30 µg/ml phenylmethylsulfonyl fluoride (PMSF), 30 µg/ml 4-(2-aminoethyl)benzenesulfonyl fluoride-HCl (AEBSF; Sigma) (Alexander et al., 1988).
LPS for 5 minutes and washed three times for 5 minutes in LPS. Non-
specific binding sites were blocked by incubation for 20 minutes in 1% 
bovine serum albumin (BSA) in LPS (80 μl/coverslip). Each coverslip 
was then incubated for 2 hours with 80 μl of a 1:500 dilution of primary 
antibody, MUD102, in 1% BSA in LPS. Parallel negative controls were 
incubated in the same solution without the primary antibody. The cov-
erslips were drained, washed three times with LPS, and incubated for 1 
hour with 80 μl of a 1:500 dilution of affinity-purified rhodamine-
conjugated goat anti-mouse immunoglobulins (IgG, IgA, IgM; whole 
molecules; Cappel/Organon Teknika) in 1% BSA in LPS. The cover-
slips were washed three times in LPS and were mounted on slides (cell 
side down) in a solution of Airvol (Air Products, Allentown, PA) in 
LPS containing 50 mg/ml DABCO (1,4-diazabicyclo-[2-2-2]-octane).
All slides were examined and photographed with a Zeiss IM inverted 
microscope using a ×100 Neofluar lens.

Germination of spores

Spores were germinated by dimethylsulfoxide (DMSO) activation. One-day-old spores of strain WS380B were harvested and washed in 10 mM potassium phosphate buffer, pH 6.7 (PB), and stored at −80°C in PB containing 20% glycerol. Thawed spores were washed in PB, activated at 10° spores/ml by shaking at 23°C at 100 rpm for 30 minutes in PB containing 20% DMSO, washed twice more in PB, and germinated by shaking at 23°C in PB (Ennis and Sussman, 1975). Samples of 100 μl were dispensed onto poly-L-lysine (1 mg/ml)-coated coverslips and allowed to settle for 10 minutes. The coverslips were fixed and immunostained as described above.

Preparation and extraction of purified spores

For large-scale spore purification (see Fig. 4), cells were grown on bacteria on trays of SM agar (21.5 cm × 28.0 cm), allowed to develop to fruiting bodies, and extracted as described (Wilkinson and Hames, 1983). Fruiting bodies were harvested into 400 ml ice-cold 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 30 μg/ml PMSF. The suspension was vacuum filtered through 100 mesh nylon. The retained material (mostly stalks mixed with some spores) was washed in 100 ml of the same buffer and filtered as before. The filtrates, which contained purified spores (>99% spores by microscopic examination), were combined and centrifuged for 10 minutes at 3000 g at 4°C. The supernatant was collected on ice and the pellet of spores was washed in 100 ml 1% SDS in the same buffer (extraction buffer), and centrifuged at 500 g for 5 minutes. The spore pellet was washed in extraction buffer two additional times. The spore pellet was then extracted three times by boiling in SDS as follows. For each extraction the spore pellet was suspended in 100 ml extrusion buffer as above, boiled for 5 minutes, and centrifuged for 3 minutes at 27,000 g at 4°C. The resulting spore pellet was suspended in 100 ml 5% 2-mercaptoethanol (2-ME) in extraction buffer (2-ME extraction buffer), boiled and centrifuged. The final pellet was suspended in 100 ml 2-ME extraction buffer. Samples were analyzed as described for Fig. 4.

To prepare purified [35S]methionine-labeled WS380B spores (see Fig. 5), cells were allowed to develop on filters. Labeling with [35S]methionine (150 μCi/filter) was begun at the early finger stage (6 hours of development) and continued for 18 hours, when the aggreg-
gates had developed into fruiting bodies. Spores from the spore mass 
at the top of fruiting bodies from six filters were carefully collected 
with a spatula into 2.5 ml 10 mM Tris-HCl, pH 8.0, 30 μg/ml PMSF 
(Tris-PMSF) on ice. Subsequent manipulations were at 4°C. 
The spores were pelleted for 5 minutes at 12,000 g, washed in 2.5 ml of 
Tris-PMSF and centrifuged. The spore pellet was then washed in 2.5 
ml 1% NP-40 in Tris-PMSF and centrifuged as before. The resulting 
pellet was suspended in 2.4 ml 0.5% SDS in Tris-PMSF, boiled 5 
minutes, and adjusted to 5% NP-40. Samples were analyzed by SDS-
PAGE, western immunoblotting and immunoprecipitation.

RESULTS

The PsB protein is secreted from vesicles during development

We previously showed that a Dictyostelium discoideum 

prespore-specific O-linked glycoprotein, PsB, exists in a develop-
mentally regulated multiprotein complex composed of at 
least six different proteins held together by both covalent and 
non-covalent bonds (Watson et al., 1993). Maximum synthesis of 
the complex occurs from the slug stage through early culmi-
nation. In order to determine the localization and possible role 
of the PsB complex in development, we immunostained 

developing aggregates with MUD102, a mAb specific for a 
protein epitope of the PsB glycoprotein (Smith et al., 1989).

Slugs were allowed to migrate onto coverslips and develop 
before fixing and immunostaining. Slug cells (Fig. 1A, phase-
contrast; and B, fluorescence) showed MUD102 staining of 
duplicate doughnut-shaped bodies, which had the same mor-
phology as previously characterized prespore vesicles 
(Maeda and Takeuchi, 1969; Devine et al., 1983). These 

bodies were randomly distributed in the cells. When mature 

fruiting bodies were examined (Fig. 1C,D), there was no staining of the extracellular matrix (m) underlying the basal 
disk or the basal disk cells (bd), but diffuse staining was seen 
where the stalk (st) of the fruiting body extends above the 
plane of focus (see bracket in Fig. 1C,D). A spore (sp) that 
had fallen away from the spore mass did not stain with 
MUD102 (Fig. 1C). Bright staining was observed in thin 
vertical tracks (see arrowheads) on the stalks of fruiting 

bodies (Fig. 1E,F). Different lines of staining were seen as 
the plane of focus was changed. In the apical spore mass of 

fruiting bodies and clumps of spores that had fallen away 
during fixation, staining was seen in the extracellular matrix 

surrounding the spores (arrowheads, Fig. 1G,H), but again 
spores that had fallen free from the matrix did not stain (Fig. 
1G,H). Controls that were immunostained without MUD102 
showed no staining (data not shown). These results indicate 
that during development the PsB protein first accumulates in 
intracellular vesicles and is secreted later during culmination 
to become part of the extracellular matrix surrounding the 
spores of the mature fruiting body. Some of this material 
appears to be deposited on the stalk as the spore mass rises 
on the developing fruiting bodies.

We used western analysis to correlate the expression of the 
PsB antigen with its secretion during development. Cells 

developing on filters were harvested into LPS at hourly 
intervals. The cells were vortexed and centrifuged to give a 

pellet containing cells and cell-associated material, and a 

supernatant containing soluble extracellular material. Analysis 
of these fractions with MUD102 revealed that most of the 

secretion of PsB occurred just before and during early culmi-
nation (Fig. 2A). Initial detection of cell-associated PsB (at 
6 hours of development) preceded detection of extracellular PsB 
(at 8 hours) by about 2 hours. In contrast to the immunofluo-
rescence results, the biochemical data indicate that the majority 
of the PsB remains associated with the maturing spores in the 

pellet fractions. Western analysis of the same fractions with 
MUD50, which recognizes an O-linked oligosaccharide on 

PsB and other proteins, showed that PsB is the primary modB 
glycoprotein secreted during culmination, although different
Fig. 1. Immunofluorescence analysis of PsB localization during development. WS380B slugs that had migrated and developed on coverslips were stained with the PsB-specific mAb, MUD102. Phase-contrast images (A,C,E,G) are presented in the left column and immunofluorescence images (B,D,F,H) are shown in the right column. The first row (A,B) shows individual slug cells after 8 hours development. The second row (C,D) shows a segment of a basal disk of a fruiting body after 24 hours of development. Individual basal disk cells (bd), extracellular slime sheath matrix (m), a free spore (sp), and the stalk (st; rising out of the plane of focus) are indicated in C. The third row (E,F) shows a segment of a stalk of a fruiting body after 24 hours of development. Vertical tracks on the stalk that stained with MUD102 are marked with arrowheads. The fourth row (G,H) shows the spore mass of a fruiting body after 24 hours of development. Arrowheads mark the extracellular matrix that stained with MUD102 around the spores in the spore mass. Bar (A), 10 µm.
MUD50 reactive proteins are secreted earlier (at 4 hours) in development (see arrows in Fig. 2B).

PsB is also located in the interior of spore coats

In the immunofluorescence experiments described above, all of the PsB detected in fruiting bodies was extracellular. However, the subsequent western analysis with MUD102 indicated that most of the PsB antigen was cell associated. This raised the possibility that, within the maturing spore, the PsB protein is also associated with the spore coat or the amoeba therein. Its location may have been undetected by the immunofluorescence staining procedure because spores are resistant to the NP-40 detergent used to permeabilize cells.

We tested this hypothesis by allowing spores to germinate and then immunostaining their inner surfaces. The preparation contained ungerminated spores, swollen spores in the process of germination, empty spore coats, and newly emerged amoebae. Samples of this preparation were deposited on poly-L-lysine-coated coverslips and subjected to immunostaining with MUD102. For comparison, Fig. 3A,B shows that the phase-dense interspore extracellular matrix (arrowhead) stains with MUD102, but the spores themselves do not stain. However, after germination the empty spore coats (sc) from germinated spores stained intensely (Fig. 3C,D). Intact spores (sp) and freshly emerged amoebae (a) did not stain. Spores that were swelling (ss) and beginning to germinate showed a thin line of MUD102 staining on the edge of the spores, where the spore coat was beginning to crack open. Controls stained in the absence of MUD102 primary antibody were negative (data not shown). These experiments demonstrate that a significant amount of PsB is incorporated into the inner layer of the spore coat, where it is not accessible to the MUD102 antibody until the spore starts to split open during germination.

The PsB protein is disulfide bonded in the spore coats of mature spores

On the basis of the localization results, we examined the manner of association of PsB with the spore coat. Spores were purified from mature fruiting bodies and extracted by the method of Wilkinson and Hames (1983) (Fig. 4A,B). Panel A is shown to document the relative amount of total proteins in each fraction. In agreement with data presented earlier, Fig. 4B shows that some PsB was found in the supernatant of freshly harvested spores (lane 1), while much of the PsB was recovered in the spore pellet (lane 2). Although a significant amount of PsB was eluted from the spore pellet with three sequential SDS washes (lanes 3–5), the majority remained in the spore pellet (lane 6). A small amount of the remaining spore-associated PsB could be extracted by boiling in SDS (lane 7), but no more PsB was removed by two subsequent boiling SDS extractions (lanes 8 and 9). Finally, the spore pellet was extracted by boiling in SDS containing 2-ME. This
treatment released most of the remaining spore-associated PsB (lane 10 and 11). These results indicate that, while some PsB is easily extracted from the spore pellets (probably from inter-spore matrix that co-sediments with the spores during washing), a large amount of the PsB is tightly associated with the spore coat by disulfide crosslinking. This extraction procedure appears to result in a significant purification of the PsB protein (compare lanes 2 and 10 in A and B).

The composition of the PsB complex changes after secretion

Developing aggregates were continuously labeled with [35S]methionine, beginning at the early finger stage, and allowed to develop into mature fruiting bodies (18 hours total labeling time). Spores were carefully harvested, washed and lysed. The samples were analyzed by SDS-PAGE and by immunoprecipitation with MUD102 (Fig. 5A,B). For comparison, the typical pattern of protein bands of the PsB complex immunoprecipitated from slug cell extracts with MUD102 (Watson et al., 1993) is shown in Fig. 5B, lane 5. Examination of the pattern of labeled proteins indicates that relatively few soluble extracellular matrix proteins were washed free from the spores despite repetitive washes (Fig. 5A, lanes 1-3). However, many proteins were recovered in the spore pellet (Fig. 5A, lane 4). Immunoprecipitation with MUD102 (Fig. 5B) shows that the PsB complex was found in both the soluble extracellular spore matrix (lane 1) and the washed spore pellet (lane 4). Compared to the PsB complex of slugs, three proteins (p112, PsB and p58) remained in the secreted PsB complex, while the p78 protein appeared greatly reduced, and the p70 and p63 proteins were not detected. Interestingly, several new bands were present in the MUD102 immunoprecipitates. New bands were present at about 102 kDa (p102) and 90 kDa (p90) in the soluble spore wash (soluble extracellular matrix) and the
Glycoprotein complex secretion in Dictyostelium washed spore pellet. Another new band of about 45 kDa (p45) was present in the spore pellet fraction only. These bands could be due to proteolytic processing of proteins from the slug cell complex or recruitment of different proteins into the complex during or after secretion of the PsB complex. These results clearly show that PsB remains in a complex during secretion and spore formation, and the composition of the PsB complex changes during these processes.

Identification of spore coat proteins in the PsB complex from slugs

Some spore coat proteins of Dictyostelium discoideum have been previously characterized and mAbs have been produced to some of them (West and Erdos, 1990; Freeze, 1992). In order to determine if the PsB complex contained previously described spore coat proteins, slug cell extracts were analyzed with several of these antibodies. The monoclonal antibody mAb16.2 (West et al., 1986) reacts specifically with a spore coat protein, SP85, which, like PsB, is found in the inner layer of the spore coat. Since PsB is polymorphic (Smith et al., 1989), we used this fact to determine if PsB is the same protein as SP85. Lysates of slug stage aggregates of strains NP73, WS380B and WS576, each with a different polymorphic form of PsB, were analyzed by western blotting with MUD102, mAb16.2 or MUD50 (Fig. 6). The SP85-specific mAb binds to the same polymorphic band that was identified with MUD102 and MUD50 as PsB (Watson et al., 1993). This result establishes the identity of the modB-dependent O-glycosylated PsB protein as the previously described SP85 protein.

Another mAb, MUD62, specifically recognizes a common fucose epitope on spore coat proteins SP96 and SP75 (Grant and Williams, 1983; Champion et al., 1991). The p112 protein of the PsB complex has properties similar to SP96. Both are heavily phosphorylated and both p112 from the PsB complex in slug cells and some SP96 from the spore coat can be released in the absence of thiol reagent (Delaney et al., 1983; Wilkinson and Hames, 1983; Watson et al., 1993). Western analysis of a slug lysate demonstrated that MUD102 and MUD62 are specific for different proteins, as expected (Fig. 7A). A lysate of [35S]methionine-labeled slugs was then immunoprecipitated with MUD102, MUD62 or MUD3, a mAb specific for a protein epitope on SP96 (Browne et al., 1989). The electrophoretic patterns of the proteins show that all three antibodies precipitated the same complex (Fig. 7B). MUD102 precipitated the complex by binding to PsB (Fig. 7B, lane 1), while MUD62 and MUD3 precipitated the same complex by binding to p112/SP96 (Fig. 7B, lanes 2 and 3). The SP75 protein was present only in the MUD62 immunoprecipitate due to the direct binding of MUD62 to the fucose epitope of SP75 (Fig. 7B, lane 2). This experiment demonstrates that p112, the largest protein in the PsB complex, is identical to the spore coat.

Fig. 4. Analysis of the association of PsB with spores. WS380B mature fruiting bodies were harvested and large-scale spore purification and sequential extraction were performed as described in Materials and Methods. Samples (all lanes contained 1.3×10^7 spore equivalents per lane, except lane 1, which contained one fifth of this amount) were subjected to SDS-PAGE on duplicate gels. One gel (A) was stained with Coomassie Blue R-250 and the other gel (B) was analyzed by western blotting with MUD102. Lanes 1 and 2 contained samples of the supernatant (S) and resuspended spore pellet (P) fractions of the spore harvest filtrate. Lanes 3-5 contained samples of the supernatant fractions of the three sequential room-temperature SDS extractions (S1-S3, respectively), and lane 6 contained a sample of the resuspended spore pellet (P) after these extractions. Lanes 7-9 contained samples of the supernatant fractions of the three sequential boiling SDS extractions of the spore pellet used in lane 6 (S1-S3, respectively); lane 10 contained a sample of the supernatant (S) of the final extraction of the spores in boiling SDS containing 2-ME; and lane 11 contains a sample of the resuspended final spore pellet (P). The order of sequential extraction treatments is indicated (left to right) above the appropriate lanes. The migration of molecular mass standards is indicated on the left and right. The position of the PsB protein is indicated on the right.
protein SP96, and that spore coat protein SP75 is not part of the PsB complex in slug cells. Moreover, these results again emphasize that some spore coat proteins are preassembled prior to secretion.

**The spore coat proteins p112/SP96 and SP75 become covalently attached to the spore coat during spore maturation**

Purified spores were extracted sequentially in boiling SDS and in boiling SDS with 2-ME, and analyzed by western blotting with MUD62 (Fig. 8). The results revealed that, although more than half of the p112/SP96 was removed from spores by boiling in SDS (lanes 1 and 2), much of the p112/SP96 was tightly associated with the spores and was released only after boiling in SDS in the presence of 2-ME (lane 3). This indicates that a significant amount of P112/SP96 associated with spores becomes covalently bound in the spore coat during spore maturation. Recall that in slug extracts p112/SP96 is easily and completely removed from the PsB complex with urea (Watson et al., 1993). In addition, MUD62 staining revealed that little SP75 is removed from spores by boiling with SDS (lanes 1 and 2). However, the bulk of SP75 was released from spores after boiling with SDS in the presence of 2-ME (lane 3). Thus, SP75, as well as p112/SP96, becomes covalently bound in the spore coat during spore maturation.

**The spore coat proteins p112/SP96 and SP75 are the two major phosphorylated proteins in slugs**

We previously showed that the largest protein, p112, of the PsB complex was heavily phosphorylated at four to eight hours of development (Watson et al., 1993). Other work has shown that two major phosphoproteins appear during *Dictyostelium discoideum* development (Devine et al., 1982; Akalehiywot and Siu, 1983; Delaney et al., 1983; Alexander et al., 1988). Therefore, we investigated the relationship of these proteins to the proteins in the PsB complex. Duplicate filters of developing cells were labeled with $[^{35}S]$methionine or $^{32}$P$_i$ at the slug stage and were lysed and immunoprecipitated with MUD50, MUD102 or MUD62. The banding patterns of the immunoprecipitates were analyzed by SDS-PAGE (Fig. 9). The immunoprecipitates of the lysate of slugs labeled with $[^{35}S]$methionine show that all three of the mAbs brought down the same multiprotein complex (lanes 1-3). In addition, MUD50 immunoprecipitated a separate MUD50-reactive protein, p104 (Watson et al., 1993), and MUD62 immunoprecipitated the separate MUD62-reactive protein, SP75 (also see Fig. 7). The two major phosphoproteins of $^{32}$P$_i$-labeled slugs can be seen in lane 4. These two proteins migrated in the gel at the same positions as the $[^{35}S]$methionine-labeled proteins p112/SP96 and SP75 that were immunoprecipitated with MUD62 (lane 3). Moreover, the immunoprecipitates of the lysate of $^{32}$P$_i$-labeled slugs show that all three mAbs brought

![Fig. 5. Analysis of the secreted PsB complex. Developing aggregates of strain WS380B were labeled with $[^{35}S]$methionine, beginning at the early finger stage (6 hours of development). After 24 hours of development, spores were collected and sequentially washed. The washed spore pellet was suspended in 0.5% SDS and samples of the washed supernatant and pellet fractions were either directly analyzed by SDS-PAGE, to examine the entire profile of $[^{35}S]$methionine-labeled proteins (A), or were analyzed by SDS-PAGE after immunoprecipitation with MUD102 to assess the composition of the PsB complex (B). (A) An autoradiograph of the SDS-PAGE of total $[^{35}S]$methionine-labeled proteins: lanes 1-4 each contains $10^6$ spore equivalents; lane 5 is a control lysate of $[^{35}S]$methionine-labeled slugs. (B) The immunoprecipitation analysis with MUD102: lanes 1-4 each contain the immunoprecipitate from $2\times10^7$ spore equivalents; lane 5 contains the immunoprecipitate from $[^{35}S]$methionine-labeled slugs used as a control to show the protein band pattern of the previously reported PsB complex (Watson et al., 1993). The samples are as follows: lane 1 (S1), first spore aqueous wash supernatant; lane 2 (S2), second spore aqueous wash supernatant; lane 3 (S3), third spore wash (NP-40 detergent) supernatant; lane 4 (P), final washed spore pellet in 0.5% SDS; lane 5 (slugs), slug lysate control. The migration of molecular mass standards is indicated in the center. The positions of the proteins previously reported in the PsB complex from slugs are shown on the right. The positions of additional protein bands that appear in the immunoprecipitates of spore fractions are indicated on the right in brackets. Note that the pellet fraction in lane 4 contains all the proteins from the intact spores including the amoebae. Therefore, this lane has more bands than Fig. 4A, lanes 10 and 11.](image-url)
down the larger of the two major phosphoproteins, p112/SP96 (lanes 5-7). In addition, MUD62 immunoprecipitates the other major phosphoprotein, SP75 (lane 7). It is interesting that the two major phosphoproteins made in slugs are spore coat proteins that eventually become covalently linked to the spore coat during spore maturation, but only p112/SP96 is associated with the PsB complex in slugs. We previously estimated that p112/SP96 has at least 50 times more phosphate incorporated than PsA, which has one phosphate per molecule due to its glycosyl phosphatidylinositol tail (Haynes et al., 1993; Watson et al., 1993). From the roughly equivalent $^{32}\text{P}$-labeling of p112/SP96 and SP75 seen in lane 7, we conclude that both of the major phosphoproteins are heavily phosphorylated.

**DISCUSSION**

O-linked oligosaccharides, although common post-translational modifications, have been studied relatively little compared to N-linked glycans (Hart et al., 1988, 1989; Freeze, 1992; Varki, 1993). This was largely due to a lack of appropriate glycosidases, glycosylation-defective mutants and O-linked oligosaccharide-specific antibodies. However, both specific mutants and antibodies are available for such studies in *D. discoideum*. Thus, we have been able to study a prespore-cell-specific O-linked glycoprotein, PsB, from *D. discoideum* in order to gain understanding of the synthesis, processing, routing and developmental function of O-linked glycoproteins. The PsB protein is a member of a family of glycoproteins that share a common O-linked oligosaccharide epitope as defined by the mAb MUD50 and the modB mutation (West and Loomis, 1985; Alexander et al., 1988; Smith et al., 1989). These glycoproteins show diverse biochemical properties and differential developmental regulation and localization. In a previous study we reported that the PsB glycoprotein exists in a developmentally regulated multiprotein complex (Watson et al., 1993).
The PsB complex is composed of at least six different proteins (p112, PsB (95 kDa), p78, p70, p63 and p58), which are held together by a combination of covalent and non-covalent interactions, and have different post-translational modifications. In this study we used mAbs in order to identify, microscopically and biochemically, the cellular localization and possible function of the PsB complex.

Immunofluorescence microscopy of slug cells stained with MUD102 revealed that the PsB antigen first accumulates in doughnut-shaped bodies dispersed throughout prespore cells. These PsB-containing bodies have the same morphology, developmental timing of appearance and cellular localization as the previously described prespore vesicles (PSVs), which have been shown to contain all the major components of spore coats except for cellulose (Maeda and Takeuchi, 1969; West and Erdos, 1990; Freeze, 1992). The identity of the PsB-containing compartment of slug cells as PSVs was confirmed by our finding that some of the proteins of the PsB complex are identical to previously reported spore coat proteins, which reside in the PSVs in slugs.

Our immunofluorescence labeling studies further revealed that the PsB antigen is secreted later in development. During culmination MUD102 stains vertical tracks on stalk tubes and the extracellular interspore matrix of mature fruiting bodies. The reason PsB antigen stained tracks on the stalk tube is not clear. The staining may represent PsB complex in the matrix, which is merely left behind on the stalk as the spore mass migrates upward. Whether it plays a role in the migration of the spore mass up the stalk tube remains to be determined. However, the specific localization to vertical tracks might indicate a specific type of association with the cellulose stalk tube. Indeed, we have recently observed that PsB is disulfide linked to purified stalks (unpublished). It is interesting to note that an antigen reactive with antibody mAb40.1 is also

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**Fig. 8.** Analysis of the association of P112/SP96 and SP75 with spores. Purified spores from strain WS380B were washed three times in aqueous buffer. The washed spore pellet (4×10^8 spores) was suspended and sequentially extracted two times by boiling in extraction buffer and one time by boiling in 2-ME extraction buffer. Samples of the spore extract supernatants (10^7 spore equivalents) and the final pellet (2×10^7 spore equivalents) were analyzed by western blotting with MUD62. The samples are shown as follows: lane 1 (S1), first boiling SDS spore extraction supernatant; lane 2 (S2), second boiling SDS spore extraction supernatant; lane 3 (S3), boiling SDS with 2-ME spore extraction supernatant; lane 4 (P), final extracted spore pellet. The positions of spore coat proteins, p112/SP96 and SP75, are indicated on the right.

**Fig. 9.** Identification of p112/SP96 and SP75 as the major phosphoproteins in developing slugs. Samples (0.5×10^9 cell equivalents) of [35S]methionine-labeled (lanes 1-3) or 32P-labeled (lanes 5-7) WS380B slug lysates were immunoprecipitated with MUD50 (lanes 1 and 5), MUD102 (lanes 2 and 6) or MUD62 (lanes 3 and 7). The immunoprecipitated samples were subjected to SDS-PAGE followed by autoradiography. Lane 4 contained a sample (10^7 cell equivalents) of total 32P-labeled proteins. The positions of the PsB complex proteins are indicated in the center. The position of another MUD50-reactive protein, p104 (Watson et al., 1993), is indicated on the left. The positions of the MUD62-reactive spore coat proteins, p112/SP96 and SP75, are indicated on the right.
deposited on the stalk tube after the spore mass has passed (West and Erdos, 1988).

Although MUD102 does not stain intact spores, it stains coats from germinated spores and swollen spores in the process of germination. These data indicate that PsB is involved in spore coat formation and ultimately becomes part of both the interspore matrix and the inner layer of the spore coat. This spore coat localization of the PsB protein agrees with earlier work on spore coat protein SP85, which in this study we demonstrate to be immunologically identical to PsB (West and Erdos, 1988, 1990). In contrast to these reports, we clearly see specific PsB/SP85 staining in the interspore matrix (Figs 1, 3). Moreover, our work demonstrates that the PsB protein in the interspore matrix remains in a specific multiprotein complex with other proteins previously localized to this extracellular matrix.

We confirmed the immunofluorescence observations by biochemical fractionation experiments. Western blots revealed that synthesis of the PsB protein began at the finger stage and that secretion of the PsB protein began about two hours later, shortly before the beginning of culmination. MUD50 staining showed that the PsB protein was the only prominent modB-dependent glycoprotein secreted during culmination, although several other MUD50-reactive proteins are secreted earlier in development. In contrast to the immunofluorescence analysis, which showed intense staining of the interspore matrix, the biochemical fractionation indicated that most of the PsB protein remained in the cell-associated fractions. We believe that this is due to two factors: (1) the PsB complex is stably assembled into the inner layer of the spore coat; and (2) PsB is associated with an opaque, phase-dense matrix that is expected to co-sediment with the cells and spores during fractionation (Fig. 3A,B).

Sequential extraction of spores from mature fruiting bodies confirmed this distribution of the PsB glycoprotein in the extracellular matrix and spore coats. While a portion of the interspore matrix is readily soluble in aqueous buffer, a larger fraction requires SDS for solubilization and presumably represents the dense matrix seen in our phase-contrast pictures (Fig. 3A). Furthermore, the PsB protein is covalently associated with the inner layer of the spore coat and requires both SDS and 2-ME for extraction. We should note that examination of the proteins recovered by these extractions indicates that the spore coat contains a large number of proteins in contrast to the relatively few that have been characterized previously by others (Orlowski and Loomis, 1979; Devine et al., 1982; see Wilkinson and Hames, 1983, for similar observations).

After determining that the PsB complex is an integral component of the spore coat, we tested whether the PsB complex might contain previously identified spore coat proteins by using mAbs specific to spore coat proteins. We show here by western analysis that PsB reacts with mAb16.2, which was previously reported to react with spore coat protein SP85 (West et al., 1986). We also compared immunoprecipitation with mAbs MUD102 and MUD62, which is specific for a fucose-containing epitope on the previously reported spore coat proteins SP96 and SP75, and MUD3, which is specific for a protein epitope on SP96 (Grant and Williams, 1983; Browne et al., 1989; Champion et al., 1991). While the antibodies are specific for different proteins by western analysis, all three precipitated the PsB complex. The complex was precipitated by MUD62 and MUD3 because these antibodies bind to p112 (via the fucose and protein epitopes, respectively). The MUD62 precipitate also contained the SP75 protein, as expected, because it contains the fucose epitope. In contrast, the MUD102 antibody precipitated the complex by virtue of its binding to PsB. This result demonstrates that p112 is identical to the previously described SP96. Therefore, we conclude that the PsB complex contains at least two previously identified major spore coat proteins, PsB/SP85 and p112/SP96. The relationship of the other proteins in the PsB complex to other spore coat proteins can be easily tested by direct protein sequencing and cross-reactivity with other antibodies as they become available.

These experiments establish that at least two spore coat proteins, PsB/SP85 and p112/SP96, exist in a preassembled complex (the PsB complex), which accumulates in prespore vesicles in slug cells. Moreover, these proteins remain in a complex after secretion. This is in contrast to previous work, which detected no preassembly of spore coat components (Erdos and West, 1989; West and Erdos, 1990). However, it is important to note that the composition of the PsB complex changes during the process of spore maturation. The p70 and p63 proteins found in the PsB complex of slugs (Watson et al., 1993) are not detected in the PsB complex immunoprecipitated from the soluble interspore matrix or from spore coats (Fig. 5). This may explain the inconsistent yield of these two proteins in immunoprecipitates in our previous work (Watson et al., 1993). Perhaps these molecules are involved in the localization of the PsB complex to the PSVs (e.g. as chaperonins or anchoring proteins) or in preventing premature assembly of the PsB complex with other spore coat components that are also sequestered in the PSV compartment. In addition, the relative amount of p78 in the PsB complex is greatly reduced after secretion. Three other proteins found in the PsB complex from slugs, PsB/SP85, p112/SP96 and p58, remain in the complex after secretion. Several new bands appeared in immunoprecipitates of the PsB complex after secretion. These bands are due to either proteolytic processing of the proteins of the PsB complex or recruitment of different proteins into the complex during secretion or spore coat assembly. Purification and protein sequencing of these bands should help resolve these issues.

The P112/SP96 and SP75 proteins, although not originally covalently bonded in the PsB complex in the PSVs, become disulfide linked in the spore coat following secretion. This observation raises the important issues of the relative topology of these proteins in the spore coat and the temporal order of their assembly. Other studies have shown that P112/SP96 is exposed to the surface of the spore (Erdos and West, 1989; Richardson and Loomis, 1992; Browne and Williams, 1993). We now have shown that it is part of the PsB complex and that the PsB protein is on the inner side of the spore coat. The inner and outer layers of the spore coat are separated by a layer of cellulose and we suggest, on the basis of all of these data, that the P112/SP96 traverses the cellulose layer.

The second issue is the order of assembly of the spore coat. Since we find the PsB complex in the interspore matrix, as well as the inner layer of the spore coat, it is unlikely that the inner layer of the spore coat is assembled last during spore formation, after cellulose deposition in the middle layer, as others have proposed (Erdos and West, 1989; West and Erdos, 1990). However, cellulose deposition during spore formation may be the necessary step for assembly of the PsB complex.
into the spore coat by directly binding to the cellulose layer as it is being formed. The disulfide bonds may be formed between the spore coat proteins during coat assembly either by a non-specific oxidative process or by the action of an enzymatic activity similar to protein disulfide isomerase, which would result in specific disulfide bonds between the proteins.

We also show that the two major phosphoproteins synthesized in slugs are p112/SP96 and SP75. This is consistent with earlier studies (Akaléhiwot and Sii, 1983; Delaney et al., 1983). It is interesting to note that both of these phosphorylated proteins ultimately become part of the spore coat. The p112/SP96 protein is incorporated into the spore coat as part of the PsB complex, which is initially synthesized and formed at the slug stage of development. The SP75 protein, on the other hand, is incorporated in an independent manner into the spore coat after secretion from the PSVs. It will be interesting to determine the mode, extent, sites and possible developmental changes of phosphorylation of these two spore coat proteins, and the role of phosphorylation of these proteins in spore coat assembly. This and our previous work establishes that p112/SP96 and SP75 are multiply phosphorylated. We would like to point out that the SP75 protein, which is phosphorylated and fucosylated, is possibly the same protein as the spore coat protein SP70, which also was reported to be phosphorylated and fucosylated (Devine et al., 1982).

In recent years Dictyostelium discoideum has proven to be a productive system for the study of protein glycosylation and its role in development. Significant progress is being made in the study of O-linked oligosaccharides in the development of this organism. Several proteins of the modB family of O-linked glycoproteins have been recently studied. Some of these glycoproteins, such as gp80 and PsA, are membrane proteins whereas others, such as the sheathins and PsB, are soluble and secreted (Freeze, 1992; Watson et al., 1993; Zhou-Chou, 1993). Although these soluble modB proteins are secreted at different developmental times and probably by different routes, those studied thus far appear ultimately to be associated with extracellular matrices (sheathins in slime sheath and PsB in interspore matrix and spore coat). A common feature of the slime sheath and spore coat matrices is that they contain cellulose, but cellulose is not found in the interspore matrix (Erdos and West, 1989; Freeze, 1992). Cellulose may be an important common feature for the assembly of these secreted O-linked glycoproteins into organized extracellular matrices that have structural integrity.

In this paper we have described the developmentally regulated secretion of the PsB complex and its assembly into extracellular matrices during spore differentiation in Dictyostelium discoideum. We have recently purified the PsB protein from spore coats for protein sequencing and production of oligonucleotide probes and additional PsB-specific antibodies (V. McGuire, N. Watson and S. Alexander, work in progress). When available, the sequence of the protein and the cloned gene should give additional insight into the structure and function of the PsB glycoprotein as well as possible consensus motifs for O-glycosylation. This information should be useful for further dissection of the structure, processing and assembly of the PsB multiprotein complex. It will be important to determine the signals and mechanism determining the developmentally regulated secretion of this complex, and how the secretion mechanism compares to other secretory routes in this and other organisms. Additional study of the PsB complex should also yield significant information about the molecular details of spore formation in this organism, the genesis of extracellular matrices, and molecular assembly processes of general biological interest.

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