SP75 is encoded by the DP87 gene and belongs to a family of modular *Dictyostelium discoideum* outer layer spore coat proteins

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Highly purified spore coats of *Dictyostelium discoideum* each contained about 5×10^6 protein molecules as determined by amino acid composition analysis. By two-dimensional gel electrophoresis the coats were found to contain nine major-abundance and numerous minor protein species, most of which were highly enriched relative to the adjacent interspore matrix. Protein was nearly quantitatively eluted by denaturants and 2-mercaptoethanol, showing that it was not irreversibly cross-linked. Because a reducing agent is required together with denaturants to elute most proteins if their free thiol groups have been prealkylated, it was concluded that the *D. discoideum* spore coat proteins are disulfide cross-linked into the matrix. One major coat protein, SP75, was partially sequenced and found to be encoded by the previously identified DP87 gene; this finding was supported by additional physical, genetic, biochemical and microscopic evidence. The five major proteins for which genes have been cloned were associated with the outer layer of the coat. In coats missing one or more of four of these proteins as a result of gene disruption, there were physical changes but, with one exception, the other major coat proteins appeared to be incorporated normally. Sequence analysis showed that these five outer layer coat proteins are homologous and consist of alternating sequence motifs related to epithelial mucin repeats, basic proline repeats found in salivary acidic proline-rich proteins, the NH₂-terminal subdomain of epidermal growth factor modules and other cysteine repeats. Based on these and other observations, outer layer coat proteins are predicted to organize indeterminately to form a cell surface microenvironment supportive of cellulose morphogenesis during spore coat formation.

Keywords: *Dictyostelium discoideum*, spore coat proteins, mucins, EGF-repeats, basic proline repeats

INTRODUCTION

The *Dictyostelium discoideum* spore coat, a 210 nm thick layer of protein and polysaccharide at the spore cell surface, constitutes an elastic (Lydan & Cotter, 1994) physical barrier that constrains osmotic swelling of the amoeba and protects it from exposure to external agents. The coat forms a permeability barrier to protein-sized molecules, but not to water and other small molecules (Cotter, 1981; West & Erdos, 1990). Coat formation is

initiated by secretion of stored protein into a soluble pool at the cell surface. As cellulose is subsequently deposited, outer and inner layers of protein become organized around a middle zone of cellulose (West & Erdos, 1990, 1992; Freeze, 1995). As previously suggested (Wilkinson & Hames, 1983) and confirmed in this paper, disulfide bonds appear to stabilize the coat via intermolecular cross-links in addition to probably stabilizing protein folding. Some intermolecular disulfide cross-links may be established prior to secretion from the prespore vesicle (Devine *et al.*, 1983; Watson *et al.*, 1993, 1994), though it is not clear to what extent observed cross-links were the result of the propensity, shown here, of coat protein

Abbreviations: MSH, 2-mercaptoethanol; IAA, iodoacetamide; EGF, epidermal growth factor.

disulfide bonds to migrate and exchange in the presence of denaturing agents.

Since five of the coat genes have been cloned (Fosnaugh & Loomis, 1989a, b; Tasaka *et al.*, 1990; Haberstroh & Firtel, 1990; Yoder *et al.*, 1994), it is possible to genetically probe the role of protein in coat formation. Deletion of each of three genes encoding proteins of the outer layer results in a coat more permeable to a protein tracer (Fosnaugh *et al.*, 1995). Strains lacking all three proteins have the highest permeability (Fosnaugh *et al.*, 1995), germinate more efficiently and have a lower buoyant density (this report). This study shows that the DP87 gene (Ozaki *et al.*, 1993) encodes another outer-layer-associated coat protein, SP75, allowing an analysis of the role of this protein in a DP87 gene disruption strain. However, in none of these mutant strains was the overall protein composition of the remaining coat examined. The role of protein in the coat has also been examined in *modB* and fucosylation mutants, in which specific protein glycosylation pathways are disrupted. These mutants have spore coats that are more permeable and have higher buoyant densities than those of the wild-type (Gonzalez-Yanes *et al.*, 1989; Aparicio *et al.*, 1990). The results indicate that coat proteins have an important role in coat permeability, exerted either during coat assembly or in the final structure.

Multiple spore coat proteins have been identified (Devine *et al.*, 1982, 1983; Wilkinson & Hames, 1983; West & Erdos, 1988; Yoder *et al.*, 1994), but the number and relative abundance of these proteins are not known. The present study establishes that coat proteins can be quantitatively extracted from the coat for two-dimensional gel electrophoretic analysis. Highly purified coats contain nine abundant protein species, and even the most protein-deficient gene disruption mutant incorporates and expresses substantial levels of all but one of the coat protein species. The result is consistent with an indeterminate rather than a crystalline lattice arrangement of outer layer proteins in the coat. Examination of the sequences of five of the abundant outer layer coat proteins not only confirms a common ancestry but also suggests that they consist of mucin-like, basic proline and epidermal growth factor (EGF)-like motifs. Thus, the outer layer of the coat consists of modular proteins with mucin-like properties that conform to the indeterminate type of organization suggested above.

METHODS

Cells. Strain TL56 is a derivative of the normal strain Ax3 of *Dictyostelium discoideum* in which the genes *cotA*, *cotB* and *cotC* (Table 1) have been disrupted by insertion of the *pyr5,6* gene (Fosnaugh *et al.*, 1995). Strain DP87⁻ is a derivative of Ax2 in which the DP87 gene has been disrupted (Nakao *et al.*, 1994). Strain PspB⁻ is a derivative of Ax4 in which the *pspB* gene has been disrupted (Powell-Coffman & Firtel, 1994). According to our confirmatory RNA and DNA blot analyses, *pspB* is a single-copy gene which is disrupted in the clone provided by J. Powell-Coffman (data not shown). Cells were passaged axenically in HL-5, or on lawns of *Klebsiella aerogenes* grown on SM agar plates (Aparicio *et al.*, 1990). To prepare spores on an analytical scale,

fruiting bodies were allowed to differentiate on lawns of *K. aerogenes*. For extraction of spores, 1-d-old sori were collected with a loop into 1 ml of KP buffer (10 mM KH₂PO₄/NaOH, pH 6.5). Spores were recovered by centrifugation at 12000 g for 10 s, resuspended in H₂O, and counted in a haemocytometer. Interspore matrix was recovered by concentrating the supernatant in a vacuum centrifuge. For germination, 1-d-old spores were transferred to the lid by slapping the inverted Petri plate onto a counter top, and recovered by jetting KP/NP-40 [KP supplemented with 0.02% (v/v) NP-40] onto the surface and collecting the suspension by decanting (Aparicio *et al.*, 1990). Contamination by stalks was reduced by vacuum filtration through a 120-mesh steel screen. For preparative-scale spore preparations from which proteins were to be purified, approximately 10¹¹ axenically grown cells were washed first in water, resuspended in PDF (20 mM KCl, 45 mM sodium phosphate, 6 mM MgSO₄, pH 5.8), and plated onto 46 × 57 cm sheets of filter paper composites, consisting of one sheet of Whatman no. 50 paper layered on top of two sheets of Whatman no. 3 paper, each premoistened with PDF. After 1–2 d incubation in a covered chamber, fruiting bodies were collected by scraping with the edge of a spatula, suspended in approximately 200 ml KP/NP-40, and vacuum-filtered through a 120-mesh screen. The resulting spore suspension was washed in KP by centrifugation.

Preparation of spore coats. Spores were recovered from *K. aerogenes* lawns and germination was induced by incubation at 45 °C for 30 min. Coats were purified by differential centrifugation and density gradient centrifugation as described previously (Aparicio *et al.*, 1990). Buoyant density was estimated from the isopycnic position of the centre of the coat band in the tube. Coats were removed from the gradient by needle puncture of the centrifuge tube wall, diluted with KP buffer into polypropylene microcentrifuge tubes, counted in a haemocytometer, and pelleted in the cold. The supernatant was aspirated and the pellets were stored at -80 °C. Since coats tend to adsorb to the wall of the tube, the entire tube contents were utilized for each procedure.

Extraction of spores for amino acid composition analysis. Coats from approximately 10⁹ spores were resuspended in 1 ml 8 M urea, 20 mM Tris/HCl (pH 7.5), 5% (v/v) MSH (2-mercaptoethanol) using a probe sonifier and heated in a boiling water bath for 5 min. Residual spore coats were recovered by centrifugation. The supernatants were transferred to new polypropylene microcentrifuge tubes and precipitated with 5 vols ice-cold acetone. Protein was recovered by centrifugation and washed with 80% (v/v) cold acetone (referred to as the 'urea extract'). The residual spore coat pellets were washed twice with water. For further extraction, coats were resuspended in 2% (w/v) SDS, 20 mM Tris/HCl (pH 7.5), 5% MSH using a bath sonifier and heated in a boiling water bath for 5 min. The residual spore coats were recovered by centrifugation and washed twice with water. The supernatants were transferred to new polypropylene microcentrifuge tubes and precipitated with 5 vols ice-cold acetone. Protein was recovered by centrifugation and washed with 80% cold acetone (referred to as the 'SDS extract').

Amino acid analysis. Duplicate preparations of samples were hydrolysed in 6 M HCl at 110 °C for 20 h, and analysed by a ninhydrin method (Ploug *et al.*, 1989). Values for Trp, Cys and Met were calculated from their proportions in five spore coat proteins of known amino acid composition (see Table 2).

Serial extraction of spores for SDS-PAGE analysis. Spores were picked with a wire loop and suspended in 1 ml of water. After counting in a haemocytometer, the spores were collected by

Table 1. Properties of spores and spore coats in various strains of *D. discoideum*

Strain*	Disrupted gene(s)	Protein(s) affected	Percentage germination†	Spore coat buoyant density
Ax3	None	None	48	1.306
X22 ^a	None	None	50	—
WS308B ^a	None	SP85 and SP86 polymorphisms	—	1.302
WS576 ^a	None	SP85 and SP86 polymorphisms	—	1.301
PspB ^{-b}	<i>pspB</i>	None	—	1.298
TL56 ^c	<i>cotA</i>	SP96 ⁻	84	1.285
	<i>cotB</i>	SP70 ⁻		
	<i>cotC</i>	SP60 ⁻		
DP87 ^{-d}	<i>cotD</i> (DP87)	SP75 ⁻	56	1.277

—, Not determined.

* Strains were generously provided by: *a*, A. Champion and K. Williams, Macquarie University, Sydney, Australia; *b*, J. Coffman-Powell and R. Firtel, University of California, San Diego, USA; *c*, K. Fosnaugh and W. F. Loomis, University of California, San Diego, USA; *d*, M. Tasaka and I. Takeuchi, Kyoto University, Japan and National Institute for Basic Biology, Okazaki, Japan, respectively.

† After heat activation for 30 min at 45 °C.

centrifugation at 12000 *g* for 10 s and resuspended in 8 M urea, 20 mM Tris/HCl (pH 7.4), containing 1 mM PMSF, 10 µg leupeptin ml⁻¹ and 18.5 µg aprotinin ml⁻¹, to give 3.3 × 10⁷ cells ml⁻¹. They were vortexed and kept at 22 °C for 15–30 min before being collected as above. The same volume of the supplemented 8 M urea solution was mixed with the pellet by vortexing, and the suspension was heated in boiling water for 3 min. Extracted spores were recovered by centrifugation, then serially extracted as above with 2% SDS in 20 mM Tris/HCl (pH 7.4) at room temperature, hot 2% SDS in 20 mM Tris/HCl (pH 7.4), and hot 2% SDS/5% MSH in 20 mM Tris/HCl (pH 7.4). The solutions of some extraction series were supplemented with 5% MSH and 5 mM EDTA, or 5% MSH and 5 mM EDTA. Where indicated, spores were prealkylated with 100 mM iodoacetamide (IAA) during the initial water wash using conditions which prevented formation of artifactual disulfide bonds during isolation of gap junction connexon proteins from tissues (Dupont *et al.*, 1989; John & Revel, 1991).

Purification and sequencing of SP75. Approximately 10¹¹ spores collected as described above were resuspended at 5 × 10⁸ ml⁻¹ in 8 M urea, 5% MSH, 10 mM NH₄HCO₃ (pH 6.0) and heated for 5 min in a boiling-water bath. After centrifugation (10000 *g* for 10 min), the supernatant was chromatographed on a TSK DEAE-5PW HPLC column (Gonzalez-Yanes *et al.*, 1989). Fractions were analysed by SDS-PAGE and immunoblotting. SP75 was identified from its apparent *M_r* and binding of mAb 83.5. After staining the gel with Coomassie Blue, the polyacrylamide fragment containing SP75 was excised and the protein was digested with endo-proteinase Lys-C, essentially as described by Stone & Williams (1993). The digestion product and undigested protein were separated in a SDS-PAGE gel using a Tris/Tricine buffer system, immunoblotted onto Immobilon-P⁸⁰ membranes and subjected to Edman degradation as described previously (Kozarov *et al.*, 1995).

Gel electrophoresis and immunoblotting. For standard one-dimensional SDS-PAGE, samples were either diluted in concentrated sample buffer or dissolved in normal strength sample buffer and applied to a 7–20% linear polyacrylamide gradient

gel using the Laemmli buffer system, as modified by West & Erdos (1992). For two-dimensional gel electrophoresis, spore coats and interspore matrix, previously dried in a vacuum centrifuge, were solubilized and resolved as described by Gonzalez-Yanes *et al.* (1989). Sample loading was based on spore coat number or on the number of spore coats with which the amount of interspore matrix loaded was originally associated. Gels were stained with Coomassie Brilliant Blue (Gonzalez-Yanes *et al.*, 1989) or by a silver method (Wray *et al.*, 1981). For immunoprobings, gels were electroblotted onto 0.45 µm pore diameter nitrocellulose paper using an apparatus consisting of parallel steel and graphite plates (Idea Scientific), or a semi-dry apparatus (Bio-Rad). The blotting buffer was Laemmli electrophoresis buffer diluted with 0.25 vol. methanol, and electroblotting was carried out at room temperature for 2–3 h at a current of 200 mA. Blots were immunoprobed as described by West & Erdos (1992).

Electron microscopic analysis. Samples were prepared and visualized as previously described for material to be immunoprobed (West & Erdos, 1992).

DP87 gene mapping. cDp87-1, kindly provided by M. Tasaka, was amplified and the DP87 coding insert was supplied to Drs A. Kuspa and W. F. Loomis for restriction enzyme mediated insertion (REMI)-RFLP mapping (Kuspa & Loomis, 1994). As described previously (Ozaki *et al.*, 1993), cDp87-1 is encoded by a unique gene as determined by Southern blotting (data not shown).

RESULTS

Protein can be quantitatively extracted from the spore coat

To determine the amount of protein in the spore coat, the amino acid content was quantified after acid hydrolysis. Values for amino acids not determined by the method were extrapolated from the cDNA-derived amino acid composition of five known spore coat proteins (Table 2, column 4). This approach estimates 0.52 pg protein per

Table 2. Amino acid composition of spores, coats, coat extracts and known coat proteins

Amino acid	Amino acid content (pmol per 10 ⁶ units)*						
	Spores	Spore coats†		Urea extraction†		SDS/urea extraction†	
		Total	5 proteins‡	Residue	Extract	Residue	Extract
Asp/Asn	10 200 (10.2)	556 (11.6)	(11.4)	75.0 (11.6)	418 (11.7)	6.8 (9.16)	—§
Thr	6040 (6.00)	365 (7.59)	(8.37)	45.4 (6.25)	334 (9.35)	4.4 (5.95)	67 (12.5)
Ser	7010 (6.98)	379 (7.92)	(12.7)	46.4 (6.62)	308 (8.60)	4.9 (6.63)	30 (5.58)
Glu/Gln	11 200 (11.1)	343 (7.16)	(7.18)	49.1 (7.54)	261 (7.29)	6.2 (8.38)	45 (8.37)
Pro	4200 (4.18)	465 (9.15)	(7.51)	48.6 (7.18)	312 (8.71)	5.3 (7.17)	14 (2.61)
Gly	8560 (8.53)	439 (9.32)	(8.78)	77.1 (11.2)	377 (10.5)	8.2 (11.1)	55 (10.3)
Ala	6730 (6.70)	232 (4.81)	(5.36)	49.8 (6.31)	206 (5.76)	2.8 (3.92)	26 (4.83)
Val	5200 (5.18)	224 (4.67)	(4.72)	26.9 (4.45)	151 (4.21)	3.4 (4.60)	24 (4.46)
Ile	4750 (4.73)	157 (3.29)	(3.20)	26.7 (4.73)	109 (3.04)	2.0 (2.70)	29 (5.39)
Leu	6200 (6.17)	151 (3.15)	(2.46)	29.2 (4.60)	111 (3.10)	2.8 (3.92)	31 (5.77)
Tyr	2520 (2.51)	145 (3.03)	(2.16)	14.7 (3.51)	84.5 (2.36)	1.5 (2.02)	19 (3.54)
Phe	3270 (3.26)	116 (2.44)	(1.79)	15.0 (2.68)	48.9 (1.37)	1.8 (2.43)	18 (3.35)
His	1860 (1.85)	213 (4.44)	(3.61)	23.2 (3.25)	185 (4.27)	3.0 (4.06)	14 (2.61)
Lys	7210 (7.18)	227 (4.74)	(5.10)	17.6 (3.28)	138 (3.86)	3.0 (4.06)	18 (3.35)
Arg	3690 (3.67)	212 (4.42)	(3.89)	32.3 (4.87)	147 (4.11)	9.0 (12.1)	23 (4.28)
Cys	—	—	(10.3)	—	—	—	—
Met	—	—	(0.41)	—	—	—	—
Trp	—	—	(1.00)	—	—	—	—
Subtotal	88 600	4220		577	3190	65	410
Total§	100 000	4770		652	3610	74	540

—, Not determined.

* Mean of two independent determinations, which differed by < 20 %. A unit represents one spore, spore coat or extracted spore coat. The percentage of total amino acids in the sample is shown in parentheses; all samples are assumed to contain 11.7 % Cys, Met and Trp, which is the summed level of these residues in five known coat proteins (see footnote ‡).

† Spore coats were purified to homogeneity after germination. Coats were extracted with urea, and the extract was separated from the residue, which was then re-extracted with SDS.

‡ Values calculated from the deduced amino acid sequences, minus the signal peptide, of five outer layer spore coat proteins, SP96, SP87, SP75, SP70 and SP60.

§ The total values for coats and extracts were calculated by subtotalling the measured amino acid levels, and then multiplying by a factor of 1.13, to correct for the values of Cys, Met and Trp, which could not be determined by the method, assuming that their proportions are the same as in the five coat proteins listed in footnote ‡. A correction was made in the same way for the missing value for Asp/Asn of the SDS extract.

spore coat [4.77 fmol amino acid per coat (Table 2, column 3, bottom row) × 109 average fg amino acid per fmol], compared to 0.58 pg per coat as determined by a Coomassie Blue dye-binding method (Aparicio *et al.*, 1990). The spore coat comprises 5 % of the total spore protein (Table 2, columns 3 and 2).

To determine how protein is attached to the coat, the coat preparation was extracted with protein denaturants and MSH. Hot urea/MSH released 85 % of the total coat protein (Table 2, column 6), and re-extraction of the insoluble residue with hot SDS/MSH solubilized 88 % of the remaining protein (Table 2, column 8). Thus, little protein is irreversibly cross-linked, and proteins can be identified, quantified and characterized by conventional biochemical methods. While these results do not rule out the possibility that proteins are extracted as multimers, it is unlikely that such multimers would be very large

because > 90 % of all protein enters the running gel during SDS-PAGE (data not shown; Gonzalez-Yanes *et al.*, 1989).

Most coat proteins are disulfide cross-linked

Previous studies have reported that extraction of most coat proteins requires reducing agents, which could be explained if proteins were cross-linked by disulfide bonds (Wilkinson & Hames, 1983). One protein, SP96 (previously termed p112), does not require reducing agents for extraction. However, in our experience, a variable percentage of most proteins are extracted from both 1-d-old and 7-d-old spores without reducing agents (Aparicio *et al.*, 1990). This indicates that there is heterogeneity in the nature of the association of individual copies of the same protein species with the coat, or that disulfide bonds

Fig. 1. Sequential extraction of proteins from spore coats. To determine how proteins were associated with the coat, 1-day-old spores were divided into three groups, and each was sequentially extracted and centrifuged from water at 22 °C (W); 8 M urea at 22 °C (CU); 8 M urea at 100 °C (HU); 2% SDS at 22 °C (CS); 2% SDS at 100 °C (HS); and 2% SDS/5% MSH at 100 °C (BME). The extracting solutions contained 5% MSH (a) or no reducing agent (control) (b). The spores of the third group were pretreated with 100 mM IAA to alkylate free thiol moieties, and no reducing agent was present until the final step (c). The supernatants from each centrifugation were collected and volumes derived from 3×10^5 original spores were analysed by SDS-PAGE, blotting and immunoprobing with the indicated antibodies.

may simply stabilize conformations which support non-covalent interactions that are resistant to denaturation. However, the data are ambiguous because protein denaturants used to extract spore coat proteins have the potential, if reduced cysteine is present, to promote disulfide exchange reactions (Creighton *et al.*, 1995). Even in the absence of a reducing agent, this could result in the release of proteins from disulfide bonds which normally tether them to the coat.

To address this issue, free thiol groups of the spore coat were first alkylated using iodoacetamide to block thiol migration. To evaluate the effect of prealkylation, three separate experimental series of spores were sequentially extracted with water, urea, hot urea, SDS and hot SDS. The solutions of the first series contained 5% MSH. The second (control) series contained no reducing agent. Spores of the third series were pretreated with IAA and contained no reducing agent. The behaviour of the five proteins monitored fell into two categories.

SP96, the sole member of the first category, elutes partially in the water wash and is completely eluted by hot urea in the control series (Fig. 1). The elution of this protein is not affected by prior alkylation or the presence of MSH. Thus, some SP96 resides in the interspore matrix and disulfide bonds are not relevant to the association of SP96 with the spore coat matrix.

The second category includes the remaining proteins examined (Fig. 1). In the control series, these proteins were partially eluted in the hot urea step, but required hot SDS/MSH for complete extraction, as previously observed (Aparacio *et al.*, 1990). When prealkylated with

IAA, however, little or no detectable SP75, SP80 or SP85 was extracted without reducing agent. In the presence of reducing agent, essentially all protein extracted in either the 22 °C or the hot urea step. Indistinguishable results were obtained for SP87 probed with anti-PL3, SP86 probed with mAb 5.1, SP70 probed with anti-SP70 (2H3) and SP60 determined by Coomassie Blue staining (data not shown). Inclusion of 10 mM EDTA in the solutions had no effect on the results for both categories of proteins (data not shown), suggesting that divalent cations are not critical for retaining proteins in the coat. Similar results were found with both 1- and 7-d-old spores (data not shown). Since prior alkylation blocked elution of protein under denaturing conditions, it is likely that the disulfide bonds affected by reduction are direct intermolecular cross-links, rather than intramolecular bonds that stabilize a conformation necessary for denaturant-sensitive intermolecular contacts. Thus, all copies of these protein species are cross-linked in a disulfide-dependent fashion. Since polysaccharides do not participate in disulfide cross-links, protein appears to form a matrix continuum which, once formed, is independent of the cellulose system of fibrils. This arrangement is reminiscent of the epithelial basement membrane, in which laminin and collagen type IV separately self- and inter-associate to form the final fabric of the matrix (Yurchenko *et al.*, 1992).

Identification of coat proteins by two-dimensional gel electrophoresis

To determine the total number of coat proteins, two-dimensional gel electrophoresis was employed to extend earlier one-dimensional gel analyses of the coat (Devine *et*

Fig. 2. Two-dimensional gel electrophoresis of spore coat proteins from normal and gene-disruption strains. Coats were highly purified from strains Ax3 (a), DP87⁻ (b), TL56 (c) and PspB⁻ (d) and solubilized in urea/SDS/MSH. Coats (2×10^7) were loaded at the basic end of standard O'Farrell two-dimensional polyacrylamide gels which were subsequently stained with Coomassie Brilliant Blue. M_r markers were applied at the acidic end of the second dimension gels. The nine most abundant and one minor-abundance proteins are labelled, and are listed with their apparent M_r and pI values in Table 3.

al., 1982; Wilkinson & Hames, 1983; Akalehiyot & Siu, 1983; Gonzalez-Yanes *et al.*, 1989). Since all protein can be extracted from the coat, this approach was expected to identify all proteins because of its ability to resolve those proteins with similar M_r values. Known coat proteins were identified based on previous one- and two-dimensional separations (Devine *et al.*, 1982, 1983; West & Loomis, 1985), immunoprobings of Western blots from parallel gels with mAbs and absence of proteins in coats from gene deletion strains.

Nine major protein species are visualized in normal strain Ax3 spore coats after staining with Coomassie Blue (Fig. 2a). The same proteins are also found in another strain, PspB⁻ (Powell-Coffman & Firtel, 1994), which is derived from strain JH010 and lacks the *pspB* gene (Fig. 2d). The positions of SP96 and SP75 were determined on immunoblots from parallel gels using mAb 83.5 (Fig. 3b), confirming previous reports (Devine *et al.*, 1982; West & Loomis, 1985). The position of SP70 was determined using the anti-SP70 (2H3) antiserum (data not shown), as previously described (Gonzalez-Yanes *et al.*, 1989). SP60 was identified on the basis of its characteristic migration compared to previous two-dimensional gel analysis (Devine *et al.*, 1982). The identities of SP96, SP70 and SP60

were confirmed by their absence in the triple gene deletion strain TL56 (Fosnaugh *et al.*, 1995) (Fig. 2c). SP87, encoded by the *pspD* (PL3) gene (Yoder *et al.*, 1994), was identified using anti-PL3 antibody (Fig. 3a). SP85 was identified using mAb 16.1 (Fig. 3a), and is equivalent to the PsB protein recognized by mAb MUD102 (Watson *et al.*, 1994). Three previously unrecognized proteins are SP52, which migrates broadly, and SP65 and SP35, which migrate as isoelectric stutters near the basic end of the gel.

The lower than predicted isoelectric points of SP96 and SP75 (Table 3), based on the algorithm of Skoog & Wichman (Hitachi MacDNAsis Pro 3.0 software) applied to their amino acid sequences, can be explained by phosphorylation of their Ser residues (Akalehiyot & Siu, 1983; Devine *et al.*, 1982; West & Erdos, 1990). The higher than predicted isoelectric points of SP87, SP70 and SP60 are possibly the result of re-formation during focussing of intramolecular disulfide bonds (see below), which the algorithm assumes do not exist. The higher than predicted M_r values for these proteins, based on their amino acid sequences (see Table 3), can be explained by their phosphorylation and glycosylation (Riley *et al.*, 1993; West & Erdos, 1990), and the presence of oligo Ser/Thr tracts.

Fig. 3. Identification of coat proteins by immunoblotting. Strain Ax3 spore coats (1×10^6) were subjected to two-dimensional electrophoresis, as described in Fig. 2 and Western blotting prior to immunoprobings. (a) Blot double-probed with mAb 16.1 and anti-PL3 antiserum showing the positions of SP85 and SP87, respectively. The minor labelling of SP60 by anti-PL3 was reproducible and probably reflects protein structural homologies. The binding of mAb 16.1 to a minor species below SP87 was also reproducible. (b) Blot double-probed with mAbs 83.5 and 16.1 showing the positions of SP96 and SP75, and SP85, respectively, in addition to numerous minor species labelled by mAb 83.5 which were reproducibly detected when the blot was overdeveloped. (c) Blot probed with mAb 5.1 showing the position of SP85, the major reactive species near the basic end, and SP86, the major reactive species toward the acidic end. This blot was overdeveloped to reveal the numerous other minor-abundance species present in this preparation which reacted with this antibody. (d) Blot probed with anti-DP87 showing a protein in the same position as SP75, which was confirmed by secondary probing with mAb 83.5 (not shown). The minor labelling of other proteins with this antibody was reproducible, consistent with sequence homologies among coat proteins.

The same nine major-abundance proteins are visualized in overloaded gels stained with Coomassie Blue (Fig. 4a). Silver staining (Fig. 4b) reveals most of the major-abundance species as either positive or negative images, though SP70 and SP85 are not identified by this technique. The high apparent abundance of SP52 is emphasized by silver staining. In general, it is difficult to compare the relative abundance of the major protein species, because of variation in either stainability or sharpness of migration during focussing and electrophoresis.

These gels also indicate reproducibly the existence of several minor proteins, including SP82, SP80, SP64, SP63 and SP51 (Figs 4a, b; Table 3). In addition, silver staining reveals a number of poorly resolved basic species over a wide range of M_r values (Fig. 4b), which do not stain with Coomassie Blue and warrant further attention. The

previously described SP86 (West & Erdos, 1992) is recognized by mAb 5.1. mAb 5.1 reacts with two major species on two-dimensional gels with the same apparent M_r (Fig. 3c). The more basic major species is SP85, recognized by mAb 16.1; the more acidic species, with an unusual blob-like image, is probably SP86, apparently present in low abundance because it is not detected by protein stains (Figs 4a, b). SP86 is polymorphic on one- and two-dimensional gels, displaying a larger apparent M_r in WS576 (unpublished data), a strain in which SP85 was previously shown to have a reduced M_r value (Watson *et al.*, 1994).

In conclusion, the *D. discoideum* spore coat consists of a finite number of major-abundance species, and additional minor species, some of which are likely to be important for coat structure and function. Genes for most of the

Table 3. Properties of spore coat proteins

Protein name/gene/linkage group	Residues after signal peptide cleavage	Polypeptide M_r after signal cleavage‡	Measured M_r §	Predicted pI	Measured pI¶	Phosphorylation**	Glycosylation††
Major abundance species							
SP96/ <i>cotA</i> /2	*21–600	57 507	96 000	5.34	4.3	yes	O (GA-X) 1 poss. N‡‡
SP87/ <i>pspD</i> /2	*22–678	69 351	87 000	6.9	7.6		1 poss. N‡‡
SP85			85 000		6.85		O (GA-XI)
SP75/(DP87) <i>cotD</i> /4	†23–555	56 143	75 000	7.29	5.2	yes	1–3 N§§ O (GA-X)
SP70/ <i>cotB</i> /2	†21–537	54 505	70 000	4.69	5.7–5.8	no	1 N§§
SP65			65 000		7.0–7.2		
SP60/ <i>cotC</i> /2	†24–424	46 927	60 000	4.67	5.6	no	no
SP52			52 000		5.3		
SP35			35 000		6.5–7.5		
Minor abundance species							
SP86			85 000		4.8		O (GA-XX)
SP82			82 000		5.15		
SP80			80 000			yes	O (GA-X) O (GA-XX)
SP64			64 000		6.7–6.8		
SP63			63 000		5.7–6.1		
SP51			51 000		5.9		

* First amino acid deduced from consensus signal peptidase recognition site (von Heijne, 1985).

† First amino acid determined by Edman degradation (Fosnaugh & Loomis, 1989b; Table 4).

‡ Calculated.

§ Taken from previous reports of SDS-PAGE determinations (see text), except for SP82, SP65, SP64, SP63, SP52, SP51 and SP35, which are from this study.

|| Based on algorithm of Skoog & Wichman, as calculated by MacDNAsis Pro 3.0.

¶ Determined by two-dimensional electrophoresis (Figs 2–4).

** Determined by metabolic labelling with $^{32}\text{PO}_4^{3-}$; a blank indicates not known (Akalehiyot & Siu, 1983; Devine *et al.*, 1982; West & Erdos, 1990).

†† O-glycosylation determined by a combination of reaction with mAbs (designated as GA) and analysis of products of β -elimination (West & Erdos, 1990; Riley *et al.*, 1993).

‡‡ Derived amino acid sequence contains the indicated number of N-glycosylation sequons.

§§ Determined by M_r shift in a *modA* (α -glucosidase-2⁻) mutant strain, M31 (C. M. West, unpublished data).

major species have been cloned, and inspection of the deduced amino acid sequences indicates the functional roles described below.

Coats and interspore matrix derive from a common extracellular space but are distinct

Superficial to the spore coat, in the interstices between spores, resides the interspore matrix fluid. Since several coat proteins have been detected in the interspore matrix (West & Erdos, 1988, 1990, 1992; Watson *et al.*, 1994; Nakao *et al.*, 1994), the protein composition of the matrix was examined to address whether what is considered a coat protein is in fact a contaminant from the interspore matrix. The protein content of the interspore matrix,

collected as a water wash from sori of strains Ax3, X22 or NC-4, grown either axenically or on *K. aerogenes*, was measured using a Coomassie Blue dye-binding assay. The values, ranging from 250–650 fg per spore, are similar to those for the protein content of the spore coat, 520 fg per coat, though a spore equivalent of interspore matrix occupies a much larger volume than the spore coat. To compare the overall protein composition of coats and matrix, a quantity of interspore matrix equivalent to the number of spore coats displayed in Fig. 4b is shown in Fig. 4c. Silver staining reveals numerous protein species over a wide range of apparent isoelectric points and M_r values. Known spore coat proteins are not abundant enough to be correlated with individual protein species with confidence, with the exception of SP96. As shown

Fig. 4. Comparison of spore coat and interspore matrix proteins. Highly purified coats and interspore matrix, i.e. the water wash fraction from sori, were isolated from strain Ax3 and subjected to two-dimensional gel electrophoresis as in Fig. 2. (a) 1×10^8 coats, gel stained with Coomassie Brilliant Blue; (b) 2×10^7 coats, gel stained with a silver method; (c) interspore matrix from 2×10^7 spores, gel stained with Coomassie Brilliant Blue followed by staining with a silver method. Both abundant and minor coat proteins are labelled in (a) and (b); the apparent M_r and pI of each protein are listed in Table 3. The stuttered spots referred to as SP35 and SP65, which varied in their apparent isoelectric points, are assumed to represent single gene products.

Table 4. Amino acid sequence of purified SP75 and endoproteinase Lys-C-derived cleavage products

Treatment	Sequence*
None	WGGGRDXE
Endoproteinase Lys-C	{K}xxRTGEIFEIWS
Endoproteinase Lys-C	{K}xxxxxxRPPRPVQCRNQ
Endoproteinase Lys-C	{K}NTRTGEIFEIxSxxKPHxDFVPYH
Endoproteinase Lys-C	{K}KEFFxVNRDxxxxxD

* Letters denote standard single letter abbreviations for amino acids; x = not determined; {K} denotes a Lys residue predicted on the basis of specificity of endoproteinase Lys-C, which cleaves on the C-terminal side of this residue. All sequences match translations of the published cDNA sequence for the DP87 gene (Ozaki *et al.*, 1993).

above (Fig. 1) and elsewhere (West & Erdos, 1990, 1992; Yoder *et al.*, 1994), only a minor proportion of the coat proteins SP87, SP86, SP85, SP75, SP70 and SP60 resides in the interspore matrix. Furthermore, the level of coat proteins detected in the interspore matrix varies substantially between strains (Zhang & West, unpublished data). Some matrix proteins are lysosomal enzymes (Seshadri *et al.*, 1986; Lenhard *et al.*, 1989), and probing immunoblots with an anti-discoidin antibody (Erdos & Whitaker, 1983) indicates that one corresponds to the lectin discoidin; discoidin is not detected in the coat (data not shown). Coat proteins represent a variable, minor fraction of the overall composition of the interspore matrix, suggesting that the specificity of intermolecular associations ensures that matrix and coat proteins are largely unique.

Identification of the coat protein SP75 as the product of the DP87 gene

To gain additional information about the major coat protein SP75, this protein was extracted from spores in 8 M urea/MSH, and purified to homogeneity using DEAE ion exchange chromatography and SDS-PAGE (see Methods). Purified SP75 was digested with endoproteinase Lys-C, and both intact and digested protein were subjected to Edman degradation after SDS-PAGE and immunoblotting. The sequences obtained (Table 4) belong to the previously characterized DP87 gene (Ozaki *et al.*, 1993). The sequence of the intact protein identifies the site of signal peptide cleavage after Gly₂₂, which conforms with consensus signal peptidase cleavage sites (von Heijne, 1985).

Consistent with the sequence data, coats from a gene disruption strain DP87⁻ lacked a protein at the position of SP75 (Fig. 2b). Both SP75 and the DP87 protein are missing from these coats as determined by labelling immunoblots with mAb 83.5 or anti-DP87, respectively (data not shown). No other abundant proteins or SP86 (data not shown) are missing. Furthermore, anti-DP87 labels a protein at the position of SP75 in immunoblots of normal spore coats (Fig. 3d). The weaker labelling of

Fig. 5. Electron microscopic immunolocalization of SP75. Thin sections from spores of strains Ax3 and TL56 were immunoprobed with anti-DP87 antibody and mAb 83.5, using an indirect colloidal gold technique. (a) Strain Ax3 spores probed with anti-DP87; (b) strain TL56 spores probed with anti-DP87; (c) strain TL56 spores probed with mAb 83.5.

other spots, which also occurs with the anti-SP87 (PL3) antibody (Fig. 3a and data not shown), is probably because these proteins share related sequences (see below) which cross-react with these anti-polypeptide rabbit antisera. Finally, DP87⁻ spore coats exhibit a reduced buoyant density (Table 1), consistent with a chemical difference in their composition.

In contrast to the biochemical data, anti-DP87 fails to label coats in thin sections (Nakao *et al.*, 1994; Fig. 5a). This raises the possibility that critical epitopes required by the anti-DP87 antibody are masked in the coat but not in the interspore matrix, where this protein was previously

localized (Nakao *et al.*, 1994). To circumvent this possibility, spores from strain TL56, which lacks three coat proteins but retains SP75 and is more permeable to macromolecules (Fosnaugh *et al.*, 1995), were examined. Immunogold antibody conjugates localized exclusively in the coat in these washed spores (Fig. 5b). Gold particles tended to associate with the inner and middle layers of the coat, whereas the fucose epitope (GA-X) localized in the outer layer of both mutant and normal coats as determined using mAb 83.5 (Fig. 5c), indicating that this aspect of organization of the coat is not affected by the absence of the coat proteins SP96, SP70 and SP60. Since SP75 is the major coat protein expressing the fucose epitope in this

strain, separate epitopes of SP75 may reside in the inner and outer layers of the coat, straddling the cellulosic middle layer.

In summary, a coat location for SP75 is supported by physical, genetic, biochemical and microscopic evidence. The high level of SP75 expression previously observed in the interspore matrix (Nakao *et al.*, 1994) does not occur in strain Ax3, but we have observed that the level of coat proteins in this compartment varies among cell strains (Y. Zhang & C. M. West, unpublished data).

Effects of coat gene deletions on spore coat assembly

The substantial molecular deficiencies of the gene disruption strain TL56, which lacks SP96, SP70 and SP60 (Fosnaugh *et al.*, 1995), and DP87⁻ (Tasaka *et al.*, 1990), which lacks SP75, are confirmed by the results shown in Figs 2(b–c). Coats are still formed by these strains, though they exhibit reduced buoyant densities (Table 1), and strain TL56 also has a relatively high germination efficiency after temperature activation (Table 1). Examination of a series of deletion strains elegantly showed that the contributions of SP96, SP70 and SP60 to the macromolecular impermeability of the coat are additive (Fosnaugh *et al.*, 1995). It is striking that the remaining major-abundance coat proteins (Figs 2b, c) and SP86 (data not shown) are incorporated normally in TL56 and DP87⁻, and that the fucose epitope is localized correctly in TL56 (Fig. 5c), despite the absence of major proteins. The one exception is that SP35 is missing from strain TL56 coats, indicating that this protein is normally retained in the coat by association with one of the missing proteins. Thus, with the possible exception of SP35, the phenotypic consequences can be ascribed to the specific proteins deleted. These observations argue against a crystalline model for protein organization in the coat, or any other model in which incorporation of proteins is strictly dependent upon associations with SP96, SP75, SP70 or SP60. An alternative indeterminate model of outer-layer-associated protein packing is supported by predicted characteristics of these proteins inferred from analysis of their sequences (see below).

DISCUSSION

The proteins of the spore coat

The germinated spore coat has a protein content slightly greater than its total carbohydrate content, including cellulose (West *et al.*, unpublished results), having an equivalent of about 5×10^6 protein molecules with an average polypeptide M_r of 60 000 [$(5.2 \times 10^{-13} \text{ g per coat} \div 60\,000 \text{ g per mol}) \times \text{Avogadro's number}$]. Since the volume of the coat, based on estimates from freeze-fracture studies (Erdos & West, 1989), is approximately $2 \times 10^{10} \text{ nm}^3$, there is a cube with an edge measuring 16 nm available for each protein, ignoring the space occupied by polysaccharide. Globular proteins of M_r 60 000 would be expected to occupy volumes less than 20 % of this value (Peters, 1986). Since crystalline cellulose is densely packed, this implies that protein may be loosely

organized and well hydrated, which is consistent with the relatively low proportion of the hydrophobic amino acids Ala, Ile, Leu, Val and Phe, and relatively high proportions of Thr, Ser, His and Pro, compared to values for the entire spore (Table 2, columns 3 and 2).

The two-dimensional gel analysis shows that there are nine major-abundance proteins and numerous, reproducibly detected, minor-abundance proteins (Table 3). However, only the proteins SP96, SP87, SP85, SP80, SP75, SP70 and SP60 have been shown to be present at similar levels and with similar apparent M_r and pI values in pregermination coats. Nevertheless, the remaining proteins listed are detected reproducibly and, altogether, the evidence suggests that the coat does not undergo dramatic changes in morphology and the biochemical composition of its protein components during germination. With the identification of SP75 as the product of the DP87 gene, the sequences of five such proteins are now known. Each is associated in some way with the outer layer of the coat as determined by proteolytic sensitivity and/or immunolocalization (Orlowski & Loomis, 1979; West & Erdos, 1988; Yoder *et al.*, 1994). Though shared sequence motifs have been previously recognized in these proteins (Fosnaugh & Loomis, 1989a, b; Ozaki *et al.*, 1993; Powell-Coffman & Firtel, 1994; Yoder *et al.*, 1994), further comparisons across the entire group document a greater than previously recognized degree of homology. Amino acid analysis indicates that the remaining proteins have, on average, a lower content of Ser and Thr, a higher content of Pro and are more hydrophobic (Table 2).

From the extraction studies, it can be concluded that the interactions of the major proteins released by urea are, with the exception of SP96, stabilized by intermolecular as well as, presumably, intramolecular (see below) disulfide cross-links. Other types of covalent intermolecular cross-linking are not detectable, and divalent cations do not seem to be important for association of proteins with the coat. It is likely that coat protein disulfide bond formation occurs at multiple stages of the secretory pathway, as it does, for example, with von Willebrand's factor (Mayadas & Wagner, 1992). At present, there is evidence for disulfide cross-linked intermolecular association prior to secretion in the prespore vesicle (Watson *et al.*, 1993, 1994), but the composition of a proposed complex is open to question because artefactual disulfide bonds may have formed during isolation, and the composition of the complex does not predict the distribution of proteins in the spore coat as determined by epitope distribution (West & Erdos, 1990). There is also evidence that some supramolecular assembly is extracellular, as some but not all proteins appear to freely and uniformly exchange between neighbouring spores during the assembly process (West & Erdos, 1992). Whatever the nature of any precursor or final protein complex in the coat, four of the outer layer proteins are not essential to its formation (Nakao *et al.*, 1994; Fosnaugh *et al.*, 1995), as the remaining proteins still coassemble with cellulose to form coats in the gene deletion constructs, though they have increased porosity.

The five cloned outer layer spore coat genes encode a family of modular proteins

Each of the cloned outer-layer-associated proteins is rich in Cys (7.4–13.2%), Pro (5.2–9.9%), Ser and Thr (18.1–34.8%; except for SP60), and deficient in the hydrophobic amino acids Phe, Val, Ile and Leu (9.3–15.6%). Structure prediction algorithms suggest only short regions of secondary structure, consistent with the low percentage of hydrophobic residues available to stabilize folded domains. All five proteins possess a predicted signal peptide, which has been shown to be cleaved in all but SP87 (Table 3). Three categories of sequence repeats, involving Cys, Ser/Thr/Gly/Pro/Ala and Arg/Lys/Pro/His, respectively, span virtually the entire length of each protein.

There are five types of Cys repeats, distinguished by the spacing of their Cys residues (Table 5). The CC motif defines a landmark occurring exactly twice in each of the proteins except for SP60: the first begins at amino acid position 124–132 (relative to the Met start), and the second near the COOH-terminus (Fig. 6). The defining characteristic of the CC motif is the consensus sequence Cys₂Xxx₅Cys (Table 5). A second repeat, the C2C motif (Table 5), is invariantly located immediately ahead of the second CC motif. The pair of CC motifs divides each protein into three regions: the NH₂-terminal region (up to residue 124–132), the central region between the two CC motifs, and the COOH-region, which follows the second CC motif (Fig. 6). Each protein conforms to this scheme except for SP60, which lacks the landmark CC and C2C motifs and consists only of the central region between them.

The C7C repeat comprises the NH₂-terminal regions (Fig. 6) of the four proteins which have CC and C2C motifs. That the only homology among the 108 residue C7C repeats from different proteins is the spacing of 12 Cys residues (Table 5) suggests a critical pattern of disulfide bonds. Each central region is comprised of 4–13 C4C and C3C motifs (Table 5), each averaging about 24 residues in length, interspersed with amino acid segments averaging 15 residues, which are generally but not always Cys-free (Fig. 6). As discussed below, these motifs may be related to EGF repeats. The intervening regions frequently consist of mucin-like (see below) and basic proline repeats. Basic proline repeats (Table 5) are 5–13 residues long and contain > 70% Arg, Lys, His and Pro. These repeats are similar in composition and spacing to those present in acidic proline-rich proteins from mammalian salivary glands (Ann *et al.*, 1987), and are distinct from Pro-rich sequences found in plant matrix proteins. Finally, mucin-like repeats tend also to be clustered COOH-terminal to the two landmark CC motifs (Fig. 6). SP70 and SP60 uniquely possess highly negatively charged stretches of 69 and 92 amino acids, containing 42–48% Asp and Glu residues, at their COOH-termini (Fig. 6).

In conclusion, the primary structure of each protein can be summarized as a similar mosaic of sequence motifs (Fig. 6) which, as discussed below, are predicted to have discrete, characteristic secondary structures. The modular

organization is reflected in the apparently vestigial retention of introns flanking the C7C repeat in the SP87 gene (Yoder *et al.*, 1994); in the other genes, an intron separates the signal peptide, together with the four AsnGlyAspTrpAspAsp (N)-repeats (Fosnaugh & Loomis, 1989b) of SP60, from the remainder of the protein. The overwhelming similarity of these five proteins, based on the order and identity of their shared motifs, confirms previous suggestions that they derive from a common ancestral gene. Examination of the relatedness of the landmark C2C and CC repeats and the sequences and distribution patterns of the other motifs suggests that the SP70 gene is ancestral, that SP75 derives from SP70, and SP96 derives from SP75. The genes for SP96, SP87, SP70 and SP60 have remained in close proximity on the right hand end of chromosome 2 (Kuspa & Loomis, 1996). However, the gene for SP75 maps to chromosome 4 (see Methods) by REMI-RFLP analysis (Kuspa & Loomis, 1994). Since three previous coat genes have been named *cotA–C*, we suggest that the DP87 gene be referred to as *cotD*.

The C2C, C4C and C3C sequence repeats resemble motifs from other proteins

The C3C and C4C motifs each contain four Cys residues with the characteristic spacing 3/5/7–9 and 4/5/7–9 (number of residues between pairs of Cys residues), respectively. They consist minimally of about 24 residues including the flanking Cys residues, and occur 36 times in the 5 proteins (Table 5). The sequence CysProXxxGly or CysProProXxx, starting at the second Cys, and CysXxx_{3–5}Gly, starting at the third Cys, are consistent features of each motif. Of the numerous Cys-rich motifs recognized in extracellular proteins (Kieffer *et al.*, 1994; Lecanidau *et al.*, 1986; Hoffman & Hauser, 1993; Wagner & Wyss, 1994), the C3C and C4C repeats most resemble the NH₂-terminal subdomain of EGF modules (Stenflo, 1991). The Cys-spacing in the C4C repeat is identical to the spacing of the first four of six Cys residues found in a major subgroup of EGF modules (Harris & Spellman, 1993). The first four Cys residues of EGF modules, which contain about 42 amino acids, form a pair of disulfide bonds that stabilize the NH₂-terminal subdomain, containing a characteristic hairpin β -motif consisting of two antiparallel β -strands separated by a β -turn (Graves *et al.*, 1994). The third pair of Cys residues forms a disulfide bond which stabilizes a second smaller hairpin β -motif, part of the COOH-terminal subdomain, which associates with the NH₂-subdomain. Thus, the *D. discoideum* motifs have Cys-spacing and other sequence characteristics of a truncated EGF module, consisting of the NH₂-subdomain. If the *D. discoideum* motif folds in the same way as the full EGF module, the Gly residues of the CysProXxxGly and CysXxx_{3–5}Gly subsequences are located at the turns flanking the first β -strand, positions which tend to be occupied by Pro and Gly residues in EGF modules. Furthermore, standard secondary structure algorithms predict, for the majority of C3C and C4C motifs, the folding of the sequences immediately surrounding the third and fourth Cys residues into

Table 5. Summary of sequence motifs in spore coat proteins

Mucin-like repeats: Ala/Gly/Pro/Ser/Thr-rich				Percentage of mucin-like sequences	
Protein	Typical repeat sequences*				
	Gly/Ser/Thr-rich	Pro/Ala/Ser-rich	Ala/Thr-rich		
SP96	GSQTTS, SSASGSSAVS	SSSPSSSAA	ATTTATTATTATT	42	
SP87	GSSSG			22	
SP75		APSSASSS		25	
SP70	GSHTTTGGSTT			28	
SP60	none			0	
Basic proline repeats: Pro/Lys/Arg/His-rich					
Protein	Position†: Sequence*				
SP96	249: PTHRPTHRRPKPPH				
SP87	385: PPKP	441: RSHRPPPP	505: RPHPPQH		
	538: HRPPPK	596: HRPPPK			
SP75	198: PHRPPPRPP	232: RDARPPH	266: PPHPPPPP		
	385: KARPPVPPPH				
SP70	327: PIRKRHWQLHP	344: RPNKIK			
SP60	110: PWRPYPP	172: RPRPHKPHRPP	207: KKKRHDR		
	235: KRTPKRP	325: PPRQPR			
CC motif: CysCysproXxxXxxproXxxCys‡					
Protein	Position†: Sequence*				
SP96	126: TACCPDVCQCKGG	352: AKCCDVQPTCIKP			
SP87	130: TSCCPGESICEGL	656: ENCCCKVPVCYST			
SP75	132: TSCCPRIPRCVGH	427: FPCCPSHPICIH			
SP70	124: TSCCPDIPQCQIP	408: EECCPIIPIICINP			
SP60	none				
C2C motif: Cys(Leu/Ser)-ThrCysXxx---hydXxx ₁₋₃ CysXxx ₃ GlyhydXxxCys‡					
Protein	Position†: Sequence*				
SP96	329: PDNCLTCNDV--NCEASGLVC				
SP87	629: RNDCLTCEDL--NCERKGLHC				
SP75	395: PPHCSTCAELSSACNHVGMIC				
SP70	387: KPKCLTCKDM--HCESNGLLC				
SP60	none				
C3C motif: CysXxxLeu+++CysProproglyhydAspCys+++Xxx ₄ GlyXxx ₂ CysCysVal++++‡					
Protein	Position†	Protein	Position		
SP96	none	SP70	none		
SP87	391, 419, 451, 480, 514, 544, 571, 602	SP60	118, 147, 185, 214		
SP75	none				
C4C motif: CysXxx ₄ CysProXxxGlyXxx ₂ CysXxx ₄ GlyXxx ₃ Cys‡					
Protein	Positions†	Protein	Positions†		
SP96	185, 221, 268, 298	SP70	158, 251, 279, 359		
SP87	189, 232, 276, 316, 356	SP60	53, 86, 300		
SP75	149, 176, 210, 241, 277, 305, 33, 363				
C7C motif: CysXxx _{7,8} CysXxx ₃₋₆ CysXxx ₈ CysXxx ₆ CysXxx _{6,7} CysXxx ₅₋₇ CysXxx _{14,15} CysXxx ₁₄₋₂₂ CysXxx ₃₋₇ CysXxx _{6,9} CysXxx ₃₋₇ CysXxx _{4,5} (Cys)‡					
Protein	Position†	Protein	Position†	Protein	Position†
SP96	3	SP70	3	SP75	7
SP87	3	SP60	none		

* Sequence examples are given in single-letter code.

† Position of the first residue of the motif in the complete protein sequence, including the signal peptide.

‡ In the consensus sequences, upper-case indicates an invariant or nearly invariant residue; lower-case indicates a predominant residue; + + +, positively charged residue, ---, negatively charged residue; hyd, hydrophobic residue; Xxx, any amino acid; (), residue from a subsequent motif.

Fig. 6. Schematic diagram depicting the arrangement of sequence repeats in the five outer-layer-associated coat proteins. The sizes and spacings of repeat elements, summarized in Table 5, are illustrated to scale. The NH₂-terminal signal peptides are not shown.

antiparallel β -strands, the location at which this folding pattern occurs in normal EGF modules. Finally, EGF modules are the only location described for post-translational modification of Ser or Thr residues with single O-linked fucose residues (Harris & Spellman, 1993). It is intriguing that SP96 and SP75, two of the coat proteins with multiple C3C and C4C motifs, also possess this O-fucose modification (Riley *et al.*, 1993).

Although there is little sequence homology among different C4C motifs aside from the key defining residues, sequence similarities imply an evolutionary path of divergence of this motif within and between proteins and possible pairwise duplication. Certain C3C repeats are more closely related and occur in only two of the proteins, where they are tandemly clustered. A search of 70847 sequences of the PIR.41 protein sequence database revealed 477 matches for the Cys-spacing of C3C and C4C motifs. Most were in extracellular proteins, and those that do not lie in EGF modules represent potential homologues of the *D. discoideum* repeats. Full EGF modules occur tandemly repeated in vertebrate extracellular matrix glycoproteins, cell surface receptors, serum proteins and extracellular enzymes, where they appear to link other protein domains, assist in the presentation of domains and participate directly in ligand-receptor interactions (Engel, 1989; Stenflo, 1991).

The C2C motif, which occurs once near the COOH-terminus just upstream of the second CC motif, contains the subsequence CysLeuThrCys or CysSerThrCys. This spacing of Cys residues forms a disulfide bond at the active site of proteins of the thioredoxin family of oxidoreductases (Mayadas & Wagner, 1992). Variation of

the two inter-Cys residues changes the redox potential of DsbA, normally an extremely oxidizing member of the thioredoxin family, over a wide range. Substitutions by the residue pairs found in the spore coat protein C2C motifs support a moderate oxidizing potential in this periplasmic protein of *Escherichia coli* (Grauschopf *et al.*, 1995). The apparent interaction of coat proteins in an extended disulfide cross-linked array indicates a need for an extracellular disulfide isomerase activity which might be provided by these vicinal Cys residues.

Ser/Thr-rich sequence repeats resemble mucin motifs

The Ser/Thr repeats, found in 4 of the 5 proteins (SP60 lacks this repeat type and the CC landmark motifs with which they tend to be associated), range from 6 to 13 residues in length (Table 5), and are often tandemly clustered. They contain exclusively Ser or Thr or a mixture of the two, and usually contain multiple Gly residues; Pro and Ala are also frequently present. The repeat sequence varies within and between proteins, but tends to be constant in a given contiguous stretch. A similar type of repeat occurs in a prespore cell-specific plasma membrane protein in which the number of repeats is polymorphic in different strains (Gooley *et al.*, 1992). The repeats are similar to those in vertebrate epithelial mucins and other animal mucin-like proteins (Verma & Davidson, 1994; Taylor-Papadimitriou & Epenetos, 1994; Devine & McKenzie, 1992), except for their relatively short length. Polymorphisms in mucin proteins have been attributed to the same genetic process of duplication and deletion. Coat proteins differ from vertebrate epithelial mucins, however, in their relatively

small size, having reduced polypeptide M_r values of $5-7 \times 10^4$, vs $1-2 \times 10^6$ (Taylor-Papadimitriou & Epenetos, 1994). Furthermore, a smaller percentage of the spore coat proteins is devoted to mucin-like repeats, ranging from 22–42% (Table 5) and, while many of these repeats are clustered in the coat proteins, smaller groups are also interspersed between truncated EGF-like modules. Like coat proteins, many vertebrate mucins also possess Cys-rich domains, at their NH_2 - and COOH -termini (Probst *et al.*, 1990; Bhargava *et al.*, 1990; Verma & Davidson, 1993; Meerzaman *et al.*, 1994; Gum *et al.*, 1992; Hansson *et al.*, 1994). Some possess characteristic Cys-spacing repeats (Hoffmann & Hauser, 1993), and are disulfide cross-linked (Hansson *et al.*, 1994). Though the *D. discoideum* mucin-like repeats have not yet been shown to share the extent of post-translational modifications typical of their mucin counterparts, two coat proteins, SP96 and SP75, exhibit M_r values which are much greater than the values predicted from their cDNA sequences (Table 3), and possess small O-linked oligosaccharides containing fucose and probably N-acetylglucosamine (Riley *et al.*, 1993). Like vertebrate mucins (Taylor-Papadimitriou & Epenetos, 1994), the coat proteins have very few N-glycans (Table 3). SP96 and SP75 are also phosphorylated on Ser residues (Akalehiywor & Siu, 1983), with approximately 50 such modifications per copy of SP96 (Watson *et al.*, 1993). This anionic modification of coat proteins may substitute for the sulfate and sialic acid modifications of vertebrate mucins. The fact that mucin-like repeats are found at the COOH -terminus of some coat proteins (Fig. 6) in addition to internal positions suggests that they are not simple interdomain spacers.

Spore coat protein sequence motifs are distinct from those of other *D. discoideum* matrix proteins

Other *D. discoideum* matrix proteins, found in the stalk tube and in the slime sheath, share amino acid composition similarities with the spore coat proteins. The prestalk cell-specific and slime sheath matrix proteins ST430 (encoded by *ecmB*), ST310 (encoded by *ecmA*), D11 (encoded by *D11*), and sheathins possess characteristic Cys-repeats but these are distinct from those of coat proteins (Ceccarelli *et al.*, 1987; Zhou-Chou *et al.*, 1995), and basic proline and mucin-like repeats are not present. The prespore-specific PspB and EB4 proteins, which possibly reside in the coat and/or interspore matrix (Powell-Coffman & Firtel, 1994; Hildebrandt *et al.*, 1991), contain multiple C4C repeats and a small number of basic proline and C3C repeats, but are devoid of CC, C2C and significant mucin-like repeats. The different sequence motifs of proteins from different *D. discoideum* extracellular matrices are consistent with the unique protein compositions of these matrices, and imply distinct functional attributes and mechanisms of intermolecular interactions for spore coat proteins.

Protein function

We suggest that the homologies between the outer layer coat proteins, and mucins, EGF modules and basic proline repeats form the basis of a model for the role of protein in

coat morphogenesis. After coat protein secretion, formation of the protein bed might be initiated by ionic interactions between the basic proline repeats and the long anionic domains at the COOH -termini of two of the proteins. The mucin-like domains presumably assume extended, rod-like conformations (Gerken *et al.*, 1989) which are spaced and may be kinked by knot-like truncated EGF modules. The mucin-like domains, together with the galuran polysaccharide, would be expected to support a hydrated, diffusion-limited micro-environment at the cell surface, which might assist in cell dehydration, compete for the cell-cell contacts (Hilkens *et al.*, 1992) that formerly linked adjacent prespore cells together, and be favourable for the subsequent morphogenesis of the cellulose fibrils that form later. Since most coat proteins are soluble after secretion in the absence of cellulose (West & Erdos, 1988) and remain soluble after extraction from the coat following dialysis (unpublished data), outer layer protein and cellulose may mutually organize each other's structures via the cellulose-binding activity of one coat protein (Zhang & West, unpublished data). The protein bed may be stabilized by intermolecular disulfide cross-links formed after secretion, as seen in mucous coatings of epithelial cells (Verma & Davidson, 1994). The absence of predictable tertiary structure of the proteins may facilitate disulfide bond migration from intrachain to interchain forms, catalysed by a single pair of vicinal Cys residues in four of the proteins during extracellular assembly. The degeneracy of the five coat proteins analysed here is more suited to the indeterminate, rather than crystalline, protein array postulated by this model. An indeterminate arrangement is consistent with the observation in the coat gene deletion strains that, with the exception of SP35, the remaining proteins appear to be incorporated normally (Figs 2, 5).

This model suggests how the coat can form near the plasma membrane without attaching to it (West & Erdos, 1990), and why a normal-appearing coat forms when cells sporulate in monolayer culture after induction with 8-bromo-adenosine cAMP (unpublished data). However, it is possible that other proteins, perhaps associated with the inner layer of the coat, link the coat to the plasma membrane. The phenotypes of the deletion mutations, and the protein glycosylation mutations targeting O-glycosylation (West & Erdos, 1990), might easily follow from changes in the amount or packing of cellulose fibrils. Because of the similarity and apparent redundancy of the outer layer coat proteins, it will be informative to delete additional members of this coat gene superfamily in order to probe how this type of protein contributes to cellulose morphogenesis.

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