Incorporation of Protein into Spore Coats Is Not Cell Autonomous in *Dictyostelium*

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Abstract. At maturity, the spores of *Dictyostelium* are suspended in a viscous fluid droplet, with each spore being surrounded by its own spore coat. Certain glycoproteins characteristic of the spore coat are also dissolved in this fluid matrix after the spore coat is formed. To determine whether any proteins of the coat reside in this fluid phase earlier during the process of spore coat assembly, pairs of strains which differed in a spore coat protein carbohydrate marker were mixed and allowed to form spore coats in each other's presence. We reasoned that proteins belonging to an early, soluble, extracellular pool would be incorporated into the spore coats of both strains. To detect trans-incorporation, spores were labeled with a fluorescent antibody against the carbohydrate marker and each spore's fluorescence was analyzed by flow cytometry. Several proteins of both the outer and inner protein layers of the coat appeared to be faithfully and reciprocally trans-incorporated and hence judged to belong to a soluble, assembly-phase pool. Western blot analysis of sorted spores, and EM localization, confirmed this conclusion. In contrast, one outer-layer protein was not trans-incorporated, and was concluded to be insoluble at the time of secretion. Three classes of spore coat proteins can be described: (*a*) Insoluble from the time of secretion; (*b*) present in the early, soluble pool but not the late pool after spore coat formation; and (*c*) present in the soluble pool throughout spore coat assembly. These classes may, respectively: (*a*) Nucleate spore coat assembly; (*b*) comprise a scaffold defining the dimensions of the nascent spore coat; and (*c*) complete the assembly process by intercalation into the scaffold.

THE spore coat of *Dictyostelium* is formed at the surface of each prespore cell as it sporulates in preparation for dormancy. Spore coats isolated after germination are composed of roughly equal proportions (by mass) of protein and polysaccharide, which includes cellulose and a polysaccharide containing galactose and *N*-acetyl-galactosamine (GPS) (1). Before spore coat formation, all known major components other than cellulose can be localized, using antibodies or a lectin, to an intracellular secretory compartment, the prespore vesicle (reviewed in reference 19). In conventional EM preparations these components tend to lie near the vesicle membrane. By using colloidal gold conjugated probes applied to thin sections, it was shown that these molecules are not homogenously intermixed in the presecretory compartment (7). Furthermore, covalent interactions are suspected to exist because some proteins coimmunoprecipitate with other proteins in a reducing agent-sensitive fashion (5). Thus there is reason to suspect some pre-assembly of spore coat glycoproteins before secretion.

Spore coat formation appears to be initiated by the exocytosis of the prespore vesicle contents into the extracellular space (19). Flow cytometric analysis indicates that the differentiating spores are smaller than prespore cells and that pre-existing plasma membrane antigens initially remain ac-cessible to antibody labeling (2). As the outer dense layer of the coat begins to assemble around the shrunken cell, cellulose deposition can be detected subjacent to this structure. Glycoproteins which carry the fucose epitope (GA-X, recognized by mAb 83.5), including the glycoproteins SP75, SP80, and SP96, become localized in the outer dense layer (see Table I for a list of proteins examined in this report). Other molecules, including the glycoprotein SP85 (which uniquely carries GA-XI, recognized by mAb 16.1) and the GPS, become concentrated in the inner dense layer, with some excursion into the middle cellulosic layer. The predominant glycoprotein which carries GA-XX (recognized by mAb 5.1), SP86, is found primarily in the outer layer. Finally, some spore coat glycoproteins and the GPS can be localized between spores (beyond the outer layer) when the soluble phase is precipitated with cetylpyridinium chloride (7, 18). The segregation of individual molecular species into separate layers of the coat implies a specificity about the assembly process. Material in the interspore matrix (soluble phase) may represent a reservoir of unincorporated molecules, which failed to incorporate into the coat proper because existing binding or attachment sites were already filled.

Most models for how protein is associated with cellulose

and other polysaccharides in plant cell walls depict an intimate interweaving (6, 16) which, it seems, would be most easily achieved by a post-secretion assembly process since cellulose deposition occurs extracellularly. Because cellulose deposition also occurs extracellularly in *Dictyostelium* (7), the plant model focuses attention on the possibility of extracellular matrix (spore coat) assembly in *Dictyostelium*. However, the *Dictyostelium* spore coat is relatively rich in protein compared to most plant cell walls (3), so it is not clear to what extent rules observed in plant matrix assembly are applicable to *Dictyostelium*.

To understand the mechanism of spore coat assembly in *Dictyostelium*, it is necessary to determine the site at which it occurs. This study has determined that certain glycoproteins produced by one cell can be incorporated into the coat of neighboring cells, showing that, like cellulose biosynthesis, entry of these proteins into spore coats is an extracellular process originating from soluble precursor pools. Differences in incorporation behavior have led to the identification of candidate molecules for nucleation, assembly, and intercalation.

Materials and Methods

Cells

Strain HL250 is unable to form GDP-fucose from GDP-mannose, which leads to an inability to fucosylate protein in the absence of exogenous fucose (10). HL250 lacks the fucose epitope (GA-X), recognized by mAb 83.5, normally found on the spore coat proteins SP75, SP80, and SP96, and consequently lower molecular weight isoforms of these proteins are accumulated (10).

Strain HW20 is a spontaneous derivative of the *modB* mutant DL118 which has haploidized and grows axenically in HL-5 medium. HW20 and DL118 lack the carbohydrate epitope GA-XX (recognized by mAb 5.1) on the spore coat protein SP86, and the carbohydrate epitope GA-XI (recognized by mAb 16.1) expressed uniquely on the spore coat protein SP85 (1). Strain Ax3 (provided by Dr. Steve Free, State University of New York, Buffalo, NY), the parent of strain HL250, and strain M28 (provided by Dr. E. Katz), which carries a round spore marker (19), were used as normal strains. Cells used for flow cytometry and Western blotting were grown axenically in HL-5, and cells processed for EM were grown in association with *Klebsiella aerogenes*. To induce sporulation, cells were washed in PDF (45 mM NaH$_2$PO$_4$/Na$_2$HPO$_4$, 20 mM KCl, 6 mM MgSO$_4$, pH 5.8), and plated on cellulose nitrate filters in PDF. Strains were mixed at the time of plating. Upright fingers (referred to as slugs in this report) were examined between 18 and 20 h of development and spores were examined after 25–40 h of development. Fluorescence microscopy of individual sori showed that mutant and normal cells were truly intermixed within the same sorus (not shown).

Immunofluorescence of Chimeric Slugs

Individual chimeric slugs (10% Ax3, normal; 90% HL250, mutant), taken at a time after spore coat proteins were synthesized and stored in prespore vesicles, were lightly digested with Pronase on a poly-L-lysine–coated slide and the dissociated cells were allowed to settle as described (12). Adsorbed cells were washed in TBS, fixed in cold methanol and labeled immunofluorescently as described below using mAb 83.5, which recognizes the fucose epitope (produced by Ax3 but not HL250 cells).

Flow Cytometry and Sorting of Spores

Spores were picked from sori with a loop and suspended in water in a 1.5-ml microcentrifuge tube. In some trials, mature spores were co-mixed to assess transfer of proteins after spore coats had formed. For this purpose, equal numbers of loop-picked 30-h sori were stirred together in a microcentrifuge tube and incubated for 24 h, and then diluted with water. Spores were recovered by centrifugation at 10,000 g for 10 s. Spores were washed by vortexing in 8 M urea in 10 mM K/NaPO$_4$, pH 6.8, containing 1 mM PMSF, 10

μg/ml aprotinin, and 10 μg/ml leupeptin. This treatment was intended to strip adventitiously adsorbed protein while allowing the spore to retain germination competence (4). After 30 min at 22°C, spores were recovered by centrifugation as above and washed 3× in TBS (10 mM Tris-HCl, 150 mM NaCl, 0.05% NaN$_3$, pH 7.4). Urea-washed spores were resuspended by vortexing in mAb 83.5 or 16.1, which had been concentrated from tissue culture supernatants using (NH$_4$)$_2$SO$_4$ and then diluted to their original concentration in 5% non-fat dried milk in TBS. After a 1–2-h incubation at 22°C, spores were washed 3× in TBS and incubated in phycoerythrin-conjugated goat anti-mouse IgG (Biomeda, Foster City, CA) at a dilution of 1:50 in the milk solution. After 1 h of incubation, spores were recovered and washed 3× as above in TBS. Labeled spores (10,000/analysis) were examined immediately in a FACSTAR Plus flow cytometer (Becton-Dickinson & Co., Mountain View, CA). Fluorescence was induced by a 250 mW argon-ion laser at 485 nm; emission was monitored at 585 ± 20 nm. The instrument was aligned before each run using 2.02-μm-diam fluorescent microbeads ('Fluoresbrite'; Polysciences Inc., Warrington, PA). In some experiments, low fluorescence and high fluorescence spores were specified, by selecting appropriate forward light scattering and fluorescence intensity windows, and sorted into conical bottom 13 × 75 mm polypropylene tubes freshly precoated with 1% BSA in TBS. Spores were recovered by centrifugation. Sorting windows were optimized for purity rather than yield. For biochemical analysis of sorted spores it was necessary to carry out manipulations in the presence of the protease inhibitors listed above, and on ice (except during antibody labeling and sorting), to inhibit proteolysis.

Electrophoresis and Western Blotting

For Figs. 1 and 3, ca. 10^7 spores, counted in a hemacytometer, were washed sequentially in 400 μl of H$_2$0 and 400 μl of urea as described above. In some cases urea-washed spores were resuspended in a fresh aliquot of 8 M urea, boiled for 3 min, and washed with TBS. For Fig. 7, spores were counted by flow cytometry. Samples were diluted to 1×-strength SDS sample buffer containing 5% 2-mercaptoethanol, boiled for 3 min, electrophoresed on a gradient SDS slab gel, electrophoretically transferred to nitrocellulose, and immunoprobed indirectly with an alkaline phosphatase-coupled second antibody as described (12).

Electron Microscopy

Spores were picked and processed for thin sectioning, and sections were immunoprobed indirectly using gold particles coated with second antibodies, as described (7).

Results

Efficiency of Incorporation of Secreted Spore Coat Proteins into Coats

EM immunolocalization studies previously showed that certain spore coat protein epitopes lie in the interspore matrix between the spores (7, 18), suggesting the existence of a soluble pool of unincorporated spore coat protein. To investigate this possibility, normal Ax3 spore masses (sori) were mixed with water, and centrifuged to separate the interspore matrix from the spores. The pelleted spores were washed with a low ionic strength 8 M urea solution to strip proteins that might be loosely adsorbed to the surface of spores, since this does not affect spore viability (4). The distributions of spore coat proteins among the washes and the intact spores were assayed by SDS-PAGE under reducing conditions and Western blotting (Fig. 1). Certain proteins, including SP70, SP75, and SP86 were found exclusively in the spore coat. Since SP86 is also found intracellularly (see below), hot 8 M urea extraction, which selectively extracts spore coat relative to intracellular proteins, was performed to confirm by biochemical criteria that SP86 is associated with the spore coat. SP96 displayed a different distribution pattern, being found in the water wash, the urea wash, and in the spore coat. SP85 was also distributed between the water wash and the spore
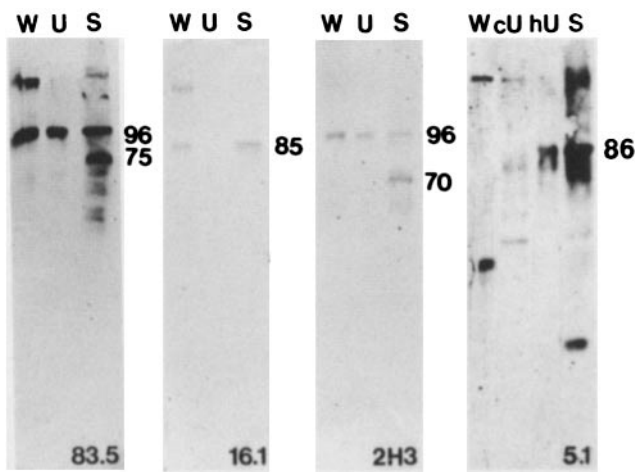
Figure 1. Individual spore coat proteins are incorporated into the coat with variable efficiencies. Sori were collected and spores washed in succession with water, cold 8 M urea, and, in one case, hot 8 M urea. Aliquots of the water (*W*), cold urea (*U*), and hot urea (HU) washes, together with an equivalent number of spores (S), 6 × 10⁵/lane, were subjected to SDS-PAGE under reducing conditions, and Western blotted. Blots were probed with the antibodies indicated to determine how proteins were partitioned among these three extracts. Individual spore coat proteins are denoted in the margins by their apparent MWs in kD.

coat, though not in the urea wash. This may be because SP85 is in the inner layer of the spore coat, where it could be more difficult to extract with urea. Finally, high molecular weight forms (150–200 kD) displayed a third pattern, being found almost exclusively in the water wash. This material appears to consist of homo- or hetero-dimers of the SP75, SP86, SP85, and SP96 polypeptides that resist complete dissociation, as determined by elution, re-electrophoresis and recovery at lower molecular weight positions (10). Properties of these proteins are summarized in Table I. Similar results were found for SP96, SP85, and SP70 in the mutant strain HL250 (not shown), suggesting that these parameters of incorporation into the spore coat are not affected by the mutation. Thus the biochemical detection of soluble spore coat proteins in sori confirms the previous EM localization studies. Because SP70, SP75, and SP86 are found only in the spore coat, their incorporation appears to be different from that of SP85 and SP96, and may be limiting for the incorporation of the latter two proteins (19).

Analysis of Transfer of Outer-layer Spore Coat Proteins by Flow Cytometry

The existence of the soluble interspore protein pool suggested that proteins might pass through this pool during spore coat assembly. This question could be addressed if an isoform of a spore coat protein could be found, and the exchange of this isoform between the cell which produced it and a neighboring cell which did not produce it could be examined. We achieved this by forming chimeras between wild-type and mutant cells, and asking whether wild-type spore coat proteins, which bear normal carbohydrate epitopes, could be detected on mutant spores, which cannot form the carbohydrate epitopes (see Table I for list of proteins, carbohydrate epitopes, and mutants). For example, to study SP75 and SP96, which normally each expresses the fucose epitope, different ratios of Ax3 (normal) and HL250 (mutant) cells were induced to sporulate together. Spore mixtures were washed, and the fluorescence properties of individual spores were quantitated after labeling with mAb 83.5, which recognizes the fucose epitope. Since the fucosylation defect leads to the formation of a lower apparent molecular weight isoform of SP96, distribution of mutant and normal SP96 was also examined on Western blots using mAb A6.2, which recognizes both molecular weight isoforms.

Before using this mutant/normal pair to study the exchange of spore coat proteins, it was necessary to show that the biochemical lesion in the mutant was cell autonomous, and that there was no preferential inclusion or exclusion of mutant spore coat protein with respect to the coat. Because the metabolic defect in HL250 is partially corrected by extracellular L-fucose (Gonzalez-Yanes, Gritzali, Brown and West, manuscript in preparation), transfer of fucose from wild-type to mutant cells could rescue fucosylation in mutant cells. Since spore coat proteins are synthesized and fucosylated in prespore cells of the slug, we asked whether mutant cells co-developed with normal slug cells were able to accumulate the fucose epitope. Individual chimeric slugs which contained 10% Ax3 (normal) cells were taken at a time after spore coat proteins were synthesized and stored in prespore vesicles, dissociated with Pronase, fixed, and labeled immunofluorescently with mAb 83.5, which recognizes the fucose epitope (produced by Ax3 but not HL250 cells). As exemplified in Fig. 2, a minority (9.1%) of cells were brightly fluorescent, the majority were negative, and cells of an intermediate level of fluorescence were uncommon. This shows that mutant cells had not acquired sufficient

Table I. Spore Coat Proteins Examined in This Report

Protein	Cognate antibody	Type of CH₂O epitope*	Location	Class‡
SP86§	5.1 (c)‖	GA-XX (*mod*B-dependent)	outer layer	1
SP75	83.5 (c)	GA-X (fucose-dependent)	outer layer	2
SP85§	16.1 (c)	GA-XI (*mod*B-dependent)	inner layer	2
SP70	2H3 (p)	–	not known	2
SP96	A6.2 (p), 83.5 (c)	GA-X (fucose-dependent)	outer layer	3

* *mod*B-dependent carbohydrate epitopes are missing in mutant strain HW20, and the fucose epitope is missing in strain HL250.
‡ Class type refers to the nature of the association of the protein with the spore coat, as described in this report. Class 1 denotes non-diffusive, i.e., strictly associated with spore of origin; class 2, diffusive between spores but no overspill into the interspore matrix; class 3, diffusive between spores and present in the soluble interspore matrix.
§ Though these two proteins comigrate on our gel system, they have been assigned slightly different apparent molecular weights, to simplify their nomenclature. SP85 and SP86 were previously assumed to be the same protein (1), but their distinctive properties reported herein show them to be different.
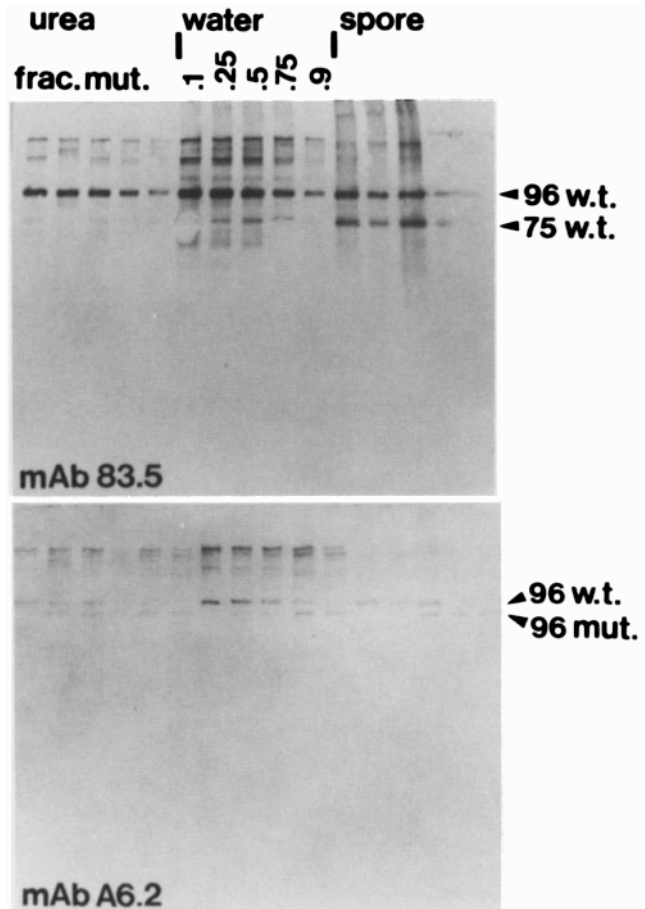‖ *c* denotes a carbohydrate-dependent epitope; *p*, a probable peptide-dependent epitope.
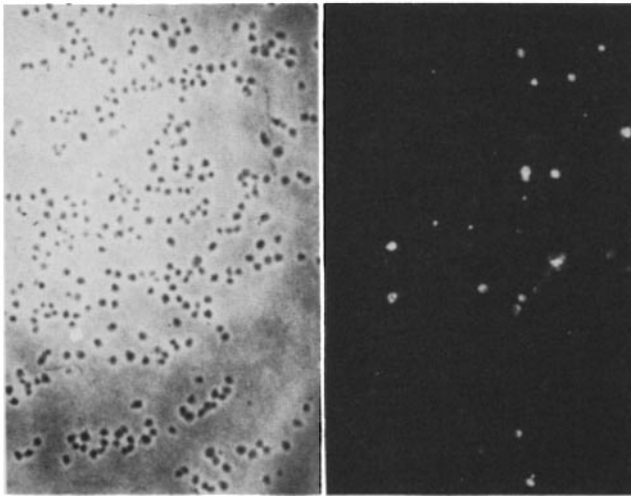
Figure 2. The fucosylation deficiency in HL250 is cell autonomous in chimeric mixtures with normal cells. Individual slugs, formed from a mixture of 10% normal (Ax3) cells and 90% mutant (HL250) cells, were dissociated on a polylysine-coated glass slide using Pronase. After fixation cells were labeled with mAb 83.5 followed by phycoerythrin-conjugated goat anti–mouse IgG, and examined by phase contrast (*left*) and fluorescence (*right*). The antibody recognizes the fucose epitope produced by normal cells on spore coat proteins and stored in prespore vesicles. Since fluorescent labeling occurred in only a subpopulation of cells, this rules out the possibility that GDP-fucose (which the mutant cells cannot make), fucose, or fucoconjugates are transferred from normal to mutant cells up through the period of spore coat protein accumulation.

Figure 3. Incorporation of spore coat proteins into the spore coat is not affected by the glycosylation mutation. Strains HL250 (mutant) and Ax3 (parent) were allowed to form fruiting bodies in the proportions indicated. Sori were picked and the water wash, urea wash and spores (3×10^5/lane) were analyzed as described in the Fig. 1 legend. After Western blotting, nitrocellulose was probed with either mAb 83.5 (*top*) which recognizes the fucose epitope expressed on the wild-type isoforms of SP75 and SP96, or mAb A6.2 (*bottom*), which recognizes both mutant and wild-type molecular weight isoforms of SP96. There is no apparent preferential incorporation of mutant or normal forms of SP96 into spores, since the same ratio of isoforms is found in the washes as with the spores, at different proportions of mutant and normal cells.

fucose from neighboring cells to permit detectable fucosylation of their spore coat protein. Thus, metabolic cooperation of a type which allows exchange of small molecules between cells does not occur in *Dictyostelium*, as expected from previous morphological studies (13).

To rule out the possibility that wild-type or mutant protein isoforms are preferentially incorporated into the coat, a range of proportions of chimeras were prepared, and water washes, urea washes and spores were examined by SDS-PAGE and Western blotting using mAb A6.2 (which detects both molecular weight isoforms of SP96). As the proportion of mutant cells increased, the relative level of the mutant isoform of SP96 found associated with spores was seen to increase proportionately (Fig. 3). Similar ratios of wild-type and mutant isoforms of SP96 were found in each of the washes and the spores. Thus the incorporation of SP96 is neutral to the mutation, suggesting that the epitope can be a neutral marker for the distribution of normal protein isoforms by immunofluorescence. Since the mutant isoform of SP96 was recovered from the chimeras at the expected level (Fig. 3), the conclusion from Fig. 2 that the fucosylation defect is cell autonomous is verified.
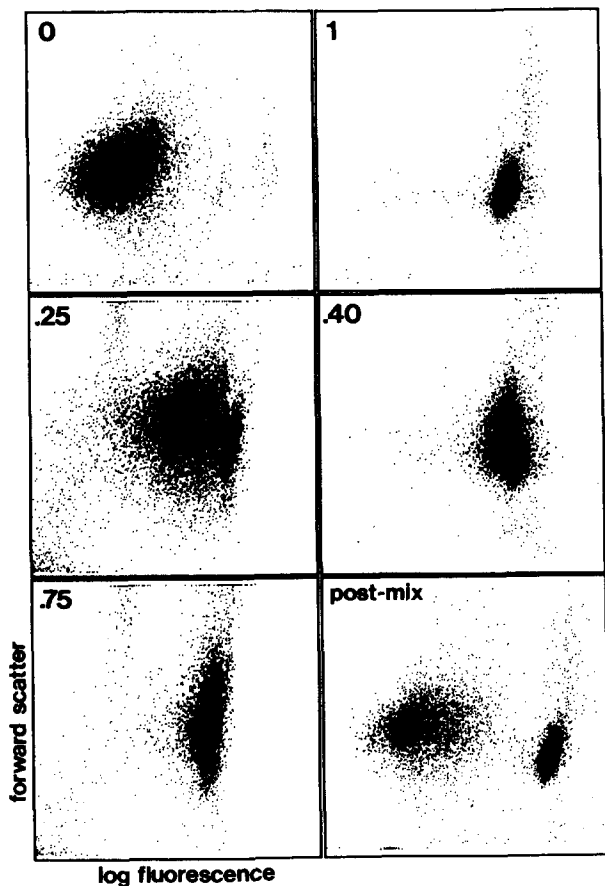
Mutant and normal spores formed in isolation from one another exhibited, as expected, a nearly 2-log difference in fluorescence labeling intensity using mAb 83.5 (Fig. 4). Normal spores were slightly smaller than mutant spores based on forward light scattering intensity. In 75%:25%; normal:mutant mixtures of co-sporulated spores, however, only a single fluorescence population was observed, with a level of fluorescence intermediate between that of either
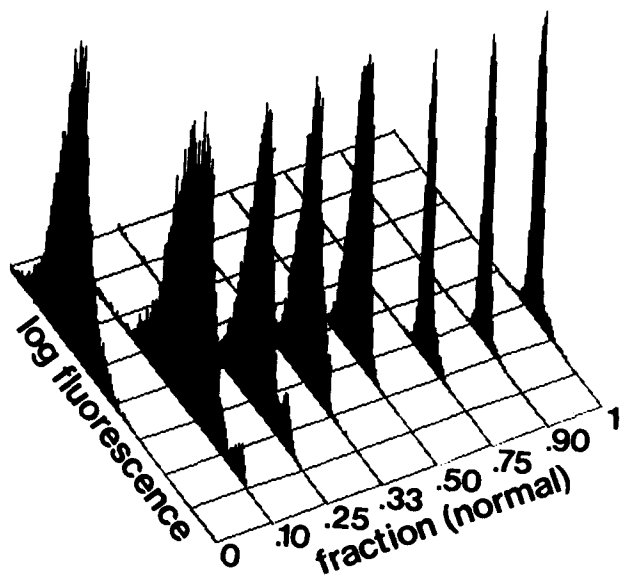
strain sporulated alone (Fig. 4). This surprising result indicated that spore coat proteins which both carry the fucose epitope (primarily SP75, SP80, and SP96), and are accessible to antibody labeling, are equally shared between spore coats of the two strains. This suggested that incorporation of these proteins into the coat occurred extracellularly, and from a soluble, secreted pool which is shared between neighboring spores. It follows that the interspore matrix components detected in the washes of Fig. 1 may be a remnant of that pool. As expected from this model of protein transfer during spore coat formation, spore mixtures which were created after spore coat formation was complete failed to transfer any proteins from normal to mutant spores (Fig. 4).

The transfer of wild-type proteins to mutant spore coats was examined further in a series of ratios of normal and mutant cells (Fig. 5) and the results are plotted in Fig. 6. Basically, with input normal cell percentages of 50% and above,
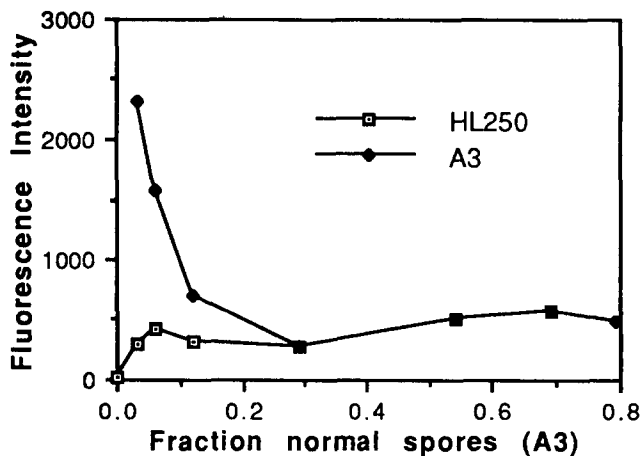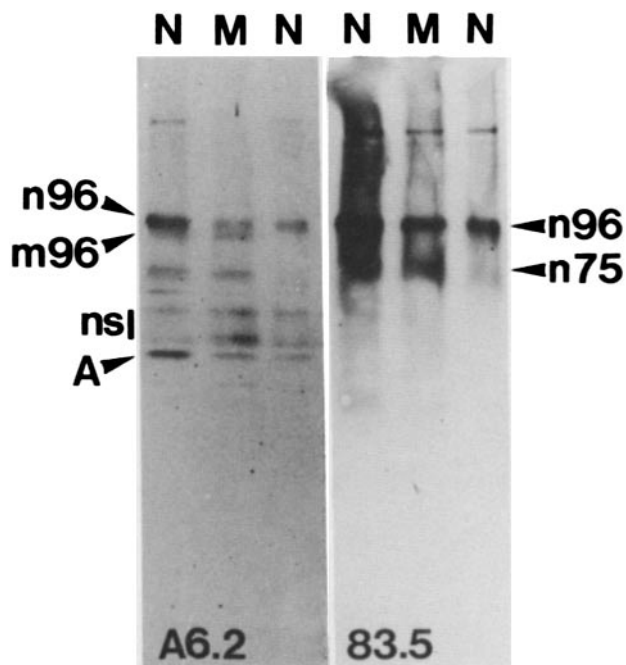
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Figure 5. Fluorescence histograms of chimeras of different normal/mutant ratios. Spore mixtures were prepared and their fluorescence distributors were quantitated by flow cytometry as in Fig. 4. A family of one-dimensional fluorescence histograms from a range of strain proportions (given as the fraction of input cells which were normal) is shown. Fluorescence intensity channels are plotted on a log scale on the left-hand axis. The fractional number of normal cells in the chimeric population for each histogram is given on the right-hand axis.

Figure 4. Spore coat proteins are shared between forming spores. Mutant (HL250) and normal (Ax3) strains were mixed at the indicated ratios, given as fraction of cells which were the normal strain, and allowed to form fruiting bodies. In one panel, sori were mixed after differentiation, in a 1:1 ratio, and incubated together for 24 h (*post-mix*). Spore mixtures were washed with 8 M urea, to strip adventitiously associated material, and immunolabeled with mAb 83.5, which recognizes the fucose epitope on spore coat protein isoforms produced by the normal spores, followed by phycoerythrin-conjugated goat anti-mouse IgG. Spore fluorescence and the intensity of forward light scattering intensity were simultaneously quantitated by flow cytometry. Mutant and normal spores which differentiated alone exhibited, as expected, monodisperse distributions of fluorescence whose mean values differed by nearly two orders of magnitude. In contrast, mixed populations consisted of spores whose fluorescent properties were different from that of cells sporulated separately. In contrast, spores mixed after spore coat formation was complete retained fluorescence properties corresponding to their type. These results indicate that spore coat molecules carrying the fucose epitope were transferred from normal to mutant spores during spore coat formation.

only one fluorescent population is observed; below 50%, two populations are observed. Above 50%, the fluorescence intensity gradually rises (except at the 100% level), reflecting the higher proportion of normal spore coat proteins. Below 50%, however, the fluorescence intensities are unexpectedly high (they do not approach zero in a linear fashion). This finding can be correlated with a previous observation that the mutant spore coat is more porous than that of the normal, as determined by the greater accessibility of labeling of the GPS, which is located in the inner layer of the coat, by a fluorescent lectin (10). The higher porosity of the mu-

tant spore coat would explain the unexpectedly high level of fluorescent labeling of the wild-type proteins transferred to it. Furthermore, since the higher porosity of the mutant is presumably associated with protein, it would be expected that the high porosity character would be shared with normal

Figure 6. Dependence of normal and mutant spore fluorescence on mutant cell input fraction. The mean value of fluorescence intensity of normal spores (the right-most peak in multi-modal distributions) and mutant spores (the left-most peak in the distributions) from chimeras in Fig. 5 were plotted as a function of the fraction of spores which were normal (based on flow cytometry data, not input numbers). As discussed in the text, the fluorescence intensity of mAb 83.5 labeling of normal and mutant spores appears to be a composite function of epitope abundance and antibody accessibility.
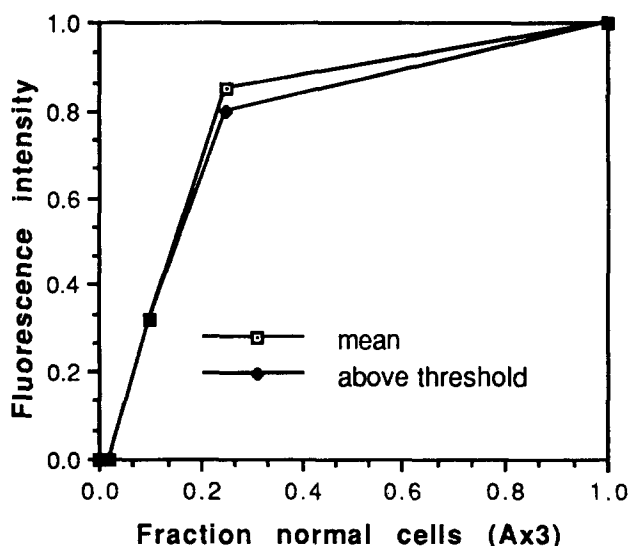
Figure 8. Chimeras between *mod*B mutant and normal spores. Cells of the *mod*B mutant HW20 and the normal stain Ax3 were codeveloped, immunolabeled with mAb 16.1, which recognizes a carbohydrate epitope on the SP85 isoform produced only by normal spores, and analyzed by flow cytometry as in Fig. 4. A bimodal distribution was obtained representing normal and mutant spores. The fraction of cells with fluorescence greater than a threshold fluorescence value defined by the minimum between the peaks, and the total mean fluorescence (each scaled to a fraction of 1), were each plotted as a function of the proportion of input normal cells. The deviation of the plots above a theoretical straight line with a slope of one indicates that normal SP85 was transferred to mutant cells.

Figure 7. Glycoprotein distributions on sorted spores. Mutant and normal spores were sorted from a chimera containing 40% normal spores (labeled with mAb 83.5 as in Fig. 4), and compared by Western blotting after SDS-PAGE. The center lane in each panel contains 5×10^5 mutant (low fluorescence) spores (*M*); the left and right lanes contain 3.2×10^5 and 1.6×10^5 normal (higher fluorescence) spores (*N*), respectively. The left panel was immunoprobed with mAb A6.2, which recognizes both mutant (m96) and normal (n96) isoforms of SP96. The right-hand panel was immunoprobed with mAb 83.5, which recognizes the fucose epitope (GA-X) found only on normal isoforms of SP96 and SP75. The bands marked NS are non-specifically labeled by the secondary antibody under the high sensitivity conditions used in the left panel. With the exception of the band marked *A*, the other bands are degradation products. Band *A* is the heavy chain of mAb 83.5, and its relative abundance in the mutant and normal samples roughly reflects the relative fluorescence of the two spore types. The results show that normal isoforms of SP96 and SP75 are efficiently transferred to mutant spores during cosporulation.

spores just as they share their proteins with mutant spores. This relationship is in fact observed: at high mutant cell proportions normal spores become even more fluorescent than when cultured alone. As the mutant proportion decreases both mutant and wild-type fluorescence tend to decrease as the proteins responsible for the porosity are diluted. Thus it is concluded that the porosity phenotype of mutant spores is efficiently shared with normal spores, and that the porosity phenotype rapidly dilutes out, consistent with its being associated with a shared protein(s). Finally, it appears that a population of spore coat proteins is preferentially associated with the spore of origin, because even when normal spores are highly diluted with mutant spores, the normal spores are more fluorescent.

Biochemical Analysis of Sorted Spores

Although the flow cytometry data showed that the antibody-accessible copies of SP96, SP80, and SP75 were efficiently shared between spores, this method could not determine the transfer of potentially inaccessible copies of these proteins. To address this question, mutant and normal spores from chimeras were sorted and their protein composition was determined by SDS-PAGE and Western blotting. Chimeras formed from input proportions of 25, 33, and 40% normal (Ax3) cells were examined, because these ratios resulted in the formation of spores which had similar fluorescence but were still separable based on dual forward scatter and fluorescence parameters. Sorting of the two strains from these chimeras was effective, because plating of separated spores yielded 11:11 colonies examined of the low fluorescence subpopulation as mutant, and 6:6 colonies examined of the high fluorescence subpopulation as normal. Similar numbers of sorted mutant and normal spores were compared by SDS-PAGE, Western blotting, and immunoprobing with mAb A6.2, which is monospecific for SP96 (Fig. 7). Sorted mutant spores contained both normal and mutant isoforms in an approximately 1:1 ratio. Although proteolysis, as evidenced by the appearance of several lower molecular weight species representing degradation products of SP96, hinders direct quantitative analysis of the extent of SP96 transfer between spores, it is clear that the ratio of SP96 isoforms associated with mutant spores roughly represents the proportion of mutant and normal spores in the comixture (40% mutant). In sorted mutant spores from a chimeric population of 25% mutant spores, the mutant isoform was in excess of the normal isoform (data not shown). Transfer of the normal isoform of SP96 to mutant spores was confirmed by immunoprobing blots with mAb 83.5 (Fig. 7). This blot indicates that the level of the normal SP96 isoform in mutant

spores is about one-half the level found on normal spores. In addition, mAb 83.5 probing also shows that the normal isoform of SP75 is efficiently transferred to mutant spores. Thus a protein (SP75) which is not present in the late pool is also transferred between forming spores.

Though immunoprobing with mAb A6.2 suggested that some mutant copies of SP96 are found on normal spores (Fig. 7) as if SP96 exchange is partially reciprocal, it is difficult to estimate the level of transfer because the band seen may be contaminated by a proteolytic fragment of the normal isoform. Since more proteolysis has occurred in this sample than in the companion mutant sample (as evidenced by more material in lower molecular weight bands), and the mutant isoform of SP96 is more proteolytically sensitive than the normal isoform (10), this evidence is not as useful as the clear evidence for transfer of normal SP96 to mutant spores. It should also be pointed out that because mutant spores were in excess during the sort, it is less likely for contamination of mutant spores by normal spores to occur than vice versa. Finally, post-immunolabeling of the same blots with mAb 16.1, which monospecifically labels the spore coat protein SP85 in both strains, verified that the relative sample loadings were accurate (not shown).

Analysis of an Inner-layer Spore Coat Protein

SP85 is located in the inner layer of the spore coat, in contrast to the glycoproteins described so far (see Table I). This protein uniquely bears a modB-dependent carbohydrate epitope (GA-XI) which is recognized by mAb 16.1 and is missing in the modB mutant HW20. Thus this antibody recognizes the wild-type isoform but not the form produced by modB mutant cells, so that by analyzing chimeras formed between these two strains we can study the potential transfer of an inner coat protein as well. Previous studies have shown that this mutation is essentially cell autonomous in the slug (12). Because of its position deep in the coat, the mAb 16.1 epitope labels less effectively than outer coat epitopes, so that there is an overlap between the fluorescent signals deriving from separate populations of strain HW20 (modB mutant) and Ax3 (normal) spores. To estimate the level of incorporation of the epitope into mutant spore coats, both the mean fluorescence and the fraction of fluorescence signal registered in fluorescence channels above the crossover point of the fluorescence distributions of mutant and normal spores were plotted relative to the proportion of normal spores (Fig. 8). These plots showed higher levels of fluorescence than expected from simple dilution of normal spore fluorescence by nonfluorescent spores, which would be described by a simple straight line reaching a maximum at 75%. At that percentage Ax3 cells completely fill the spore cell compartment, which they enter preferentially relative to modB mutant cells (12). Regardless of whether increased fluorescence is a result of increased incorporation of normal SP85 into mutant spores, or also to increased labeling of normal spores as a result of transfer of mutant spore coat permeability characteristics (1), we conclude that at least some copies of SP85 are also incorporated from a shared extracellular pool.

Microscopic Analysis of Spore Coat Protein Transfer

To obtain additional evidence for trans-incorporation, and to determine whether proteins from normal cells were properly localized to their respective layers in mutant coats, chimeric sori were sectioned and the position of normal protein isoforms in mutant spores was determined by immunogold labeling. The normal strain M28, which carries a round spore marker, was used to facilitate identification of mutant spores, which are elongate. Strain M28 and either mutant strain HL250 or HW20 were mixed in a 2:1 ratio. By flow cytometry, M28 and HW20 spores were distinctive by both side scatter and forward scatter intensity measurements, though there was some overlap of the distributions (data not shown). The overlap between M28 and HL250 was greater since HL250 spores are less elongate. However, since one in three spores were of the HL250 strain, it was possible to infer the labeling properties of HL250 spores simply by observing multiple spores.

All spores from the M28/HL250 mixture were labeled similarly by mAb 83.5 (Fig. 9, a and b). The similar density of gold particles was consistent with data from flow cytometry showing identical fluorescence labeling, and Western blot analysis of sorted spores. The distribution of gold particles in the outer layer of the spore coat showed that normal glycoproteins were incorporated into mutant spore coats in a spatially correct manner. The continuous character of the labeling indicates that mutant spores did not incorporate preformed patches of wild-type spore coat proteins. In control labelings strain HL250 was not labeled, and all cells of strain M28 were labeled (data not shown).

All spores from the M28/HW20 mixture were labeled similarly by mAb 16.1 (Fig. 9, c and d). There was a similar density of particles over each spore indicating that transfer of SP85, which is specifically labeled by mAb 16.1, was efficient. The distribution of gold particles in the inner and middle layers of the spore coats showed that trans-incorporated SP85 was located correctly. In control labelings, strain HW20 spores were not labeled by either mAb 16.1 or 5.1 (see below); all M28 spores were labeled by each of these antibodies.

In contrast, some spores were not labeled by mAb 5.1 in the M28/HW20 chimeras (Fig. 9, e and f). Since the elongate spores (HW20) were unlabeled, SP86, the predominant glycoprotein carrying the glycoantigen (GA-XX) recognized by mAb 5.1 (see Fig. 1), remains associated with its cell of origin. SP86, which is concentrated in the outer layer of the spore coat (7), is thus not diffusive and, in contrast to SP75, SP85, and SP96, is not located in the soluble pool during spore coat assembly.

Discussion

Incorporation of Certain Proteins into Spore Coats Is Communal In Sori

The basic observation is that certain spore coat glycoproteins are efficiently exchanged between neighboring spores during spore coat assembly, whereas one is not. This conclusion is based on mixing two strains of cells, which produce isoforms of several glycoproteins differing in apparent MW and expression of glycoantigens. After differentiating together, the newly formed spores were immunolabeled with an antibody against one of the glycoantigens, which only one of the two strains had been able to synthesize, and analyzed by flow
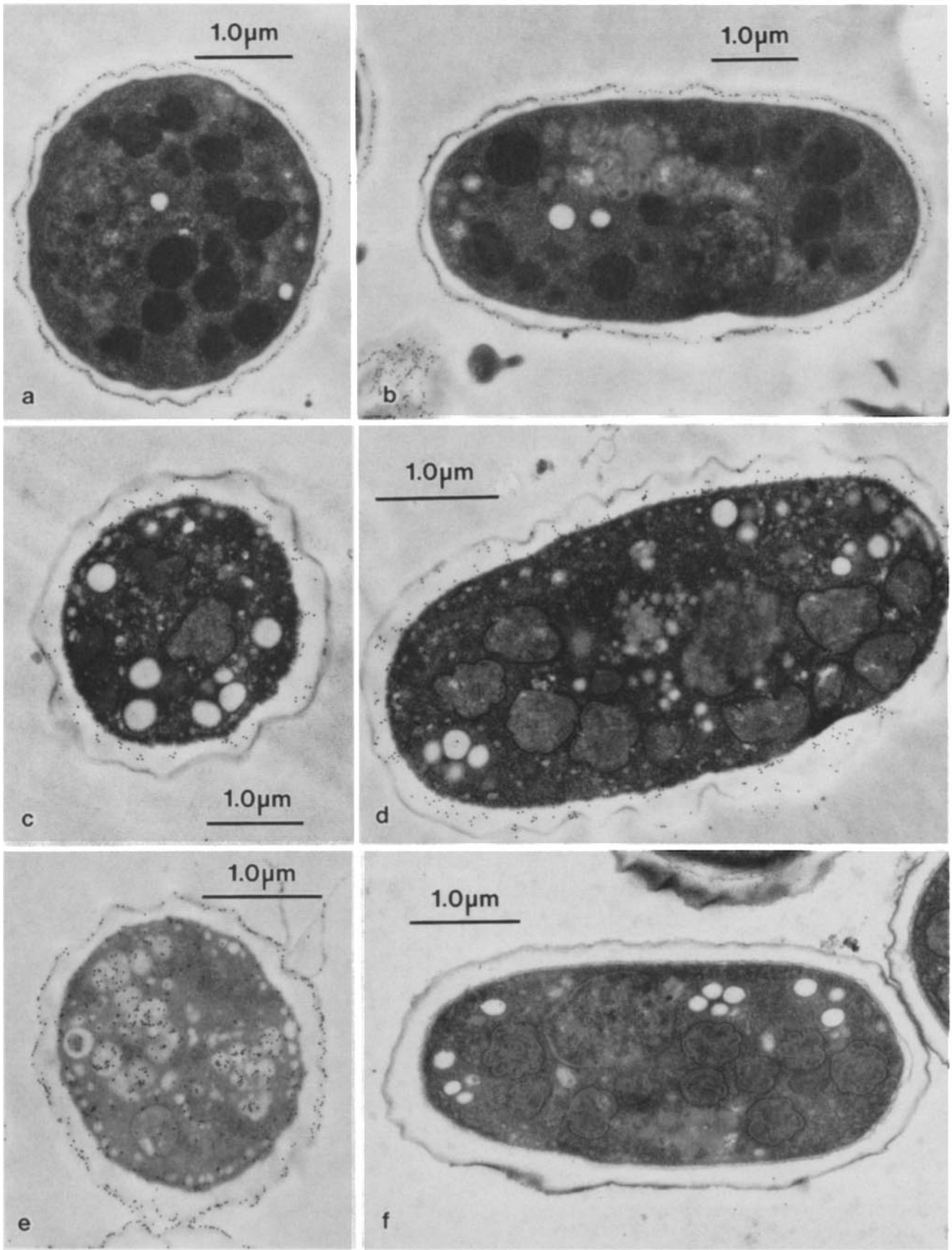
cytometry. When normal cells, which synthesize the gly-coantigen, are in excess, nearly all of the cells have the same fluorescence (Fig. 4), as if they each equally share the glycosylated spore coat proteins. When normal cells are in the minority, quantitation of spore fluorescence revealed that more protein remains associated with the cell of origin (Figs. 4–6). At these ratios the two strains can be sorted on the basis of their fluorescence differences, and Western blot analysis showed that SP75, SP85, and SP96 are each shared, and that sharing does not simply involve a small, antibody-accessible subpopulation of glycoproteins (Fig. 7). Shared proteins are, as expected, resistant to extraction by 8 M urea at room temperature (Fig. 3), but released by boiling in 8 M urea. EM immunogold labeling verified that trans-incorporation is uniform, substantial, and of proper topography for both inner and outer layer glycoproteins (Fig. 9). This method also showed that SP86, located primarily in the outer layer, is not shared between forming spores.

These observations indicate that assembly of proteins other than SP86 occurs from an exchangeable pool of precursors. Consistent with this model, the acquisition of glycoantigen by mutant cells occurs at a time after the glycoantigen is formed and stored in secretory vesicles, as determined by immunofluorescence analysis of prespore cells (Fig. 2). It also occurs at a time prior to final assembly of the spore coat, because mixing spores after this time does not allow transfer of glycoantigen (Fig. 4).

Protein Pools in Spore Coat Formation

The model for communal sharing of spore coat proteins is consistent with earlier observations regarding spore coat genesis (reviewed in 19). The first observable event of spore coat formation is the exocytosis of glycoproteins and the GPS which contribute to the coat. This material accumulates extracellularly before the cell shrinks and cellulose is deposited, as determined by EM immunocytochemistry. Immuno-flow cytometry reveals that plasma membrane antigens can still be labeled after the cells are separated by papain digestion (2). The glycoantigen associated with SP96 and SP75 is initially diffusely distributed with no indication of layering. Secreted protein may be retained by the viscous properties of the GPS. Concomitant with the advent of cellulose deposition, SP75 and SP96 become closely associated with the electron-dense outer layer of the future spore coat. This time period after secretion but before assembly of the electron-dense layer represents the most likely interval for exchange of coat proteins between neighboring cells. The prediction that spore coat precursors are initially soluble after secretion is consistent with our earlier observation that these

molecules resist sedimentation at 100,000 g after being secreted from cells developing in suspension (18). This proposed water-soluble pool of precursors would be expected to be shared among neighboring cells since there are no extracellular boundaries evident ultrastructurally at this time (7). The solubility of these precursors is incompatible with the idea that patches of preassembled spore coats are exteriorized and connected together at the surface, and the continuous distribution of shared epitopes on spores (Fig. 9) rules out the possibility that mutant spores acquire protein as preformed patches.

The exchange of glycoproteins between normal and mutant cells is at least partly reciprocal, based on the increase of intensity of fluorescent labeling of normal spores formed with an excess of mutant spores (Fig. 6). Since mutant spores are unusually porous when probed with fluorescent lectins, we assume that it is the reciprocal incorporation of these underglycosylated isoforms by normal spore coats that renders them more porous and accessible to fluorescent antibody labeling. However, since at high mutant ratios, normal spores are more fluorescent than mutant spores, it must be concluded that the cell of origin preferentially retains some copies of the normal glycoprotein isoforms. For this reason we have considered that there may be two pools of these glycoproteins: a readily exchanged pool and a weakly exchanged pool. However, the Western blot data and the EM immunogold analysis argue against the postulated tightly associated pool being significant in size. These two postulated pools may merely reflect a gradient of isoform concentrations between adjacent cell surfaces.

After the spore coat is fully assembled, material not incorporated into the coat can be detected between spores after trapping by precipitation with cetylpyridinium chloride (7). Biochemical analysis of this interspore matrix by Western blotting confirms that several glycoproteins, including SP85 and SP96, are not quantitatively incorporated into the coat (Fig. 1). The detection of a water-soluble pool of glycoproteins between spores is consistent with the earlier existence of a soluble pool of precursors during the time of assembly of the electrondense layer, and may be its remnant.

Nucleation, Assembly of a Scaffold, and Intercalation

The non-exchangeability of SP86 (a class 1 glycoprotein; see Table I) suggests that it plays a special, perhaps primary role in spore coat assembly. SP86 resides in the presecretory prespore vesicle compartment together with the other glycoproteins of the coat (7). Since it is not shared between spores, it cannot belong to the soluble pool of other coat precursor glycoproteins after secretion, or it becomes insoluble before

Figure 9. EM immunolocalization of shared glycoproteins. Chimeras between the normal strain M28 (*round spores*) and glycosylation mutant strains HL250 (*a* and *b*) or HW20 (*c–f*) (*elongate spores*) were formed at a 2:1 (normal/mutant) ratio and allowed to sporulate as in Figs. 4 and 8. Spores were fixed and processed for immunogold labeling to localize carbohydrate antigens produced by M28 (but not by mutant) cells. The M28/HL250 chimera shown in *a* and *b* was labeled with mAb 83.5. All spores were labeled equivalently, demonstrating that glycoproteins produced by M28 cells and bearing this epitope, were shared with HL250 spores, and inserted properly into the outer layer of the spore coat. The M28/HW20 chimera shown in *c* and *d* was labeled with mAb 16.1, which monospecifically labels M28 SP85. All spores, including elongate (mutant) spores (*d*), were labeled equivalently, showing that SP85 was also shared, and properly assembled into the inner layer of the spore coat. The M28/HW20 chimera shown in *e* and *f* was labeled with mAb 5.1, which labels GA-XX associated with the normal SP86 glycoform produced by M28 cells. Since the elongate (mutant) spores (*f*), which cannot produce GA-XX, were not labeled with this antibody, SP86 was not transferred from normal to mutant spores.

it has time to diffuse. Thus SP86 is the best candidate among the studied spore coat molecules for initiating spore coat assembly. Its localization in the outer layer is consistent with the observation that this is the first layer visualizable during assembly. The distance between its final position in the outer layer and the plasma membrane may be explained by hydration of the GPS, which is concentrated in the inner layer, and growth of the cellulose layer, which begins at the time that the future outer layer is organized (19). A second class of glycoproteins (class 2) can be defined which resides in the early assembly pool (detected by trans-incorporation), but not in the post-assembly pool. All copies of these proteins, including SP70 and SP75, are assembled into the coat, and they may comprise a scaffold determining the full extent of the coat. A third class of glycoproteins (class 3) can be defined based on residence in both the preassembly and post-assembly pools. These glycoproteins, including SP85 and SP96, would appear to be intercalated into sites made available by the assembly of the scaffold, with excess copies accumulating in the late pool (interspore matrix). Based on strong binding between each copy of the less abundant SP75 species and SP96, we have previously suggested that SP75 is limiting for incorporation of SP96 into the spore coat (19). The recent cloning of several of the major spore coat protein genes (8, 9, 11, 17), and the fact that some assembly steps occur extracellularly, suggest that molecular mechanisms of assembly will be amenable to experimental manipulation.

Spores can also differentiate in small clusters or as solitary cells under certain conditions (14, 15). It is not known whether the spore coats of cells which have sporulated individually possess their normal complement of proteins. The answer may provide an independent measure of the size of the protein pool which is preferentially associated with the spore of origin, or indicate the ability of spore coat proteins to diffuse beyond a zone of high viscosity presumably conferred by the mucopolysaccharide (GPS) component of the coat.

Isolation of Mutants

The present study grew out of an effort to isolate fucose epitope revertants from HL250 by cell sorting. It was initially noted that when 1 or 10% wild-type cells were mixed with mutant cells and allowed to co-sporulate, the fluorescence of both mutant and normal spores increased (Fig. 6). The observation that incorporation of protein is not absolutely nonautonomous means that, in principle, it should still be possible to isolate many kinds of spore coat protein mutants from heterogeneous spore populations, and for natural selection to have a basis for optimizing spore coat properties in individual, rare spores of chimeric sori.

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