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Differential synthesis of spore coat proteins in prespore and prestalk cells of *Dictyostelium*

(pattern formation/fucosylation/phosphorylation/two-dimensional gel electrophoresis)

KEVIN M. DEVINE, JAMES H. MORRISSEY*, AND WILLIAM F. LOOMIS

Department of Biology, B-022, University of California at San Diego, La Jolla, California 92093

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ABSTRACT **The major spore coat proteins (SP60, SP70, and SP96) of *Dictyostelium discoideum* have been analyzed by using two-dimensional nonequilibrium pH gradient electrophoresis. These proteins have been characterized with respect to electrophoretic behavior, metachromatic staining with silver, and post-translational modifications; these techniques allow unambiguous identification of these proteins in silver-stained gels and autofluorograms of total cell extracts. They are synthesized by cells in the prespore region of migrating slugs, ultimately becoming major components of mature spores, but are not detectable either in cells in the prestalk region of slugs or in mature stalk cells. The ease with which these relatively abundant proteins can be recognized on two-dimensional gels makes them very useful markers for spatial and temporal differentiation in this organism.**

Starvation of *Dictyostelium discoideum* cells initiates a developmental program during which the amoebae aggregate in response to pulses of cAMP and form a slug, which, depending on environmental conditions, undergoes extended migration or immediate formation of a fruiting body. This latter structure is composed of a droplet of spores sitting atop a long tapering stalk. The stalk in turn is attached to a basal disk, which rests on the substratum and is composed of stalk-like cells (1, 2). Each spore is encapsulated in a spore coat, which is composed of four layers: an inner 100- to 300-Å-thick amorphous layer, which can be disrupted by treatment with cellulase followed by Pronase; two middle layers, which together are 700–900 Å thick and can be removed by cellulase; and a 200- to 300-Å-thick outer layer whose exact composition is unknown, but which is thought to contain mucopolysaccharide (2, 3, 4). This spore coat is shed upon germination of the spore and can be purified by a variety of methods (5, 6). Electrophoretic analysis of spore coat extracts revealed that they contained six proteins with apparent molecular weights ranging between 60,000 and 200,000 (5). In this study we have sought to characterize the spore coat proteins further and to evaluate their patterns of synthesis and accumulation in slugs and fruiting bodies. Our results indicate that (*i*) the spore coat proteins are highly specific markers for terminal differentiation, being found only in spore cells and not in stalk cells; (*ii*) they are found in cells of the prespore region but not in cells of the prestalk region of migrating slugs, demonstrating that, with respect to spore coat protein expression, slugs are patterned in a manner that prepares them for fruiting body formation; and (*iii*) synthesis of these prespore and spore-cell specific proteins begins between the tipped aggregate (14-hr) and standing slug (16-hr) stages of development.

MATERIALS AND METHODS

Cell Development. Strains HL100 and NC4 were grown on nutrient agar plates in association with *Klebsiella aerogenes* (7). Cells were freed of bacteria by five cycles of washing in distilled deionized water at 4°C and low-speed centrifugation. Synchronous development was achieved according to the method of Sussman (8).

Radiolabeling and Harvesting of Cells. At particular stages of development quadrants of filters were placed on the appropriate radiochemical for the designated period of time: (*i*) 15 μCi of L-[1-^{14}C]fucose (New England Nuclear, 40–55 mCi/mmol) in 30 μl of sterile 10 mM potassium phosphate buffer, pH 6.5 (KP_i); (*ii*) 50 μCi of L-[5,6-^{3}H]fucose (New England Nuclear, 56 Ci/mmol) in 50 μl of sterile water; (*iii*) 500 mCi of $H^{32}PO_4^{2-}$ (Amersham, carrier-free) in 63 μl of water; (*iv*) 200 μCi of L-[^{35}S]methionine (New England Nuclear, 1,226 Ci/mmol) in 25 μl of water (1 Ci = 3.7×10^{10} becquerels). Slugs were prepared by depositing washed cells in streaks across a plate of 2% non-nutrient Noble agar (Difco). The plates were maintained in a humid darkened box at 22°C and the slugs were allowed to migrate toward a window in the box for 40 hr. The slugs were radiolabeled by depositing 1 μCi of L-[^{35}S]methionine in 10 nl of KP_i on either the anterior fifth or the posterior four-fifths of a slug, using a brake pipette (9). Migration was allowed to proceed for a further 2 hr at which time the anterior and posterior regions were separately harvested by sucking them into a drawn-out capillary tube. Spores were harvested according to Orlowski and Loomis (5) and were washed three times with KP_i containing 0.1% Nonidet P-40 to lyse and remove unencapsulated amoebae before being processed for two-dimensional gel electrophoresis. To isolate stalks, cells of strain NC4 were allowed to form fruiting bodies on Millipore filters. After the sori were removed by suction, the stalks were collected with watchmakers' forceps and washed with KP_i until the number of adherent spores was estimated by microscopy to be less than 1% of the total number of cells. Spores and stalks were radiolabeled by placing developing cells at the Mexican hat stage of development onto 200 μCi of L-[^{35}S]methionine and allowing them to complete culmination. The sori and stalks were individually harvested with watchmakers' forceps. All cells were washed twice with KP_i prior to being processed for two-dimensional gel electrophoresis.

Purification of Spore Coats. Spore coats were prepared by the method of Lam and Siu (6) and processed for two-dimensional gel electrophoresis.

Preparation of Samples for Two-Dimensional Gel Electrophoresis. Samples were prepared for electrophoresis by boiling in 0.5% $NaDodSO_4$ in 20 mM Tris·HCl, pH 7.4, for 10 min. 2-Mercaptoethanol was added to a final concentration of 1% and the samples were reboiled for 5 min. One-tenth volume of DNase I/RNase A solution (containing DNase I at 1 mg/ml, RNase A at 0.5 mg/ml, and 50 mM $MgCl_2$ in 0.5 M Tris·HCl,

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Abbreviation: NEPHGE, nonequilibrium pH gradient electrophoresis.

* Present address: Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU England.

pH 7.0) was added and the resultant solutions were incubated at 20°C for 10 min. Nonidet P-40, 2-mercaptoethanol, pH 5–7 ampholytes, and urea were added to final concentrations of 4%, 5%, 2%, and 10 M respectively, and samples were stored at −70°C.

Two-Dimensional Nonequilibrium pH Gradient Electrophoresis. Two-dimensional nonequilibrium pH gradient electrophoresis (NEPHGE) (10) was adapted to separate acidic and neutral proteins by (*i*) loading the samples at the basic end of the gel and (*ii*) electrophoresing the samples towards the anode. Electrophoresis in the first dimension was performed essentially by the method of Garrels and Gibson (11) with the following modifications: (*i*) gels were electrophoresed in 14-cm-long capillary tubing (inside diameter 1 mm) for 3,000 V·hr; (*ii*) the gels contained 2% ampholytes (LK_i) consisting of 10% pH 2.5–4, 5% pH 4–6, 20% pH 5–7, 30% pH 6–8, and 35% pH 3.5–10 (designated mix A ampholytes). The second-dimension $NaDodSO_4$ gels were 10% polyacrylamide.

Gel Staining. Silver staining of gels was performed according to the method of Morrissey (12). Coomassie blue staining of gels was performed by soaking the gels for several hours in 0.2% Coomassie blue R-250/45% methanol/10% acetic acid (vol/vol) and destaining in several changes of 10% methanol/7% acetic acid (vol/vol).

Fluorography. Silver-stained gels were treated with EN^3HANCE (New England Nuclear) according to the manufacturer's instructions, dried, and exposed to Kodak prefogged X-Omat AR x-ray film at −70°C. Intensifying screens were used for ^{32}P autofluorography (13).

Peptide Mapping. Peptide mapping was performed according to the method of Cleveland *et al.* (14).

RESULTS

Identification of the Spore Coat Proteins on Two-Dimensional Gels. The major spore coat proteins of *D. discoideum* have previously been identified by one-dimensional gel electrophoresis of extracts from purified spore coats (5). With whole cell lysates, the spore coat proteins are not abundant enough to be unequivocally identified against the complex background of other cellular proteins of similar size. Unequivocal identification of the spore coat proteins is a prerequisite for analysis of their synthesis and accumulation during development. Thus, two-dimensional gel electrophoresis, because of its very high resolution of complex protein mixtures, was used throughout this study. The spore coat proteins were readily identified on two-dimensional gels by comparing the protein patterns of spore coat extracts with those obtained from identical samples run on one-dimensional gels (Fig. 1). It can be seen that the major spore coat proteins of the one-dimensional Coomassie blue-stained profile and the two-dimensional silver-stained profile correspond with SP96, SP70, and SP60 described on one-dimensional gels by Orlowski and Loomis (5). Minor spore coat proteins SP200, SP94, and SP68 could not be reliably identified on two-dimensional gel patterns. In order to stain SP96 with silver, it is necessary to use the silver stain method of Morrissey (12), because other silver stain methods (15, 16) stain this protein faintly or not at all. In addition to having greater sensitivity, the procedure of Morrissey gives metachromatic staining of the major spore coat proteins. The bulk cellular proteins stain dark brown, whereas SP60 stains yellow-orange, SP70 blue-gray, and SP96 reddish-brown. This greatly facilitates the localization of these proteins in two-dimensional electrophoretograms of total cellular protein extracts.

SP96 was found to be a very acidic protein which migrates to the acidic extremity of isoelectric focusing gels (and in some cases out of the gel altogether). To reproducibly identify this protein, it is necessary to use NEPHGE (10) modified to separate acidic proteins. SP96 also displays anomalous migration behavior during two-dimensional NEPHGE, streaking through the first dimension. The extent of the streaking varies among different gels. This unusual characteristic can aid in the identification of this protein, but its cause is not known.

SP60 and SP70 appear as diffuse spots when either isoelectric focusing or NEPHGE is used in the first dimension, a phenomenon of unknown significance, but observed in over 100 samples of developing cells, spores, and spore coats.

Cell Type Specificity of the Spore Coat Proteins. Because spore coats are associated only with spores, it was decided to examine the spatial distribution of the spore coat proteins in fruiting bodies to see if they might be useful as markers for terminal differentiation. Spores and stalks were prepared as previously described and analyzed by two-dimensional gel electrophoresis. The spore coat proteins were identified in two-dimensional gel electrophoretograms of total cell extracts by their metachromaticity, characteristic migratory behavior, and

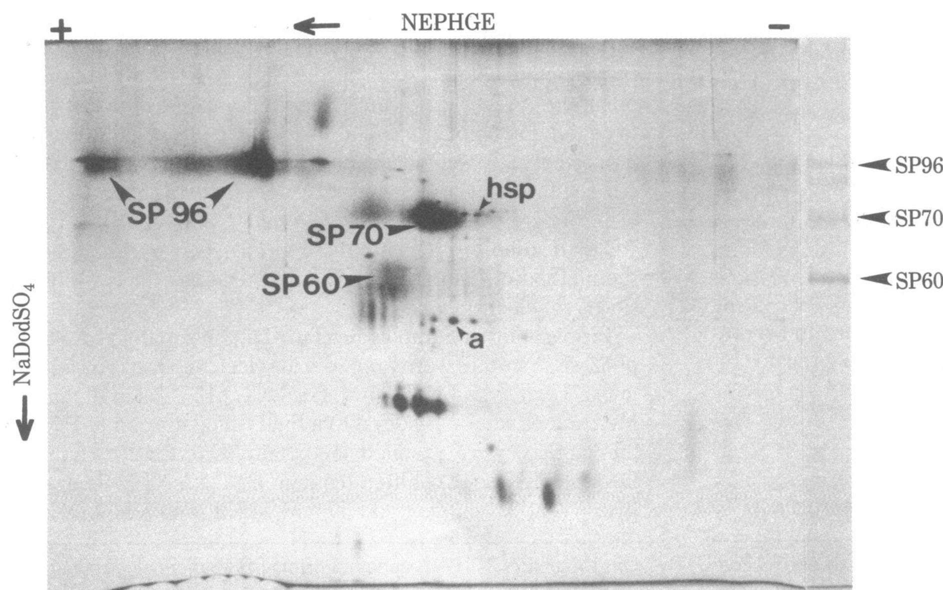

FIG. 1. Identification of the major spore coat proteins on two-dimensional gels. Spore coat extracts were subjected to NEPHGE for 3,000 V·hr in the first dimension, with mix A ampholytes and electrophoresis in the second dimension on 10% acrylamide/$NaDodSO_4$ gels. The silver-stained two-dimensional electrophoretogram is compared with a one-dimensional $NaDodSO_4$ gel protein profile of an identical extract stained with Coomassie blue (right). The gels were loaded with 4×10^6 and 2.5×10^6 spore coats for the Coomassie blue and two-dimensional gel silver staining, respectively. a and hsp indicate the positions of actin and the M_r 70,000 heat shock proteins, respectively (20). RNase and DNase added during sample preparation are the major spots below actin.

position in gels with respect to actin and the heat shock proteins. Identification of these proteins in autofluorograms was achieved by direct superimposition onto the silver-stained gel. The results (Fig. 2) demonstrate very high levels of all three major spore coat proteins in spore cells. SP60, SP70, and SP96 were not detected in stalk cells. The autofluorogram (Fig. 2*B*) and silver-stained gel (Fig. 2*D*) are from different experiments and demonstrate the variation in streaking behavior of SP96.

It has been demonstrated that cells in the anterior fifth of the slug (prestalk region) ultimately form stalk cells and cells in the posterior four-fifths of the slug (prespore region) form spore cells (17). Thus it was decided to see if the major spore coat proteins are likewise patterned. The results (Fig. 3) demonstrate that all three major spore coat proteins are synthesized by cells in the prespore region of the slug but are not synthesized by cells in the prestalk zone.

Partial proteolytic cleavage of the protein appearing as a diffuse spot of apparent molecular weight 96,000 (labeled x in Fig. 3) with *Staphylococcus aureus* V8 protease revealed a peptide profile differing from that of SP96 (data not shown). Analysis of spore coat proteins in migrating slugs by silver staining of two-dimensional gels (Fig. 3) demonstrated that the spore coat proteins accumulated to detectable levels in the prespore zone but not in the prestalk zone. SP96 was observed in every experiment, whereas SP60 and SP70 could be unequivocally identified in only half of the experiments. Perhaps the levels of SP60 and SP70 vary in migrating slugs such that in some experiments these levels are below the sensitivity of silver staining.

Spore Coat Proteins During Development. In order to determine the time of synthesis of the spore coat proteins, cells were labeled with [^{35}S]methionine for 2-hr intervals at successive stages of development and extracts were analyzed by two-dimensional gel electrophoresis. The results (Table 1) indicated that synthesis of SP60, SP70, and SP96 was first detected during the 14- to 16-hr pulse labeling period (tipped aggregate to standing slug stage), increased to a maximal level during the 18- to 20-hr pulse labeling period (early culmination), and decreased as culmination was completed (22- to 24-hr pulse labeling period). The proteins SP60 and SP96 could first be detected by silver staining at the early culmination stage of development (20 hr), whereas SP70 was not detected until the 22-hr stage (midculmination). All three spore coat proteins were visible throughout the remaining periods of development.

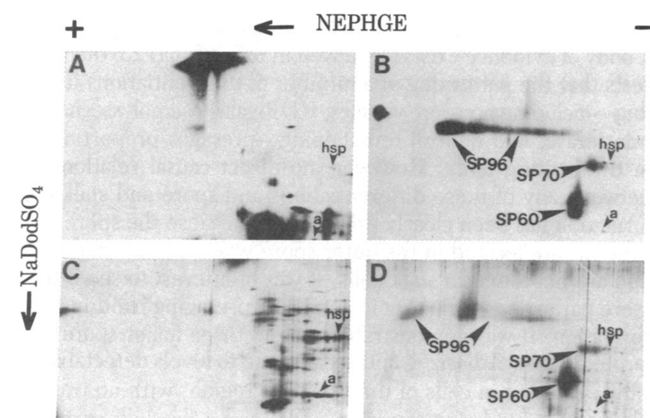

FIG. 2. Spatial distribution of spore coat proteins in fruiting bodies. Conditions for electrophoresis were as described in Fig. 1. Stalk (*A*) and spore (*B*) cell extracts were radiolabeled by placing cells onto 200 μCi of [^{35}S]methionine at the Mexican hat stage of development and allowing them to culminate (18–24 hr). The gels were autofluorographed for equivalent periods of time. Separate stalk (*C*) and spore (*D*) extracts were similarly electrophoresed and stained with silver. The areas shown correspond to the high molecular weight acidic quadrant of the gel shown in Fig. 1. See the positions of actin (a) and the heat shock proteins (hsp) for reference points.

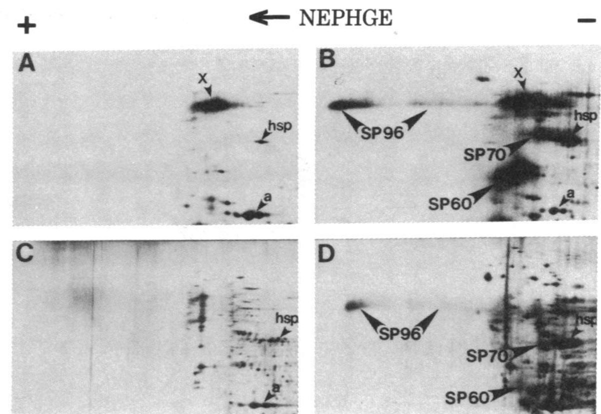

FIG. 3. Spatial distribution of spore coat proteins in migrating slugs. Conditions for electrophoresis were as described in Fig. 1. Cells from the prestalk (*A*) and prespore (*B*) regions were radiolabeled in slugs (40–44 hr). The gels were autofluorographed for equivalent periods of time: prestalk (*C*) and prespore region (*D*) cells were similarly electrophoresed and stained with silver. a, Actin; hsp, M_r 70,000 heat shock proteins. The areas shown correspond to the high molecular weight acidic quadrant of the gel.

Further Characterization of the Major Spore Coat Proteins. Modification of the spore coat proteins by fucosylation and phosphorylation was examined to further characterize these proteins so that they might be unambiguously identified and also to tie in with previously published studies on phosphoproteins (18) and fucoproteins (6, 19) in *Dictyostelium*. HL100 cells were labeled with [^{14}C]fucose between the Mexican hat (18-hr) and early culmination (20-hr) stages of development. The results (Fig. 4*A*) indicate that SP96 and SP70 are the major glycoproteins labeled under such conditions. Similar analysis (Fig. 4*B*) of HL100 cells pulse labeled with $H^{32}PO_4^{2-}$ between the Mexican hat (18-hr) and early culmination (20-hr) stages of development revealed that SP96 was the major phosphorylated protein, whereas SP60 and SP70 were unlabeled. In order to determine the time of modification of the spore coat proteins, cells were separately labeled with [^{3}H]fucose and $H^{32}PO_4^{2-}$ for 2-hr intervals at successive stages of development and the cell extracts were analyzed by two-dimensional gel electrophoresis

Table 1. Expression of the spore coat proteins during development

Time interval, hr	Synthesis			Detection by silver staining		
	SP60	SP70	SP96	SP60	SP70	SP96
8–10	−	−	−	−	−	−
10–12	−	−	−	−	−	−
12–14	−	−	−	−	−	−
14–16	+	+	+	−	−	−
16–18	+	+	+	−	−	−
18–20	++	++	++	+	−	+
20–22	+	+	+	++	++	++
22–24	+	+	+	++	++	++

HL100 cells were radiolabeled by placing cells onto 200 μCi of [^{35}S]methionine at successive stages of development for 2-hr periods. Cell extracts were analyzed by two-dimensional gel electrophoresis under the conditions described for Fig. 1. Approximately 3×10^6 cell equivalents of protein was loaded onto each gel. The gels were silver stained (12) and then autofluorographed for equivalent periods of time. −, Not detectable; +, detectable; ++, maximal levels.

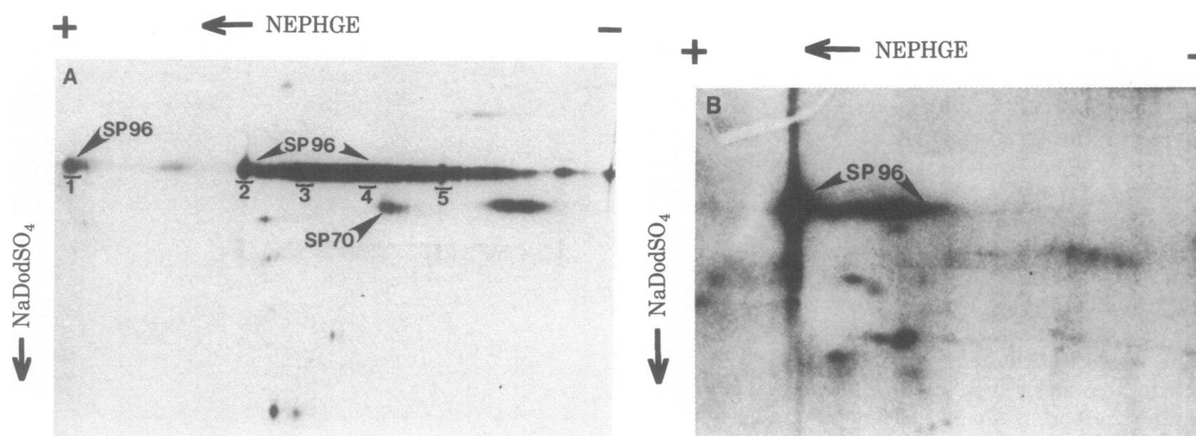

FIG. 4. Two-dimensional gel electrophoresis of extracts of fruiting bodies radiolabeled with [^{14}C]fucose (*A*) and $H^{32}PO_4^{2-}$ (*B*). Cells were labeled between the Mexican hat and early culmination stages of development.

followed by autofluorography. Results indicated that incorporation of radiolabeled phosphate and fucose into SP96 and radiolabeled fucose into SP70 began coincidently with the onset of synthesis of the polypeptide portion of these spore coat proteins and could be detected at all subsequent stages of development.

One unusual feature of SP96 is that when analyzed by NEPHGE followed by $NaDodSO_4$/polyacrylamide gel electrophoresis it streaks horizontally across the gel. This is especially evident when SP96 is radiolabeled and could result from heterogeneity of the protein. To examine the nature of the glycoprotein at different positions along this streak, one-dimensional peptide analysis after limited proteolysis using V8 protease was performed on gel slices taken from the positions of the fucosylated SP96 streak as indicated in Fig. 4A. The resultant profiles (Fig. 5) revealed that these samples had identical fucopeptide profiles.

DISCUSSION

In this study, the major spore coat proteins of *D. discoideum* were characterized by examining their spatial and temporal patterns of synthesis during development. It was demonstrated that although SP60, SP70, and SP96 are present in mature spore cells, they were not detectable by fluorography or silver staining of two-dimensional gels of stalk cell extracts, indicating that these proteins are specific molecular markers for terminal differentiation. Their usefulness is enhanced by a number of characteristics that allow their unambiguous identification in two-dimensional gel analysis of complex protein mixtures: (*i*) they exhibit anomalous electrophoretic behavior, SP60 and SP70 migrating as diffuse spots and SP96 migrating as a streak (on NEPHGE); (*ii*) they stain metachromatically with silver, SP96 appearing reddish brown, SP70 blue-gray, and SP60 yellow-orange; (*iii*) their position on two-dimensional gels is readily determined, relative to that of the well-known markers actin and the heat shock protein complex (20); (*iv*) SP96 and SP70 are fucosylated; (*v*) SP96 is a very acidic protein that separates well from other cellular proteins; (*vi*) SP96 is phosphorylated. These points are important because it has been found difficult to precisely reproduce two-dimensional gel patterns from different laboratories. The spore coat proteins are detectable on two-dimensional gels by autofluorography and by silver staining, an important feature because it allows unambiguous identification of these proteins by superimposition of the autofluorograph onto the dried silver-stained gel.

Bonner and Slifkin (17) and Sampson (21) have demonstrated a predisposition of cells from the anterior fifth and posterior four-fifths of the slug to form stalk cells and spores, respectively. A body of evidence exists (reviewed in refs. 22 and 23) that suggests that the patterning of a number of differentiations in the slug—including prespore vesicles, UDP-galactose:polysaccharide transferase, and neutral red staining—presages proportioning in the fruiting body. However, no direct causal relationship between any of these differentiations and spore and stalk cell formation has been clearly established. Because the spore coat proteins are located in the outer spore wall (unpublished data) and are not found in stalk cells, it was of interest to see if they were patterned in the slug in a manner presaging fruiting body formation. It was demonstrated that all three major spore coat proteins are synthesized and accumulate to levels detectable by silver staining in cells of the prespore region, with no trace of these proteins being found in cells in the prestalk region of migrating slugs. This then is a clear demonstration of a differentiation exclusive to cells of the prespore region that appears to participate directly in spore formation.

Several other two-dimensional analyses of the synthesis of proteins during development of *Dictyostelium* have been reported (24, 25, 26, 27). Only in the study of Morrissey *et al.* (24) were we able to recognize the major spore coat proteins, their spots 12 and 13 being SP70 and SP60, respectively. The absence of these proteins in the other studies may be a con-

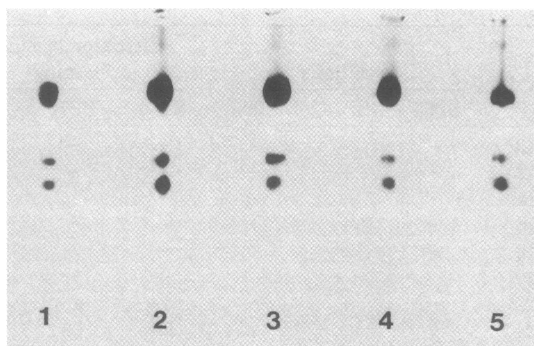

FIG. 5. Analysis of fucosylated SP96 by partial peptide hydrolysis. Slices of gel taken from the positions of the fucosylated SP96 streak as indicated in Fig. 4*A* were partially hydrolyzed by using 1.2 μg of V8 protease per well as described by Cleveland *et al.* (14). The cleavage products were separated by $NaDodSO_4$/12.5% polyacrylamide gel electrophoresis. The gel was subsequently autofluorographed.

sequence of the difficulty in solubilizing them by techniques other than those used in this study. The solubilization conditions used in this study, particularly boiling in $NaDodSO_4$ and reducing agent, are essential for optimal extraction of spore coat proteins from spores.

Synthesis of the major spore coat proteins begins between the tipped aggregate and standing slug stages of development (14- to 16-hr labeling period). However, they do not accumulate to the level of stainability with silver until the early culmination stage of development. This is in contrast to other proteins specific to cells of the prespore region of migrating slugs that accumulate to levels detectable by silver staining in the pulse labeling period in which synthesis begins (unpublished data). Explanation for the lag period in the detection of the spore coat proteins by silver staining would include (*i*) that the spore coat proteins must accumulate to higher levels than these other proteins before they can be detected by silver staining; (*ii*) that synthesis of the spore coat proteins is initiated but somehow maintained at a low level in migrating slugs with the rate of synthesis increasing during early culmination; (*iii*) that rapid turnover of the spore coat proteins occurs in migrating slugs, this process being terminated at early culmination, allowing them to accumulate; or (*iv*) they could be secreted by the cells. Coloma and Lodish (26) reported that the synthesis of the majority of spore- and stalk-specific proteins commenced at culmination (19–26 hr). In contrast, we have demonstrated that the major spore coat proteins (which are spore specific) are synthesized beginning at the tipped aggregate to standing slug stages of development (14–16 hr). A global study of spore and stalk as well as prespore- and prestalk-specific proteins during development should further resolve these points (unpublished data).

In this study, we have demonstrated that the spore coat proteins SP96 and SP70 are fucosylated. The patterning of these fucosylated proteins is consistent with observations made by Gregg and Karp (9, 19) that in whole slug autoradiography prespore cells are more heavily labeled with $[^3H]$fucose than are prestalk cells and that radioactive fucose incorporated into spores is associated predominantly with the spore wall. In addition, Lam and Siu (6), have reported that some proteins, which are fucosylated during early culmination, comigrate on one-dimensional gels with proteins extracted from radioiodinated spore coats. Although it is very difficult to equate bands on one-dimensional gels with two-dimensional protein profiles, it is possible that their gp103 is SP96 because it coincides with a very faint band in the Coomassie blue-stained profile, behavior that is consistent with our observations that SP96 stains very poorly with Coomassie blue and is very easy to over-destain.

We have also demonstrated that SP96 is highly phosphorylated, a modification that no doubt contributes to the very acidic nature of this protein. This is the major phosphoprotein synthesized during each 2-hr labeling period from the beginning of the period of SP96 synthesis (14–16 hr) until the completion of culmination (24 hr). Coffman *et al.* (18) reported synthesis of phosphoproteins (with molecular weights in the 90,000 region) beginning at 14 hr of development. However, their analysis utilized one-dimensional gels, making correlation with our study difficult.

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