Multiple O-Glycoforms on the Spore Coat Protein SP96 in Dictyostelium discoideum

Fuc(α1–3)GlcNAc-α-P-Ser IS THE MAJOR MODIFICATION*

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A decreased level of fucosylation on certain spore coat proteins of Dictyostelium discoideum alters the permeability of the spore coat. Here the post-translational modifications of a major spore coat protein, SP96, are studied in a wild type strain (X22) and a fucosylation-defective mutant (HU2470). A novel phosphoglycan structure on SP96 of the wild type strain, consisting of Fuc(α1–3)GlcNAc-α1-P-Ser, was identified by electrospray ionization mass spectrometry and NMR. It was shown using monosaccharide and gas chromatography mass spectrometry analysis that SP96 in the mutant HU2470 contained approximately 20% of wild type levels of fucose, as a result of a missing terminal fucose on the novel glycan structure. The results support previous predictions, based on inhibition studies on different fucose-deficient strains, about the nature of monoclonal antibody epitopes identified by monoclonal antibodies MUD62 and MUD166, which are known to identify O-linked glycans (Champion, A., Griffiths, K., Gooley, A. A., Gonzalez, B. Y., Gritzali, M., West, C. M., and Williams, K. L. (1995) Microbiology 141, 785–797). Quantitative studies on wild type SP96 indicated that there were approximately 60 sites with phosphodiester-linked N-acetylglucosamine-fucose disaccharide units and a further approximately 20 sites with fucose directly linked to the protein. Over 70% of the serine sites are modified, with less than 1% of these sites as phosphoserine. Threonine and tyrosine residues were not found to be modified.

Protein glycosylation is being increasingly recognized as an important protein modification with diverse roles (1). N-Linked glycosylation has long been known, and the fundamentals of the synthetic steps leading to the transfer of a dolichol phosphate GlcNAc-Man-Glc complex to an asparagine residue in the synthetic steps leading to the transfer of a dolichol phosphate GlcNAc-Man-Glc complex to an asparagine residue in the transferase B complex (2). The peptide motif Asn-Xaa-Ser/Thr/(Cys) of eukaryote cell surface and secreted proteins is well known (3). Indeed, there is at least 2% of antigenic distinct classes identified by monoclonal antibodies MUD50 (PsB or SP85) and MUD62 (SP75 and SP96). While the glycosylation structure identified by mAb1 MUD50 is becoming well understood (12, 13) as being a fucose-decorated N-acetylglucosamine O-linked to threonine residues in a proline/threonine-rich domain (14), less is known about the epitope recognized by mAb MUD62 (15).

Some information about the epitope can be obtained by competition studies with different sugars or by screening and analyzing mutants that lack a particular sugar (15, 16). Three genes have been defined in relation to fucosylation in D. discoideum.

1 The abbreviations used are: Mab, monoclonal antibody; COSY, correlated spectroscopy; PACE, polyacrylamide gel electrophoresis; ESI, electrospray ionization; MS, mass spectrometry; TOF, time of flight detection; GC, gas chromatography; HPAEC-PAD, high performance anion exchange chromatography with pulsed amperometric detection; HSQC, heteronuclear single quantum coherence; ROESY, rotating frame Overhauser enhancement spectroscopy; SP, spore coat protein; TOCSY, total correlation spectroscopy; HPLC, high pressure liquid chromatography; DIPSI, composite-pulse decoupling in the presence of scalar interactions; GARP, globally optimized alternating phase rectangular pulse; TPPI, time-proportional phase incrementation.

2 N. E. Zachara, D. R. Jardine, N. H. Packer, and A. A. Gooley, manuscript in preparation.
O-Glycosylation and Phosphoglycosylation in Dictyostelium discoideum (modC, modD, and modE) (16). These mutants lack the MUD 62 (GA-X) epitope, which is competed by L-fucose. Strains carrying a modC or a modE mutation lack another epitope, GA-XII, which is recognized by mAbs MUD3 and MUD141. Lack of fucosylation is sufficient to affect the spore coat permeability, leading to premature loss of viability (17). Strains carrying these mutations expose a new carbohydrate epitope, competed by N-acetylglucosamine and recognized by mAb MUD166 (16).

The genes encoding major spore coat proteins SP96 and SP75 have been sequenced, and the predicted sequence shows extensive, mucin-like serine- and threonine-rich regions that may be sites for phosphorylation or O-glycosylation (18–20). Both of these glycoproteins carry the glycosylation epitope identified by mAb MUD62. The modifications on the different spore coat proteins can partly be characterized by radiolabeling experiments with phosphate, sulfate, or different tritiated sugars. It has been shown that SP96 and SP75 are phosphorylated, and these proteins also label with [3H]fucose and [3H]GlcNH2 (21, 22).

While these results gave information about the composition of the post-translational modifications, they do not provide the exact structure of the attached glycoforms. Here we elucidate the glycosylation structures found on spore coat protein SP96 in a wild type and a fucosylation-defective strain and quantify the differences in the glycosylation pattern. Having determined several of the glycoconjugates found in mutant and wild type SP96, we correlate antigenicity, based on several mAbs, with sugars as part of their recognition domains. Three different glycoforms are found on wild type SP96, and a further truncated glycoform is found on the fucosylation-defective mutant.

**EXPERIMENTAL PROCEDURES**

Materials—Horseradish peroxidase-conjugated sheep anti-mouse antibody was from Silenus Laboratories (Melbourne). Nitrocellulose (pore size 0.2 μm) was from Schleicher & Schuell, and prestained molecular mass markers were purchased from NOVEX. The Immun-Blot® molecular mass markers were purchased from NOVEX. The Immun-Blot® Kit for glycoprotein detection was obtained from Bio-Rad, and CNBr-activated Sepharose was from Amersham Pharmacia Biotech. Both steps were carried out essentially as described in the protocols of the companies. After a 2-h incubation at 20 °C on the MUD3 affinity column, SP96 was eluted with 100 mM diethylamine (pH 11) because the SP96 was eluted on the basis of high pH. After it was neutralized with 1 M Tris (pH 8), it could be bound immediately again on the column.

Approximately 1.2–1.6 nmol of SP96 was extracted from 5 × 109 spores of each strain. **Phosphoamino Acid Analysis—**Affinity-purified SP96 of strains X22 and HU2470 (20–60 pmol) was gas phase-hydrolyzed with 6 M HCl for 4 h at 110 °C and analyzed for the presence of phosphoamino acids (25).

**Monoasaccharide Determination—**To release the monosaccharides, 25 pmol of affinity-purified SP96 from each strain was hydrolyzed with 2 m trifluoroacetic acid for 4 h at 100 °C. To quantitate the amino sugars, the hydrolysates were separated as above.

Electrospray Ionization Mass Spectrometry—Glycans released by β-elimination of X22-SP96 (300 pmol) were desalted through a Hypercarb® (Shandon Scientific, Runcorn, United Kingdom) guard column (26). After washing with MilliQ water to remove the salt, the carbohydrates were eluted from the column into the mass spectrometer using a 15-min linear gradient of 0.05% (v/v) trifluoroacetic acid to 90% (v/v) acetonitrile, 0.05% (v/v) trifluoroacetic acid at a flow rate of 10 μl/min. Spectra were collected in negative ion mode on an ESI-TOF (LCT; Micromass, Manchester, UK). Samples were introduced into the electrospray source through a capillary held at a potential of 3.5 kV. The sample cone voltage was 50 V, or 50 V for in-source collision-induced dissociation. Full scan spectra were acquired over a mass range of 70–1200 Da with 0.7-s scan duration and 0.7-s interscan delay. All spectra were externally calibrated with NaI.

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**Nuclear Magnetic Resonance Spectroscopy—**The NMR sample was prepared from the HPLC-purified disaccharide (∼100 μg) by dissolving it in D2O (99.96 atom % D, Aldrich) after repeated lyophilization from D2O and filtered into an NMR tube (PP272; Wilmad). The sample was degassed and equilibrated under an atmosphere of nitrogen.

The NMR data were acquired on a Bruker DRX600 (600 MHz) NMR spectrometer at 27 °C and processed using xwinNMR (version 2.6). All two-dimensional NMR experiments were run with quadrature detection with an H spectral width of 6000 Hz and a recycle delay (dipolar) of 1 s. Chemical shifts were referenced to the fucose methyl (δH 1.166 ppm; δC 62.16 ppm). High power 1H-1H pulses were determined to be 9.5 μs, and low power for (DIPSI spin lock) were at 25.15 μs. DIPSI sequences were flanked with trim pulses at the same low power of 2-m duration. 1H high power 2H pulse was 10.5 μs, and a low power pulse of 65 μs was used for GARP decoupling. Gradient pulses were delivered along the z axis using a 100-step sine program.

Data for one-dimensional experiments were acquired using a WATERRAGE 3-9-19 pulse sequence (27) enhanced with gradient selection (28). All pulses were delivered at the same power of 0 decibels, and gradients were at a ratio of 50:50 of full power. Two dummy scans were used before the acquisition of 128 scans, using a recycle delay of 2 s. 32,768 real points were acquired and zero-filled to 64,000 and then Gaussian multiplied for resolution enhancement. Carbon-hydrogen correlation (HSQC) was achieved via a sensitivity-enhanced double INEPT transfer using echo/antiecho-TIPPI gradient (80:20.1) selection (29–31).

Data for one-dimensional ROESY spectra were measured using a selective Gaussian pulse on the anomeric protons. A 1000-s Gaussian program (60 ms, 64.6 decibels) was used to achieve a 2H/H pulse. A mixing time of 250 ms (13 decibels) was used for a continuous wave spin lock. Gradient selection was achieved with a 15% gradient along the z axis. 10,000 transients were accumulated over 6008 Hz, rotating frame Over-
O-Glycosylation and Phosphoglycosylation in Dictyostelium

Two-dimensional homonuclear Hartman-Hahn transfer spectra (TOCSY) were measured using the MLEV17 (32) pulse sequence flanked with 2-ms low power trim pulses. Water suppression was achieved using a gradient-assisted WATERGATE (27, 28) pulse sequence. A gradient ratio of 50:50 was used in the WATERGATE sequence. A mixing time of 30 ms was used for the Hartman-Hahn transfer. Sine bell-shifted (90°) apodization was used in the processing of both dimensions. Homonuclear shift correlation (COSY) with double quantum filter phase sensitive using the States-TIPPI method was supplemented with a gradient-assisted WATERGATE sequence (27, 28) to achieve water suppression. A gradient ratio of 30:30 was used in the WATERGATE sequence.

Determination of Reducing Terminal Linkages of SP96—To determine the reducing terminal linkages of the glycans on SP96, sugar residues were subjected to reductive β-elimination and analyzed by gas chromatography/mass spectrometry (GC-MS) after acetylation. SP96, from strains X22 and HU2470 (both strains 0.2–0.5 nmol), was dissolved in 400 μl of 100 mM NaOH, 1 μl sodium borohydride and incubated at 45 °C for 18 h. The samples were carefully neutralized with 4 M acetic acid and desalted by anion exchange chromatography (Bio-Rad AG 50W-X8, 200–400 mesh). Dried samples were acetylated with excess anhydrous acetic anhydride (50 μl) and anhydrous pyridine (50 μl) at 100 °C for 1 h. The acetylated alditols were extracted into dichloromethane and analyzed on a Fisons MD800 GC-MS using a BPX5 (0.22 mm × 25 m) column. Samples (1 μl) were analyzed by injection onto the column at 150 °C. The temperature was held for 5 min and then increased to 300 °C over 30 min and held for 10 min.

Dephosphorylation with HF—After reductive β-elimination and desalting, the samples were lyophilized, cooled, and mixed with precooled 40% (w/v) HF (50 μl). After 48 h at 0 °C, the HF was neutralized with LiOH in equimolar concentration, the LiF was pelleted, and the supernatant and the supernatant of a wash with 200 μl of water were combined and used for further analysis.

Total Phosphate Analysis after Acid Hydrolysis—The degree of phosophorylation of SP96 was determined by measuring the amount of inorganic phosphate released by strong acid hydrolysis using the method of Lanzetta et al. (33), which was modified for microplate analysis. 20-μl protein samples (20–100 pmol) of SP96 were hydrolyzed with 4 μl of 10 M H2SO4 at 170 °C in a sealed tube for 4 h. The samples were transferred into a 96-well microplate, and 100 μl of phosphate reagent was added and carefully mixed. After 1 min, the reaction was stopped by adding 10 μl of a 25% (w/v) trisodium citrate solution, and after a 20-min incubation, the absorbance was measured at 630 nm. A potassium dihydrogenphosphate standard curve (0–4.0 nmol) was used to calculate the concentration of inorganic phosphate in the samples. To determine the background phosphate in the samples, nonhydrolyzed SP96 protein samples were also assayed.

Detection of Inorganic Phosphate after Treatment with Alkaline Phosphatase—To determine the enzymic release of phosphate, 30 μl of 100 mM ammonium bicarbonate (pH 8.3), 10–20 μl of SP96 sample (100–200 pmol), and 10 μl (15 milliunits) of alkaline phosphatase were incubated for 16 h at 37 °C. The reaction was stopped with 10 μl of 4 M HCl before the released phosphate was detected as before.

Miscellaneous Methods—Protein concentrations were determined by amino acid analysis (34). SDS-PAGE was performed according to Laemmli et al. (35) and Western blotting according to Khayse-Anderson et al. (36). Glycoproteins electroblotted to nitrocellulose membrane were detected using the Bio-Rad Immun-Blot Kit as described by the manufacturer.

RESULTS

The difference in the post-translational modification of the spore coat protein SP96 in the wild type (X22) and a fusosity-defective mutant (HU2470) of D. discoideum was determined by chemical and mass spectroscopic methods on mAb MUD3 affinity-purified protein extracted in hot 8M urea from spores of both strains.

Purification of SP96 from X22 and HU2470—The mAb MUD3 (23) recognizes only SP96 in both strains, which makes it a perfect tool for monitoring the purification process. After washing the spores (4 × 10^12) with 50 mM Tris, 8 μl urea, SP96 was extracted with hot 50 mM Tris, 8 μl urea. The extract was approximately 10-fold partially purified on a DEAE-anion exchange column as judged by the protein content of the single fraction that reacted with the MUD3 antibody. The MUD3-positive fraction eluted by 250 mM NaCl contained 22 mg of protein derived from 200 mg of crude extract.

SP96 was finally purified by affinity chromatography on a MUD3 monoclonal antibody column. The eluted antibody positive fractions were repurified by a second chromatography step on the same column. After the second affinity purification, silver stain visualized SP96 as the major band at an apparent mass of approximately 110 kDa in the X22 sample (Fig. 1A, 1) with only one very minor contaminating bands at around 60 kDa. In the HU2470 sample (Fig. 1A, 2), SP96 was visible at a slightly lower apparent molecular mass (~100 kDa), with two minor protein contaminating bands at 85 and 60 kDa visible. The difference of about 10 kDa between the X22-SP96 protein and HU2470-SP96 is the result of the altered migration due to decreased fusocylation of the mutant protein. Specific staining for glycoproteins (Fig. 1, B1 and B2) visualized only the SP96 protein band in each sample, and the Western blot with MUD3 displayed the same result (Fig. 1, C1 and C2). Based on these results, the degree of purity of SP96 in both samples was approximately 95–97%. Amino acid analysis of the protein preparations showed a yield from 5 × 10^8 spores of 1.2 nmol of protein from the X22 wild type and 1.6 nmol of protein from the mutant HU2470. This is equivalent to approximately 10^8 molecules of SP96 recovered in the hot urea extraction of each mutant and wild type spore.

Phosphoamino Acid Analysis—In the early 1980s, it was shown by 32P radiolabeling experiments that some spore coat proteins of D. discoideum were phosphorylated. A major spore coat phosphoprotein was identified by several groups as a 103-kDa (37), 95-kDa (38) and 96-kDa (39) spore coat protein. These proteins correspond to the protein now named SP96. By comparing the radiolabeled, hydrolyzed protein with standard phosphoamino acids using thin layer chromatography, serine was identified as the only phosphorylated amino acid (21).

We confirmed that serine was the only phosphorylated amino acid in SP96 from both strains using the more sensitive Fmoc phosphoamino acid analysis method (25). The chromatogram of the acid-hydrolyzed protein from both strains (approximately 20 pmol) showed the presence of serine phosphate with no evidence of threonine phosphate or tyrosine phosphate. To confirm that serine is the only amino acid with an O-linked modification, SP96 protein samples of each strain were analyzed by amino acid analysis pre- and post-β-elimination. By
**Analysis of the Glycan Linkage to the Protein**—To analyze the linkage of the sugars to the protein, the sugars were β-eliminated, reduced, and peracetylated for analysis by GC-MS. After β-elimination, a single reducing terminal sugar, which is directly attached to the protein, is detected as an acetylated alditol, an open chain sugar form. Glycans larger than monosaccharides and phosphorylated sugars will not be detected by this method. The composition of these larger glycans can then be determined by hydrolysis of the reduced β-eliminated sugars prior to acetylation for GC-MS. Any reducing terminal sugar is detected as an alditol, whereas any sugar attached to a linkage other than the peptide will not be reduced and will maintain an acetylated pyranoside ring structure. The alditos and pyranoses can be readily separated by GC and identified by their different fragmentation spectra by electron impact mass spectrometry. Phosphorylated sugars will not be seen in the conditions of GC-MS, so that dephosphorylation of the monosaccharides prior to peracetylation is required to determine the presence of any sugar phosphates.

The glycans of SP96 of each strain were released by β-elimination using reducing alkaline conditions and aliquoted into four samples, each for a different treatment. To see whether a single monosaccharide was directly attached to the protein, the first sample was directly peracetylated (treatment a). The second sample was hydrolyzed and peracetylated to see the whole monosaccharide composition (treatment b). The presence of phosphorylated sugars in the third aliquot was determined after treatment with HF to dephosphorylate the sugars prior to peracetylation (treatment c), and the fourth aliquot was a hydrolysate of a similarly dephosphorylated sample (treatment d).

As an example, the GC chromatograms of the acid-hydrolyzed, β-eliminated sample (treatment b) of X22-SP96 (A) and HU2470-SP96 (B) are shown in Fig. 3. In both samples, there is a large fucitol acetate peak (peak 2) and a peak of GlcNAc acetate in the pyranoside form (peak 4). The peaks collectively labeled as I are the multiple isomers of deoxyhexosepyranoside (fucose acetate). The peak at around 16 min is of contaminating glucose peracetate, which varied in all samples. The corresponding electron impact mass spectrometry fragmentation spectra (data not shown) were used to identify these sugars. Quantitation of the GC peaks in each sample was carried out relative to an internal standard myoinositol (peak 3) that was added to each sample prior to β-elimination. The released sugar compositions are shown in Table II.

The results after reductive β-elimination and acetylation (treatment a) showed a deoxysugar in the alditol acetate form,
which from the monosaccharide analysis must be fucitol, with approximately the same amount in both the wild type and the mutant. Single fucoses are thus attached via a glycosidic O-linkage directly to about 28 sites of serine in SP96. In the mutant, there was a small amount of nonreduced aminosugar in the pyranoside acetate form, which from the monosaccharide analysis was GlcNAc. The amount of GlcNAc pyranoside in the mutant after this treatment (treatment a) could not be calculated, because of a large contaminating peak, but it was in insufficient quantity to be significant, suggesting that GlcNAc occurs in the mutant in an alkali-labile linkage that could not be reduced.

The GC of the acid hydrolysate of the reductively β-eliminated glycans (treatment b) showed approximately the same amount of fucitol as prior to hydrolysis. There was also a large amount of acid-released GlcNAc in the pyranoside form in both the wild type and mutant strains. The increased amount of this form of GlcNAc in the mutant, compared with the wild type as shown in the GC-MS analysis (Table II), does not correlate with the amount of this sugar found by monosaccharide analysis (Table I) and may be anomalous due to contamination by a peak with the same retention time on the gas chromatogram. The presence of the acetylated pyranoside form of the amino sugar reveals that GlcNAc is contained in the glycans of SP96 but is not at the reducing terminal attachment site to the protein. The presence of a deoxysugar in the pyranoside form was also detected in the wild type after hydrolysis, which suggests the presence of additional fucose residues that are not directly linked to the protein. This peak was dominant (approximately 30-fold more) in the wild type with very little detectable in the mutant.

There are 121 serine and 83 threonines residues in SP96. In the 20-amino acid signal sequence that is cleaved from the mature protein, there is only one serine and one threonine. Therefore, 120 serine and 82 threonine residues have the potential to be O-glycosylated. Since fucose has been found in both the mutant and wild type in a reducing terminal linkage and it is found only at a low level of outer decoration on the glycans of the mutant, it can be deduced that at least 18 serine residues are occupied by a reducing terminal fucose and that the fucose mutation in HU2470 does not affect this modification. Since fucose is susceptible to acid degradation in the monosaccharide analysis, these values are necessarily less reliable, and the GC quantitative data (an average of 28 sites) is probably a more accurate estimate of O-fucose. The remainder of the fucose is located as a nonreducing terminal decoration on the glycans of the wild type, but not the mutant, and presumably its absence in the mutant reflects the modD352 mutation.

### TABLE II

**Quantitation of β-eliminated sugars from SP96 by GC-MS**

Sugars were released by reductive β-elimination from the purified SP96 protein (0.2–0.5 nmol) of the X22 and HU2470 strain, neutralized, desalted, and converted to a volatile form before- and after- hydrolysis with 4 M trifluoroacetic acid for 4 h at 100 °C, before they were analyzed by gas chromatography mass spectrometry. Quantitation of the GC peaks was carried out relative to an internal standard myoinositol.

| Treatment                  | Fucitol acetate | Fucose acetate | GlcNAc acetate |
|----------------------------|-----------------|----------------|---------------|
| Reductive β-elimination    | 28              | 29             | —             |
| Reductive β-elimination    | 33              | 23             | 56            |
| after acid hydrolysis      | 33              | 23             | 56            |

* Could be identified but not quantitated.

* Signal was partly overlapped with a contaminating peak.
in HU2470. Since there was no evidence of N-acetylglucosaminitol after reductive $\beta$-elimination, it can be concluded that none of the N-acetylglucosamine is linked directly to the protein. Instead, in both the wild type and mutant, GlcNAc is located as a distal residue of the sugar modification on the protein, at between 52 and 66 mol/mol of protein (Table I).

Dephosphorylation of the reduced $\beta$-eliminated glycans (treatment c) also showed the fucitol peak, but, contrary to the nondephosphorylated sample, GlcNAc pyranoside was present in both strains. This result strongly suggests that GlcNAc exists as a phosphorylated nonreducing terminal monosaccharide. The phosphate must be attached at the anomic carbon (C-1) position, since its location on any other carbon would result in an alditol acetate, rather than an acetylated pyranoside, appearing after reductive $\beta$-elimination and dephosphorylation. This implies that GlcNAc is attached to the protein via a phosphodiester bond. Fucose pyranoside was also detected in the wild type at a low level after dephosphorylation, but it was not detected in the mutant protein, although the same amount of protein was used for the analysis. The same rationale would indicate the presence of a small amount of fucose in a phosphodiester linkage to the protein in the wild type SP96.

Hydrolysis of the dephosphorylated $\beta$-eliminated glycans (treatment d) yielded the same GC-eluted sugar forms (fucitol, GlcNAc pyranoside, and Fuc pyranoside) as in treatment b (data not shown), since 2 M trifluoroacetic acid hydrolysis removed the phosphate from the sugars to the same extent as the HF treatment. The quantitation of the amount of the sugars in the HF-treated samples is not shown, since it was apparent that destruction of the monosaccharides was occurring over the long period of incubation in the HF acidic conditions, even at 0 °C.

**Release and Identification of Phosphorylated Mono- and Disaccharides**—The phosphoamino acid analysis indicated the presence of phosphorylated serines, and the above data imply that the sugar phosphates are linked to the protein via a phosphodiester bond. Phosphodiester and fucose linkages have both been shown to be susceptible to mild acid hydrolysis (40), and furthermore, some of the mAb epitopes on SP96 have been shown to be mildly acid-labile. To analyze the mild acid-labile sugar linkages, SP96 protein from both the wild type and mutant strains (500 pmol) was treated with mild acid (40 mM trifluoroacetic acid, 8 min, 100 °C), and the released sugars were separated on a CarboPac PA1 column. In both cases, a large peak at 4 min was observed, which eluted at the same time as a GlcNAc-$\alpha$-1-P standard.

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**FIG. 4. Separation of released phosphorylated sugars by HPAEC-PAD.** After mild alkaline $\beta$-elimination of the SP96 protein, the released phosphorylated sugars were separated on a CarboPac PA1 column. A, chromatogram of the released sugar ($P_1$; 14.4 min) of the X22 strain (~1 nmol). B, chromatogram of the released sugar ($P_2$; 18.1 min) of the HU2470 strain (~1 nmol). This latter peak eluted at the same time as a GlcNAc-$\alpha$-1-P standard.

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3 A. Champion and L. Browne, unpublished results.
time as monosaccharides on a gradient that is capable of resolving monosaccharides from monosaccharide phosphates and disaccharides (data not shown). The acid treatment appeared to remove the phosphate from the sugar as well as hydrolyzing any disaccharides present.

Nonreductive β-elimination using 0.1 M NaOH was also used to try to release the glycans from the SP96 protein (1 nmol) of both strains. The reducing terminus of nonsubstituted sugars undergoes a degradative “peeling” reaction under alkaline conditions and would not be detected. The treatment of X22-SP96 with 0.1 M NaOH (Fig. 4A) released one glycan (P1; 14.4 min). The same treatment released a single different glycan (P2; 18.8 min) from HU2470-SP96 (Fig. 4B), with a shift of retention time of over 4 min and a weaker response factor.

Peak P1 was collected and desalted over a graphitized carbon column, and an aliquot of the sample was hydrolyzed in 2 M trifluoroacetic acid at 100 °C for 4 h. Fucose and GlcNAc were detected by monosaccharide analysis and quantified by comparison with an internal standard (Fig. 5). Phosphate was assayed after both strong acid hydrolysis and alkaline phosphatase treatment of the released P1 glycan. A molar ratio of Fuc/GlcNAc phosphate of 1:1:1 was obtained in the glycan released by β-elimination of the wild type SP96.

The HPLC peak (Fig. 4B, P2) at 18.8 min from the alkaline treatment of the mutant HU2470-SP96 showed the same retention time as a standard of GlcNAc-α-1-P. Chromatographing a mixture of the sample with a GlcNAc-α-1-P standard gave one peak. The small shoulder on the P2 peak was also observed after treatment of the GlcNAc-α-1-P standard with alkali. This might be due to the cyclization of some of the phosphate on the sugar under alkaline conditions (41).

Treatment of both samples with mild acid (40 mM trifluoroacetic acid, 8 min, 100 °C) caused the peaks of the P1 and P2 glycans to disappear with a concomitant large increase of monosaccharide eluting at 4 min (data not shown). To investigate this apparent acid lability of GlcNAc-α-1-P, the standard was treated with two different mild acid hydrolysis conditions that have been reported in the literature (namely 40 mM trifluoroacetic acid at 100 °C for 8 min (42) and 10 mM HCl at 100 °C for 8 min (43)). After both treatments, GlcNAc-α-1-P could no longer be detected by CarboPac PA1 chromatography. Glycan P1 apparently lost both the phosphate and fucose under these conditions, thus confirming the similar sensitivity of these bonds to mild acid hydrolysis (40) and explaining why no acidic or disaccharides were detected by the analysis of the mild acid hydrolysate.

A further sample of X22-SP96 protein was treated with alkaline conditions (0.1 M NaOH) and desalted over cation exchange resin. The sample was passed through an in-line graphitized carbon cartridge and was analyzed by liquid chromatography-MS-TOF in negative ion mode. The cone voltage was chosen at 30 V (Fig. 6, 1) and 80 V (Fig. 6, 2) for collision-induced dissociation. The major signal of mass m/z 446.05 correlates with the negative ion mass of a Fuc-GlcNAc-α-1-P saccharide, and the signal at m/z 300.05 is the result of the loss of fucose from this structure. It is not possible to definitively differentiate as to whether this loss results from the mass spectrometry ionization or reflects the native heterogeneity of the structures. The former interpretation is favored however, since only the disaccharide phosphate was seen in the HPAEC-PAD chromatogram of the eliminated wild type glycans (Fig. 4A). In both spectra, masses of dimers of these saccharides were detected at m/z 893.13 (Fuc-GlcNAc-α-1-P)2 and the defucosylated dimer m/z 747.10 (Fuc-GlcNAc-α-1-P plus GlcNAc-α-1-P). Because of the high mass accuracy of an ESI-TOF mass spectrometer, these masses cannot be accounted for by larger oligosaccharide structures. If the glycosylation was composed of repeats of these structures, then a mass of m/z 875.12, representing the structure Fuc-GlcNAc-α-1-P-Fuc-GlcNAc-α-1-P, would result. This mass was not observed. The trifluoroacetic acid adduct of Fuc-GlcNAc-α-1-P at m/z 560.08 and of GlcNAc-α-1-P at m/z 414.05 and the trifluoroacetic acid dimer at m/z 226.98 were evident in the 30-V ionization spectrum (Fig. 6, 1) but were not seen when the cone voltage was increased to 80 V (Fig. 6, 2).

**Determination of Disaccharide Phosphate Linkages**—The α-linked phosphate was confirmed by NMR spectroscopy of the disaccharide. This revealed an anomeric proton with a chemical shift (δ_H 5.37) indicative of α-anomeric sugars. In addition, the small coupling constant to GlcNAc H-2 (J = 3.3 Hz) is not consistent with an axial proton at H-1, where a much larger coupling would be expected. The carbon chemical shift of C-1 (δ_C 94.4) is also consistent with an α-linked phosphate (Table III). In comparison, for GlcNAc (β-1-P, C-1 resonates at 96.7 ppm (44), very similar to β-GlcNAc (δ_C 96.2) (44). The phosphate has very little effect on the carbon resonances of the sugar except...
for C-3 (Table III), which is shifted upfield by ~3 ppm compared with α-GlcNAc due to the shielding effect of the phosphate.

Similarly, the fucose anomeric proton was coupled to Fuc H-2 ($J = 4.1$ Hz), indicating that fucose was also in an α-configuration. Values of 6–9 Hz are typical for β-linkages. The site of attachment of Fuc to GlcNAc required the full 13C and 1H assignment of the disaccharide. This was achieved using HSQC (Fig. 7), TOCSY (Fig. 8), and COSY spectra (Table III). Assignment of the Fuc signals was aided by the HSQC (Fig. 7) and the COSY (data not shown) spectra. In particular, H-6 was coupled to H-5 ($d_H 4.36$). No correlation was observed in the COSY spectrum between H-5 and H-4, but the TOCSY spectrum (Fig. 8) showed strong correlation between H-1 and H-2/H-3 and a weak correlation to H-4 of Fuc. For GlcNAc, H-1 was assigned on the basis of the coupling to H-2 and phosphorus ($J_{HP} 6.9$ Hz). The HSQC (Fig. 7) was used to help assign the remaining signals. GlcNAc C-6 protons were evident as the only correla-

**Table III**

| Assignment | 13C | 1H |
|------------|-----|-----|
| Ac         | 23.1| 2.04|
| GlcNAc C-2 | 54.6| 4.10|
| GlcNAc C-6 | 61.7| 3.84|
| Fuc C-6    | 67.9| 3.86|
| Fuc C-2    | 69.5| 3.70|
| GlcNAc C-4 | 71.9| 3.69|
| Fuc C-3    | 70.7| 3.84|
| Fuc C-4    | 72.0| 3.80|
| GlcNAc C-5 | 73.7| 3.96|
| GlcNAc C-3 | 79.3| 3.85|
| GlcNAc C-1 | 94.4| 5.37|
| Fuc C-1    | 100.8| 5.03|

for C-3 (Table III), which is shifted upfield by ~3 ppm compared with α-GlcNAc due to the shielding effect of the phosphate.

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tion of two protons to the same carbon (δ 61.7). Both C-6 protons were coupled to H-5 (COSY, δH 3.96), which in turn coupled to H-4 (δH 3.59). The TOCSY spectrum (Fig. 8) showed correlation from H-1 to H-2 and H-3 and a weak correlation to H-4 to complete the assignments. The chemical shifts of Fuc were similar to those previously reported (45), while those of GlcNAc were affected by the α-1-phosphate. In particular, C-2 and C-5 were shifted ~2 ppm upfield by the shielding effect of the phosphate. This was also observed in the GlcNAc-α-1-P standard (Table III). Comparisons of the C-3, C-4, and C-5 chemical shifts with those of similar disaccharides (45) indicated that substitution was at C-3 (Table III). The chemical shifts of C-4 (δ 69.5) and C-3 (δ 79.3) of GlcNAc were similar to those of an α1–3 linkage (δ 69.7 and δ 83.4, respectively). As noted earlier, the only effect of phosphorylation of C-1 on the carbon chemical shifts of α-GlcNAc is to shift C-3 upfield by about 3 ppm.

A similar shift is noted here for the disaccharide (i.e. δ 83.4 in Fuc(α1–3)GlcNAc compared with δ 79.3 in Fuc(α1–3)GlcNAc-α-1-P). This was confirmed by the ROESY spectrum (Fig. 9), which showed a strong correlation between Fuc H-1 and GlcNAc H-3 (4.5%) but not GlcNAc H-4. In addition, approximately equal (~1%) rotating frame Overhauser enhancements were observed between Fuc H-1 and GlcNAc H-2 and GlcNAc H-4. These data established conclusively the substitution and stereochemistry of the disaccharide as Fuc(α1–3)GlcNAc-α-1-P (Fig. 9).

**DISCUSSION**

The spore is the dormant stage of the asexual developmental cycle of *D. discoideum*. It is a resistant structure capable of surviving for long periods. Spores are surrounded by three different layers, in which proteins are embedded, which build up a shield and protect the dormant amoeba from environmental stress. Some of the proteins carry post-translational modifications, and previous studies have shown that a lack in fucosylation on these results in increased permeability of the spore coat, which makes the spores less viable with time (17, 46). There is emerging a picture of the assembly of the spore coat, involving a complex of proteins (7) and cellulose binding (9, 10).

In order to better understand the nature of the spore coat, detailed structural information is required for each of the component glycoproteins. We purified a major spore coat protein, SP96, from the wild type X22 and the modD fucose mutant HU2470 and characterized, quantified, and compared the post-translational modifications.

Our goal in these studies was to identify the glycan structures and to elucidate the difference in glycosylation of the wild type and the fucose mutant. The quantification, which is here done for the first time on SP96, in combination with the already known sequence of SP96 (18), is an important step toward understanding how the protein is embedded in the spore coat and how it protects the spore.

Before commencing these studies, there was a significant amount of information, although it was fragmentary. The presence of phosphoserine was an early discovery (21, 39), and this helped explain the acidic pI observed by two-dimensional PAGE (17, 39). The presence of more than one SP96 spot on two-dimensional gels could indicate differently phosphorylated forms of the protein. In the early studies, it was already speculated that the phosphate group could also be part of a phosphodiester linkage. We show here that if there is phosphoserine in SP96 it represents only a minor part of the modified serine residues. Most of the phosphate-modified serine residues are further modified by the addition of N-acetylglucosamine and fucose in the wild type.

This study makes clear that the novel phosphoglycan structure (Fuc(α1–3)GlcNAc-α-1-P-Ser) is a major component of the spore coat.
More than 50% of serine residues in SP96 are modified with this glycan. A further 15–20% of the serines are modified by O-linked fucose, and phosphoserine accounts for less than 1% of the modifications. The phosphoglycosylation of SP96 may be analogous to phosphoglycan assembly observed in the protozoan parasite *Leishmania*, although the glycans are different. Here more than 60% of the serine residues of a serine/threonine-rich domain of an acid phosphatase are modified by Man-α-1-P-Ser and elongated by other sugars (47, 48).

Mutants have helped our understanding of the O-glycosylation of SP96. Comparing the structures of the modification on the *modD* fucose mutant confirmed our ideas about the nature of the epitopes of the mAbs MUD62 and MUD166, which were developed from a study on fucose mutants (16) (Fig. 10). Champion *et al.* (16) observed that the GA-X epitope, recognized by MUD62 on wild type SP96, was competed by fucose, as was a previous, independently isolated anti-carbohydrate antibody, mAb 83.5 (15). It was also reported that the antigen GA-X is an O-linked GlcNAc containing oligosaccharide (17). The *modD* fucose mutant HU2470 lost this epitope, and a new epitope appeared (GA-XIII), which was competed by GlcNAc on mutant-SP96; this epitope is recognized by MUD166. All of these observations can be explained by our results. The *modD* mutant HU2470-SP96 is missing the terminal fucose from the phosphodiester-linked Fuc(α1–3)GlcNAc-α1–P-Ser disaccharide. This fucose residue is necessary for recognition by MUD62. Therefore, the *modD* mutant HU2470-SP96 presents a new terminal sugar structure, GlcNAc-α1–P-Ser, which is recognized by MUD166. All of these observations can be explained by our results. The *modD* mutant HU2470-SP96 is missing the terminal fucose from the phosphodiester-linked Fuc(α1–3)GlcNAc-α1–P-Ser disaccharide. This fucose residue is necessary for recognition by MUD62. Therefore, the *modD* mutant HU2470-SP96 presents a new terminal sugar structure, GlcNAc-α1–P-Ser, which is recognized by MUD166. Specifically, the *modD* mutation appears to affect the activity of the α1–3-fucosyltransferase. In previous studies, it was already postulated that there was a glycan consisting of Fuc and GlcNAc on the wild type SP96 (17, 22). Srikrishna *et al.* (43) suggest that the mAb 83.5 epitope involves Fuc-β1–P-Ser, and we found evidence that there are minor amounts of this structure on SP96. However, the evidence to date suggests that mAb 83.5 and MUD62 have similar recognition, and we show here that MUD62 identifies Fuc(α1–3)GlcNAc-α1–P-Ser. On this basis, we suggest that mAb 83.5 is likely to identify this structure, which is another structure on SP96. However, Metha *et al.* (49) have dismissed this structure as the epitope for mAb 83.5 on the basis of α-fucosidase treatment. Certainly, most studies with these two antibodies on later stage proteins conclude that both antibodies recognize fucose as part of the epitope. Other work in our laboratory with vegetative stage proteins of wild type and fucosylation mutants

**FIG. 9.** One-dimensional ROESY spectrum of the released disaccharide. Proton NMR spectra of Fuc(α1–3)GlcNAc-α1–P showing the non-anomeric region. The normal WATERGATE spectrum (A) shows a quartet (δH 4.36) for Fuc H-5. The singlet at (δH 3.77) is an unidentified impurity. Spectrum B is a one-dimensional ROESY obtained by selective irradiation of Fuc H-1. Enhancements of GlcNAc H-3 (4.5%) and GlcNAc H-2/H-4 (1%) indicated an α1–3 linkage.

**FIG. 10.** Model of mAb binding sites on SP96. Identified post-translational modifications and their postulated antibody epitopes on the spore coat protein SP96 are shown. 1, on spore coat proteins from the wild type; 2, on some spore coat proteins from the fucose mutants HU2470, HU2471, and HU2733 with a mutation in *modD*, *modE*, and *modC*; 3, on spore coat protein SP96 from the wild type and the *modD* mutant HU2470. The mutants HU2471 and HU2733, with further decreased amounts of fucose, lose this epitope.
suggests that developmental regulation may control the presence of these glycosylation types. In Dictyostelium discoideum, it has been shown that on cysteine proteinases, a serine-rich domain is the target for GlcNAc-α-1-P-Ser modification (50). The cysteine proteinase CP6 has 44% serine in 100 amino acids, and CP7 contains 43% serine in its 130-amino acid domain. The domain contains several short repeated motifs (SGSG, SQSQ, SQSA, and SGSA) (50). In the case of SP96, our preliminary data indicate that most of the phosphoglycans are on the last 180 amino acids, where the molecule has a serine/threonine-rich tail (74 of the 121 serines in SP96 and 47 of the 83 threonines are in this region). In this tail region of SP96 (Swiss-Prot number P14328) are two repeat regions; one is serine-rich (ASSSSAPPSSPA), and the last 65 amino acids show a threonine-rich repeat (TTTATTA). Further upstream is a repeat with both serine and threonine (AGSQTSGGSTSG).

In this report, we clearly demonstrate the presence of O-fucose in addition to the phosphodiester-linked GlcNAc-fucose. There is some evidence of a minor amount of phosphodiester-linked fucose. The O-fucose glycans is almost certainly part of the epitope recognized by the monoclonal antibody MUD3, since this epitope is absent from strains carrying a mutation at the epitope recognized by the monoclonal antibody MUD3, since this epitope is absent from strains carrying a mutation at modE loci (16). The location of the O-fucosylation modification on the molecule is still not clear. Strains carrying the modE mutation are unable to transform GDP-Man to GDP-Fuc; therefore, they lose all fucose dependent epitopes. Strains with modE show a reduced fucosylation to <5% of the wild type (16). Whether the O-linked fucose is also in the tail region or further upstream on the protein is still unknown. It is possible that a heavily glycosylated, hydrophilic tail of SP96 could protrude from the spore coat and help to protect the dormant amoeba from protease digestion and other environmental influences.

We suspect that the Fuc-Ser is more important to the structural integrity of the spore coat than the Fuc(α1-3)GlcNAc-α-1-P-Ser structure, since mutants carrying mutations in the modC or modE genes (which lack all or nearly all fucose containing structures) exhibit shorter viability on storage than strains carrying a modD mutation (which only lacks the phosphodiester-linked disaccharide containing fucose). This is the first identification of three different distinct serine glycosylations on a single protein. The glycoforms were characterized by the combination of mutations, antibodies, and chemical analysis.

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Multiple O-Glycoforms on the Spore Coat Protein SP96 in *Dictyostelium discoideum*: Fuc(α1–3)GlcNAc-α-1-P-Ser IS THE MAJOR MODIFICATION
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