Immunochemical, genetic and morphological comparison of fucosylation mutants of *Dictyostelium discoideum*

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Mutations in three loci in *Dictyostelium discoideum* which affect fucosylation are described. Mutations in two of these loci resulted in the simultaneous loss of two separate carbohydrate epitopes. The GA-X epitope, which was competed by L-fucose, was absent in strains carrying a *modC354*, *modD352* or *modE353* mutation. These strains exposed a new carbohydrate epitope, competed by N-acetylglucosamine, and the size of several glycoproteins was reduced. A second epitope (GA-XII) was also absent in strains carrying the *modC354* or *modE353* mutations, reducing the size of the glycoprotein which normally expresses it. Fucose content was reduced in the three mutants, suggesting that each mutation affected a separate step in fucosylation. The lesions did not appear to inhibit synthesis of the underlying carbohydrate, because detergent extracts of mutant vesicles were more active than normal vesicles at transferring [¹⁴C]fucose from GDP-[¹⁴C]fucose to endogenous acceptor species. The *modD352* and *modE353* mutant strains incorporated exogenous [³H]fucose poorly, suggesting that lesions in the *modD* and *modE* genes interfere with the biosynthesis of fucoconjugates downstream from the previously described GDP-fucose synthesis defect of the *modC* mutation. Intact *modE353* mutant vesicles were relatively inefficient in *in vitro* assays, suggesting a global fucosylation defect (which is consistent with the loss of both glycoantigens, GA-X and GA-XII, in this mutant). Finally, the *modC354* mutation led to delayed accumulation of slime sheath *in vitro*. The three genetic loci define a fucosylation pathway in *D. discoideum* comprising defined biochemical steps which contribute to multicellular morphogenesis in this organism.

Keywords: *Dictyostelium discoideum*, fucosylation mutants, carbohydrate epitopes

INTRODUCTION

Biochemical assays now exist for most of the known steps of fucosylation, including biosynthesis of the sugar nucleotide donor GDP-fucose, its transport into the Golgi apparatus and transfer of fucose to acceptor sites in glycolipids and glycoproteins. Manipulation of the protein-mediated steps of these pathways necessitates isolation of the genes encoding these proteins (Stanley,

1992). Several fucosyltransferase genes have been isolated by expression cloning in animal cell lines by virtue of detection of unique antigens or lectin reactivities that normally result from the presence of the appropriate fucosyltransferase. A drawback to the analysis of these cell lines is that they are limited in their utility to associate changes in carbohydrate structure with function, especially at a multicellular level. For this reason, we use the cellular slime mold *Dictyostelium discoideum*, intending not only to correlate losses of specific fucosylation reactions with morphogenesis, but also to uncover genes associated with earlier steps of fucosylation pathways involved in the biosynthesis and transport of GDP-fucose. These genes

Abbreviations: FITC-SAM, fluorescein isothiocyanate-conjugated sheep anti-mouse antibody; GlcNAc, N-acetyl-D-glucosamine; GlcNH₂, glucosamine; GalNAc, N-acetyl-D-galactosamine; mAb, monoclonal antibody.

promise to be useful for dissecting how the supply of GDP-fucose regulates the accumulation of fucose-conjugates in these cells (C. West, B. Gonzalez & M. Gritzali, unpublished work).

In *D. discoideum*, fucose has been detected on N-linked oligosaccharides in both peripheral and proximal locations, on O-linked oligosaccharides of secretory and plasma membrane proteins, as well as on a cytosolic glycoprotein (Freeze, 1991; Gonzalez-Yanes *et al.*, 1992; Haynes *et al.*, 1993). *modC354* mutants are completely deficient in fucosylation, and manifest a number of phenotypes, including slow proliferation (C. West, B. Gonzalez & M. Gritzali, unpublished work), increased spore coat permeability (Gonzalez-Yanes *et al.*, 1989) and delayed slime sheath deposition. Gaining access to genes responsible for the fucosylation of specific glycoproteins would be useful for dissecting the role of specific oligosaccharide types to these cellular processes. For example, disruption of a gene required for GDP-fucose transport would allow study of functional consequences of cytosolic relative to Golgi fucosylation events.

Several monoclonal antibodies (mAbs) which, based on competitive inhibition studies using L-fucose, recognize fucose as part of their epitopes have been generated against *D. discoideum* glycoproteins, and provide useful reagents to evaluate the fucosylation activity of mutants (West *et al.*, 1986; Champion *et al.*, 1991). Other mAbs monospecific for individual fucoproteins allow an assessment of how fucosylation is affected in these mutants (Gonzalez-Yanes *et al.*, 1989; Champion *et al.*, 1991). Here we report the use of a combination of mAbs and mutants to discover new features of fucosylation in *D. discoideum*.

METHODS

***D. discoideum* culture and strains.** Haploid strains of *D. discoideum* used in this study for genetic and morphological analysis are described in Table 1. Strain HW51 is a growth-temperature-sensitive mutant of strain HL250 (Gonzalez-Yanes *et al.*, 1989), obtained after mutagenesis with N-methyl-N'-nitro-N-nitrosoguanidine to 10% survival. Strains AX3, HU51, HU2711 and their derivatives which are capable of axenic growth, were grown on axenic medium (Watts & Ashworth, 1970). Non-axenic growth was on a lawn of bacteria (*Klebsiella pneumoniae*) on SM agar (Sussman, 1987). Slug production for Western blot analysis was as described in Gregg *et al.* (1982).

mAb production. mAbs MUD62, (Grant & Williams, 1983), MUD3 (Voet *et al.*, 1985) and MUD141 (Champion *et al.*, 1991) have been described previously. Fusion techniques for the production of mAb MUD166 were similar to those described elsewhere (Goding, 1986; Harlow & Lane, 1988). The antigen was partially purified from slug cell membranes of 'double mutant' strain HU2522 (*modB501/modE353*). An initial intraperitoneal injection into a female BALB/c mouse (age 8 weeks) of 50 µg total protein in 0.2 ml given in Freund's complete adjuvant (CSL) was followed by another intraperitoneal injection, also 50 µg total protein, in Freund's incomplete adjuvant (CSL) after 3 weeks. Three weeks after the second injection, the mouse was given an intravenous boost of the same antigen. After 3 d the spleen cells were fused with NS1 cells in the presence of 35% polyethylene glycol 1500 (Boehringer-

Mannheim) (Harlow & Lane, 1988). Hybridomas were screened using a dot immunobinding assay (Smith *et al.*, 1984).

mAbs were further characterized by Western blotting on mutant and wild-type *D. discoideum* antigens separated by SDS-PAGE using previously described methods (Champion *et al.*, 1991).

Genetic analysis. Standard parasexual genetic techniques were used to construct diploids of mutant strains to be analysed (Loomis, 1987). Haploids were obtained on SM agar containing thiabendazole (2 µg ml⁻¹). Details of phenotypes used for mapping are given in Table 2.

Flow cytometry. Spores were analysed for mAb binding on the surface by flow cytometry using a well established second antibody technique (Voet *et al.*, 1985). Between two and four asexual spore heads (approx. 3×10^5 spores) were harvested from mature fruiting bodies into 50 µl 20 mM potassium phosphate buffer (pH 6.5) (KPO₄ buffer) and washed once with KPO₄ buffer. After washing, the spores were resuspended in 50 µl KPO₄ buffer before adding an equal volume of mAb-containing tissue culture supernatant, and 4 µl fluorescein isothiocyanate-conjugated sheep anti-mouse antibody (FITC-SAM) (Silenus Laboratories) per 100 µl. The samples were then vortexed and incubated in the dark for 30 min at room temperature. Analysis was carried out on a Coulter Electronics EPICS V, EPICS ELITE or XL flow cytometer, with an argon-ion laser operating at 488 nm.

Flow cytometric assays for the inhibition of binding of antibodies to the surface of spores in the presence of various sugars were performed by adding appropriate volumes of a stock solution of each sugar to spore samples in 50 µl KPO₄ buffer to make 100 µl before the addition of 100 µl mAb supernatant and 8 µl FITC-SAM. Final sugar concentrations were 100–200 mM. If inhibition was detected with any of the sugars, the assay was repeated with final sugar concentrations of 10, 1 and 0.1 mM. Sugars used in these assays were galactose, α-D-fucose, α-L-fucose (α-L-fuc), mannose, N-acetyl-D-galactosamine (GalNAc), N-acetyl-D-glucosamine (GlcNAc) (all from Sigma) and glucose (BDH). The effects of sulphate (as 100 mM sodium sulphate) and the fucose polymer compound fucoidan (equivalent to 200 mM fucose subunits) (Sigma) were also tested.

Development in axenic cells. Shaking cultures of axenic strains were grown in 20 ml axenic medium up to a concentration of 1×10^6 – 5×10^6 cells ml⁻¹. Cells were centrifuged at 300 g and washed once in salt solution (10 mM NaCl, 10 mM KCl, 3 mM CaCl₂) before being resuspended at approximately 10^7 cells (ml salt solution)⁻¹ in one well of a sterile 24-well tissue culture plate (Costar). The plate was sealed with parafilm, and rotated at 180 r.p.m. on an orbital shaker for 16–20 h to stimulate aggregation of cells in liquid.

Biochemical analysis of fucosylation. Spores were collected, washed in distilled H₂O by centrifugation and acid-hydrolysed, before analysis of the fucose content was performed by high pressure anion exchange chromatography monitored by a pulsed amperometric detector (Gonzalez-Yanes *et al.*, 1989). Entire spores were analysed because treatments which might release unincorporated fucose precursors might also release macromolecular fucose, and unincorporated fucose is a small fraction of the total fucose in vegetative cells (C. West, B. Gonzalez and M. Gritzali, unpublished work). In cases in which an estimate of the upper limit of fucose content was given, interference in the chromatogram prevented more accurate quantification.

Metabolic labelling. Fucosylation was assessed by metabolically labelling slug cells with [³H]fucose. Cells were grown on K.

Table 1. Relevant genotypes of haploid strains referred to in this study

Phenotypes for mutations at these loci are: *acr*, resistance to acriflavin (100 µg ml⁻¹) or 2% (v/v) methanol; *axe*, ability to grow in axenic medium; *bsg*, inability to grow using *B. subtilis* as a food source; *bwn*, formation of brown pigment; *cou*, sensitivity to 1.3 mM coumarin; *cyc*, resistance to cycloheximide (500 µg ml⁻¹); *man*, α-mannosidase-1-deficient; *mod*, glycosylation-defective mutation, loss of either MUD50 or MUD62 epitope; *spr*, altered spore shape; *tsg*, temperature-sensitive for growth; *whi*, white spores, absence of normal yellow spore pigment; + denotes the wild-type (non-mutant) allele. Strains designated as AX, NC, NP, X, HL, HU or HW are haploid, and DU or DP are diploid. All strains carry the *matA1* mating type allele. Diploids have been constructed using parasexual techniques between the following haploid strains: DP4 (M28/NP14); DU584 (XP99/NP187); DU3006 (HU2421/HU2471); DU3018 (NP2/HU2470); DU3034 (X22/HW51).

Haploid strain	Parent	Genotype											Reference
		<i>acr</i>	<i>axe</i>	<i>bsg</i>	<i>bwn</i>	<i>cou</i>	<i>cyc</i>	<i>man</i>	<i>mod</i>	<i>spr</i>	<i>tsg</i>	<i>whi</i>	
NP2	AX3	+	<i>A1,B1,C1</i>	+	+	+	+	+	+	+	<i>A1</i>	+	Kessin <i>et al.</i> (1974)
X22	DP4	<i>A1</i>	+	+	+	+	+	+	+	<i>A1</i>	<i>D12,E13</i>	<i>A1</i>	Williams & Newell (1976)
HU407	DU584	+	+	<i>A5</i>	<i>A1</i>	<i>A351</i>	<i>A1</i>	<i>A2</i>	+	+	<i>K21</i>	+	Welker & Williams (1980)
HU2470	X22	<i>A1</i>	+	+	+	+	+	+	<i>D352</i>	<i>A1</i>	<i>D12,E13</i>	<i>A1</i>	Champion <i>et al.</i> (1991)
HU2471	X22	<i>A1</i>	+	+	+	+	+	+	<i>E353</i>	<i>A1</i>	<i>D12,E13</i>	<i>A1</i>	Champion <i>et al.</i> (1991)
HW51	HL250	+	<i>A1,B1,C1</i>	+	+	+	+	+	<i>C354</i>	+	–399	+	This study
HU2733	DU3034	<i>A1</i>	+	+	+	+	+	+	<i>C354</i>	<i>A1</i>	<i>D12,E13*</i>	<i>A1</i>	This study
HU2711	DU3018	+	<i>A1,B1,C1</i>	+	+	+	+	+	<i>D352</i>	+	<i>A1</i>	+	This study
HU2522	DU3006	<i>A1</i>	+	+	+	+	+	+	<i>B501,B353</i>	<i>A1</i>	<i>D12,E13</i>	<i>A1</i>	This study
HL244	AX3		<i>A1,B1,C1</i>	+	+	+	+	+	–357	+	+	+	Knecht <i>et al.</i> (1984)

* The presence or absence of *tsg-399* has not been scored in this strain.

Table 2. Linkage analysis of 43 haploid segregants of diploid DU2981 which is heterozygous for the *modD352* mutation

Diploid (parental haploids)	<i>mod</i> genotype	No. of segments	I		II		III		IV		VI		VII	
			+	<i>cycA1</i>	<i>whiA1</i>	+	+	<i>bsgA5</i>	+	<i>bwnA1</i>	+	<i>manA2</i>	+	* <i>tsgK21</i>
			* <i>tsgE13</i>	+	* <i>tsgD12</i>									<i>couA351</i>
			<i>sprA1</i>	+	<i>acrA1</i>									
DU2981	<i>modD352</i>	6	2	4	2	4	0	6	6	0	5	1	6	0
(HU2470, HU407)	+	37	9	28	18	19	5	32	0	37	23	14	3	34

* Because there are several growth temperature sensitivity mutations in the above diploids, temperature sensitivity was not used in the mapping of the segregants.

pneumoniae, developed on a 0.2 µm pore diameter Nucleopore filter to the early tip stage of development (about 11 h) and labelled for a 4 h period by incubation with [³H]fucose according to Riley *et al.* (1993), at the end of which cells were beginning to culminate. The cells were then harvested into 0.25 M sucrose in 50 mM HEPES (pH 7.4) and a P100 particulate fraction was prepared according to Gonzalez-Yanes *et al.* (1992). The particulate fraction was weighed before being subjected to SDS-PAGE, fixed, sliced and counted to determine d.p.m. (µg protein)⁻¹.

In vitro fucosyltransferase reactions. Incorporation of exogenous fucose *in vitro* was determined for 16 h (upright finger stage) cells. A P100 particulate fraction was incubated with GDP-[¹⁴C]fucose in 5 mM MgCl₂, with or without the addition of 0.1% Tween 20 to assess the difference in incorporation between solubilized and intact vesicles. Fucosylation was determined as described by Gonzalez-Yanes *et al.* (1992). In the absence of Tween 20, vesicles were essentially

latent as determined by resistance of UDP-GlcNAc transferase activity to inhibition by 5 mM EDTA, based on transfer of label from UDP-[³H]fucose to endogenous acceptors (Gonzalez-Yanes *et al.*, 1992). In the presence of Tween 20, vesicles were entirely non-latent by this test.

RESULTS

The mAbs 83.5 and MUD62 are similar, but independently isolated anti-carbohydrate mAbs which are specifically competed by high concentrations of L-fucose (West *et al.*, 1986; and below). The epitope for mAb 83.5, expressed by normal *D. discoideum* slug prespore cells and spores, has been designated GA-X (West & Erdos, 1988). Slug proteins of two strains (HW51 and HL250) which carry the *modC354* allele are not recognized by mAbs 83.5 and MUD62, and thus lack GA-X. Elsewhere it has been

Fig. 1. Complementation analysis between *modC* and *modD* or *modE* mutations using Western blots of multicellular slug stage proteins extracted from approximately 10^5 parent haploid or diploid cells and then probed with mAbs MUD62 (a, lanes 1–8) or MUD50 (b, lanes 9–16). Lanes: 1 and 9, X22; 2 and 10, HW51 (*modC*); 3 and 11, DU3034 (X22 $\times$ HW51); 4 and 12, HU2470 (*modD*); 5 and 13, DU3032 (HW51 $\times$ HU2470); 6 and 14, HU2471 (*modE*); 7 and 15, DU3033 (HW51 $\times$ HU2471); 8 and 16, X22.

Table 3. Linkage analysis of 41 haploid segregants of diploid DU3035 which is heterozygous for the *modD354* mutation

Diploid (parental haploids)	<i>mod</i> genotype	No. of segments	I		II		III		IV		VI		VII	
			+	<i>cycA1</i>	<i>whiA1</i>	+	+	<i>bsgA5</i>	+	<i>bwnA1</i>	+	<i>manA2</i>	+	* <i>tsgK21</i> <i>couA351</i>
DU3035	<i>modD354</i>	14	5	9	12	2	14	0	14	0	11	3	10	4
(HU2470, HU407)	+	27	8	19	20	7	1	26	0	27	13	14	13	14

* Because there are several growth temperature sensitivity mutations in the above diploids, temperature sensitivity was not used in the mapping of the segregants.

shown that strain HL250 lacks GA-X because it cannot synthesize GDP-fucose from GDP-mannose, and that it possesses a null fucosylation phenotype (Gonzalez-Yanes *et al.*, 1989).

Mutant strains HU2470 and HU2471 also fail to bind mAbs MUD62 and 83.5, owing to mutations at the *modD* and *modE* loci, respectively (Champion *et al.*, 1991). This report defines the *modC354*, *modD352* and *modE353* loci and shows how the wild-type products of these loci contribute to protein fucosylation in normal cells.

Genetic analysis identifies three independent loci associated with fucosylation

A diploid strain (DU3034), prepared from the haploid mutant HW51 (*modC354*) and normal haploid strain X22, produced slug proteins (including spore coat proteins) recognized by MUD62 (see Fig. 1a, lane 3), showing that the mutation in strain HW51 is recessive to wild-type. Diploid strains DU3032 [HW51 (*modC354*) $\times$ HU2470

(*modD352*)] and DU3033 [HW51 (*modC354*) $\times$ HU2471 (*modE353*)] exhibited complementation, each being reactive with mAb MUD62 (Fig. 1a, lanes 5 and 7). In addition, Fig. 1(b) shows that the apparent M_r of prespore glycoprotein PsA was reduced by approximately 2000 by the *modC354* and *modE353* mutations in strains HW51 and HU2471 (Fig. 1b, lanes 10 and 14, respectively, and summarized in Table 4). Fig. 1(b) lanes 11 and 15 show that this phenotype is also complemented in the same pattern as MUD62 reactivity. Together with previous data demonstrating complementation between HU2470 and HU2471 (Champion *et al.*, 1991), these results show that at least three loci are involved in the assembly of the MUD62 epitope.

The mutation in HU2471 (*modE353*) has been previously mapped to linkage group II and the mutation in HU2470 (*modD352*) tentatively mapped to linkage group III or IV (Champion *et al.*, 1991). Haploidization of DU3033 (*modC354* $\times$ *modE353*) produced both MUD62-reactive and non-reactive haploids, demonstrating that *modC354*

Fig. 2. Two parameter flow cytometry histograms (forward angle light scatter vs FITC fluorescence) demonstrating the competitive effect of α -L-fucose and sodium sulphate on MUD62 binding (a–c) and GlcNAc on MUD166 binding (d–f). Antibody binding was assayed with FITC-SAM. Panels (a–c) show wild-type (X22) spores incubated with MUD62 only (a), MUD62 + 100 mM α -L-fucose (b) and MUD62 + 100 mM α -L-fucose and 100 mM sodium sulphate (c). Panels (d–f) show HU2733 (*modC354*) spores incubated with MUD166 only (d), MUD166 + 100 mM GlcNAc (e) and 200 mM GlcNAc (f). Results for other mutant strains HU2470 (*modD352*) and HU2471 (*modE353*) were similar to the results for HU2733.

and *modE352* loci are unlinked and therefore that *modC354* is not on linkage group II. No haploids expressing the MUD62 epitope were obtained from the segregation of DU3032 (*modC354* $\times$ *modD352*), implying that the *modC354* and *modD352* loci are linked.

Full parasexual mappings of the *modD* (strain HU2470) and *modC* (strain HU2733, derived from DU3034, see above) loci, using diploids formed with the tester strain HU407, are listed in Tables 2 and 3, respectively. The

resulting diploids DU2981 (HU2470 $\times$ HU407) and DU3035 (HU2733 $\times$ HU407) bound mAb MUD62, confirming that both *modD352* and *modC354* mutations are recessive to wild type. Segregation of DU2981 shows that *modD352* maps to linkage group IV, as the *bwnA1* marker (formation of brown pigment) on linkage group IV segregates in opposition to absence of recognition by MUD62 (*modD352*) (Table 2). Similar analysis of DU3035 showed absence of MUD62 recognition to segregate in opposition to both the *bwnA1* marker on linkage group IV and the *bsgA5* marker (inability to grow on *Bacillus subtilis*) on linkage group III (Table 3). This pattern of co-segregation was observed in a repeat of the above cross using an earlier clone of HU407 (data not shown). This is most likely due to a translocation between linkage groups III and IV in HU2733, as linkage groups III and IV segregate independently in DU2981 and other diploids formed with the other partner HU407. Therefore, based on complementation/segregation analyses of DU3032 (above), the *modC* locus is on linkage group III or IV.

Antibody analysis of the mutants

To obtain additional information about the nature of the mutant glycosylation defects, the binding of a panel of mAbs to spores and individual glycoproteins was compared. As described above, the three mutants share the common property of failure to bind mAbs MUD62 and 83.5, and thus lack GA-X defined by mAb 83.5. GA-X is competed by L-fucose (West *et al.*, 1986), and flow cytometric analysis shows that MUD62 was also selectively competed by this sugar (Fig. 2); 100 mM L-fucose substantially inhibited binding of MUD62 to wild-type (X22) spores, 10 mM L-fucose inhibited marginally whereas 1 mM L-fucose did not inhibit. In contrast, 100 mM GlcNAc, D-fucose, galactose, mannose, GalNAc and glucose, and the fucose polymer fucoidan at a concentration equivalent to 200 mM fucose, each failed to

Table 4. Antibody definition of fucosylation mutants of *D. discoideum*

The table shows presence or absence of epitopes recognized by mAbs MUD62, 83.5, MUD3, MUD141, A6.2 and MUD166, and the apparent molecular mass (M_r) of spore coat protein SP96 (as recognized by MUD141, MUD166 or A6.2) and the prespore-specific cell surface molecule PsA [as recognized by MUD50 (Grant & Williams, 1983)] in *modD352*, *modE353* and *modC354* mutants of *D. discoideum*. +, present at same or similar level to normal; –, absent or almost absent; +/–, present, but at reduced levels.

<i>mod</i> mutation	Strains	mAbs				$10^{-3} \times M_r$	
		MUD62 ^a 83.5 ^b	MUD3 ^c MUD141 ^d	A6.2 ^b	MUD166 ^c	SP96	PsA
+	X22	+	+	+	+/–	96	30
+	AX3	+	+	+	+/–	96	30
<i>modD352</i>	HU2470	–	+	+	+	93	30
<i>modE353</i>	HU2471	–	–	+	+	90	28
<i>modC354</i>	HW51	–	–	+	+	90	28

References: a, Grant & Williams (1983); b, West *et al.* (1986); c, Voet *et al.* (1985); d, Champion *et al.* (1991); e, this study.

Fig. 3. Two parameter flow cytometry histograms (forward angle light scatter vs FITC fluorescence) of spores of wild-type (X22) and mutant [HU2470 (*modD352*), HU2471 (*modE353*) and HU2733 (*modC354*)]. The spores were labelled with three antibodies, MUD62, MUD3 and MUD166, and binding was assayed with FITC-SAM.

inhibit MUD62 binding, although the presence of sulphate (in the form of 100 mM sodium sulphate) did cause marginal inhibition (at the same approximate degree as 10 mM L-fucose), and enhanced the inhibitory effect of the 100 mM L-fucose considerably (Fig. 2).

GA-X is normally expressed on SP96 and other spore coat proteins. SP96 was specifically identified by mAb A6.2 on Western blots, and the apparent M_r of SP96 was reduced from 96000 to 93000 by the *modD352* mutation (Table 4). The *modC354* and *modE353* mutations reduced the SP96 apparent M_r from 96000 to 90000. The *modC354* mutation totally abolishes fucosylation (Gonzalez-Yanes *et al.*, 1989). The mAbs MUD141 and MUD3 recognized the 96000 and 93000 forms of SP96, but not the 90000 glycoform (Table 4). Thus MUD141 and MUD3 define what is probably a new glycoantigen, GA-XII, which is present only on the 93000 and 96000 glycoforms of SP96 and is abolished by the *modC354* and *modE353*, but not the *modD352*, mutations. Failure of these mAbs to bind SP96 in Western blots of proteins from the *modC354* and *modE353* mutants is corroborated by flow cytometry analysis of spores of these two strains (Fig. 3). Binding of MUD3 or MUD141 was not affected by the presence of any of the compounds used in competition studies (data not shown). All haploids containing either *modC354* or *modE353* lacked both MUD3 and MUD62 epitopes when screened by flow cytometry.

A fourth mAb, MUD166, also recognizes SP96. MUD166 was prepared against partially purified slug membranes of HU2522, a *modB501/modE353* double mutant, which lacks the immunodominant MUD50/54.2 and MUD62/83.5 reactive glycoantigens. MUD166 reacted only weakly with several bands in Western blots of wild-type (X22) slug extracts, but reacted strongly with bands of apparent M_r 93000 in a *modD352* strain (HU2470) and 90000 in *modC354* (HU2733) and *modE353* (HU2471)

Fig. 4. Western blot of slug proteins from wild-type (X22) and mutant strains of *D. discoideum* labelled with MUD166. A Western blot of X22 probed with MUD62 is included on the left (strains carrying *modC*, *modD*, or *modE* mutations do not carry the MUD62 epitope). Note the absence of label of MUD166 on SP96 of wild-type (position indicated by arrow) and minor amounts of a molecule of approximately 93 kDa (*).

strains, corresponding to the M_r of SP96 in these strains (Fig. 4 and Table 4). This reactive band was selectively extracted from spores in the presence of 1% (w/v) SDS under non-reducing conditions, which is diagnostic for SP96 (Wilkinson & Hames, 1983). Flow cytometry of wild-type and mutant spores labelled with MUD166 showed a low level of binding on wild type when compared with mutants, thus confirming results obtained with Western blots (Fig. 3). Thus, MUD166 recognizes a

Table 5. Fucose content of normal and mutant spores

Spores were collected, washed in water, acid-hydrolysed, and analysed by high pressure anion exchange chromatography monitored by a pulsed amperometric detector (Gonzalez-Yanes *et al.*, 1989). In some cases an estimate of the upper limit of fucose content is given, because interference in the chromatogram prevents quantification of the absolute level.

Strain	<i>mod</i> mutation	Fucose content (molecules per spore)
AX3	+	3.6×10^{10}
HL250	<i>modC354</i>	$< 0.75 \times 10^7$
X22	+	7.7×10^{10}
HU2470	<i>modD352</i>	1.3×10^{10}
HU2471	<i>modE353</i>	$< 2.1 \times 10^9$

core epitope partially masked by modifications disabled by the *modC*, *modD* and *modE* mutations examined in this report, and defines a new glycoantigen, GA-XIII. Since GA-XIII is expressed strongly on SP96 of all three mutants, it is associated with the oligosaccharide which normally carries GA-X, and possibly also GA-XII. GA-XIII consists, at least in part, of a GlcNAc-like moiety, since 100–200 mM GlcNAc inhibits binding of MUD166 to mutant spores in a flow cytometry assay (Fig. 2). At a concentration of 100 mM, L-fucose, D-fucose, galactose, mannose, GalNAc, glucose and sodium sulphate each failed to inhibit MUD166 binding, as did the fucose polymer fucoidan at a concentration equivalent to 200 mM fucose.

The analysis of SP96 in these mutants indicated that products of all three *mod* loci are required to construct GA-X, but that products of only the *modC* and *modE* loci are required for construction of GA-XII. Discrete increases in protein M_r were associated with the formation of each epitope type (Table 4). The functions disabled by the *modC354* and *modE353* mutations also contributed to the modification of slug prespore cell surface glycoprotein PsA (Table 4), although the GA-X, GA-XII or GA-XIII epitopes were not expressed on this glycoprotein. It should be noted that the glycoform expressed on PsA (also known as SP29), the *modB* dependent GA-XX, also occurs on other proteins such as CsA (gp80) and PsB (WGA80B) (West *et al.*, 1986), but the M_r of these molecules was not altered detectably by the *modC* (unpublished observations) or *modE* (Champion *et al.*, 1991) mutations.

Biochemical analysis of fucosylation in the mutants

To verify that fucosylation was affected in the mutants, the fucose content of spores was determined by direct acid hydrolysis and high pressure anion exchange chromatography. As shown in Table 5, the fucose content was reduced in all three mutants, with values ranging from < 0.1 % of wild-type levels for HL250 (*modC354*) and 5.8 %

Table 6. Incorporation of L-[³H]fucose by cells

Cells were grown on *K. aerogenes*, developed to the early tip stage of development, and labelled for 4 h in the presence of [³H]fucose. Cells were harvested in 0.25 M sucrose, and a particulate fraction was subjected to SDS-PAGE and fixed. Gels were sliced and counted to determine total d.p.m.

Strain	<i>mod</i> mutation	Incorporation [$10^{-3} \times$ d.p.m. (μ g protein) $^{-1}$]
X22	+	11.4
HU2470	<i>modD352</i>	5.1
HU2471	<i>modE353</i>	2.2

for HU2471 (*modE353*) to 17 % for HU2470 (*modD352*). These results are consistent with a partial loss of fucosylation pathways in mutants carrying *modD352* and *modE353*, compared to total loss in *modC354* noted previously (Gonzalez-Yanes *et al.*, 1992).

To independently assess fucosylation in the mutants, slug cells were metabolically labelled with [³H]fucose for 4 h, and incorporation of extracellular fucose into macromolecular material was determined after SDS-PAGE of the sample by counting gel slices. Efficiency of incorporation in this test would depend on fucose uptake, conjugation with GTP via the salvage pathway, transport into the Golgi apparatus and transfer to coordinately synthesized and compartmentalized macromolecular acceptors by a specific fucosyltransferase(s). As shown in Table 6, mutant strains HU2470 (*modD352*) and HU2471 (*modE353*) were 45 and 19 % as efficient, respectively, as the parental strain X22 in utilizing extracellular fucose. HL250 was equally efficient as wild-type cells in this assay (C. West, B. Gonzalez & M. Gritzali, unpublished work) as expected because only the conversion pathway of GDP-fucose synthesis, which is bypassed by metabolic labelling, is mutated in this strain. Under these conditions negligible fucose is interconverted to other metabolites (Gonzalez-Yanes *et al.*, 1992). Thus [³H]fucose incorporation roughly parallels the direct chemical measurements of *modD352* and *modE353* strains and suggests that these mutations interfere with the biosynthesis of fucoconjugates. The variation between the level of fucose incorporation and the level of accumulated activity, relative to normal levels, may be because the uptake study reflects activity during a 4 h window following tip formation, whereas fucose levels in the spore would be expected to be the sum of all synthetic activities during development in addition to any residual levels not turned over after growth cessation.

Although the results are consistent with a direct defect in a fucosylation pathway, the data do not exclude a block in the accumulation of appropriate fucosyltransferase acceptor substrates, at the level of biosynthesis of the acceptor oligosaccharide. Although the appearance of GA-XIII in the *modC*, *modD* and *modE* mutants suggests

Table 7. *In vitro* fucosylation activity in cell extracts

A particulate fraction was prepared from slug cells as described in Table 6, and fucosylation in the presence of GDP-[14 C]fucose, 5 mM MgCl₂, and $\pm 0.1\%$ Tween 20, was determined as described by Gonzalez-Yanes *et al.* (1992). $\pm$ refers to SEM of triplicate samples.

Strain	<i>mod</i> mutation	Incorporation [pmol(mg protein) ⁻¹ (30 min) ⁻¹]	
		+ Tween 20	Intact
X22	+	2.0 $\pm$ 0.3	7.0 $\pm$ 0.2
HU2470	<i>modD352</i>	5.0 $\pm$ 1.6	14.2 $\pm$ 0.2
HU2471	<i>modE353</i>	7.0 $\pm$ 0.3	4.0 $\pm$ 0.4

that an acceptor species is present, GA-XIII might be the product of an alternative modification pathway suppressed by normal fucosylation (Schachter &

Brockhausen, 1992). These possibilities can be distinguished by asking whether suitable acceptors are in fact available for fucosylation in cell extracts provided with the sugar nucleotide donor GDP-[14 C]fucose. As shown in Table 7, detergent-solubilized vesicle preparations from HU2470 and HU2471 mutant slug stage cells incorporated 2.5–3.5 times as much [14 C]fucose from GDP-[14 C]fucose as did a similar fraction from normal cells, suggesting that mutant cells either contained higher fucosyltransferase activity or higher concentrations of acceptor substrates. Higher fucosyltransferase activity is unlikely because there is less fucose and lower incorporation (see above), whereas higher acceptor substrate concentration is consistent with the above data, as seen previously in strain HL250 (Gonzalez-Yanes *et al.*, 1992). A prediction from this hypothesis is that strain HU2471, which had a lower fucose content and incorporated less fucose *in vivo* than strain HU2470, would have a higher acceptor substrate concentration; this is consistent with the difference in activities observed between these two mutant extracts.

Intact vesicles from both strains X22 (normal) and HU2470 (*modD352*) transferred 2.8–3.5 times as much

Fig. 5. Photomicrographs of wild-type and mutant strains of *D. discoideum* cells after shaking for 16 h under starvation conditions (see Methods). (a) Wild-type (NP2), (b) HL244 (*mod-257*) aggregation-defective negative control, (c) HL250 (*modC354*), (d) HU2711 (*modD352*). Field width is 2.5 mm.

[¹⁴C]fucose from GDP-[¹⁴C]fucose as did detergent-solubilized vesicles, presumably because of the ability of the former to concentrate GDP-[¹⁴C]fucose and because acceptor substrate was not diluted under these conditions. However, vesicles of strain HU2471 actually showed a reduction in incorporation of > 40%. This result is consistent with the possibility that these vesicles take up GDP-fucose inefficiently or that the GDP-fucose transporter, fucosyltransferase(s) and/or acceptor substrates are not correctly co-compartmentalized. These kinds of defects might be rescued by detergent solubilization as observed.

Both *modD352* and *modE353* mutant strains were deficient in L-fucose and inefficiently incorporated it from the medium into protein, but possessed increased levels of fucose-accepting substrates in vesicle extracts. It is therefore likely that the mutations directly disable separate steps between uptake and acceptance of the fucosylation pathway. Since the decrease in fucosylation was quantitative rather than qualitative, it is not clear from the biochemical studies whether the mutations target specific fucosyltransferases, or decrease the quantity or efficiency of an overall rate-limiting enzyme or transporter. The selective effect of the *modD352* mutation on GA-X over GA-XII suggests that this mutation may target a fucosyltransferase responsible for the GA-X modification.

Mutant phenotypes

When wild-type amoebae (strain NP2) were resuspended in salt solution at 10^7 cells ml⁻¹, and rotated on an orbital shaker for 16–20 h, the cells aggregated to form tight, well-defined spheres, completely surrounded by sheath (Fig. 5a). In contrast, cells of axenic strain HW51 (*modC354*) failed to form tight aggregates containing sheath under these conditions (Fig. 5c). Strains carrying the *modD352* mutation, however, developed normally under these conditions (Fig. 5d). Fig. 5(b) shows the complete lack of aggregation of *mod-357* mutant HL244 in contrast to the *modC* phenotype, under the same conditions. Strains carrying *modE353* could not be tested as the *modE* locus is carried on linkage group II from a non-axenic strain, and an axenic mutation on this linkage group (*axeA*) is required for this test. The effect of *modC354* on slime sheath formation was subtle, because sheath did appear after 48 h in the assay and slugs of normal appearance with sheath were formed on agar surfaces.

DISCUSSION

Classification of *Dictyostelium* glycans

In classifying types of O-linked glycosylation, priority should be given to the proximal-to-distal order of sugars, for glycosylation is a sequence of dependent events. In the absence of knowledge of complete structure, genetic analysis can be used to classify glycosylation types because the attachment of specific sugars can be blocked by mutations in glycosyltransferase genes. Selective blockage can also result from mutations in genes for sugar

nucleotide synthesis, sugar nucleotide transport, and compartmentalization of glycosyltransferases and/or their donor/acceptor substrates (Stanley, 1984; Ripka *et al.*, 1986). mAbs which distinguish glycoantigens occurring on specific glycan types can be used to screen and classify mutants. Mutational studies have distinguished three types of glycosylation in *D. discoideum*, and structural studies have resolved two further types, as summarized in Table 8.

Type 1 glycosylation is N-linked, and structural studies subdivide this type into two broad subgroups. Initial assembly is identical between these subgroups, with the differences being in post-translational trimming and substitution of the peripheral sugars, as extensively reported on N-linked sugars in many different organisms (Stanley, 1984). Two types of O-linked glycosylation (identified by mAbs) have been distinguished by expression on generally separate groups of proteins, suggesting the existence of two families of protein defined by distinctive carbohydrate structures (West & Erdos, 1988; Champion *et al.*, 1991). One type of O-linked glycosylation, previously termed type 2 glycosylation (Hohmann *et al.*, 1987), is altered by mutations at the *modB* locus. Type 2 glycosylation is marked by glycoantigen GA-XX, which is identified by mAbs E28D8, 54.2, MUD50 and 20-121-1 (Table 8). Another type of O-linked glycosylation, termed type 3 in this report, is altered by mutations at the *modC*, *modD* and *modE* loci, but not by *modB*. The type 3 glycosylation is marked by GA-X and is defined by mAbs 83.5 and MUD62 (Table 8).

Comparison of type 2 and type 3 O-linked glycans

Mutations at the *modC*, *modD* and *modE* loci were generated as a result of chemical mutagenesis and detected based on absence of binding of mAb 83.5 or MUD62 to cells or extracts. In the case of strains carrying a *modB* mutation, the mutation blocks the addition of the reducing terminal sugar (possibly GlcNAc) to threonine in PsA (Gooley *et al.*, 1992). Unlike *modB* mutant strains (which apparently lack the entire type 2 glycan), strains carrying the *modC*, *modD* or *modE* mutations express a vestigial type 3 glycan. This vestigial glycan is defined by a new carbohydrate-specific mAb, MUD166, which was generated against purified membranes from a *modB/modE* double mutant, which lacks type 2 and part of the type 3 glycan. Biochemical studies of the mutants indicate that the vestigial type 3 glycan lacks fucose.

Analysis of a type 2 glycan-bearing glycopeptide of the prespore cell surface glycoprotein PsA suggests that it contains glucosamine (GlcNH₂) (possibly as GlcNAc), fucose and phosphate (Haynes *et al.*, 1993). Glycoproteins which carry type 3 glycans in the multicellular stages of *D. discoideum* become part of the spore coat (including the spore coat glycoprotein SP96) and slime sheath. SP96 contains small, O-linked oligosaccharides, some of which have been shown by metabolic labelling and exoglycosidase treatment to contain GlcNH₂ (possibly as GlcNAc) and L-fucose (Riley *et al.*, 1993). Despite these apparent compositional similarities between type 2 and type 3

Table 8. Glycosylation types reported in *D. discoideum*

Type	Nature of linkage	Description and examples	Glyco-antigen	Mutations	Example mutant strains	CHO mAbs (epitope absent)	Phenotype of mutant lesion
Type 1a	*N-Linked	Classical high mannose N-linked structure with dolichol-P-P precursor. Trimmed, sulphated and/or phosphorylated and/or fucosylated, but probably no sialic acid ^{1,2} . Endo-H-resistant if fucose present (fucose on core) ² . Found on vegetative stage proteins, most studied on lysosomal enzymes ¹ .		<i>modA531</i> ³	M31 ³	None	Failure to cleave Glc residues from dolichol precursor leads to reduced sulphation ¹ .
				<i>mod-355</i> <i>mod-356</i>	HL242 ⁴ HL243 ⁴	CA1 ⁴	Dolichol precursor truncated, reduces sulphation. Development impaired ¹ .
				<i>mod-357</i>	HL244 ⁴		Normal dolichol precursor, but no sulphation ¹ . Development impaired.
Type 1b	N-Linked	As above, but more highly trimmed and less substituted. Endo-H-sensitive, fucose may be present peripherally. Found on developmentally regulated protein, e.g. cell adhesion molecule csA ^{5,6*} .		<i>modA531</i> ³	M31 ³	None	M_r of csA increased ⁷ .
Type 2	O-Linked	Clustered. Suspected GlcNAc linkage to threonine in cell surface molecule PsA ⁸ . Contains fucose, GlcNAc and probably phosphate in PsA ⁹ . Present on PsA, csA, PsB and others.	GA-XX ⁷	<i>modB501</i> ¹⁰	HL205 ¹⁰ HU2428 ¹¹ HU2460 ¹¹	E28D8 ¹⁰ MUD50 ¹² 54.2 ⁷ 20-121-1 ⁵	Prevents linkage of reducing terminal sugar to protein in PsA ⁸ , linkage in other proteins not studied.
Type 3	O-Linked	Details of linkage unknown. Contains fucose and GlcNAc ^{13,14} . Found on spore coat proteins SP96, SP75 ⁷ , and vegetative cysteine proteinases, CP4 and CP5 ¹⁵ .	GA-X ⁷ (GA-XII ¹³) (GA-XIII ¹³)	<i>modC354</i> ¹³	HL250 ¹⁴ HW51 ¹³	83.5 ⁷ MUD62 ¹²	Cannot transform GDP-Man to GDP-Fuc ¹⁴ . Epitope absent on CP's ¹⁵ , and M_r of PsA reduced ¹⁰ . GA-XIII exposed on SP96, GA-XII absent ¹³ .
				<i>modD352</i> ¹³	HU2470 ¹¹	MUD62 83.5	Fucosylation reduced to about 15% of wild type ¹³ . PsA of normal M_r ¹¹ and epitope present on CP's ¹⁵ . GA-XIII exposed on SP96, GA-XII present ¹³ .
				<i>modE353</i> ¹³	HU2471 ¹¹ 83.5	MUD62	Fucosylation reduced

Table 8. (cont.)

Type	Nature of linkage	Description and examples	Glyco-antigen	Mutations	Example mutant strains	CHO mAbs (epitope absent)	Phenotype of mutant lesion
Type 4	O-Linked	Solitary, cytoplasmic ¹⁶ . Contains Xyl, Gal and Fuc ¹⁷ .		<i>modC354</i> ¹³	HL250 HW51		to < 5% of wild type, possible transport-defective ¹³ . Epitope on CP's absent ¹⁵ , and <i>M_r</i> of PsA reduced ¹¹ . GA-XIII exposed on SP96, GA-XII absent ¹³ .
Type 5	O-Linked	Fucose directly O-linked to protein. Found on SP96 ¹⁸					As for <i>modC354</i> above.

References: 1, Freeze (1986); 2, Ivatt *et al.* (1984); 3, Free *et al.* (1978); 4, Knecht *et al.* (1984); 5, Bertholdt *et al.* (1985); 6, Hohmann *et al.* (1985); 7, West *et al.* (1986); 8, Gooley *et al.* (1992); 9, Haynes *et al.* (1993); 10, Murray *et al.* (1984); 11, Champion *et al.* (1991); 12, Grant & Williams, (1983); 13, this study; 14, Gonzales-Yanes *et al.* (1989); 15, A. Champion, G. Harrison, M. Wilkins, M. North, A. Gooley & K. Williams (unpublished work); 16, Gonzales-Yanes *et al.* (1992); 17, Kozarov *et al.* (1995); 18, Riley *et al.* (1993).

* For a more complete treatment of N-linked glycosylation in *D. discoideum*, the reader is referred to the review by Freeze (1991).

glycoforms, the immunological, genetic and spatial distinctions between them justify their classification as fundamentally different (Champion *et al.*, 1991). In the *modB* genetic background, type 3 glycoproteins are normally processed, indicating either different glycosyltransferases(s) or different recognition sites controlling the processing.

Fucose linkages in *Dictyostelium*

Evidence exists for several types of fucose linkages in *D. discoideum*. Fucose is apparently attached to the core GlcNAc of N-linked glycans in an α -1,3 linkage based on sensitivity of vegetative stage, endoglycosidase-H-resistant glycans to N-glycopeptidase A (E. Henderson, personal communication). Fucose is also found in α -linkage to peripheral sites on N-linked glycans, as indicated by sensitivity to epididymal α -fucosidase (*ibid.*). Fucose is found on some type 2 glycans as suggested by the *M_r* shift of PsA in *modC354* and *modE353* mutants. It is a hallmark of type 3 glycans and, as noted above, there may be heterogeneity in fucosyl-linkages on type 3 glycans based on separate effects of the *modD352*, and the *modC354* and *modE353* mutations on GA-X and GA-XII expression. Fucose is present in the α -linkage at the periphery of type 4 glycans found on the 21 kDa cytoplasmic glycoprotein FP21 (Gonzalez-Yanes *et al.*, 1992). The type 4 glycan contains xylose and galactose as well as fucose (Kozarov *et al.*, 1995), and is presumably assembled by independent cytosolic glycosyltransferases (Gonzalez-

Yanes *et al.*, 1992). Finally, the type 5 glycan involving direct O-linkage of fucose has been reported on the basis of the detection of fucitol after alkaline borohydride treatment of spore coat protein SP96 (Riley *et al.*, 1993). This type of linkage is known to occur in the epidermal growth factor (EGF) domains of several human blood proteins (Harris & Spellman, 1993) and has been described recently in CHO cells (Stults & Cummings, 1993). Since formation of each of the above linkages is potentially catalysed by a distinct fucosyltransferase, there are likely to be many more mutable genetic targets having fucosylation phenotypes in this system.

The presence and peripheral location of fucose on type 3 O-glycans indicated by fucosidase susceptibility is consistent with fucose being an essential part of GA-X. The uncovering of a GlcNAc-competed epitope, GA-XIII, on glycoproteins from the *modC*, *modD* or *modE* mutants (which carry GA-X in wild-type cells) suggests that GlcNAc may be in the core of the type 3 oligosaccharide. The MUD62, MUD141 (and presumably MUD3) as well as MUD166 epitopes on SP96 are susceptible to mild acid hydrolysis, in contrast to the MUD50 (*modB*-dependent) epitope which is resistant to such treatment (A. Champion & L. Browne, unpublished work). Either the type 3 structure is diverse, or parallel pathways exist to assemble the same structure at different sites, because two different glycoantigens (GA-X and GA-XII) are associated with the type 3 structures on SP96, one of which (GA-XII) is not affected by the *modD352* mutation.

Biochemical defects in fucosylation mutants

This report characterizes the contributions of three genes to the formation of type 3 oligosaccharides containing GA-X or GA-XII. A defect in the *modC* locus has been shown previously to disable formation of GDP-fucose from GDP-mannose, and this gene may encode the dehydratase or epimerase enzyme activities responsible for this conversion (Gonzalez-Yanes *et al.*, 1989). As such, fucosylation is globally blocked in this mutant. The *modE353* mutation appears to inhibit fucosylation globally as well, though incorporation persists at 5–20% of wild type, rather than being absent as in strains carrying the *modC354* mutation. Whether this reflects incomplete inactivation of the gene product or indicates that there are multiple, parallel pathways for the affected step, is presently unknown. However, it seems unlikely that the *modE* locus encodes a fucosyltransferase because that enzyme would be required to control 80–95% of all incorporation. A possible step affected by this gene is transport of GDP-fucose into the Golgi apparatus. The best candidate for a specific fucosyltransferase gene is *modD*, because of its selective effect on GA-X in developing cells, as evidenced by retention of GA-XII in the *modD352* mutant. This mutation only partially inhibits fucosylation in prespore/spore cells, and two cysteine proteinases isolated from vegetative cells of this mutant still express the GA-X epitope (A. Champion, M. North & K. Williams, unpublished work). One possible explanation for this result is that there are two such fucosyltransferases, one transcribed in vegetative cells, the other during development. The results presented here are consistent with a defect in the *modD* strain being in the developmental gene.

Fucosylation phenotypes

Three phenotypes (slow proliferation, permeable spore coat and defective slime sheath) are now associated with loss of fucosylation due to the *modC354* allele. The slow proliferation phenotype is partially rescued, as expected, by exogenous L-fucose (C. West, B. Gonzalez & M. Gritzali, in preparation). Further studies are expected to lead to a new understanding of the regulation of slime sheath and spore coat assembly, and regulation of cell proliferation at the molecular level.

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