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Mass Spectrometric Analysis of Neutral and Anionic N-Glycans from a *Dictyostelium discoideum* Model for Human Congenital Disorder of Glycosylation CDG IL

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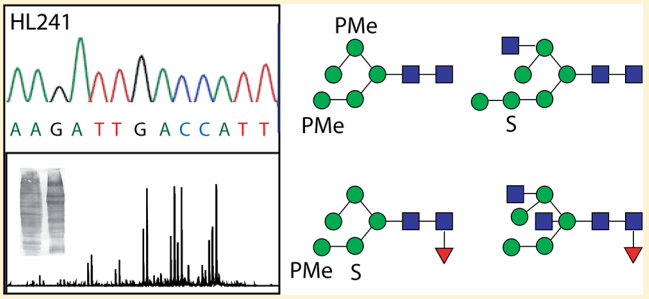
Supporting Information

ABSTRACT: The HL241 mutant strain of the cellular slime mold *Dictyostelium discoideum* is a potential model for human congenital disorder of glycosylation type IL (ALG9-CDG) and has been previously predicted to possess a lower degree of modification of its N-glycans with anionic moieties than the parental wild-type. In this study, we first showed that this strain has a premature stop codon in its *alg9* mannosyltransferase gene compatible with the occurrence of truncated N-glycans. These were subject to an optimized analytical workflow, considering that the mass spectrometry of acidic glycans often presents challenges due to neutral loss and suppression effects. Therefore, the protein-bound N-glycans were first fractionated, after serial enzymatic release, by solid phase extraction. Then primarily single glycan species were isolated by mixed hydrophilic-interaction/anion-exchange or reversed-phase HPLC and analyzed using chemical and enzymatic treatments and MS/MS. We show that protein-linked N-glycans of the mutant are of reduced size as compared to those of wild-type AX3, but still contain core α1,3-fucose, intersecting *N*-acetylglucosamine, bisecting *N*-acetylglucosamine, methylphosphate, phosphate, and sulfate residues. We observe that a single N-glycan can carry up to four of these six possible modifications. Due to the improved analytical procedures, we reveal fuller details regarding the N-glycomic potential of this fascinating model organism.



KEYWORDS: *Dictyostelium, fucose, sulfate, methylphosphate, mannosyltransferase, glycan*

INTRODUCTION

In studies stretching over some twenty or more years, the N-glycans of the cellular slime mold *Dictyostelium discoideum* have been examined in both wild-type and mutant strains, initially by use of radiolabeling and only in part by mass spectrometry. The various observed modifications of the N-glycans can be grouped into neutral and anionic substitutions of types often absent in the more familiar glycans of mammals. The first category includes the addition of core α1,3-fucose and of both bisecting and intersecting *N*-acetylglucosamine residues, compatible with the presence of relevant enzymatic activities.[1,2] The second category of glycan modifications is exemplified by addition of methylphosphate, as shown by mass spectrometry and NMR,[3] and sulphation claimed on the basis of radiolabeling as well as the use of antibodies;[4] the occurrence of sulfate was only recently verified by mass spectrometry on the basis of a moiety conferring an increase in mass of 102 Da in the positive-ion mode.[5] Overall, previous studies based on anion-exchange chromatography suggested occurrence of up to six negatively charged moieties on a single N-glycan (maximally four methylphosphate and five sulfate residues in various combinations) in the wild-type.[6] However, this high degree of modification with anionic groups has not, to date, been verified by mass spectrometry.

Indeed, in general, the analysis of anionic glycans, such as sulphated, phosphorylated and sialylated forms, presents challenges; in mixtures, anionic glycans are suppressed in the positive-ion mode.[7] (To avoid confusion with the negatively charged ions observed in mass spectrometry, the terms "acidic" or "anionic" are used here to indicate glycans with "negatively charged moieties" such as sulfate and phosphate.) Furthermore, in-source fragmentation often occurs, resulting in a loss of the anionic moieties.[8] Particularly, sulphated glycans have proven difficult to analyze and recently specific permethylation conditions have been developed for this class of oligosaccharide.[9] However, the substitution of the hydroxyl groups of a glycan with methyl residues precludes subsequent enzymatic

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treatments, which aid definition of the overall glycan structure. Therefore, adequate fractionation is a prerequisite in order to analyze anionic glycans from either mammals or lower eukaryotes in their native state.

In the present study, we compared the N-glycans of a mutant defective in the formation of the dolichol-linked oligosaccharide precursor (HL241) with those of a standard axenic "pseudowild-type" parental strain (AX3). The mutant strain is viable, but slower growth and secretion rates have been reported.[10,11] Older radiolabeling studies indicated a reduction in the size of the protein-linked glycans of this mutant as well as a decreased degree of modification with anionic residues;[11] we not only determine the nature of the genetic defect, but also have analyzed in depth the structures and isomeric status of both neutral and acidic glycans in order to assess the impact of the mutation in the HL241 strain. Solid-phase extraction into different pools of N-glycans coupled to hydrophilic-interaction/anion-exchange (HIAX) and reversed-phase (RP) HPLC was required to fractionate the N-glycome in order to facilitate the structural analysis, including detection of characteristic fragment ions upon MS/MS before and after exoglycosidase treatments. In the case of one glycan, mass spectrometry coupled to Fourier transform ion cyclotron resonance (FTICR) was employed in order to define the presence of sulfate and methylphosphate with ultrahigh mass resolution.

EXPERIMENTAL SECTION

Cultivation of Slime Molds and Isolation of Dolichol Linked Glycans

The strains HL241[4] and AX3 were obtained from the Dictyostelium Stock Centre and grown in HL5 medium. The extraction of the lipid linked oligosaccharides (LLOs) was performed based on the procedure of Gao.[12] After organic extraction, the final supernatant containing the LLOs was subject to mild acid hydrolysis (0.1 M HCl in 50% isopropanol, for 1 h at 50 °C) and the released oligosaccharides were purified on nonporous graphitized carbon (NPGC, see below) and eluted with 40% acetonitrile prior to drying, pyridylamination, and analysis by MALDI-TOF MS (MS/MS).[13]

Cloning and Sequencing of the alg9 Gene

The open reading frame encoding *Dictyostelium* ALG9 (EC 2.4.1.259), based on the sequence provided from Dictybase (DDB_G0279349) and GenBank (XM_636716), was isolated by RT-PCR of RNA isolated from wild-type AX3 and mutant HL241 cells using TRIZOL (Invitrogen) and reverse transcribed using SuperScript (Invitrogen). For the PCR reactions combinations of the two forward primer and four reverse primers were used with Expand polymerase (Roche) using an increased concentration of $MgCl_2$ (2.5 mM). The primer sequences were as follows:

5DdAlg9O_1, 5′-TGAAAATTGTGATCATACAC 3′;
5DdAlg9I_2, 5′-TAGAAAATGGAGTGGTAG-3′;
5DdAlg9I_3rev, 5′-ATGGATAAATTACGAAAAGGAA-3′ ;
5DdAlg9I_4rev, 5′-AATCTTTCTTCTTTATGTGG-TA-3′;
5DdAlg9O_2, 5′-AAATTGGTTCAAATTATTCTC-3′;
5DdAlg9_Seq2, 5′-TTATATTTTTTCTAAAATGTAA-TAG-3′.

The purified PCR products (GFX purification kit, GE Healthcare) were ligated into the pGEM-T vector (Promega) and transformed into *E. coli* TOP 10 F′ cells. The sequencing was performed by MWG or LGC Genomics. The sequence alignments were done using the Multalin server at http://multalin.toulouse.inra.fr/multalin/.[14]

Western Blotting

Crude whole cell extracts were analyzed by Western blotting after separation by SDS-PAGE on 12.5% gels and transfer to nitrocellulose membrane using a semidry blotting apparatus. After blocking with 0.5% (w/v) bovine serum albumin in Tris-buffered saline, the membranes were incubated with rabbit antihorseradish peroxidase (anti-HRP, Sigma-Aldrich; 1:10 000) or biotin-conjugated wheat germ agglutinin lectin (WGA, Vector Laboratories; 1:2000). After washing the membrane, either alkaline phosphatase-conjugated goat anti rabbit antibody (1:2000) or alkaline phosphatase-conjugated antibiotin antibody (1:10 000) were used with subsequent color detection with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (SigmaFAST BCIP/NBT). For detection of mannose-6-phosphate modifications, the recombinant myc-tagged scFv M6P-1 antibody fragment (5 μg/mL) was employed, followed by sequential incubation with monoclonal mouse antimyc antibodies (9E10, Sigma-Aldrich; 1:1000), HRP-conjugated goat antimouse IgG (Dianova; 1:5000) and enhanced chemiluminescence (Pierce).[15]

Release of N-Glycans

N-glycans were prepared by a modification of our previously published procedures.[1,13,16] Initially, cellular material (5−6 g, wet weight) was heat inactivated by boiling in deionized water prior to cooling and addition of formic acid (up to 5% [v/v]) and 3 mg porcine pepsin. After Dowex (AG50) and gel filtration (Sephadex G25) chromatography, glycopeptides were subject to PNGase F treatment followed by a further round of Dowex chromatography. The unbound fraction contained the released N-glycans, whereas the bound fraction was subject to another round of gel filtration, digestion with PNGase A (an enzyme capable of releasing core α1,3-fucosylated glycans) and subsequently Dowex chromatography. The analytical workflow is depicted in Supporting Information Figure S1.

Glycan Purification

Glycans released with PNGase A or PNGase F were subject to nonporous graphitized carbon (NPGC) chromatography using a modification of the procedures of Packer[17] and Lebrilla.[18] In brief, NPGC material (SupelClean ENVICarb, Sigma-Aldrich) was pre-equilibrated with 40% (v/v) acetonitrile and then water. The aqueous samples were applied; predominantly neutral N-glycans were first eluted with 40% (v/v) acetonitrile, whereas subsequent elution with 40% (v/v) acetonitrile containing 0.1% (v/v) trifluoroacetic acid was employed to yield a pool of glycans enriched in anionic species. The samples were dried by vacuum centrifugation prior to labeling with 2-aminopyridine followed by gel filtration (Sephadex G15) to remove excess labeling reagent.[16]

High Pressure Liquid Chromatography

The conditions for hydrophilic-interaction/anion-exchange (HIAX) were adapted from those previously described by Neville and colleagues,[19] using an IonPac AS11 column (Dionex), with a reduction in the number of solvent systems from four to two and an alteration in the gradient without comprising the ability to separate glycans effectively. Buffer A was 0.8 M ammonium acetate, pH 3 (i.e., 0.8 M ammonia adjusted with acetic acid) and buffer B 80% acetonitrile. The

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following gradient was applied at a flow rate of 1 mL/min: 0−5 min, 99% B; 5−50 min, 90% B; 50−65 min, 80% B; 65−85 min, 75% B. The HIAX column was calibrated using a mixture of oligomannosidic glycans ($Man_{3,6,7,9}GlcNAc_2$) derived from white beans. Reverse-phase (RP) HPLC (Agilent Hypersil ODS 4 mm ×250 mm, 5 μ) was performed using buffer C (0.1 M ammonium acetate, pH 4.0; i.e., 0.1 M acetic acid adjusted with ammonia) and the gradient was a 1% increase of D (30% MeOH) per minute for 30 min at 1.5 mL/min. To calibrate the RP-HPLC column in terms of glucose units (g.u.), PA-labeled forms of partial dextran hydrolysates were employed (3−11 g.u.). For both columns, the fluorescent-labeled glycans were measured at 320 nm (extinction) and 400 nm (emission) using a Shimadzu RF 10 AXL fluorescence detector. Collected fractions were dried and reconstituted in 5−10 μL depending on the fluorescence intensity.

MALDI-TOF MS Analysis

Fluorescent peaks eluting from gel filtration or HPLC columns were collected, dried, and analyzed by MALDI-TOF MS using 6-aza-2-thiothymine as matrix. A peptide ladder was used as an external mass calibrant (Bruker Daltonics) for glycan samples. Glycans were analyzed using either an Ultraflex II MALDI-TOF/TOF (equipped with a 50 Hz nitrogen laser, 337 nm) or an Autoflex Speed MALDI-TOF/TOF (equipped with a 1000 Hz Smartbeam-II laser; Bruker Daltonics) in reflectron positive or negative ion modes; typically, 1000 shots from different areas of each sample spot were summed. MS/MS experiments were performed using the LIFT cell; fragment ions were generated by laser-induced dissociation (LID) in positive and negative ion modes. Ultrahigh resolution MALDI-FTICR (Fourier transform ion cyclotron resonance) MS of a selected anionic glycan fraction was performed using a 15 T solariX FTICR mass spectrometer. The instrument was controlled by Compass solariXcontrol software and equipped with a 1000 Hz Bruker Smartbeam-II Laser System. Mass spectrometric data were assessed manually after initial processing using Bruker FlexAnalysis 3.3 software.

Exoglycosidase Digestions and Dephosphorylation Treatments

Pyridylaminated N-glycan structures whose composition had been confirmed by MALDI TOF MS/MS were subject to exoglycosidase or chemical treatments. The N-glycans were incubated with either 0.2 μL *Aspergillus saitoi* α-1,2 mannosidase (4 μU, Prozyme), 0.2 μL *Xanthomomas* α-1,2/3 mannosidase (6.4 U, New England Biolabs), 0.2 μL *Canavalia ensiformis* (jack bean) α-mannosidase (0.06 U, Sigma-Aldrich), or 0.2 μL *Bos taurus* (bovine) α-1,6 fucosidase (0.2 mU, Sigma-Aldrich). All of the digestions were performed in 1 μL of 50 mM ammonium acetate pH 5 and incubated for 16−36 h at 37 °C. For digestion of a standard $Man_9GlcNAc_2$ glycan (Takara), an aliquot of *Caenorhabditis elegans* endoplasmic reticulum (ER) mannosidase MANS-3 expressed in *Pichia pastoris* was incubated with the glycan at room temperature prior to RP-HPLC.[20] Furthermore, phosphodiester bonds were cleaved by treating the dried glycan fractions with 3 μL of 40% hydrofluoric acid (on ice in the cold room) for 36 h prior to repeated evaporation. The digests were analyzed using MALDI-TOF MS and MS/MS.

RESULTS

Genetic Basis for the Mannosylation Defect in the HL241 Strain

Earlier studies using size-fractionation HPLC indicated that the HL241 strain possesses truncated N-glycans containing predominantly five or six mannose residues.[11] To verify that this is due to altered precursor formation, glycans released from the dolichol-linked oligosaccharides extracted from amoebae were fluorescently labeled and separated by RP-HPLC. We confirmed by mass spectrometry that the major lipid-linked oligosaccharide in the HL241 mutant was of the form $Hex_6HexNAc_2$, with some traces of $Hex_{7-8}GlcNAc_2$, whereas in the AX3 wild-type, a number of oligosaccharides were identified, the largest of which was $Hex_{12}HexNAc_2$ (Figure 1A). The data suggest a defect in the luminal endoplasmic reticulum

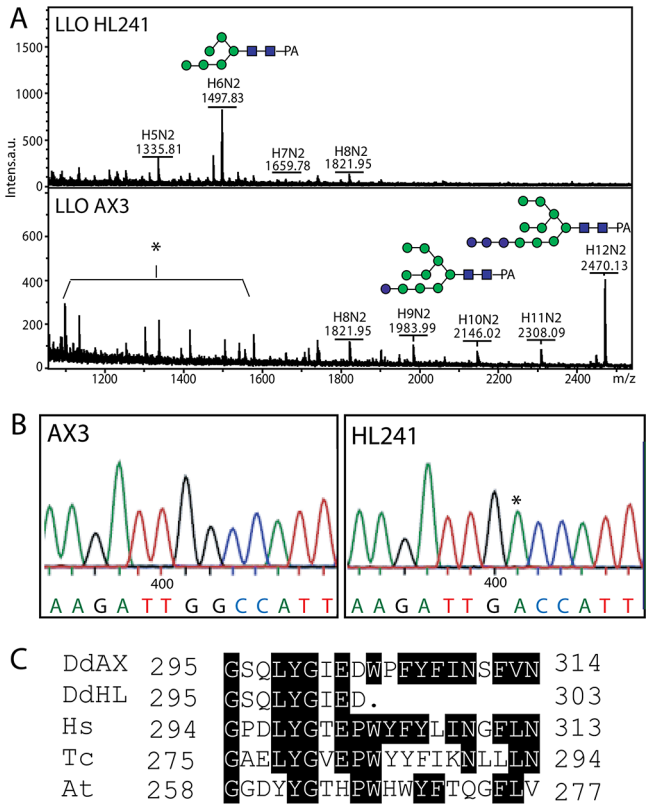

Figure 1. The mutant HL241 has a truncated precursor and possesses a defective *alg9* gene. (A) The lipid-linked oligosaccharides (LLO) were analyzed by MALDI TOF MS in positive ion mode with the major quasimolecular ions being in sodiated forms. The HL241 strain has a major precursor of m/z 1497, corresponding to a composition of $Hex_6HexNAc_2$ (H6N2), whereas the major precursor in the AX3 strain (m/z 2470) has the composition $Hex_{12}HexNAc_2$ (H12N2); unknown contaminants are indicated with an asterisk. Intensities are shown in arbitrary units (a.u.). (B) The cDNA of both strains was used to amplify the *alg9* open reading frame; as indicated in the section of the sequencing chromatogram shown, one base pair is exchanged in HL241 from guanine (G) to adenine (A, highlighted with an asterisk) leading to a premature stop codon in the third exon instead of a tryptophan (W). (C) An alignment of the relevant region of the Alg9 proteins of DdAX (*Dictyostelium discoideum* AX3), DdHL (*Dictyostelium discoideum* HL241), Hs (*Homo sapiens*), Tc (*Trypanosoma cruzi*), and At (*Arabidopsis thaliana*) shows that this amino acid is conserved throughout the presented species; the stop codon present in the mutant form (DdHL) is indicated by a ".".

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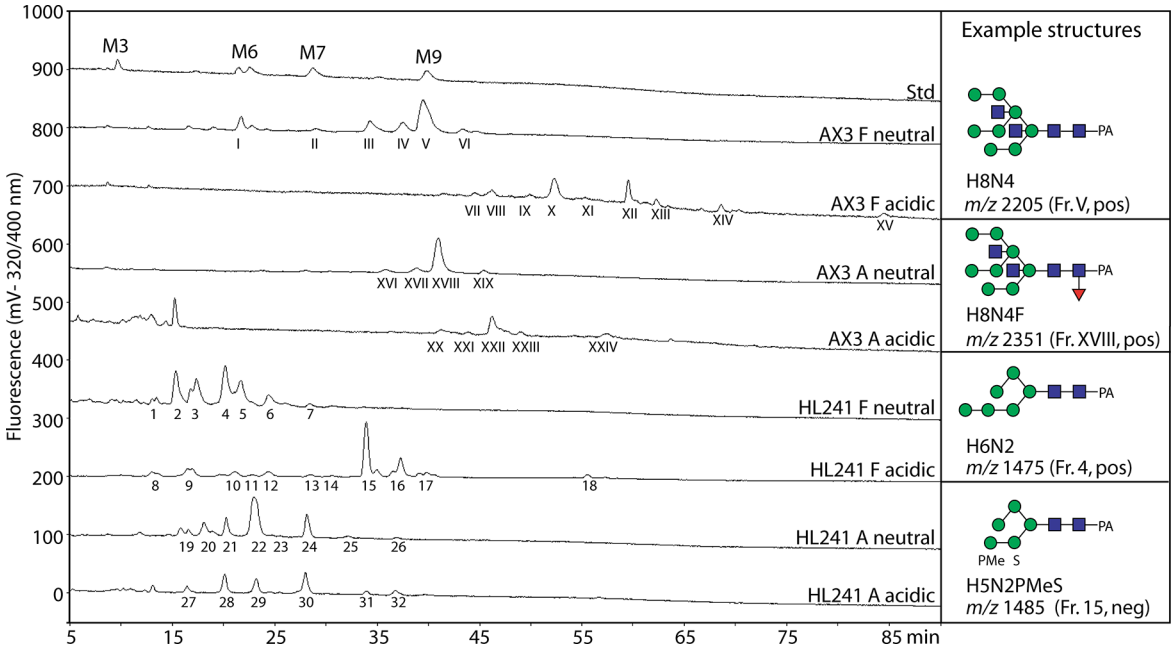

Figure 2. HPLC Fractionation of pyridylaminated neutral and acidic N-glycans. (A) HIAX-HPLC chromatograms of the NPGC-fractionated pools (20% of each neutral- or acidic-enriched pool of PNGase F or A released glycans) are annotated with roman and arabic numerals respectively to define fractions of AX3 and HL241 glycans; these fractions were then analyzed with MALDI-TOF MS in positive and negative ion modes as summarized in Tables 1 and 2. Selected pyridylaminated oligomannosidic N-glycans from beans were used as standards; the peaks eluting between 5 and 15 min in the "AX3 A acidic-enriched" chromatogram are nonglycan impurities as judged by MALDI-TOF MS. Two example major structures from each strain (with the relevant fraction numbers and *m/z* values from positive or negative mode MALDI-TOF MS analyses) are depicted according to the pictorial nomenclature of the Consortium for Functional Glycomics using the abbreviations: H, hexose (circles); N, *N*-acetylhexosamine (squares); F, fucose (triangles); PMe, methylphosphate; PA, pyridylamino; and S, sulphate.

mannosyltransferase, which transfers the seventh mannose of the $Glc_3Man_9GlcNAc_2$ dolichol-linked precursor for N-linked glycosylation. As the *Saccharomyces cerevisiae alg9* mutant is also defective in this mannosylation step and accumulates $Man_6GlcNAc_2$–PP-Dol as an intermediate,[21] we set out to identify the *alg9* gene in *Dictyostelium discoideum* and sequenced both the wild-type and HL241 forms.

Using either AX3 or HL241 cDNA as templates, we amplified the open reading frame of *alg9* as two overlapping sequences by PCR, before ligation into a standard cloning vector. The sequences obtained from both strains were identical to the published genome sequence from the AX4 strain,[22] except in one codon; in two independently isolated clones, the HL241 *alg9* cDNA has at this position a nonsense mutation (G→A). Thereby, the wild-type Trp_{304} codon in the third exon of the gene is replaced by a stop codon in the mutant (Figure 1B,C); the resulting encoded polypeptide would be of 303 aa instead of 649 aa, a truncation predicted to disrupt the catalytic function.

Western Blotting to Screen Glycan Epitopes

In an initial comparison of the glycomes of HL241 and the parental AX3 strain, the presence of key epitopes (bisecting β1,4-GlcNAc, core α1,3-fucose and Man-6-P) in both the wild-type and mutant were demonstrated by Western blotting (Supporting Information Figure S2) using wheat germ agglutinin,[23] antihorseradish peroxidase,[24] and the scFv M6P-1 antibody fragment,[15] respectively. The strains differ in the pattern and/or intensity of reactivity based on predicted bisecting β1,4-GlcNAc, α1,3-fucose, and Man-6-P glycoprotein modifications. Noticeable is the somewhat higher reactivity of HL241 extracts toward wheat germ agglutinin, which may be explained by possibly lower steric hindrance of the bisecting GlcNAc in the relatively smaller N-glycans of this strain (see below) as compared to the larger glycans found in AX3.

Analytical Approach for Studying Amoebal N-Glycomes

In order to more specifically examine the differences in the N-glycomes of the two strains, mass spectrometry was employed. Particularly with the goal of examining the nature of any anionic glycans, a modification of our previously described approach for analyzing the N-glycome of the slime mold was required, as previously we had only analyzed neutral glycans of *Dictyostelium* by MALDI-TOF MS.[1] Particularly, a change of matrix employed for mass spectrometry and a new set of purification procedures, including the use of a different HPLC column appeared to be important prerequisites.

After release and solid phase extraction (see also flowchart, Supporting Information Figure S1), a total of four pools (PNGase F- and A-released, "neutral-enriched" and "acidic-enriched" N-glycans) were obtained for each strain and analyzed by MALDI-TOF MS in both positive and negative modes (Supporting Information Figure S3). These initial MS data show that the glycans of the HL241 cells generally contain three hexose residues less than the wild-type AX3. Furthermore, the majority of the anionic glycans appeared in the PNGase F digests. However, fucosylated glycans were only observed in the pools of glycans released with PNGase A; in the negative mode spectra of the acidic-enriched pools derived from PNGase A digests, an increase in mass of 80 Da, potentially either phosphate or sulfate, is apparent for the major peaks as compared to those in the positive spectra of the neutral pools. For more detailed analysis, the four glycan pools from each strain were fractionated by HIAX chromatography

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Table 1. Summary of the N-Glycan Structures Analyzed from *Dictyostelium* AX3 with HIAX-HPLC[a]

		fract. no.	$[M+H]^+$	$[M+Na]^+$	$[M-H]^-$	$[M-H+Na]^-$	$[M-H+2Na]^-$	$[M-H+3Na]^-$	composition
AX3	F neutral	I	1475	1497					H6N2
		II	1637	1659					H7N2
		III	1799	1821					H8N2
		III	2043	2065					H7N4
		IV	2002	2024					H8N3
		V	1961	1983					H9N2
		V	2205	2227					H8N4
		VI	2123	2145					H10N2
		VI	2164	2186					H9N3
	F acidic	VII		2307	2283				H8N4S
		VIII	2096	2118	2094				H8N3PMe
		VIII	2299	2321	2297				H8N4PMe
		IX	2055	2077	2053				H9N2PMe
		IX	2258	2280	2256				H9N3PMe
		X	1987	2009	1985				H8N2(PMe)2
		X	2190	2212	2188				H8N3(PMe)2
		XI	2176	2198	2174				H8N3PMeP
		XI	2393	2415	2391				H8N4(PMe)2
		XII		2292	2268				H8N3(PMe)2S
		XII		2089	2065				H8N2(PMe)2S
		XIII				2167	2189		H8N2(PMe)2S2
		XIV				2370	2392		H8N3(PMe)2S2
		XV					2269	2291	H8N2(PMe)2S3
	A neutral	XVI	1945	1967					H8N2F
		XVI	2189	2211					H7N4F
		XVII	2148	2170					H8N3F
		XVIII	2351	2373					H8N4F
		XIX		2453	2429				H8N4FS
	A acidic	XX		2291	2267				H7N4FS
		XXI		2250	2226				H8N3FS
		XXII	2242	2264	2240				H8N3FPMe
		XXII		2453	2429				H8N4FS
		XXIII	2445	2467	2443				H8N4FPMe
		XXIV				2531	2553		H8N4FS2

[a]Pyridylaminated glycans from the axenic wild-type AX3 strain (fractions I–XXIV) were fractionated by HIAX (see Figure 2) and analysed by MALDI-TOF MS. The predicted compositions are given using the abbreviations: H, hexose; N, *N*-acetylhexosamine; F, fucose; P, phosphate; PMe, methylphosphate; and S, sulphate. The terminologies "neutral" and "acidic" in the tables refer to the "neutral-enriched" and "acidic-enriched" PNGase F or A released glycan pools.

(see Figure 2 and Tables 1 and 2), which tended to result in a later elution time for anionic glycans, and by RP-HPLC, which yields complementary data to HIAX especially in terms of different isomers (Supporting Information Figure S4). All fractions were subject to mass spectrometry and MS/MS, with the primary focus on the analysis of the N-glycans of the mutant.

Isomers of Oligomannosidic Glycans in *Dictyostelium*

The positive-ion mode spectra of the pools of neutral-enriched N-glycans released with PNGase F are dominated by oligomannosidic species including structures with one or two additional HexNAc residues (Supporting Information Figure S3A). As previously reported[1] and as shown in the present study, the major neutral AX3 nonfucosylated glycans are $Hex_8HexNAc_{2-4}$ and $Hex_9HexNAc_2$. It is expected that the Hex_8 structures are based on $Man_8GlcNAc_2$ (Man8); the question nevertheless is as to which Man8 isomer dominates. In yeast and in higher eukaryotes, the initial trimming step in the endoplasmic reticulum, after removal of the glucose residues, is catalyzed by a class I mannosidase to yield the so-called Man8B isomer.[20]

As different isomers of oligomannosidic glycans can be distinguished by RP-HPLC,[25] the elution position of the major putative $Man_8GlcNAc_2$ structure from AX3 was compared to that of the Man8B product (5.2 g.u.) resulting from *C. elegans* ER mannosidase digestion of $Man_9GlcNAc_2$[20] and of isomaltooligosaccharide standards. This experiment (Supporting Information Figure S5A) indicated that the major $Man_8GlcNAc_2$ (5.8 g.u.) in AX3 is not Man8B, but probably Man8A, in which one α1,2-mannose from the A branch has been removed (the third possible isomer, Man8C, would be expected to elute beyond 6.5 g.u.). This suggests that processing of $Man_8GlcNAc_2$ in *Dictyostelium* may occur via a different route as in other eukaryotes studied (see also the Discussion). MS/MS spectra of the Man8B and Man8A isomers are shown in Supporting Information Figures 5C,D.

In HL241, the major neutral-enriched glycans released with PNGase F are $Hex_5HexNAc_{2-4}$ and $Hex_6HexNAc_2$. On the HIAX column, the $Man_6GlcNAc_2$ (fraction 4; Figure 2 and Table 2) coelutes with neither of the two isomers present in the employed calibrant (a pool of oligomannosidic N-glycans isolated from beans). These data, in addition to the RP-HPLC

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Table 2. Summary of the N-Glycan Structures Analyzed from *Dictyostelium* Mutant HL241 with HIAX-HPLC[a]

		fract. no.	$[M+H]^+$	$[M+Na]^+$	$[M-H]^-$	$[M-H+Na]^-$	$[M-H+2Na]^-$	composition
HL241	F neutral	1	1151	1173				H4N2
		1			1229			H4N2S
		1	1354	1376				H4N3
		2	1313	1335				H5N2
		2			1391			H5N2S
		3	1516	1538				H5N3
		4	1475	1497				H6N2
		4			1553			H6N2S
		4			1594			H5N3S
		5	1719	1741				H5N4
		6	1637	1659				H7N2
		6			1715			H7N2S
		6	1610	1632	1608			H5N3PMe
		6			1797			H5N4S
		6	1678	1700				H6N3
		7	1813	1835	1811			H5N4PMe
		7	1840	1862				H7N3
	F acidic	8		1253	1229			H4N2S
		9		1415	1391			H5N2S
		10		1618	1594			H5N3S
		10	1407	1429	1405			H5N2PMe
		11		1577	1553			H6N2S
		12	1610	1632	1608			H5N3PMe
		12		1821	1797			H5N4S
		13	1813	1835	1811			H5N4PMe
		13		1780	1756			H6N3S
		14	1487	1509	1485			H5N2PMeP
		14	1934	1956	1932			H7N3PMe
		15		1509	1485	1507		H5N2PMeS
		15				1493	1515	H5N2S2
		16				1655	1677	H6N2S2
		16		1712	1688	1710		H5N3PMeS
		17		1671	1647	1669		H6N2PMeS
		17		1915	1891	1913		H5N4PMeS
		17				1858	1880	H6N3S2
		18		1603	1579			H5N2(PMe)2S
		18				1749	1771	H6N2PMeS2
	A neutral	19	1297	1319				H4N2F
		19		1399	1375			H4N2FS
		19	1662	1684				H5N3F
		20	1703	1725				H4N4F
		21	1459	1481				H5N2F
		21		1561	1537			H5N2FS
		22	1865	1887				H5N4F
		22		1764	1740			H5N3FS
		23		1805	1781			H4N4FS
		24		1967	1943			H5N4FS
		25	2189	2211				H7N4F
		26			2267			H7N4FS
	A acidic	27			1375			H4N2FS
		28			1537			H5N2FS
		29		1764	1740			H5N3FS
		30		1967	1943			H5N4FS
		31		1509	1485	1507		H5N2PMeS
		32	1553	1575	1551			H5N2FPMe
		32			1631	1653		H5N2FPMeS

[a]Pyridylaminated glycans from the HL241 strain (fractions 1−32) were fractionated by HIAX (see Figure 2) and analysed by MALDI-TOF MS.

elution position of 6 g.u. (Supporting Information Figure S5B), are indicative of an unusual isomeric structure. The sensitivity of this glycan to three different mannosidases was tested. First, α1,2-mannosidase removed two residues and α1,2/3-mannosi-

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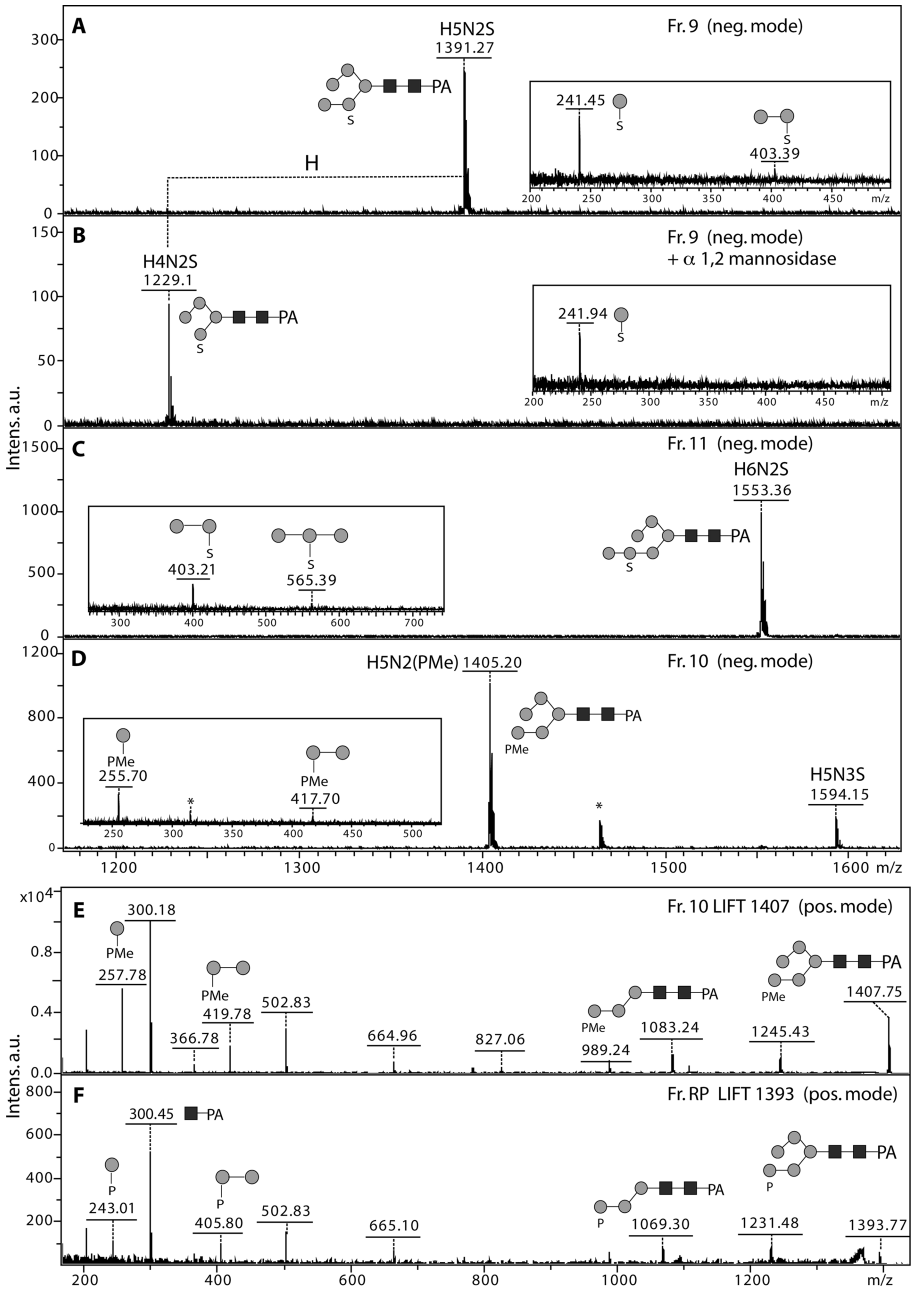

Figure 3. Positive and negative ion mode MALDI-TOF MS/MS analysis of monoacidic N-glycans from HL241. The HIAX-HPLC fractions of the mutant strain HL241 were analyzed with MALDI-TOF MS/MS in positive and negative ion modes with the PNGase F released glycans being detected in their $[M+H]^+$ and $[M-H]^-$ forms. The fragments indicating the presence of sulfate linked to a mannose were m/z 241 (HexS) and 403 (Hex_2S). The methylphosphate residues were detected in negative and positive ion modes as the following respective fragments: m/z 255 or 257 (HexPMe) and 417 or 419 (Hex_2PMe). The fragments m/z 243 (HexP), 405 (Hex_2P) and 1069 ($Hex_3HexNAc_2P$-PA) confirmed the peripheral single phosphate modification. (A, B) Negative-ion mode spectra of untreated and α1,2-mannosidase treated $Hex_5HexNAc_2S$ (HIAX fraction 9) with the low-range MS/MS spectra as insets. (C, D) Negative-ion mode spectra of $Hex_6HexNAc_2S$ (HIAX fraction 11) and $Hex_5HexNAc_2PMe$ (HIAX fraction 10) with the low-range MS/MS spectra as insets. (E, F) Positive-ion mode MS/MS spectra of $Hex_5HexNAc_2PMe$ (HIAX fraction 10) and $Hex_5HexNAc_2P$ (RP-HPLC fraction at 5.8 min, see Supporting Information Figure S4). The asterisks in panel D indicate nonglycan impurities.

dase three (not shown), whereas jack bean α-mannosidase cleaved up to five residues resulting in a product with m/z 665 (Supporting Information Figure S6A).

The $Man_5GlcNAc_2$ structure from HL241 (HIAX fraction 2; Figure 2 and Table 2) elutes at 6.3 g.u. on RP-HPLC, as compared to 7.2 g.u. for the standard Golgi-processed isomer which lacks any α1,2-mannose residues (Supporting Information Figure S5B). Furthermore, a preflipping isomer of $Man_5GlcNAc_2$, as isolated from *Trichomonas*, with two α1,2-mannose residues elutes even earlier at 5.8 g.u.[16] Compatible with the intermediate elution properties of the HL241 $Man_5GlcNAc_2$ isomer, α1,2-mannosidase removes only one residue (data not shown); also, the MS/MS spectrum of the mutant $Man_5GlcNAc_2$ isomer is different from that of the standard (Supporting Information Figure S5E,F). Thus, we postulate a Manα1,2Manα1,3(Manα1,3Manα1,6)-Manβ1,4GlcNAcβ1,4GlcNAc for this glycan, with an additional α1,2-linked mannose on the $Man_6GlcNAc_2$ from the mutant.

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Glycans modified with bisecting and intersecting GlcNAc residues, e.g., $Hex_5HexNAc_{3-4}$ or $Hex_6HexNAc_3$, were analyzed by MS/MS, which revealed key diagnostic fragments (Supporting Information Figure S7A–C); in particular, the fragments of m/z 569 and 868 are indicative of the bisected structure. Jack bean mannosidase treatment of these structures resulted in a loss of one hexose residue from $Hex_5HexNAc_4$, two from $Hex_5HexNAc_3$ and three from $Hex_6HexNAc_3$ (data for the second of these structures is shown in Supporting Information Figure S6A); the inability to remove all unsubstituted mannoses is compatible with the steric hindrance caused by the bisecting and intersecting GlcNAc residues.

Fucosylated N-Glycans in the Dictyostelium HL241 Strain

Fucosylated glycans were found only in the PNGase A-released pool and not in the prior PNGase F-released pool (see Supporting Information Figure S3); the MS/MS data of glycans from individual HPLC fractions (especially the fragment of m/z 446; Supporting Information Figure S7D) indicate that the fucose is on the core GlcNAc residue. Although core α1,6-fucosylation has been reported for a single *Dictyostelium* protein,[26] the fucose residue in the HL241 glycome is concluded to be core α1,3-linked as (i) PNGase A, and not PNGase F, can release core α1,3-fucosylated glycans[27] and (ii) the fucosylated glycans elute earlier on RP-HPLC than their nonfucosylated forms (Supporting Information Figure S4), as also seen for AX3 glycans. This is compatible with the reactivity of both *Dictyostelium* strains with antihorseradish peroxidase (Supporting Information Figure S2). Another proof of the core α1,3-linkage is the sensitivity of the fucose of the $Hex_5HexNAc_4Fuc_1$ structure to hydrofluoric acid and its resistance to bovine kidney α-fucosidase (Supporting Information Figure 6B); similar to its nonfucosylated relative, jack bean mannosidase can only remove one hexose residue from this glycan (data not shown).

Monoacidic N-Glycans

Negative-ion mode spectra of the acidic-enriched pools of both AX3 and HL241 indicate the presence of glycans with modifications of 80 and 94 Da (Supporting Information Figure S3B). In positive-ion mode, the modification with 80 Da is not obvious, but low-intensity species of 102 Da higher than the major ions are observed; on the other hand, the modification of 94 Da is clearly observed in positive-ion mode. Considering previous studies on *Dictyostelium*, as well as the properties of anionic glycans when analyzed by MALDI-TOF MS,[28] these modifications may be assigned as sulfate and methylphosphate, respectively. The species observed in positive-ion mode can be interpreted as being primarily $[M-SO_3+H]^+$ (with some $[M+Na]^+$) for sulphated glycans, indicative of in-source loss of sulfate, and $[M+H]^+$ for methylphosphorylated glycans; both types of monoacidic glycans are observed in negative-ion mode as $[M-H]^-$ (Tables 1 and 2).

When examining individual HIAX and RP-HPLC fractions, further anionic glycans can be detected by mass spectrometry; probably some suppression of charged glycans occurs in the presence of more dominant structures, thus explaining their apparent absence from the spectra of unfractionated pools. From the extra information gained by HPLC fractionation (Tables 1 and 2; Figure 2 and Supporting Information Figure S4), it became obvious that the prefractionation by NPGC chromatography results in some monoacidic glycans being present in the neutral-enriched fraction, whereas the acidic-enriched fraction contained both mono- and multiacidic species.

A number of other lessons can also be learnt from the HPLC fractionation patterns. For instance, monosulphated oligomannosidic glycans of the form $Hex_nHexNAc_2Fuc_{0-1}S$ coelute with the unsulphated form when fractionated by HIAX (Table 2); however, glycans with inter- and/or bisecting GlcNAc of the form $Hex_nHexNAc_{3-4}Fuc_{0-1}S$ elute later on the HIAX column than the corresponding unsulphated versions, consistent with its anion-exchange characteristics. Glycans modified with methylphosphate elute consistently later than the "parental" structures (compare the elution of $Hex_5HexNAc_2(PMe)_{0-1}$ in HIAX fractions 2 and 10; Figure 2). Moreover, multiacidic glycans elute later on the HIAX column than their monoacidic "relatives". When fractionating by RP-HPLC, sulphated and methylphosphorylated glycans elute earlier than their neutral counterparts; glycans with multiple acidic groups are especially poorly retained (Supporting Information Figure S4).

As sulfate and phosphate modifications would result in very similar mass differences in negative mode, high-accuracy analyses of a major HIAX-purified fraction were performed by MALDI-FTICR MS (Supporting Information Figure S8), which allowed the assignment of a species at m/z 1405.4651 as $Hex_5HexNAc_2PMe$-PA. In addition, its sulphated counterpart was observed in the same spectrum at m/z 1485.4220, with the observed mass difference of 79.9569 Da being in very good agreement with the theoretical value of 79.9568 for a sulfate modification, while ruling out a phosphate modification (theoretical mass increase of 79.9663 Da). Hence, these data unambiguously show that the negatively charged substituent of 80 Da is sulfate. In MALDI-TOF MS/MS analyses, sulphation was also associated with fragments in negative-ion mode of m/z 241 and 403 ($Hex_{1-2}S$), whereas methylphosphorylation is associated with positive-ion mode fragments of m/z 257 and 419 ($Hex_{1-2}PMe$) or negative-ion mode fragments of m/z 255 and 417 (Figure 3).

The next challenge was to examine the positions of these modifications on the N-glycans; thereby MS/MS spectra of putatively sulphated N-glycans from the HL241 strain were compared before and after mannosidase treatment. In general, sulphated glycans based on $Hex_5GlcNAc_{2-4}Fuc_{0-1}$ showed the presence of m/z 403 fragments in negative-ion mode (Figure 3A and Supporting Information Figure S7E), which were replaced by m/z 241 upon successful α1,2-mannosidase digestion of nonbisected structures (Figure 3B). Considering the structure of the $Man_5GlcNAc_2$ isomer in HL241 (see above), these data suggest that the sulfate group on monoanionic glycans is on the penultimate (α1,3-linked) mannose of the A-antenna. In the case of the $Man_4GlcNAc_2S$ which lacks any α1,2-linked mannose residues, only the m/z 241 fragment is observed in negative-ion mode (data not shown). The sulphated form of $Man_6GlcNAc_2$ displays fragments of m/z 403 and 565 ($Hex_{2-3}S$) consistent with the presence of three mannose residues on a sulphated form of the A-antenna (Figure 3C); the position of the sulfate on this glycan could be localized to the penultimate mannose residue as judged by the sensitivity to jack bean and fungal mannosidases and concomitant loss of the m/z 565 fragment (data not shown).

In the case of methylphosphorylated glycans from the HL241 strain, the dominance of the m/z 255 and 257 MS/MS fragments (HPMe) in negative and positive-ion modes (Figure 3D,E) is taken as being an indication that the methylphosphate

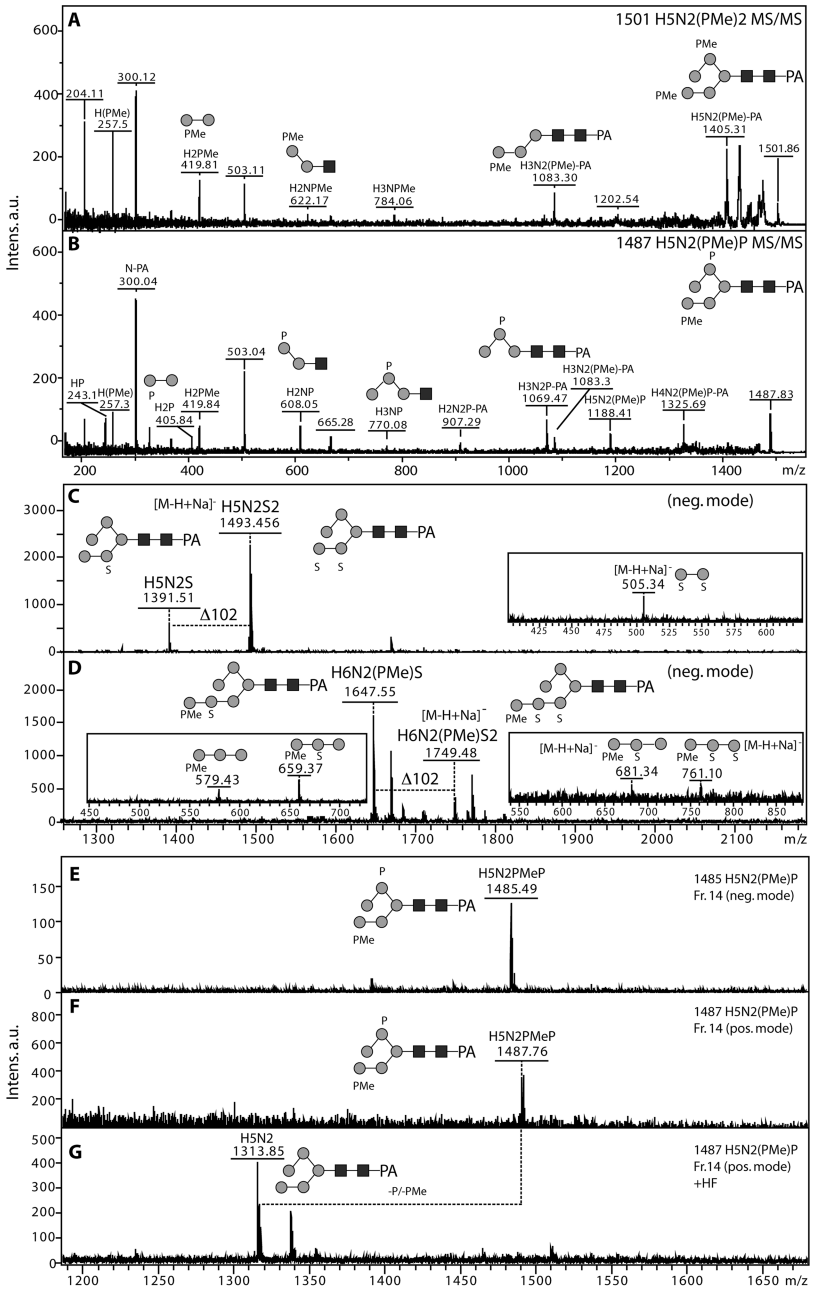

Figure 4. The multianionic structures of the mutant strain HL241. Three combinations of diacidic structures from early eluting RP-HPLC fractions were analyzed in (A, B) positive ($Hex_5HexNAc_2[PMe]_2$ and $Hex_5HexNAc_2PMeP$; 4.2 and 3.8 min, see Supporting Information Figure S4) and (C) negative ($Hex_5HexNAc_2S_2$; 5.5 min) ion modes. The methylphosphate residues were terminal and associated with trimannosyl core fragments, but were not on the same antenna, in contrast to the double sulphated m/z 505 (Hex_2S_2) combination. (D) Three anionic residues were identified on the structure $Man_6GlcNAc_2(PMe)S_2$ (HIAX fraction 18, see Table 2); the fragments m/z 681 and 761 confirmed that the three anionic groups are linked to mannoses of the same "lower" arm (α1,2Manα1,2Manα1,3Man linked to β-linked Man). (E, F, G) MALDI-TOF MS analysis of HIAX fraction 14, containing the same $Hex_5HexNAc_2PMeP$ glycan as in the 3.8 min RP fraction, before and after HF treatment. The abbreviations of the annotated structures are as follows: H, hexose; N, *N*-acetylhexosamine; P, phosphate; PMe, methylphosphate; PA, pyridylamino; and S, sulphate. The glycans are in their $[M+H]^+$ forms in positive mode and $[M-H]^-$ in the negative mode; disulphated glycans are annotated as $[M-H+Na]^-$.

group is attached to a terminal mannose residue, a supposition supported by the resistance to α1,2-mannosidase. One monoacidic glycan (m/z 1393), observed when analyzing an early eluting RP-HPLC fraction in the positive-ion mode ($Hex_5HexNAc_2P$; Figure 3F), contains an unmodified phosphate which yields $Hex_{1-2}P$ fragments of m/z 243 and 405; a loss of 80 Da occurred upon treatment with hydrofluoric acid (data not shown). These properties contrast with those of sulphated glycans which neither are HF-sensitive nor yield such fragments in positive-ion mode.

Monoacidic glycans from AX3 were also examined by MS/MS and the presence of key fragments of m/z 241 (sulfate; HexS) and 257 (methylphosphate; HPMe) in negative and positive modes was used as a verification of the proposed composition (Table 1); example spectra of AX3 anionic glycans with one or two methylphosphate or one sulfate residues are shown in Supporting Information Figure S9. However, isomeric

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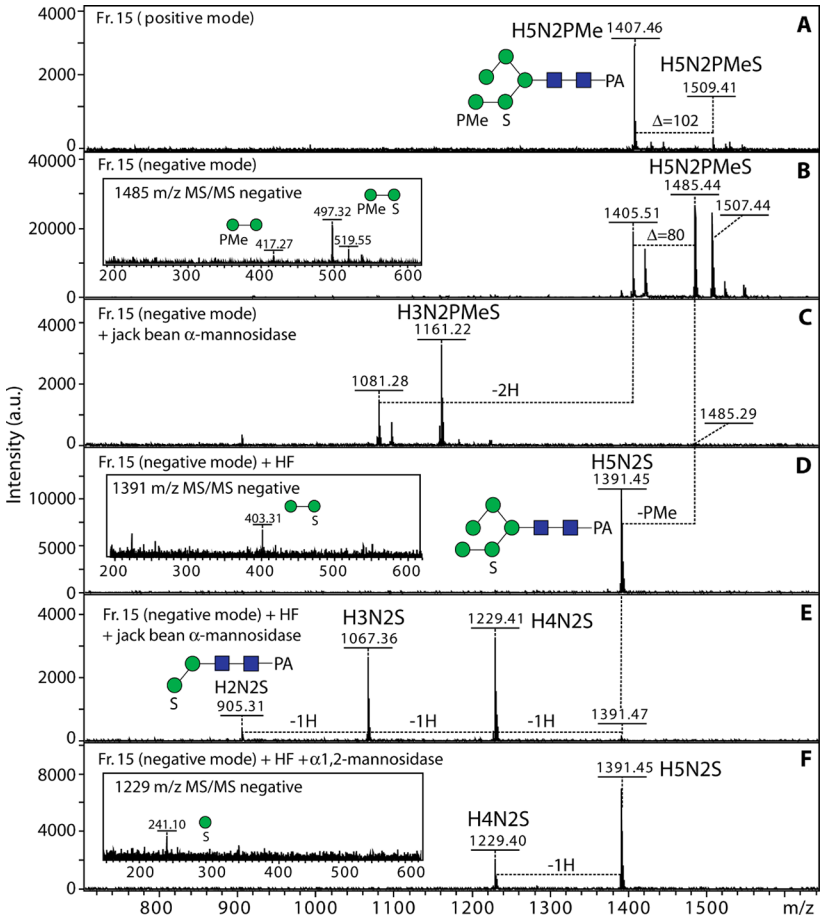

Figure 5. Structural determination of the major anionic N-glycan in the HL241 strain. After purification, the N-glycan structure $Hex_5HexNAc_2PMeS$ (HIAX fraction 15) was subjected to several treatments to determine the position of the anionic groups (PMe and S) and analyzed in the (A) positive-ion and (B–F) negative-ion modes. Jack bean α-mannosidase digested two mannose residues groups from the glycan resulting in the structure m/z 1161 ($Hex_3HexNAc_2PMeS$) detected in the negative ion mode. After HF treatment and release of the methylphosphate group, the α-mannosidase digests enabled the identification of the position of the sulfate group. The glycan is predominantly observed as $[M+H-SO_3]^+$ in the positive mode (together with some $[M+Na]^+$, $\Delta m/z$ +102) and as $[M-H]^-$ (with some $[M-SO_3]^-$ and $[M-2H+Na]^-$, $\Delta m/z$ −80 or +22) in the negative mode. The positive mode spectra for the mannosidase and/or HF treated forms of this glycan are shown in Supporting Information Figure S10.

and positional information was more complex than for HL241 and, therefore, no exact structures are proposed for anionic AX3 glycans, especially as to whether the terminal methylphosphate is on the B or C branch.

Di- and Triacidic N-Glycans

Among the doubly- and triply anionic glycans from the HL241 strain, which eluted exclusively in the acidic-enriched fraction from nonporous graphitised carbon, were structures carrying various combinations of modifications of 80 (sulfate or phosphate) or 94 Da (methylphosphate); multiply sulphated glycans were detected in the negative mode in their sodiated forms. In the HPLC fractions, maximally three anionic modifications were observed for the HL241 strain and five for the AX3 strain (Tables 1 and 2, Figure 2 and Supporting Information Figure S4); this does not preclude the presence, in low abundance, of glycans with further anionic substitutions.

One example of a dianionic glycan of m/z 1501, eluting at 4.2 min (RP-HPLC), is proposed to be modified with two methylphosphate groups. The presence of a positive-ion mode fragment of m/z 622 ($Hex_2HexNAc_1PMe$; Figure 4A) suggested that one of the methylphosphate groups was two hexose residues distant from the distal core GlcNAc residue. On the basis of removal of only one mannose residue from the upper arm by jack bean mannosidase (data not shown) and the fragmentation pattern, we propose that the $Hex_5HexNAc_2(PMe)_2$ glycan carries methylphosphate groups on both the "upper" α1,6-linked mannose and "lower" α1,2-linked mannose residues; the latter is a known position for the methylphosphate modification in glycans from the "sulphation-defective" HL244 strain.[3] A further dianionic glycan from the HL241 strain, from which only jack bean mannosidase removed two residues (data not shown), is presumed to carry two sulfate groups on the α1,3-arm as shown by a negative-ion mode sodiated fragment at m/z 505 (Hex_2S_2; Figure 4C).

The next class of anionic glycans in the PNGase F-released pool displayed mixed modifications (e.g., $Hex_5HexNAc_2PMeP$, $Hex_5HexNAc_{2-4}PMeS$ and $Hex_6HexNAc_2PMeS_{1-2}$). Small amounts of such mixed diacidic glycans were also found in the PNGase A-released pool of HL241 glycans ($Hex_5HexNAc_2Fuc_1PMeS$; see Table 2 and Supporting Information Figure S4). To illustrate the distinctive modifications and their positions, two glycans in two different RP-HPLC fractions (PNGase F acidic-enriched; 3.8 and 6.2 min) as well as in two different HIAX fractions (14 and 15) were analyzed which apparently possess the same mass. The first,

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$Hex_5HexNAc_2PMeP$, is observed as m/z 1485 in negative mode and m/z 1487 in positive-ion mode (Figure 4E,F). For this glycan, fragments of m/z 243/257 ($Hex_1P[Me]_{0-1}$), 405/419 ($Hex_2P[Me]_{0-1}$) and 1069/1083 in positive mode (Figure 4B) as well as its HF sensitivity (Figure 4G) suggested the presence of a "methylphosphate/phosphate pair" on a glycan with two mannose residues on each arm. The modification with phosphate is presumed to be on the subterminal α1,6-linked mannose residue as judged by the presence of a presumed $Hex_2HexNAcP$ fragment (m/z 608) and the removal of one hexose residue by jack bean mannosidase; analogous to the structures of other glycans such as $Hex_5HexNAc_2(PMe)_2$ (see Figure 4A), we propose that the methylphosphate modification is attached to the terminal mannose on the A-antenna.

The second glycan, $Hex_5HexNAc_2PMeS$, was detected in positive mode as $[M+H-SO_3]^+$ and $[M+Na]^+$ species (Figure 5A; m/z 1407 and 1509); however, it was best observed in negative mode (Figure 5B) in its $[M-H]^-$ form (m/z 1485) and was also the glycan proven to contain sulfate in the aforementioned FTICR MS experiment (Supporting Information Figure S8). Compatible with the insensitivity of sulfate toward HF, this glycan only lost one modification of 94 Da (methylphosphate) when treated with this reagent (Figure 5D; see also Supporting Information Figure S10). In terms of the MS/MS analyses, particularly interesting was the negative-ion mode fragment of m/z 497 (Hex_2PMeS, Figure 5B) which suggested that there was a methylphosphate on the terminal α1,2-mannose and sulfate on the penultimate α1,3-linked mannose of the A-antenna. Indeed, after hydrofluoric acid treatment (resulting in a Hex_2S fragment; m/z 403, Figure 5D), α1,2-mannosidase could remove one terminal mannose (resulting in a Hex_1S fragment; m/z 241, Figure 5F), whereas jack bean mannosidase treatment resulted in the loss of two mannose residues before acid treatment and three mannose residues afterward (Figure 5C,E; see also Supporting Information Figure S10). The putative subterminal position of the sulfate residue and terminal position of methylphosphate is compatible with previously hypothesized models for glycan structures in the HL241 strain.[29]

A triacidic glycan from the HL241 strain was predicted to have the structure $Hex_6HexNAc_2PMeS_2$; a key fragment to support its structural identification was one of m/z 761 suggestive of Hex_3PMeS_2 in sodiated form, thus indicating that all three anionic moieties are present on the same arm (Figure 4D). Multiacidic glycans, such as $Hex_8HexNAc_2(PMe)_2S_{1-3}$ and $Hex_8HexNAc_3(PMe)_2S_{1-2}$, were also observed in the PNGase F-released pool derived from the AX3 strain (Table 1). In contrast, no triacidic glycans were found in the PNGase A-released pools of fucosylated HL241 or AX3 glycans.

DISCUSSION

Dictyostelium As a Model for Congenital Disorders of Glycosylation

Previous data, employing HPLC, exoglycosidase digestion and methylation analysis regarding the lipid-linked glycans from the mannosyltransferase-defective HL241 strain,[11] indicating a major structure of Manα1,2Manα1,2Manα1,3-(Manα1,3Manα1,6)Manβ1,4GlcNAcβ1,4GlcNAc, are upheld by our MS-based data on both lipid- and protein-linked glycans. Therefore, we predicted that the *alg9* gene of the HL241 strain carries a mutation. Indeed, sequencing of the *alg9* gene of this strain revealed that there is a nonsense mutation resulting in a premature stop codon; the identified defect is compatible with the known biochemical effects of *alg9* mutations in yeast and in humans.

In the yeast *alg9* mutant, $Man_6GlcNAc_2$ is the dominant lipid-linked glycan, but the leaky specificity of Alg12p results in the formation of some $Man_7GlcNAc_2$ species if ALG12 is overexpressed in the yeast *alg9* strain.[30] Indeed, the traces of $Hex_{7-8}HexNAc_2$ in the lipid-linked glycan fraction and of the mannosidase-sensitive (data not shown) $Hex_7HexNAc_3$ in the protein-linked glycan fraction of the slime mold HL241 strain may indicate some "leaky" specificity of another mannosyltransferase (possibly also Alg12) in *Dictyostelium*. In the case of human *ALG9* homozygous point mutations, premature stop codons have not been found, but the missense mutations E523K and Y286C, affecting residues also conserved in the *Dictyostelium* ALG9, have been reported in patients with the type I congenital disorder of glycosylation IL (recently renamed ALG9-CDG).[31,32] In these patients, the dolichol-linked oligosaccharides are primarily $Man_6GlcNAc_2$ and $Man_8GlcNAc_2$;[31] this is suggestive of an incomplete defect in the activity of ALG9, compatible with the presence of a "faulty" but complete open reading frame. This contrasts to the case of the truncated predicted reading frame of the HL241 mutant.

Anionic Modifications of Dictyostelium N-Glycans

The N-glycome of *Dictyostelium discoideum* is striking due to the presence of glycan structures unknown in other organisms. The range of proven modifications encompasses core fucosylation, intersected and bisected *N*-acetylglucosamine, methylphosphate and sulfate. However, mass spectrometric evidence that a single N-glycan can contain up to four of these features has been lacking to date. We have previously shown that a major neutral N-glycan of wild-type *Dictyostelium* released by PNGase A is a core α1,3-fucosylated glycan with both intersecting and bisecting residues;[1] furthermore, NMR and mass spectrometric data indicated the presence of one or two methylphosphate and one intersecting residues on endoglycosidase H-released N-glycans.[3,33] Only recently was mass spectrometric evidence obtained for the modification of *Dictyostelium* N-glycans by sulfate.[5] A monoclonal antibody recognizing clusters of mannose-6-sulfate, known as the common antigen 1 (CA1) epitope, has been described to bind wild-type proteins;[34] while still sulphated to some degree, such clusters are apparently absent from the glycans of the HL241 mutant.[11] In the present study, complementary digestion and fragmentation data enable us to propose the locations of sulfate modifications.

Results from anion-exchange chromatography of radiolabeled glycans indicated that these contain maximally two methylphosphate residues and, in total, up to three acidic groups in the HL241 strain,[10] in contrast to maximally four methylphosphate residues and six anionic modifications in three wild-type axenic strains.[6,10,35,36] Our new mass spectrometric data demonstrates up to respectively two or three methylphosphate moieties on glycans of the mutant HL241 ($Hex_5HexNAc_2[PMe]_2$) and of the wild-type AX3 ($Hex_8HexNAc_2[PMe]_3$). At least in the fractions analyzed, a maximum of two sulfate residues is detected ($Hex_8HexNAc_4Fuc_1S_2$ in the wild-type and $Hex_{5-6}HexNAc_2S_2$ in the mutant) in the absence of methylphosphate. We also found glycans with both sulfate and methylphosphate possessing maximally three acidic groups in HL241

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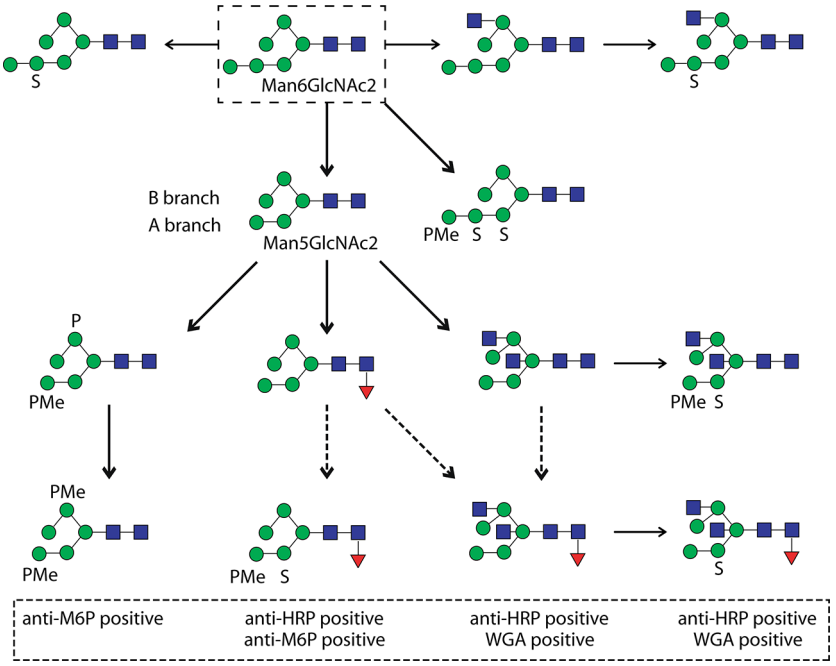

Figure 6. Post-transfer processing of N-glycans in the *Dictyostelium* HL241 strain. A proposed scheme for the modification of N-glycans in the HL241 strain commencing after the transfer of $Man_6GlcNAc_2$ (boxed). Whereas the anionic modifications and the intersecting *N*-acetylglucosamine residue can be found on both $Man_5GlcNAc_2$ and $Man_6GlcNAc_2$ scaffolds, the transfer of fucose and bisecting *N*-acetylglucosamine appears to be dependent on the prior removal of one terminal α1,2-mannose residue from the A branch. Reactions for which there is evidence from the literature are shown with *solid arrows*; for some reactions involving fucosylated glycans, the order of processing is unclear and so these are indicated with *dashed arrows*. The predicted antibody or lectin reactivity status (anti-Man6P, anti-HRP or WGA) of the four glycans at the foot of the diagram is also indicated.

($Hex_5HexNAc_2[PMe]_2S_1$ and $Hex_6HexNAc_2PMeS_2$) and five in AX3 ($Hex_8HexNAc_2[PMe]_2S_3$).

Previous radiolabeling data suggested that sulphation can occur on fucosylated glycans or on phosphorylated glycans in a mutually exclusive manner.[37] This, however, is not in accordance with our new data; indeed, sulfate is present not only on PNGase F-released glycans, but also on fucosylated ones released by PNGase A. Due to the use of the latter enzyme rather than endoglycosidase H, we also reveal, for the first time, that methylphosphorylated glycans can also carry core α1,3-fucose. Furthermore, we have detected one glycan of low abundance carrying fucose, sulfate, and methylphosphate ($Hex_5HexNAc_2FucPMeS$) in HL241; however, no glycan carrying all three of these modifications contained bisecting or intersecting GlcNAc. In the wild-type AX3, we have detected glycans with fucosylation, bisecting and intersecting GlcNAc with either sulfate ($Hex_{7-8}HexNAc_4FucS_{1-2}$) or methylphosphate ($Hex_8HexNAc_4FucPMe_1$). Thus there seems to be an "upper limit" for the number of modifications on a *Dictyostelium discoideum* N-glycan, at least in terms of those structures detected.

Analyzing Anionic Glycans in Dictyostelium

That a greater range of acidic structures is identified in our study, as compared to maximally monoacidic species found by Feasley et al.,[5] is possibly due to a range of factors. First, we analyzed complete cells as compared to a single overexpressed protein (gp130), thus there is a potential for greater variability. Second, we have performed solid phase extraction to enrich the anionic N-glycans. Third, we have employed both reversed-phase and mixed sizing and anion exchange (HIAX) columns, the latter having the advantage of excellent compatibility with later MALDI-TOF MS analyses. Fourth, the choice of ATT as a matrix for both positive and negative-mode MS resulted in better sensitivity compared to our previous use of DHB. Our data indicate that both solid phase extraction prior to labeling and HPLC fractionation afterward are important for maximizing the estimation of the glycomic potential of a species. The use of two HPLC columns results in complementary elution patterns, with RP-HPLC resulting in, e.g., early eluting fractions of mixtures of anionic glycans, as well as facilitating determination of the isomeric status of oligomannosidic glycans[25] and the type of core fucosylation (α1,3- or α1,6),[38] and HIAX which results in late-eluting fractions of often pure glycans with multiple anionic groups.

We have verified all the structures by MS/MS, in positive and negative modes, in order to define the positions of the sulfate and methylphosphate moieties in the HL241 *alg9* mutant; we have also detected simple phosphorylation as another modification in low abundance. It is not clear whether the strong reactivity of both AX3 and HL241 glycoproteins with the single-chain anti-Man6P antibody fragment is due to detection of these traces of nonmethylated phosphate or to cross-reactivity with the more abundant methylated phosphodiester. Interestingly, in the $Hex_5HexNAc_2PMeP$ glycan, unmodified phosphate is present on the α1,6-linked mannose of the trimannosyl core and not on a terminal mannose; this position was not shown to be modified in an NMR analysis of a doubly methylphosphated slime mold glycan[3] and was also not found to be phosphorylated in mammalian lysosomal proteins.[39] Indeed, 6-phosphorylation of this residue is only possible when the Alg9 α1,6-mannosyltransferase, which would otherwise modify the same hydroxyl, is defective.

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Biosynthesis of N-Glycans in Dictyostelium

In general, in eukaryotes transferring $Glc_3Man_9GlcNAc_2$ to proteins, the first events in the endoplasmic reticulum are removal of the three glucose residues and of the mannose from the so-called B branch to result in the Man8B isomer.[20] However, in the AX3 wild-type *Dictyostelium*, we observe Man8A as the dominant form of $Man_8GlcNAc_2$, which would result from trimming the A branch. On the basis of our data, we also observe a form of $Man_5GlcNAc_2$ in HL241 which would result from processing of the A branch (Figure 6). This processing step opens the way for the action of the bisecting and intersecting *N*-acetylglucosaminyltransferases to result in $Man_5GlcNAc_{3-4}$; when, however, the A-branch is not processed, then only the intersecting GlcNAc is preferentially transferred as evidenced by the lack of $Man_9GlcNAc_4$ in the wild-type and $Man_6GlcNAc_4$ in the mutant. Indeed, in vitro data indicates that $Man_9GlcNAc_2$ is a substrate for the intersecting, but not bisecting, enzyme.[2] Apparently, the A-branch also controls core α1,3-fucosylation as evidenced by the lack of $Man_9GlcNAc_2Fuc$ in AX3 and $Man_6GlcNAc_2Fuc$ in HL241.

The presence of unmodified phosphate in the HL241 strain is also noteworthy as this was previously undetected in *Dictyostelium*; however, its occurrence is not unexpected considering the biosynthetic pathway. First GlcNAc-1-phosphate is transferred to mannose residues,[40] possibly in the *cis*-Golgi by comparison to mammals.[41] Then the capping GlcNAc is removed and finally methylation occurs;[42] thus, unmodified phosphate is an intermediate during biosynthesis of the methylphosphate modification. The exact order of reactions to result in sulphated glycans is not clear, but both fucosylated and nonfucosylated glycans are modified; at least in higher organisms, sulphation is often a late event during glycan processing in the Golgi.[43] Furthermore, early biosynthetic studies in *Dictyostelium* suggested that fucosylation precedes sulphation.[44]

CONCLUSIONS

Our study probably presents the most detailed study of the neutral and acidic N-glycans of the slime mold to date, with a focus on the glycans of a *Dictyostelium* model for a human disease, and indicates the necessity of adequate fractionation of the glycome in order to analyze single oligosaccharide species. Such work is a necessary foundation for later studies in order to explore the glycoproteins modified with the different glycans as well as partners recognizing these glycans and the biological function of such moieties; indeed, specific N-glycopeptides (of albeit unknown structure) may be involved in aggregation of *Dicytostelium*,[45] a process necessary for development of fruiting bodies. In the future, it will also be of interest to identify specific glycoproteins carrying the fucose, methylphosphate, phosphate, and sulfate epitopes in this species.

ASSOCIATED CONTENT

Supporting Information

Summary of the analytical workflow (Figure S1); detection of different epitopes in the AX3 and HL241 strains (Figure S2); N-glycans of the cellular *Dictyostelium discoideum* wild type (AX3) and Alg9 mutant strain (HL241) (Figure S3); RP-HPLC of HL241 glycans (Figure S4); RP-HPLC and MS/MS of different oligomannosidic structural isomers from HL241 and AX3 (Figure S5); chemical and enzymatic treatments of neutral N-glycans from the mutant strain HL241 (Figure S6); MS/MS analysis of truncated N-glycans of the *alg9* mutant HL241 carrying fucose, bi and/or intersecting GlcNAc modifications (Figure S7); MALDI-FTICR-MS analysis of the $Man_5GlcNAc_2(PMe)S$ structure (Figure S8); analysis of the major acidic N-glycans in *Dictyostelium discoideum* wild type strain AX3 (Figure S9); and positive mode MALDI-TOF MS analysis of the major anionic N-glycan in the HL241 strain (Figure S10). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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