Published in final edited form as:

Electrophoresis. 2014 August ; 35(15): 2116–2129. doi:10.1002/elps.201300612.

N-glycomic profiling of a glucosidase II mutant of *Dictyostelium discoideum* by ‘off-line’ liquid chromatography and mass spectrometry

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Abstract

In this study, we have performed the first mass spectrometric analysis of N-glycans of the M31 mutant strain of the cellular slime mould *Dictyostelium discoideum*, previously shown to have a defect in glucosidase II. Together with glucosidase I, this enzyme mediates part of the initial processing of N-glycans; defects in either glucosidase are associated with human diseases and result in an accumulation of incorrectly-processed oligosaccharides which are not, or only poor, substrates for a range of downstream enzymes. To examine the effect of the glucosidase II mutation in *Dictyostelium*, we employed off-line LC-MALDI-TOF-MS in combination with chemical and enzymatic treatments and MS/MS to analyse the neutral and anionic N-glycans of the mutant as compared to the wild-type. The major neutral species were, as expected, of the composition Hex₁₀₋₁₁HexNAc₂₋₃ with one or two terminal glucose residues. Consistent with the block in processing of neutral N-glycans caused by the absence of glucosidase II, fucose was apparently absent from the N-glycans and bisecting N-acetylglucosamine was rare. The major anionic oligosaccharides were sulphated and/or methylphosphorylated forms of Hex₈₋₁₁HexNAc₂₋₃, many of which surprisingly lacked glucose residues entirely. As anionic N-glycans are considered to be mostly associated with lysosomal enzymes in *Dictyostelium*, we hypothesise that glycosidases present in the acidic compartments may act on the oligosaccharides attached to such slime mould proteins. Furthermore, our chosen analytical approach enabled us, via observation of diagnostic negative-mode MS/MS fragments, to determine the fine structure of the methylphosphorylated and sulphated N-glycans of the M31 glucosidase mutant in their native state.

Keywords

Glycans; glycomics; sulphate; methylphosphate

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Conflicts of Interest

None

1. Introduction

N-glycosylation is almost ubiquitous in eukaryotes and is also known in bacteria and archaea ⁽¹⁾. However, a high variability in terms of finally-processed structures is obvious when comparing different species, which is reflected also in the types of enzymes present. Nevertheless, amongst eukaryotes which are not unicellular obligate parasites, the initial stages of the N-glycosylation process (precursor formation, oligosaccharyl transfer and initial trimming) are highly conserved ⁽²⁾. After transfer of a tetradecasaccharide containing three glucose, nine mannose and two *N*-acetylglucosamine residues (Glc₃Man₉GlcNAc₂) to protein, the three glucose residues are removed by α -glucosidases I and II, which are resident in the endoplasmic reticulum and have a neutral pH optimum ^(3, 4). Two human diseases are associated with defects in these enzymes: mutations of glucosidase I are associated with a congenital disorder of glycosylation ⁽⁵⁾, whereas defects of the β -subunit of glucosidase II are found in some patients with polycystic liver disease ⁽⁶⁾. Homologues of genes encoding glucosidase I and the α and β subunits of glucosidase II ^(7, 8) are present in a range of organisms including the cellular slime mould (alternatively known as a social amoeba) *Dictyostelium discoideum*.

The biological role of glucosidase II (EC 3.2.1.84) has been assessed with a range of mutant cell lines and organisms, including *Arabidopsis thaliana*, *Candida albicans*, *Saccharomyces cerevisiae* and *Trypanosoma brucei* knock-outs as well as a hypercellulolytic strain of *Trichoderma reesei*, a mouse lymphoma cell line and the *Dictyostelium discoideum* *modA* (M31) mutant ⁽⁹⁻¹⁵⁾. A recurring feature of the plant and fungal mutants is the occurrence of cell wall phenotypes suggesting that glucosidase II is required for proper formation of cell walls; indeed in *Dictyostelium*, which also produces cellulose, stunted fruiting bodies are the obvious morphological phenotype of the *modA* strain. Otherwise, the *modA* mutant displays some growth retardation and alterations in the levels of some intracellular and secreted glycosidases ⁽¹⁶⁾. It is possible that the binding to ER lectins involved in quality control in the secretory pathway is affected by a lack of glucosidase II activity; certainly, calnexin binds Glc₁Man₉GlcNAc₂ (the intermediate product of glucosidase II during trimming or the product of UDP-glucose:glycoprotein glucosyltransferase), but not Glc₂₋₃Man₉GlcNAc₂ ⁽¹⁷⁾ and an absence of glucosidase II may in some cases prevent association with calnexin and so result in increased degradation of proteins in the endoplasmic reticulum ⁽¹⁸⁾.

D. discoideum has proven to be an interesting model organism as it undergoes, under conditions of environmental stress, the transition from a single-cell amoeba to a multicellular organism with fruiting bodies; it also has an alternative sexual lifecycle ⁽¹⁹⁾. Over the past thirty years, there have been a number of studies regarding the N-glycosylation of this species. The consensus is that the finally processed N-glycans contain a number of features unusual for oligomannosidic-type structures: methylphosphate, sulphate, bisected GlcNAc, intersected GlcNAc and core α 1,3-fucose residues are found, whereas there are shifts in the N-glycome during development from amoebae to fruiting bodies ⁽²⁰⁾. Some mutants with defects in N-glycosylation are known, but much of the available information on the glycans of wild-type and mutant strains was based on radiolabelling studies and not on mass spectrometry. Recently, though, studies from two laboratories have used a combination of HPLC and MALDI-TOF MS to elucidate the structures of many N-

glycans from *D. discoideum* including some from glycosylation mutants ⁽²¹⁻²³⁾. Key to the workflow in our own laboratory has been the use of a number of separation techniques, including solid-phase extraction early on in the analyses followed by either reversed phase (RP) and hydrophilic-interaction/anionic-exchange (HIA-X) HPLC in order to yield fractions with lower complexity, thus avoiding suppression of a number of the glycans in the subsequent mass spectrometric (MS and MS/MS) analyses.

In this study, we apply our methodology to examination of the N-glycans of a glucosidase II mutant of *D. discoideum*; this M31 strain has been previously shown to have altered size and charge of its N-glycans, which are rescuable effects phenocopied by a specific knock-out of the gene encoding the glucosidase II catalytic α subunit ⁽²⁴⁾. Thereby we note a reduced glycomic complexity as compared to the wild-type, but also the presence of anionic oligosaccharides with unexpected backbone structures. The most likely explanation is that glycosidases in cell compartments other than the endoplasmic reticulum and Golgi apparatus contribute to the final spectrum of N-glycans present in the slime mould.

2. Experimental Procedures

2.1 Slime mould cultivation

The M31 (glucosidase II mutant) and Ax3 (pseudo axenic wild type) strains were obtained from the Dictyostelium Stock Centre and grown in HL5 liquid medium according to standard techniques ⁽²⁵⁾.

2.2 Cloning and sequencing of the glucosidase II (*modA*) gene

Based on the sequences in Dictybase (gene ID: DDB_G0269154) and GenBank (accession U72236), the open reading frame cDNA encoding *modA* was isolated from mutant and wild type cells using TRIZOL and SuperScript RT (both from Invitrogen). For the PCR reaction combination of two forward *modA*-1 (GGATCGATTGAATCAGTAGAT), *modA*-3 (GAATCTGGTATTATCGATGTATTC) and two reverse primers *modA*-2 (ATATTTGTTTGTTCCTTTTATTTAT), *modA*-4 (CAGTATCAACTAATAAATCTGG) were used with GoTaq polymerase. The purified PCR products (GFX purification kit, GE Healthcare) were ligated into pGEM-T vector (Promega) and transformed into *E. coli* JM 109 cells. The sequencing of the plasmid DNA was performed by MWG and sequence alignments were done using the Multalin server.

2.3 Expression of *P. pastoris* ER mannosidase Mns1

The *Pichia* homologue of *S. cerevisiae* ER mannosidase I was identified by blasting the *S. cerevisiae* *MNS1* coding sequence (gene ID 853595) against the *P. pastoris* GS115 genome (www.pichiagenome.org). The *P. pastoris* *MNS1* gene was amplified from genomic DNA (Q5 polymerase, NEB) using the following primers: MNS1_KpnI_fw (ATCGGTACCATTACAAATTTCTGGCCGG) and MNS1_XbaI_rv (ATTCTAGATCCGATGATAAAGGTGAT). After digestion with the respective enzymes, the partial open reading frame encoding the catalytic region without the original leader peptide was ligated into the pPICZαA vector to produce a C-terminal His-tagged fusion protein. The sequence was confirmed by Sanger sequencing and after linearization using

DraI, the linearized plasmid was used to transform *P. pastoris* X-33 cells. Selected positive clones were grown in YPD medium (yeast extract, peptone and dextrose) overnight prior to transfer to YP medium containing 1% methanol for a total of 48 hours at 25°C. Expression of secreted recombinant mannosidase in the medium was verified by Western blotting using a mouse His-tag specific primary antibody in combination with an anti-mouse-IgG alkaline phosphatase conjugated secondary antibody. Protein activity was confirmed using PA-labeled Man₉GlcNAc₂ glycan as substrate (data not shown). His-tag affinity purification was essentially performed as described previously using Ni-NTA agarose (Qiagen); recombinant protein was eluted with 500 mM imidazole and then desalted using Vivaspin columns (30 kDa NMWL, Millipore) prior to storage in 20 mM TrisCl, 25 mM NaCl, pH 7.5 at 4°C.

2.4 Expression of GH99 endo- α -mannosidase

The plasmid encoding *Bacteroides xylanisolvens* (BxGH99) endo- α -mannosidase in pET30a was kindly provided by Prof. Gideon J. Davies⁽²⁶⁾ and used to transform *E. coli* Rosetta (DE3) cells. Starter cultures were grown at 37°C overnight in LB medium containing kanamycin and used to inoculate 10 ml of LB/kanamycin at an OD₆₀₀ = 0.05. Cells were grown at 37°C until an OD₆₀₀ of 0.6 was reached. The temperature was reduced to 16°C and protein expression was induced overnight by adding IPTG (isopropyl β -D-1-thiogalactopyranoside) to a final concentration of 500 μ M. Cells were harvested by centrifugation and resuspended in 1 ml lysis buffer (400 mM NaCl, 100 mM KCl, 40 mM Tris, 10 mM imidazole, pH 7.8). Cellular protein was released by three consecutive freeze-thaw treatments and by a single sonication step. Cell debris was collected by centrifugation and the supernatant was kept for protein purification. Recombinant protein was detected by Western blotting using a His-tag sensitive primary antibody and purified on Ni-NTA agarose as described above.

2.5 Release and purification of N-glycans

Cellular material (2-3 g wet weight) was heat-treated, homogenised by sonication and proteolysed with pepsin, prior to purification of the (glyco)peptides as in our previously-published procedures^(27, 28). After cation-exchange and gel filtration chromatography, (glyco)peptides dissolved in 20 mM ammonium bicarbonate (pH 8) were incubated with PNGase F (Roche) overnight at 37 °C, prior to addition of an excess of 20 mM ammonium acetate (pH 5) and incubation with PNGase A overnight. Subsequent Dowex chromatography enabled the separation of the free N-glycans from the residual (glyco)peptides. The free N-glycans were subject to solid phase extraction using non-porous graphitised carbon (NPGC) as previously published⁽²³⁾; the resulting neutral and anionic pools of N-glycans were labelled with 2-aminopyridine prior to subsequent HPLC and MALDI TOF MS/MS analysis.

2.6 Separation of neutral and anionic and N-Glycans

Separation of the labelled N-glycans was performed using one of two HPLC columns. The hydrophilic-interaction/anion-exchange (HIAx) system was successfully applied for *Dictyostelium* N-glycans using an IonPac AS11 column (Dionex) as described

previously⁽²³⁾. A two solvent gradient was applied using buffer A (0.8 M ammonium acetate, pH 3) and buffer B (80% acetonitrile) 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. A pool of pyridylaminated oligomannosidic N-glycans from white beans (containing Man₇₋₉GlcNAc₂) was used to calibrate the column. Reverse-phase (RP) HPLC (Agilent Hypersil, ODS 5 μ) was performed using buffer C (0.1 M ammonium acetate, pH 4) and the gradient was a 1% increase of buffer D (30% methanol) per minute for 30 minutes at 1.5 ml/min. The column was calibrated with a dextran standard (2-22 g.u.). The fluorescence was measured at 320 nm (excitation) and 400 nm (emission) using a Shimadzu RF-20A XS detector.

2.7 N-glycan analysis with MALDI-TOF MS

Collected peaks from HPLC were dried and reconstituted in deionized water and analysed by MALDI-TOF MS using 6-aza-2-thiothymine as matrix. The peptide standard calibration II was used as a mass calibrant for the N-glycans. The MS and MS/MS (laser-induced dissociation) spectra were generated using either an Ultraflex MALDI TOF/TOF or an Autoflex Speed MALDI TOF/TOF in positive and negative reflectron ion modes. Spectra were processed with the manufacturer's software (Bruker Flexanalysis 3.3.80) using the SNAP algorithm with a signal/noise threshold of 6 for MS (unsmoothed) and 3 for MS/MS (smoothed four times) and thereafter analysed manually.

2.8 Chemical and enzymatic treatments of the N-glycans

Digestions of pyridylaminated N-glycans with commercial mannosidases were performed at 37 °C in 50 mM ammonium acetate (pH 5) buffer using either 0.2 μ l *Aspergillus saitoi* α 1,2 mannosidase (4 μ U, Prozyme) or 0.2 μ l *Canavalia ensiformis* (jack bean) mannosidase (0.02 U, Sigma Aldrich). Recombinant *B. xylanisolvens* (BxGH99) endo- α -mannosidase was incubated directly with 1 μ l aliquots of HPLC purified N-glycan for overnight at 37 °C. The recombinant *P. pastoris* ER mannosidase I was incubated with N-glycans at room temperature overnight in 80 mM MES pH 6.9 buffer containing 25 mM CaCl₂. Aliquots of glycans of the glucosidase-containing supernatant from an insect cell line (Hi5; 0.5 μ l) were incubated with purified N-glycans in 40 mM MES pH 6.9 at 30°C overnight. The methylphosphorylated N-glycans were subject to hydrofluoric acid (48% HF) treatment to cleave phosphodiester bonds on ice at 4 °C for 36 h prior to evaporation. The digests were either measured directly with MALDI-TOF MS or after a purification step using either C18 or NPGC solid phase extraction or RP-HPLC prior to analysis.

3. Results

3.1 Determination of the genetic defect in the M31 glucosidase II mutant

The *Dictyostelium* M31 mutant⁽¹⁶⁾ was previously shown to be phenocopied by a specific mutation of the *modA* glucosidase II gene in the DG1033 strain as well as be rescued by transformation with the wild-type gene under control of an actin promoter⁽²⁴⁾. However, the exact nature of the mutation in M31 was not known. RT-PCR using mRNA from the M31 strain was performed using primers specific for the *modA* gene and the fragments were ligated into a standard cloning vector; sequencing of clones derived from two independent PCR reactions showed the same G→A mutation at nucleotide 1931 of the open reading

frame which would result in a substitution of a conserved glycine residue by aspartate (Gly644→Asp; Figure 1). This amino acid is in a region associated with glucosidase II mutations in other organisms – in particular, the Ser599→Phe alteration in the *Arabidopsis* *rsu3* mutant⁽⁹⁾ and a frameshift in the *Trichoderma* Rut-30C strain⁽¹³⁾. We propose that this alteration results in a diminution or abolition of glucosidase II activity, which in turn has an impact on the N-glycome of *Dictyostelium*; indeed, in an initial screen for glycomic differences in M31, Western blotting with anti-horseradish peroxidase (anti-HRP), wheat germ agglutinin (WGA) and a single-chain antibody specific for mannose-6-phosphate showed, respectively, that core α 1,3-fucosylation is absent, that WGA reactivity (possibly to intersecting and bisecting GlcNAc) is reduced and that mannose-6-phosphate is still present (data not shown).

3.2 Glycomic screening workflow

Glycopeptides from axenically-grown cells of the M31 mutant strain were treated with both PNGase F and PNGase A prior to fractionation of the N-glycans by solid phase extraction: one pool, eluted with 40% acetonitrile alone from non-porous graphitised carbon, contained predominantly neutral N-glycans as judged by MALDI-TOF MS in positive and negative modes, whereas the second pool, eluted with 40% acetonitrile containing 0.1% trifluoroacetic acid was enriched in a set of anionic N-glycans. Similar to our study on another *Dictyostelium* mutant⁽²³⁾, the two pools were then subject to fluorescent labelling with 2-aminopyridine prior to MALDI-TOF MS (Figure 2), RP-HPLC and HPLC chromatography. Whereas HPLC is particularly useful for separation by size and charge (Figure 3), RP-HPLC can discriminate isomeric structures (Figure 4). Each HPLC fraction was analysed by MALDI-TOF MS and MS/MS; also selected glycans were incubated with enzymes, whereas many anionic glycans were treated with hydrofluoric acid to remove phosphodiester prior to re-analysis by mass spectrometry. A comparison to the corresponding pools of the wild-type AX3 was made; in terms of fluorescence of the two pools, the ratio of neutral- and anionic-enriched glycans was approximately 3:1 for both AX3 and M31.

3.3 Neutral N-glycans of the M31 mutant

As shown in Figure 2C and summarised in Table 1, neutral N-glycans ranging from m/z 1637 (Hex₇HexNAc₂) to m/z 2691 (Hex₁₁HexNAc₄) were observed. The two most dominant glycans of the mutant strain had the composition Hex₁₁HexNAc₂₋₃, as compared to Hex₈HexNAc₄Fuc₀₋₁ for the wild-type. The presence of the Hex₁₁HexNAc₂₋₃ as the major glycans would be compatible with a loss of glucosidase II activity which should theoretically result in a block in N-glycan processing at the stage of Glc₂Man₉GlcNAc₂; therefore, we presumed that two of the hexose residues of these structures were indeed terminal α -linked glucose moieties which would result in partial mannosidase resistance of the N-glycans. Glycans in the range Hex₈₋₁₁HexNAc₂₋₃ were analysed by MS and MS/MS before and after a range of enzymatic treatments. In the absence of relevant commercially-available glucosidases, an *N*-butyldeoxynojirimycin-sensitive α -glucosidase activity present in supernatants from insect cell cultures as well as a recombinant bacterial endomannosidase were used to treat selected glycans. Digestions with jack bean α -mannosidase or *Aspergillus*

or *Pichia* α 1,2-specific mannosidases with and without prior glucosidase or endomannosidase treatments were also performed.

As mentioned above, the two major neutral N-glycans as judged by intensity were Hex₁₁HexNAc₂₋₃ (m/z 2285 and 2488); these were the primary components of the two major RP-HPLC peaks (6.2 and 8.8 g.u.; Figure 4) as well as the two major HPLC peaks (fractions XI and XII; see Figure 3 and 5). MS/MS of these glycans results in a major Hex₆HexNAc₂-PA (m/z 1475) fragment (Figure 5L). Both were sensitive to glucosidase and endomannosidase digestions (Figure 5D and I), which resulted in a loss of the m/z 1475 fragment and appearance of ones of m/z 1151 and 989 respectively (not shown). Furthermore, the number of residues removed by jack bean α -mannosidase digestion of Hex₁₁HexNAc₂ increased from four (Figure 5C) to eight after glucosidase treatment (Figure 5E). Consistent with the predicted structure, an α 1,2-specific mannosidase removed two residues (not shown) and *Pichia* ER mannosidase only one (Figure 5B). Due to the presence of the 'intersecting' GlcNAc, Hex₁₁HexNAc₃ was more resistant to mannosidase treatments (i.e., only two hexose residues were removed by jack bean mannosidase and only one by *Aspergillus* α 1,2-specific mannosidase; Figure 5 G and H). On the basis of the fragmentation and digestion data, the conclusion is that the Hex₁₁HexNAc₂₋₃ structures are, as expected, capped with two glucose residues on the 'A' branch (see Figure 4 for a definition of the A, B and C branches).

The next most common neutral N-glycans were Hex₁₀HexNAc₂₋₃ (m/z 2123 and 2326). Hex₁₀HexNAc₂ was only found in one RP-HPLC fraction (5.8. g.u.) and digestions of Hex₁₀HexNAc₂ with glucosidase, ER mannosidase, α 1,2-mannosidase, endomannosidase and jack bean α -mannosidase digestion, analogous to those for Hex₁₁HexNAc₂ mentioned above, were compatible with a composition of Glc₁Man₉GlcNAc₂ (data not shown). Hex₁₀HexNAc₃ was detected in three RP-HPLC fractions (7.2, 8.2 and 9.4 g.u.; Figure 4); these forms could be distinguished on the basis of the results of glucosidase, endomannosidase and jack bean mannosidase digestions and by MS/MS. While MS/MS of the major isomer with 8.2 g.u. results in a dominant m/z 1313 fragment (Figure 5K), both minor forms show m/z 1475 fragments when subject to MS/MS. Based on the fragmentation pattern, the third HexNAc is assumed to be an intersected GlcNAc which also blocks the action of mannosidase; the major isomer is concluded to be a monoglucosylated form of Man₉GlcNAc₃ and the two minor isomers are suspected to be diglucosylated versions of Man₈GlcNAc₃ based on Man8B and Man8C scaffolds (see Figure 4 for a definition of Man8 isomers).

RP-HPLC also separated two isomers of Hex₉HexNAc₂ (eluting at 5.4 and 7.1 g.u.; see Figure 4). Again MS/MS and mannosidase digestion could distinguish these forms: the later eluting one carries two terminal glucose residues and has the composition Glc₂Man₇GlcNAc₂, whereas the earlier-eluting isomer has the standard Man₉GlcNAc₂ structure with which it shares the elution time in terms of glucose units. The Hex₈₋₉HexNAc₃ structures (m/z 2002 and 2164; 7.2 g.u. and 6.8 g.u.) lost four or five residues after jack bean mannosidase digestion, consistent with a lack of terminal glucose residues and the presence of an intersected GlcNAc. While the Hex₉HexNAc₃ possessed an m/z 1151 fragment (Figure 5J), the Hex₈HexNAc₃ glycan has an m/z 989 fragment and a

later elution time than Hex₉HexNAc₃; therefore, the latter is presumed to be a form of Man₈A carrying an intersected GlcNAc residue as in the wild-type.

3.4 Anionic N-glycans of the M31 mutant

The most dominant anionic N-glycans of the M31 mutant in the overall negative-mode mass spectrum carry one methylphosphate or one sulphate residue with traces of glycans carrying both modifications; this compares to the very obvious presence of dianionic and trianionic glycans in the overall spectrum for the anionic-enriched fraction of glycans from the wild-type AX3 strain (Figure 2 and Table 1). The surprise was that the major anionic glycans of the M31 mutant contained nine or ten hexose residues, as compared to the eleven hexose residues present in the most dominant neutral species; in the case of the wild-type, the majority of the neutral and the anionic glycans contain eight hexose residues. This was a first hint that unexpected processing occurs prior to the definition of the final glycosylation pattern of proteins carrying anionic glycans in the mutant.

As for the neutral glycans, HPLC fractionation was able to reveal a greater complexity in the anionic N-glycome of M31 than apparent from the complete spectrum and MALDI-TOF MS of the individual fractions showed the presence of glycans also containing two or three anionic residues in combinations such as Hex₉₋₁₀HexNAc₂(PMe)₂, Hex₈₋₁₁HexNAc₂₋₃PMeS, Hex₈₋₁₀HexNAc₂PMeS₂ and Hex₈₋₁₀HexNAc₂S₂₋₃. Whereas methylphosphate modified glycans were well observed in positive and negative ion modes, sulphated glycans were only detected in their intact form in the negative mode. As previously described, diagnostic for the presence of methylphosphate and sulphate were fragments of m/z 255 (Hex₁PMe) and 403 (Hex₂S) in the negative mode. A selection of MS/MS of anionic Hex₉-based structures is shown in Figure 6 and data on a number of anionic Hex₈-based glycans are presented in Figure 7.

The two most dominant anionic HPLC fractions contained Hex₉HexNAc₃PMe and Hex₁₀HexNAc₃PMe (fractions 9 and 11; see Figure 3). MS/MS of these showed the presence of a m/z 1106 fragment in negative mode (Hex₅HexNAc₁PMe; Figure 6D) and either 1151 or 1313 (Hex₄₋₅HexNAc₂-PA) fragments in positive mode (not shown); in contrast, a negative-mode fragment of m/z 1083 (Hex₃HexNAc₂PMe-PA) typical of wild-type glycans was not observed in any structure from the M31 strain. Hydrofluoric acid treatment resulted in loss of the methylphosphate moieties and after subsequent jack bean mannosidase digestion, respectively up to five or two mannose residues were removed, whereas subsequent α 1,2-mannosidase treatment resulted in loss of respectively three or one residues (data not shown). As prior to hydrofluoric acid treatment only four mannose residues could be lost upon jack bean mannosidase treatment from Hex₉HexNAc₃PMe (just two in the case of α 1,2-mannosidase), a terminal position for the methylphosphate is proposed. MS/MS of the related Hex₉HexNAc₂PMe glycan, lacking the intersecting GlcNAc, resulted in appearance of an m/z 903 fragment (Figure 6B) also indicative of a similar position of the methylphosphate on the α 1,6-arm.

The Hex₈HexNAc₂₋₃PMe structures were each found in single HPLC fractions, but also in two RP-HPLC fractions each. Analogous to the difference in retention time between Man₈ isomers⁽²⁹⁾ and based on the different fragmentation patterns upon MS/MS (especially the

presence of either m/z 989 or 1151; Figure 7 D and E), we propose that these are based on respective Man8A and Man8B scaffolds for the 3.7 and 4.5 g.u. RP-HPLC fractions of Hex₈HexNAc₃PMe and for the 3.7 and 3.3 g.u. fractions for Hex₈HexNAc₂PMe (see Figure 7F). RP-HPLC of HIAx fraction 6 following HF treatment indeed showed that the resulting Hex₈HexNAc₃ isomers from M31 eluted at 5.4 and 7.2 g.u., the latter co-eluting with the Hex₈HexNAc₃ derived from AX3 which is presumed to be based on Man8A (Figure 7C); similarly for Hex₈HexNAc₂PMe, HF treatment of HIAx fraction 5 resulted in two RP-HPLC peaks of 4.8 and 5.4 g.u. (data not shown), which correspond to literature values for the elution of Man8B and Man8A ⁽²⁹⁾.

Monosulphated Hex₈₋₁₁HexNAc₂₋₃S were detected in negative mode of individual HPLC fractions, but the levels of these were far lower than those carrying a single methylphosphate residue; example MS/MS spectra are shown in Figure 6A and C. Within the negative-mode spectrum of the entire anionic pool, the Hex₁₁HexNAc₃S species was most obvious monosulphated glycan; this lost three residues (i.e., two glucose and one mannose) upon endomannosidase digestion (data not shown). Unlike the mixture of isomers for methylphosphate-containing Hex8 structures, MS/MS data indicated that the Hex₈HexNAc₂S was a sulphated form of the Man8A isomer (data not shown).

Di- and tri-anionic glycans eluted later on the HIAx column than mono-anionic species, but earlier when performing RP-HPLC. Hex₉₋₁₀HexNAc₂(PMe)₂, resulted in a fragment of m/z 997 (Hex₅[PMe]₂) suggesting that both the B and C branches were modified with terminal methylphosphate residues (Figure 6G). An alternative form of di-anionic glycan species is represented by Hex₈₋₁₀HexNAc₂₋₃PMeS structures; MS/MS showed the presence of m/z 255 (Hex₁PMe) and 403 (Hex₂S) and, in some cases, 497 (Hex₂PMeS) fragments (Figure 6E and H). Jack bean mannosidase digestion of Hex₉HexNAc₂PMeS resulted in the loss four mannose residues; the subsequently observed m/z 741 and 821 fragments are proposed to be Hex₄PMeS₀₋₁ units (Figure 6F). In this case, the sulphate and methylphosphate are not on adjoining residues, but are respectively on the B and C antennae. Similarly, disulphated Hex₈HexNAc₂S₂ is predicted to be a Man8A structure also with subterminal sulphates on the B and C arms (Figure 7F).

A significant portion of the anionic glycans contained one methylphosphate and two sulphate moieties. MS/MS of Hex₉HexNAc₂PMeS₂ resulted in fragments of m/z 403, 1021 and 1101 in negative mode indicative of three anionic modifications of the α 1,6-branch (Figure 6I). The position of the second sulphate was more difficult to discern, but jack bean mannosidase digestion of Hex₈₋₉HexNAc₂PMeS₂ resulted in the loss of three or four mannose residues; the subsequently observed m/z 921 fragment is suggestive of a Hex₄PMeS₂ unit as [M-2H+Na]⁻. After hydrofluoric acid treatment of Hex₈₋₉HexNAc₂PMeS₂ to remove the methylphosphate, a further mannose residue could be lost upon jack bean mannosidase resulting in Hex₄HexNAc₂S₂ (data not shown). The 'lower' α 1,3-branch of the glycan is thereby concluded not to be modified; both sulphates are proposed to be on sub-terminal mannose residues of the non-digested structure in the context of standard Man8A and Man9 scaffolds (Figure 6I and 7F). The trisulphated Hex₈HexNAc₂S₃ is also concluded to be based on Man8A, due to its sensitivity to jack bean mannosidase (loss of three hexoses) as well as the m/z 989 positive-mode and m/z 504

(Hex₂S₂) and 1108 (Hex₅S₃) negative-mode fragments of the untreated glycan (see Figure 7F).

4. Discussion

4.1 Analysis and chromatography of N-glycans of lower eukaryotes

Recent years have seen an increase in the possibilities for glycomic analyses of high sensitivity⁽³⁰⁾; a primary tool is mass spectrometry, especially of LC-ESI-MS and MALDI-TOF-MS of native and permethylated N-glycans. As in a number of our previously-published studies, we have opted here for an off-line LC-MALDI-TOF MS approach in order to separate the N-glycans into fractions of lower complexity and facilitate their analysis by chemical and enzymatic treatments on a small scale. This enables us to also examine the structures of even low abundance structures in their native state. Due to the wide variability in the N-glycans of various protists and invertebrates there is no single analytical workflow, but glycomic analyses of each species type very often have to be tailored to suit the properties of the glycans present. Thus, whereas N-glycans of nematodes, cestodes or trichomonads are either neutral or zwitterionic⁽³¹⁻³³⁾, the protein-linked oligosaccharides of insects can be neutral, acidic or zwitterionic⁽³⁴⁻³⁶⁾ and the N-glycans of oysters are neutral or sulphated⁽³⁷⁾; in the case of trematodes apparently only neutral glycans have been reported^(38, 39).

Keeping in mind the historic literature on the N-glycans of *Dictyostelium*, primarily based on chromatography of radiolabelled glycans⁽⁴⁰⁻⁴²⁾ and one NMR/MS-centered study⁽⁴³⁾, the presence of both neutral and anionic glycans has to be considered. Although these classes are the same as in the oyster⁽³⁷⁾, a different approach is used by us when analysing glycans from these different sources; while no solid-phase extraction was necessary prior to HPLC of oyster N-glycans, we needed to pre-fractionate neutral and anionic N-glycomic pools from the slime mould prior to MS and HPLC analysis⁽²³⁾. This acts not only as a polishing step during the N-glycan purification protocol, which is not necessary in every case, but separates different types of glycans and so the type of solid-phase extraction varies. Graphitised carbon is a good choice for separating neutral from anionic glycans⁽⁴⁴⁾, whereas reversed-phase materials may be appropriate for clean-up of mixtures of neutral and zwitterionic N-glycans (unpublished observations). Subsequent fractionation by HPLC is also important to uncover the complexity of invertebrate glycomes; here, we have used both RP-HPLC, which is very suitable for examining isomeric glycans with the same mass, and HPLC, for size/charge separation. In other cases, 'pure' NP-HPLC may also be appropriate, as in the case of the oyster glycome⁽³⁷⁾, or a '2D'-combination of NP- and RP-HPLC as applied to *Caenorhabditis* and *Trichomonas*^(33, 45) in order to achieve almost complete separation into single structures; particularly the latter greatly simplifies interpretation of exoglycosidase digests and avoids co-fragmentation of other glycans in the same sample when performing MS/MS.

4.2 The glycomic impact of inhibition or ablation of glucosidase II

Glucosidase II is a key enzyme in the standard biosynthetic pathway for eukaryotic N-glycans whether from yeast or mammals⁽⁴⁶⁾. Inhibition or ablation of this enzyme is

expected to prevent further processing by glycosidases and glycosyltransferases in the secretory pathway. Previous data on the *Dictyostelium discoideum* M31 glucosidase II mutant have indicated that radiolabelled N-glycans are increased in size (as judged by gel filtration) and have a decreased degree of sulphation and phosphorylation (as shown by anion-exchange chromatography) in comparison to the wild-type^(15, 24, 47). Our new mass spectrometric data confirm that the N-glycome of *Dictyostelium* is strongly affected in the M31 glucosidase II mutant; nevertheless, our study indicates that a remarkable degree of processing still takes place. An increased degree of resistance of mannosidase digestion of M31 glycans was hypothesised to be due to two 'blocking' groups⁽¹⁵⁾: one being glucose and the other remained then undefined, but which is probably due to the intersecting GlcNAc detected in the present study.

The main neutral structures ($\text{Hex}_{11}\text{HexNAc}_{2-3}$ as compared to $\text{Hex}_8\text{HexNAc}_4\text{Fuc}_{0-1}$ in the wild-type) contain, as expected, the glucose residues which would otherwise be removed by glucosidase II; however, the modification with intersecting GlcNAc is seemingly not affected. On the other hand, core α 1,3-fucosylation is abolished and processing by mannosidases and the bisecting *N*-acetylglucosaminyltransferase is strongly reduced. The previous data on wild-type $\text{Hex}_8\text{HexNAc}_3(\text{PMe})_2$ ⁽⁴³⁾ and our own unpublished data regarding the major wild-type anionic N-glycans ($\text{Hex}_8\text{HexNAc}_2(\text{PMe})_2$ and $\text{Hex}_8\text{HexNAc}_3\text{PMeS}_2$) indicate the presence of methylphosphate on the A and C branches of a Man8A scaffold. The surprise in the mutant is with the anionic N-glycans; in this case the most dominant structure is $\text{Hex}_9\text{HexNAc}_3\text{PMe}$, with methylphosphate on the B or C branches, but which lacks any glucose residues. Therefore the question arises as to how the glucose residues are removed before the glycoproteins reach their final molecular form in the mutant.

Many studies on slime mould glycosylation have been performed on lysosomal enzymes which are induced to be secreted upon transfer of amoebae to a dilute buffer; these enzymes carry predominantly anionic N-glycans. Although the exact role of mannose-6-phosphorylation in targetting of lysosomal enzymes in *Dictyostelium* is unclear, it would appear that the presence of 6-methylphosphorylation and 6-sulphation is a characteristic of lysosomal enzymes in this phagocytic organism^(41, 48, 49). Thus it may be that a high proportion of glycoproteins which are modified by at least one anionic residue are directed to the lysosome where acidic glucosidases and mannosidases can act. Thereby, as summarised in Figure 8, we hypothesise that while an absence of the glucosidase II (which has a neutral pH optimum and resides in the endoplasmic reticulum) results in a reduction in the degree of modification by methylphosphate and sulphate (due to 'blocking' of the A branch), the action of glycosidases in the acidic lysosome results in removal of the glucose residues from the A branch and, therefore, the oligomannose scaffolds on the mature lysosomal glycoproteins of the M31 mutant are not unlike those of the wild-type (unlike the abolition of neutral glucosidase II activity, the levels of acidic α -glucosidase are not affected in the mutant⁽¹⁵⁾). The traces of deglycosylated neutral glycans may also result from the action of lysosomal hydrolases on proteins which have an anionic glycan at a separate N-glycosylated site; however, examination of the *Dictyostelium* genome would appear to rule

out the possibility of an endomannosidase capable of generating, as in vertebrates ⁽⁵⁰⁾, the Man8A isomer from a glucosylated Man9 glycan.

Some of the anionic glycans of the M31 strain contain only eight mannose residues with and without intersecting GlcNAc. Those containing solely methylphosphate were present as isomers based on Man8A and Man8B; the Man8A form is that isomer seemingly typical for *Dictyostelium* ⁽²³⁾, whereas Man8B is rare in this organism. In other organisms, these isomers result from the action of different enzymes: Man8A will result either from the action of endomannosidase on glucosylated glycans or from Golgi mannosidase I processing of deglycosylated glycans, whereas Man8B is the product of the endoplasmic reticulum (ER) mannosidase ⁽⁴⁶⁾. As deglycosylation in the endoplasmic reticulum does not occur in the M31 strain and there is no apparent endomannosidase homologue in the slime mould, an ER mannosidase in *Dictyostelium* has the chance to remove the α 1,2-linked mannose from the B branch, whereas the removal of mannose from the A branch by Golgi mannosidase I is blocked. According to our aforementioned hypothesis, removal of glucose and one mannose from the A branch is then only possible in the lysosome. Interestingly, there are three neutral isomers of Hex₁₀HexNAc₃ which lack either a mannose from the B or C branch or a glucose from the A branch.

4.3 Specificity of glycan-modifying transferases in *Dictyostelium*

The variability of the impact of the lack of glucosidase II on the different types of modifications (mannose trimming, fucosylation, bisecting and intersecting GlcNAc, methylphosphorylation and sulphation) enables us to make conclusions regarding the specificity of the relevant enzymes. Obviously, the action of the core α 1,3-fucosyltransferase, presumably located in the Golgi apparatus, is completely blocked if deglycosylation in the ER cannot take place; also, the bisecting *N*-acetylglucosaminyltransferase is significantly inhibited if the glucose residues are not removed. This may well be explained by the three-dimensional structure of the neutral M31 oligosaccharides. On the other hand, the position of the intersecting *N*-acetylglucosamine is still accessible to the relevant transferase ⁽⁵¹⁾ and so still around half of the N-glycans carry this residue in the mutant. For the anionic modifications, the lower A branch is no longer an acceptor for either the GlcNAc-1-phosphotransferase, which catalyses the first step in formation of the methylphosphate moieties ^(52, 53), or the sulphotransferases; however, the B and C branches can still be modified. Thus, the numbers of anionic groups on the glycans decreases, even though we do not detect a drastic shift towards neutral structures. The question of mannose trimming pathways in the presence of the glucose residues is more complicated as the removal of mannose by mannosidases outside the ER and Golgi is, as discussed above (see also Figure 8), probable. In the absence of detailed enzymatic data, there remain many unanswered questions regarding these pathways.

4.4 Conclusion

The ablation of glucosidase II has a major impact on the N-glycome of the slime mould *Dictyostelium discoideum* as well as on the morphology of its fruiting bodies; especially for the anionic glycans, the mass spectrometric definition of the structures resulted in some surprises. In terms of major structures, it was predominantly neutral glycans which were still

carrying diglucosylated ‘caps’, which may interfere with lectin-based quality-control mechanisms in the endoplasmic reticulum and so may have an impact on cell wall morphology. On the other hand, the scaffolds of the anionic glycans tended to be trimmed more than expected, thus suggesting that removal of glucose and mannose residues may occur in *Dictyostelium* in a manner independent of glucosidase II. The final definition of N-glycan processing in this model organism requires a set of recombinant enzymes and of substrates currently not available. However, by our studies, the chromatographic and mass spectrometric tools to analyse the glycomic complexity of this ‘not so simple’ system are now established.

Acknowledgments

The authors are grateful to Prof. Gideon Davies for the kind gift of the BxGH99 expression plasmid, to Dr. Shi Yan for help with preparing Figure 8 and to ao. Univ-Prof. Ebrahim Razzazi-Fazeli (Veterinärmedizinische Universität Wien) for access to the Ultraflex I mass spectrometer. This work was funded by grants from the Austrian Fonds zur Förderung der wissenschaftlichen Forschung (Austrian Science Fund) to D.R. (TRP127), I.B.H.W. (P19615 and I391) and K.P. (P21946).

Abbreviations

g.u.	glucose units
HIAX	hydrophilic-interaction/anionic-exchange
PA	pyridylamine

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Figure 1. Determination of the mutation in the M31 *modA* glucosidase II gene

(A) Electrophoretogram of the sequencing reaction of one of the partial M31 glucosidase II cDNA clones showing the location of the G→A mutation at nucleotide 1931 (marked with an asterisk) as compared to the wild-type cDNA and protein sequence. (B) Partial alignment of the *Dictyostelium*, *Trichoderma*, *Arabidopsis*, *Homo sapiens* and *Saccharomyces* predicted protein sequences showing also the positions of the amino acid changes resulting from mutations in the glucosidase II open reading frames isolated from M31 (missense), Rut-30C (frameshift) and *rsw3* (missense); amino acids conserved in all or all but one species are shown in blue and alterations are shown in red.

Figure 2. MALDI-TOF mass spectra of neutral and anionic N-glycans from two *Dictyostelium* strains

Complete MALDI-TOF mass spectra of pyridylaminated forms of the neutral (C) and anionic (D) N-glycans of the M31 strain, released using PNGase A and F and enriched by non-porous graphitised carbon solid-phase extraction, as compared to those of the wild-type AX3 (A and B). The glycan species are annotated with the m/z values (generally as $[M+H]^+$ or $[M+H]^-$, except for sulphated species as $[M-2H+Na]^-$) and the abbreviated glycan compositions of the form H_xN_y , where F is fucose, H is hexose, N is *N*-acetylhexosamine, PMe, methylphosphate and S sulphate.

Figure 3. HPLC chromatograms for neutral and anionic N-glycans from the M31 strain
 Sections of the chromatograms for the two pools of pyridylaminated N-glycans, with the major peaks, are shown and annotated with fraction numbers; in both cases, 20% of the pyridylaminated pools were applied, but the scale for the anionic chromatogram was multiplied by a factor of two for clarity. The elution positions for Man₇₋₉GlcNAc₂ standards isolated from beans are indicated; fraction numbers 1-26 (anionic) and I-XV (neutral) are annotated and the results of MALDI-TOF MS analyses of each fraction are summarised in Table 1. The two peaks marked with an asterisk are probable contaminants as judged by MALDI-TOF MS.

Figure 4. RP-HPLC of neutral N-glycans from the M31 strain

The section of chromatogram including all major peaks is shown annotated with a number of the significant Hex₉₋₁₁HexNAc₂₋₃ glycans depicted according to the nomenclature of the Consortium for Functional Glycomics (green circles, mannose; blue squares, *N*-acetylglucosamine). Also shown (right-hand panel) are the structures for standard Man8A, Man8B, Man8C and Man9 scaffolds and the positions of the A, B and C branches.

Figure 5. Analysis of major neutral N-glycans from the M31 strain

(A-E) MALDI-TOF MS of Hex₁₁HexNAc₂ before and after treatment by *Pichia* ER mannosidase I (ER Man I), jack bean α-mannosidase (JB Man), a glucosidase activity derived from insect cells and a combination of jack bean mannosidase and glucosidase. (F-I) MALDI-TOF MS of Hex₁₁HexNAc₃ before and after treatment by jack bean α-mannosidase (JB Man), *Aspergillus* α1,2-mannosidase (α1,2Man) and *Bacteroides xylanisolvens* endo-α-mannosidase (EndoMan). (J-L) MALDI-TOF MS/MS of Hex₉₋₁₁HexNAc₃ present in RP-HPLC fractions showing the *m/z* 1151, 1313 and 1475 fragments indicative of loss of the entire α1,6-arm as indicated by the red dashed line.

Figure 6. MS/MS of anionic N-glycans from the M31 strain

Negative-mode MS/MS spectra for a range of mono-, di- and tri-anionic N-glycans based on Man9 scaffolds are shown and annotated with the major diagnostic fragments. Glycans are depicted according to the nomenclature of the Consortium for Functional Glycomics (green circles, mannose; blue squares, *N*-acetylglucosamine; PMe, methylphosphate; S, sulphate). Selected differences of 80 Da are indicated with red dashed lines annotated with $\Delta=S$.

Figure 7. Definition of isomers of anionic Man8-based N-glycans

(A and B) HPLC fraction 6 of M31 glycans before and after HF treatment showing loss of 94 Da from the original glycan. (C) RP-HPLC of the same HPLC fraction after HF (M31) in comparison to a previously isolated Hex₈HexNAc₃ glycan from the wild-type (AX3) showing the presence of two isomers in the mutant. (D and E) MALDI-TOF MS/MS of the two dephosphorylated glycans found in the two RP-HPLC fractions. (F) Schematic RP-HPLC profile showing the relative elution positions in terms of glucose units (g.u.) of different Hex₈-based mono-, di- and tri-anionic N-glycans from the M31 mutant strain; red-dashed lines indicate positions of fragmentation and resulting major fragments in negative (blue) and positive (green) modes are annotated.

Figure 8. Hypothesised pathway for biosynthesis and post-Golgi processing of N-glycans in *Dictyostelium*

Based on the structural data, known biosynthetic data and our interpretation, we propose that the precursor $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ protein-bound glycans are processed differently in the M31 strain as compared to the AX3 strain (the latter pathways are shown within the red-dashed box). Initially, in the endoplasmic reticulum (ER, green) removal of glucose is incomplete and so in the mutant, some $\text{Glc}_2\text{Man}_9\text{GlcNAc}_2$ species are processed by removal of one mannose in the ER, whereas others reach the ER with no mannose removal. In the Golgi, methylphosphorylation occurs via a three-step process (GlcNAc-1-phosphorylation, removal of GlcNAc and methylation); also sulphation takes place in the Golgi apparatus (blue), but fucosylation is only apparent in the wild-type. Subsequently after transport of glycoproteins carrying anionic groups to the lysosomes (red), further trimming of glucose and mannose by acid hydrolases takes place in the mutant; potentially, the degree of processing in the wild-type Golgi is such that 'lysosomal processing' is minimal.

Table 1
Summary of the N-glycan structures analysed from the *Dictyostelium* M31 strain

HIAX fraction numbers (arabic for neutral and roman for anionic) are as annotated in Figure 3. The m/z values are from MALDI-TOF MS analyses of each fraction; the adduct status and the proposed compositions are given (H, hexose; N, N-acetylhexosamine, PMe, methylphosphate and S, sulphate).

Fraction (Neutral)		[M+H] ⁺	[M+Na] ⁺
I	H6N3	1678	1700
II	H7N2	1637	1659
III	H7N3	1840	1862
IV	H8N2	1799	1821
	H7N3	1840	1862
V	H8N2	1799	1821
VI	H8N3	2002	2024
VII	H9N2	1962	1984
VIII	H9N3	2164	2186
IX	H10N2	2123	2145
X	H10N3	2326	2348
XI	H11N2	2285	2307
XII	H11N3	2488	2510
XIII	H10N3PMe	2420	2442
XIV	H12N3	2650	2672
XV	H11N4	2691	2713

Fraction (Anionic)	Composition	[M+H] ⁺	[M-H] ⁻	[M-2H+Na] ⁻	[M-2H+K] ⁻	[M-3H+2Na] ⁻	[M-3H+Na+K] ⁻	[M-3H+2K] ⁻
1	H6N3PMe	1772	1770					
2	H8N2S		1877					
3	H7N3PMe	1934	1932					
4	H9N2S		2039					
5	H8N2PMe	1893	1891					
	H8N3S		2080					
6	H8N3PMe	2096	2094					
7	H10N2S		2201					
8	H9N2PMe	2055	2053					
9	H9N3PMe	2258	2256					
10	H10N2PMe	2217	2215					
11	H8N2PMeS		1971	1993	2009			
	H10N3PMe	2420	2418					
12	H8N2S2			1979	1995			
	H10N3S		2404					
13	H11N2PMe	2379	2377					
	H10N4PMe	2623	2621					
14	H9N2PMeS		2133	2155	2171			
	H11N3S		2566					

Fraction (Anionic)	Composition	[M+H] ⁺	[M-H] ⁻	[M-2H+Na] ⁻	[M-2H+K] ⁻	[M-3H+2Na] ⁻	[M-3H+Na+K] ⁻	[M-3H+2K] ⁻
	H11N3PMe	2582	2580					
15	H9N3S2			2344	2360			
16	H9N2PMe2	2149	2147	2169	2185			
	H10N2PMeS		2295	2317	2333			
17	H10N2S2			2303	2319			
	H10N2PMe2	2311	2309	2331	2347			
18	H11N2PMeS		2457	2479	2495			
19	H9N3PMeS		2336	2358	2374			
20	H11N2S2			2465	2481			
	H10N3PMeS		2498	2520	2536			
	H11N3S2			2668	2684			
21	H11N3PMeS		2660	2682	2698			
22	H8N2PMeS2					2095	2111	
23	H9N2PMeS2					2257	2273	
24	H10N2PMeS2					2419	2435	
25	H11N2PMeS2					2581	2597	
26	H8N2S3						2097	2113