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SWEET SECRETS OF A THERAPEUTIC WORM: MASS SPECTROMETRIC N-GLYCOMIC ANALYSIS OF *TRICHURIS SUIS*

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Abstract

Trichuris suis, a nematode parasite of pigs, has attracted attention as its eggs have been administered to human patients as a potential therapy for inflammatory diseases. However, the immunomodulatory factors remain molecularly uncharacterised, but in vitro studies suggest that glycans on the parasite excretory/secretory proteins may have a role. Using an off-line LC-MS approach in combination with chemical and enzymatic treatments, we have examined the N-linked oligosaccharides of *T. suis*. In addition to the paucimannosidic and oligomannosidic N-glycans typical of many invertebrates, a number of glycans carry *N,N′*-diacetyllactosamine (LacdiNAc) modified by fucose and/or phosphorylcholine. Such antennal epitopes are similar to ones previously associated with immunomodulation by helminths, but here we can propose phosphorylcholine modifications predominantly of terminal *N*-acetylgalactosamine as well as of subterminal α1,3-fucosylated *N*-acetylglucosamine. Exact knowledge of the glycome of *T. suis* will facilitate more targeted studies on glycan receptors in the host as well enable engineering of cell lines to produce correctly-glycosylated recombinant forms of candidate proteins for future studies on immunomodulation.

Keywords

HPLC; mass spectrometry; fucose; N-glycans; nematode; phosphorylcholine

The relationship between mammals and helminths is both ancient and complex; thus, some have referred to parasitic worms as ‘old friends’ [1]. In developed countries, nematode parasites are no longer ubiquitous in the human population, but are of at least agricultural relevance. While mortality may not be high upon nematode infections, morbidity and poor response to vaccinations in the developing world are definite ‘negative’ effects; on the other hand, there is a debate as to whether increased levels of allergy and autoimmune diseases in more developed countries reflect that nematodes have a ‘positive’ side-effect on their hosts [2-4]. The co-evolution of mammalian immune systems and helminths may be an explanation for the disbalance when nematode infections are lacking. The difficulties in treatment of some autoimmune diseases have led to trials with nematodes in order to relieve inflammation and a few publications have appeared regarding the use of eggs of the porcine

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parasite, *Trichuris suis*, or the larvae of *Necator americanus* as potential therapies for, e.g., Crohn's disease, coeliac disease or allergic rhinitis [5-7]; the therapeutic value of *T. suis* has also been disputed [8]. However, very often the molecular nature of the immunomodulatory substances from nematodes is unknown [9]; an exception is the phosphorylcholine-modified excretory/secretory protein ES-62 from *Acanthocheilonema viteae* [10]. In other cases, there are data to indicate that helminth glycans play a role and affect signalling pathways in cells of the immune system [11,12]; this has been reported also specifically for *T. suis* excretory-secretory products and their glycans, but without a thorough molecular characterisation [13,14].

The glycans of many helminth parasites have been characterised, especially the N-linked oligosaccharides attached to asparagine residues of proteins. Regardless of evolutionary distance (trematode, cestode or nematode), both recurring and unique glycosylation motifs have been found. However, it is probably correct to state that fucose and/or phosphorylcholine residues are found in most cases: examples include the antennal fucosylated epitopes of the trematode *Schistosoma mansoni* and the nematode *Trichinella spiralis* [15,16], the phosphorylcholine modifications of the nematodes *Ascaris suum* [17], *Onchocerca volvulus* [18] and *Trichinella spiralis* [19] or the core fucosylated motifs in *Haemonchus contortus* [20,21]. Occasionally individual glycoproteins such as ES-62 from the nematode *Acanthocheilonema viteae* [22] and Ag5 from the cestode *Echinococcus granulosus* [23] were specifically characterised. It is interesting to note that phosphorylcholine-modified N-glycans have been found in a cestode and in many nematodes, whereas the occurrence of fucosylated *N,N'*-diacetyllactosamine (fucosylated LacdiNAc) has been reported in only a few nematodes as well as in trematodes [15,16,20]. Nevertheless, the N-glycomic variation between species is very high and different biases in epitope abundance lead to a unique glycan population in each species, which may reflect also the patho-ecological niche, lifecycle or developmental stage.

Considering the evidence that glycans play a role in host-parasite interactions, including those of the porcine whipworm *T. suis*, we have performed the first glycomic analysis of this species using an off-line LC-MALDI-TOF MS strategy that we have applied to a number of other parasitic and non-parasitic nematode species [24,20,25-27]. Thus, we reveal that *T. suis* not only possesses a core fucosylation capacity, which is less complex than in some nematodes, but that it has fucosylated forms of phosphorylcholine-modified antennae which are, to date, unique to this species.

Experimental Procedures

N-glycan preparation and fractionation

Trichuris suis adults (kindly supplied by Dr. Stig Thamsborg of the Department of Veterinary Disease Biology, University of Copenhagen, Denmark) were homogenised and proteolysed with pepsin. N-glycans were then released from peptic peptides using peptide:*N*-glycosidase A (Roche) according to the procedures described previously [28]. Free glycans were labelled with 2-aminopyridine [29,28] prior to MALDI-TOF MS and fractionation by reversed-phase HPLC (RP-HPLC). Separation of PA-labeled glycans was performed on a Shimadzu HPLC system equipped with a fluorescence detector (RF 10

AXL; excitation at 320 nm and emission at 400 nm) and an Ascentis® Express 2.7 μ RP-Amide column (150 × 4.6 mm; Sigma-Aldrich). A gradient of 30% methanol (solvent B), with 100 mM ammonium acetate, pH 4.0, as buffer (solvent A), up to 35% over 34 minutes was applied at a flow rate of 0.8 ml/min as follows: 0-4 min, 0% B; 4-14 min, 0-5% B; 14-24 min, 5-15% B; 24-34 min, 15-35% B; 34-35 min, return to starting conditions [27]. The RP-HPLC analysis of the glycan preparation was performed twice together with MALDI-TOF MS of each fraction.

Mass spectrometry

Monoisotopic MALDI-TOF MS was performed in positive and negative reflectron modes with a Bruker Autoflex Speed instrument (equipped with a 1000 Hz Smartbeam™-II laser) and 6-aza-2-thiothymine (ATT) as matrix. MS/MS was performed by laser-induced dissociation. 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 (four-times smoothed). Interpretation of glycan spectra was performed manually on the basis of the masses of the predicted component monosaccharides, relative elution times as compared to previous studies, differences of mass in glycan series and fragmentation patterns. Glycans were primarily detected as $[M+H]^+$, except for instances of $[M+Na]^+$ in some digested fractions. As part of this study, some 600 MS and MS/MS spectra were recorded and approximately fifty selected mzXML files with the MS/MS spectra for all annotated glycans, prior to any chemical or enzymatic treatment, are available in a Supplementary ZIP file.

Enzymatic and chemical treatments

Selected fractions were subject to treatment overnight, prior to MALDI-TOF MS, with either α-mannosidases (jack bean [30] from Sigma or *Xanthomonas* α1,2/3- or α1,6-specific [31] from New England Biolabs), α-fucosidases (almond α1,3-fucosidase [32] from Prozyme or the more general bovine kidney α-fucosidase from Sigma-Aldrich) or β-*N*-acetylhexosaminidases (recombinant insect FDL prepared in-house, encoded by the *fused lobes* gene and specific for the *N*-acetylglucosamine attached to core α1,3-mannose [33], recombinant *Caenorhabditis elegans* HEX-4 prepared in-house, specific for terminal GalNAc residues [33], or recombinant *Streptomyces* β1,3/4/6-*N*-acetylhexosaminidase, also known as a chitinase [34], from New England Biolabs) in 25 mM ammonium acetate, pH 4.5 (or pH 6.5 in the case of HEX-4), at 37 °C. The substrate preferences of HEX-4 and the chitinase were confirmed by incubations with GlcNAcβ1,4GlcNAc-R and GalNAcβ1,4GlcNAc-R (where R is a methoxyamino-conjugate) in which HEX-4 was specific for the latter, while the chitinase cleaved both substrates. For removal of core or antennal α1,3-linked fucose or of phosphorylcholine, selected fractions were dried and incubated overnight at 0 °C with 3 μl 48% (v/v) hydrofluoric acid prior to evaporation [23]; the samples were diluted in water and re-evaporated, prior to redissolving once again prior to re-analysis by HPLC and/or MALDI-TOF MS.

Results

Overall glycomic characteristics

N-glycans from *Trichuris suis* adults were released using PNGase A from almonds as this enzyme can cleave N-glycans, regardless of the type of core fucosylation, from glycopeptides; bacterial PNGase F was not used as this enzyme cannot cleave core α1,3-fucosylated N-glycans [35], which are commonly found in plants, insects and nematodes [36]. The N-glycans were then fluorescently labelled using 2-aminopyridine, which is a well-established method and suited for reversed-phase chromatography in order to obtain also isomeric information [37,38]. In this study, we used a fused core RP-amide column calibrated with a partial dextran hydrolysate in terms of glucose units (g.u.) in conjunction with MALDI-TOF MS (see Figure 1) as previously employed for separation and analysis of insect and nematode N-glycans [27,39]. Approximately 40 RP-HPLC peaks were collected and individually analysed with the focus on those glycans whose MS/MS spectra indicated the presence of fucose and/or phosphorylcholine. A detailed analysis of glycans predicted to be oligomannosidic was not performed, but they were assigned (see Figure 2) due to being comparable in terms of retention time and MS/MS fragmentation pattern with either commercial standards or structures from another nematode (*Pristionchus pacificus*) analysed in parallel using the same RP-amide column [27].

Fucosylated paucimannosidic N-glycans

Initial perusal of the *Trichuris* full pyridylaminylated N-glycome by RP-HPLC and MALDI-TOF MS indicated the presence of a major fucosylated peak ($Hex_3HexNAc_2Fuc_1$-PA; 9.4 g.u.; *m/z* 1135) as well as a range of glycans of lower abundance with predicted compositions $Hex_3HexNAc_2Fuc_1$-PA (5.2 g.u.; *m/z* 1135), $Hex_2HexNAc_2Fuc_1$-PA (8.2 and 9.0 g.u.; *m/z* 973) or $Hex_3HexNAc_2Fuc_2$-PA (6.8 g.u.; *m/z* 1281). According to the positive ion mode MS/MS spectra yielding fragments of *m/z* 446 and 592 ($HexNAc_1Fuc_1$-PA and $HexNAc_1Fuc_2$-PA; Supplementary Figure 1 A and E), the fucose residues were linked to the reducing terminal GlcNAc. Based also on the sensitivity to α-mannosidases, bovine α-fucosidase and hydrofluoric acid (see Supplementary Figure 1 B, C, F and G), these glycans are $Man_{2\text{-}3}GlcNAc_2$ paucimannosidic structures modified with either core α1,3- or α1,6-fucose or both in the case of $Hex_3HexNAc_2Fuc_2$ with the most abundant being the α1,6-fucosylated form of $Man_3GlcNAc_2$. Due to the high abundance of this glycan, MS/MS was also performed in the negative ion mode (*m/z* 1133; Supplementary Figure 1D), resulting in typical N-glycan fragmentation ions at *m/z* 323, 545 and 688 respectively corresponding to an unprocessed antenna (Hex_2), cross-ring cleavage of penultimate GlcNAc ($^{2,4}A_3$) and glycosidic cleavage within the chitobiose unit (B_3, loss of $GlcNAc_1Fuc_1$-PA from the parent ion) [40]; these fragments as well as the *Y1α* and *Z1α* ions at *m/z* 444 ($GlcNAc_1Fuc_1$-PA) and 426 are also compatible with core fucosylation.

Hybrid and pseudohybrid N-glycans

A range of *Trichuris* oligosaccharides had putative compositions of $Hex_3HexNAc_{3\text{-}5}Fuc_{0\text{-}2}$-PA, very often in two separate HPLC fractions. Thus, there were typical isomeric pairs of $Hex_3HexNAc_3$-PA (*m/z* 1192; 6.7 and 9.0 g.u.) and $Hex_3HexNAc_3Fuc_1$-PA (*m/z* 1338; 8.8 and 12.0 g.u.) with retention times, MS/MS fragmentation patterns (Supplementary Figure

2) and enzymatic digestion patterns compatible with the presence of antennal non-reducing GlcNAc on either the α1,3- or α1,6-arm (summarised in Figure 2); the pattern of respective early or later retention for the so-called MGn and GnM isomers (*m/z* 1192) has been reported previously [41]. Other pairs of glycans, possessing an additional *N*-acetylhexosamine residue, were $Hex_3HexNAc_4$-PA (*m/z* 1395; 8.0 and 10.5 g.u.) and $Hex_3HexNAc_4Fuc_1$-PA (*m/z* 1541; 10.5 and 14.0 g.u.); the earlier eluting isobaric structure in each case had a retention time and MS/MS spectrum compatible with that of an 'uncapped' biantennary glycan, whereas MS/MS of the later eluting structures in both cases yielded *m/z* 407 fragments (i.e., $HexNAc_2$) suggestive for a HexdiNAc antennal modification. While the 10.5 g.u. form of *m/z* 1541 was resistant, the 14.0 g.u. species lost one HexNAc when incubated with a chitinase displaying β1,3/4/6-, but no β1,2-, hexosaminidase specificity (Supplementary Figure 3 A-D). The late retention time of the latter is suggestive for an upper arm HexdiNAc modification, a presumption confirmed by the loss of one hexose upon subsequent incubation with α1,2/3-specific mannosidase. The same *m/z* 407 fragment was also observed in the case of the single form of $Hex_3HexNAc_5Fuc_1$-PA (*m/z* 1744; 11.0 g.u.; Supplementary Figure 2J); as also judged by the relative retention times, this glycan is probably identical to one isolated from human urinary kallikrein [42]. Furthermore, GalNAcβ1,4GlcNAc is a known motif for N-glycans and glycolipids from other nematodes, it is probable that the *m/z* 407 fragment derives from a LacdiNAc modification, as described below for fucosylated or phosphorylcholinylated antennae.

A further glycan variant had the composition $Hex_3HexNAc_4Fuc_2$-PA (*m/z* 1687; 8.0 g.u.). Considering the lack of an *m/z* 592 fragment consistent with core difucosylation (compare with Supplementary Figure 1E), but the presence of ones of *m/z* 407 and 553 ($HexNAc_2Fuc_{0-1}$; Supplementary Figure 2I and 3E), it was probable that this structure carries an α1,3-fucosylated LacdiNAc motif. Therefore, the glycan was treated with hydrofluoric acid or with almond α1,3-fucosidase; both treatments resulted in the loss of one fucose residue (Figure 3D and Supplementary Figure 3F). Thereby, the fucosylated LacdiNAc fragment (*m/z* 553) was no longer apparent, while the core fucose-related one at *m/z* 446 was retained in both cases. When reinjected onto the RP-amide column (Figure 3A), the hydrofluoric acid treated form of the glycan eluted later (9.4 g.u.:, *m/z* 1541), but still earlier than the aforementioned forms of *m/z* 1541 present in the original glycome, which is consistent with a lower arm modification; furthermore, the defucosylated glycan was sensitive to subsequent bacterial chitinase and insect FDL-hexosaminidase (Figure 3 E and F). Due to the FDL-sensitivity of the HF/chitinase-treated form, the fucosylated LacdiNAc motif is concluded to be on the α1,3-arm, whereby the definition of the terminal HexNAc as GalNAc is verified by its removal by *C. elegans* HEX-4 (Supplementary Figure 3G), an enzyme specific for GalNAc residues. Furthermore, based on the shared elution properties, the glycan has the same structure as ones proposed in *Haemonchus* [20], mosquitoes [39] and honeybee venom phospholipase A_2 [43].

Phosphorylcholine-modified N-glycans

MALDI-TOF MS analysis of various fractions of the *Trichuris* N-glycome contained glycans whose masses were 165 Da greater than some of the hybrid and pseudohybrid N-

glycans discussed above which indicated modification by phosphorylcholine. MS/MS of these oligosaccharides, which all contained at least four *N*-acetylhexosamine residues, revealed fragment ions at *m/z* 369, 572 and 734 compatible with $HexNAc_{1-2}PC_1$ and $Hex_1HexNAc_2PC_1$ motifs (Figure 4). The general lack of *m/z* 531 fragment ions ($Hex_1HexNAc_1PC_1$, showing close vicinity of the PC-HexNAc to a hexose) contrasts with glycans of the same mass in, e.g., *Pristionchus* (see Supplementary Figure 4A-C) and was indicative of a different location for the phosphorylcholine moiety as compared to many other nematodes. Together with the relatively higher *m/z* 369 fragment (as compared to that at *m/z* 572) for most phosphorylcholine-modified glycans of *Trichuris*, the occurrence of [M +H-$HexNAc_1PC_1$] fragment ions in the case of structures carrying two phosphorylcholine residues (Figure 4 H and I) and the general lack of [M+H-$HexNAc_1$] fragments, we conclude that it is the terminal HexNAc of the HexdiNAc motif which is most often modified by phosphorylcholine. We therefore postulate that the phosphorylcholine moieties on $Hex_3HexNAc_{4-6}Fuc_{0-1}PC_{1-2}$ glycans (*m/z* 1560, 1706, 1909, 2131 and 2278; Figure 4 A, C, F, H and I) are generally separated from a core α-mannose by two *N*-acetylhexosamine residues.

The loss of a terminal HexNAc upon MS/MS was only observed for a minority of phosphorylcholine-modified glycans. For example, MS/MS of the 8.8 g.u. isoform of *m/z* 1706 yielded an [M+H-$HexNAc_1$] fragment (*m/z* 1503); furthermore, the MS/MS shows a relatively high intensity of the *m/z* 572 fragment as well as an ion at *m/z* 531, contrasting to the case for the 12.3 g.u. isoform (compare Figures 4 B and C). This suggests that the former has a subterminal phosphorylcholine modification (i.e., on a GlcNAc directly substituting a mannose), a presumption in keeping with the ability to remove one HexNAc with the *C. elegans* HEX-4 hexosaminidase (Supplementary Figure 4E). The terminal loss of a HexNAc is also observed for *m/z* 1909 (Figure 4F), which anyway possesses one non-modified GlcNAc on one arm.

Other cases of a subterminal position for the phosphorylcholine modification are two glycans also carrying a fucose on the antennae ($Hex_3HexNAc_{4-5}Fuc_2PC_1$; *m/z* 1852 and *m/z* 2055; 7.5 and 8.5 g.u.). For these glycans, fragment ions at *m/z* 515, 718 and 880 were observed ($HexNAc_1Fuc_1PC_1$, $HexNAc_2Fuc_1PC_1$ and $Hex_1HexNAc_2Fuc_1PC_1$; Figure 4 E and G) suggestive of trisubstitution of the subterminal GlcNAc. Consistent with the non-substitution of the terminal GalNAc, the MS/MS spectra of *m/z* 1852 and 2055 displayed fragments ion compatible with the loss of a single HexNAc (respectively, *m/z* 1650 and 1852). The antenna was also sensitive to almond α1,3-fucosidase and subsequently to HEX-4 (as summarised in Figure 2). There was nevertheless a second form of $Hex_3HexNAc_4Fuc_2PC_1$ (*m/z* 1852; 6.8 g.u.) for which a terminal phosphorylcholine modification is presumed on the basis of the lack of the *m/z* 515 fragment (Figure 4 D).

Other than fragmentation, another proof for the modification by phosphorylcholine was sensitivity towards hydrofluoric acid, which not only cleaves α1,3-fucose linkages (as described above), but also effectively removes phosphodiesters [44]. For example, hydrofluoric acid treatment of the 12.3 g.u. form of $Hex_3HexNAc_4Fuc_1PC_1$ (*m/z* 1706) yielded a product with *m/z* 1541 ($Hex_3HexNAc_4Fuc_1$-PA), which eluted at 14.0 g.u. when reinjected onto the RP-amide column (thus co-eluting with the aforementioned ‘upper’ arm

HexdiNAc-modified isomer occurring in the original glycome; Figure 3A and B) and which displayed a definite *m/z* 407 fragment ion (Figure 3H). This product lost, as for the HexdiNAc glycans discussed above, one HexNAc residue when incubated with chitinase (Figure 3I) and then a hexose when subsequently treated with α1,2/3-mannosidase (Figure 3J). Furthermore, recombinant *C. elegans* HEX-4 could remove one HexNAc after, but not before, hydrofluoric acid treatment (Supplementary Figure 4I); as HEX-4 only removes GalNAc residues from standard N-glycan substrates [33], the overall conclusion is that the phosphorylcholine modification of this isomer is on the terminal GalNAc of a LacdiNAc motif on the α1,6-arm. Another example of the effect of hydrofluoric acid is exemplified by the analysis of the *m/z* 1852 glycan eluting at 8.5 g.u., for which the loss of antennal fucose and phosphorylcholine is accompanied by the appearance of the otherwise suppressed core fragments (e.g., $HexNAc_1Fuc_1$-PA, *m/z* 446, rather than the $HexNAc_2Fuc_1PC_1$ fragment ion at *m/z* 718; compare Supplementary Figure 3 H and I).

Discussion

In this study, we reveal the variety of N-glycans present in adults of the porcine whipworm, *T. suis*, whose eggs have been used as a potential therapy for autoimmune and allergic disorders. Using the ability of a fused core RP-amide HPLC column to fractionate isomeric forms of pyridylaminated N-glycans prior to MALDI-TOF MS and MS/MS, we could distinguish core α1,3- from core α1,6-fucosylation or the antennae carrying GlcNAc or HexdiNAc modifications (summarised in Figure 2). Based on the HEX-4 hexosaminidase sensitivity and analogies to glycans from insects or from other nematodes (as discussed below), we consider it most probable that the HexdiNAc is indeed LacdiNAc. Targetted digests with enzymes or hydrofluoric acid, as well as comparisons to the N-glycans of other nematodes, enable us to propose some forty oligosaccharide structures, including some modified by antennal fucose or phosphorylcholine; a number of these have not been previously described by any method or only now were analysed by ‘off-line’ LC-MALDI-TOF MS for the first time. As many invertebrates, the N-glycome of *T. suis* contains a large percentage of paucimannosidic and oligomannosidic glycans [45]; however, it is remarkable for its range of antennal fucose and phosphorylcholine modifications based on HexdiNAc motifs. Indeed, we did not detect phosphorylcholine on a single terminal HexNAc, as common in *Caenorhabditis elegans*, *Pristionchus pacificus, Acanthocheilonema viteae, Ascaris suum* or *Haemonchus contortus* [46,27,22,17,20]. We propose that, in contrast to these organisms, which are members of nematode clades III and V [47], *T. suis* tends to add phosphorylcholine to terminal GalNAc of the putative LacdiNAc motif unless the subterminal GlcNAc is fucosylated.

The association of phosphorylcholine solely with HexdiNAc may be shared with *Trichinella spiralis*, as the smallest phosphorylcholine-modified glycan in this species may be $Hex_3HexNAc_4Fuc_1PC_1$ as judged by analysis of perdeuteroacetylated forms by FAB-MS; although it was not determined in *T. spiralis* as to whether the modification was on the terminal or subterminal HexNAc, a relatively high amount of terminal GalNAc was detected by GC-MS of the hydrofluoric acid-treated *T. spiralis* N-glycan pool suggesting the presence of LacdiNAc [19]. Thus, as *T. suis* and *T. spiralis* are both members of the Dorylaimia (clade I nematodes, rather distant phylogenetically from *C. elegans*), it could be that they share

some phosphorylcholine-modified N-glycan structures. On the other hand, no longer HexNAc repeats were observed as described for some filarial nematodes [18] which are members of clade III and no disubstitution of HexdiNAc by phosphorylcholine was obvious.

Antennal fucosylation in *T. suis* also occurred in the absence of a phosphorylcholine modification and is probably in the form of fucosylated LacdiNAc as present in insects and trematodes [43,48]. As judged by retention times and example digests, antennal fucose is predominantly on the lower arm and we recently found co-eluting N-glycans in both *H. contortus* and *Anopheles gambiae* [39,20]; both these species express compatible fucosyltransferase activities [49,50]. However, an antibody recognising fucosylated LacdiNAc only poorly reacted with *T. suis* soluble products [13], which could be due to the localisation of this epitope only in certain tissues or proteins of the parasite or steric hindrance when this motif also carries phosphorylcholine. Although fucosylated LacdiNAc is also known from *T. spiralis*, all glycans in that organism with this motif were apparently capped with tyvelose [16], a sugar modification not found during any other N-glycan analysis of a eukaryote. Thus, our off-line LC-MALDI-TOF MS study is the first to demonstrate uncapped and phosphorylcholine-modified fucosylated LacdiNAc in any clade I nematode.

Our glycomic analyses suggest that there are at least three N-glycan modifying fucosyltransferase activities in *T. suis* (core α1,3-, core α1,6- and Lewis-like α1,3-fucosyltransferases). Indeed, homology searching of the genome [51] indicates perhaps fifteen possibly relevant predicted gene products, although key protein motifs are in some cases not conserved (unpublished data). Missing from this repertoire are almost certainly an α1,3-fucosyltransferase modifying the distal core GlcNAc or any galactose-modifying α1,2-fucosyltransferase. Whereas distal core fucosylation and galactosylation of core α1,6-fucose is known from *C. elegans, A. suum, Oesophagostomum dentatum* or *H. contortus* [25,20,52] and bisecting galactose is seemingly a unique feature of *C. elegans* [26], there is no sign of such modifications in *T. suis*. Indeed, as there is a complete lack of tri- and tetrafucosylated N-glycans, it can be argued that the N-glycomic potential of *T. suis* is rather simple as compared to some other nematodes, but this may be related to its phylogeny and its pathoecology.

The identification of the N-glycome of *T. suis* should aid studies regarding interactions of the proteins secreted by this and related parasites with components of the immune system. Indeed, there are reports regarding binding of *T. muris* and *T. suis* proteins to various C-type lectins such as the mannose receptor, macrophage galactose receptor and DC-SIGN [53,13]; although the nature of the *T. suis* structures recognised is not known, in other studies fucose-containing glycans are proven ligands for all three lectins. Also potentially relevant to the fucose-containing glycans, it has been shown by antibody cross-reactivity that fucosylated LacdiNAc is present on a host-protective protein fraction from *H. contortus* [54], whereas core α1,3-fucose is an epitope for IgE from sheep infected with *H. contortus* [55]. Furthermore, nematode-derived phosphorylcholine is a ligand for C-reactive protein as well as from antibodies raised against nematodes [56-59], whereas core fucose modifications of nematode parasite N-glycans are also targets of nematotoxic lectins derived from fungi [60]. While some of the relevant glycan ligands can be synthesised chemoenzymatically, synthetic

approaches are required for those containing phosphorylcholine. Thus, knowledge of the actual N-glycans of *T. suis* is the starting point for targeted studies (e.g., with glycan arrays) to understand the molecular basis of carbohydrate-mediated effects of this parasite in potential therapeutic settings.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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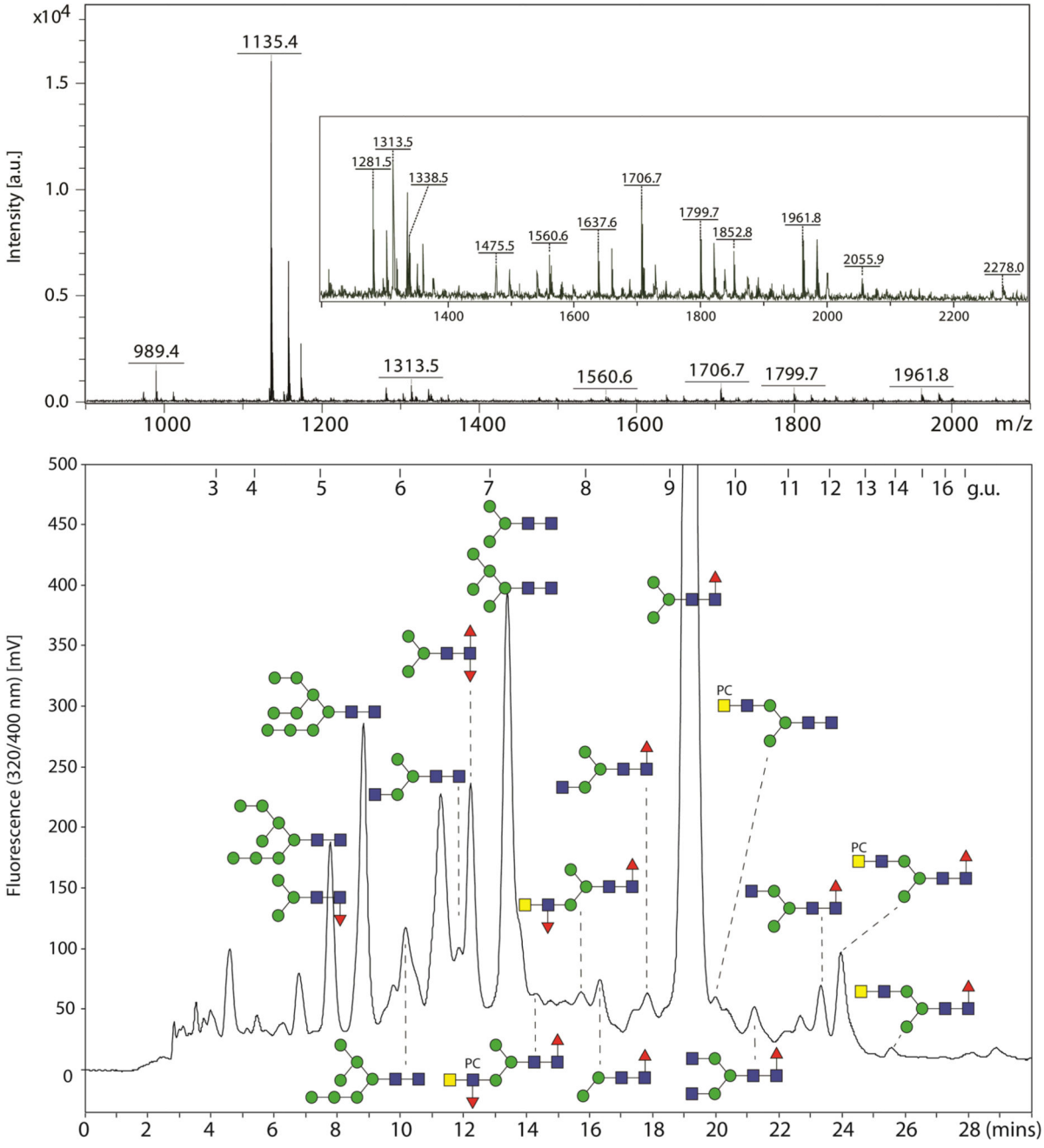

Figure 1. MALDI-TOF MS and RP-HPLC analysis of *Trichuris suis* N-glycans

Pyridylaminated N-glycans derived from a PNGase A digest of *T. suis* glycopeptides were analysed by MALDI-TOF MS in positive ion mode and by RP-HPLC on an fused core Ascentis RP-amide column calibrated in terms of glucose units (g.u.). The MALDI-TOF MS spectrum is dominated by a $Man_3GlcNAc_2Fuc_1$-PA glycan (*m/z* 1135 as the protonated quasimolecular ion); the region *m/z* 1200-2300 is also shown magnified with further protonated ions annotated. Selected HPLC fractions are annotated according to the

nomenclature of the Consortium for Functional Glycomics (circles, hexose; squares, *N*-acetylhexosamine; triangles, fucose; PC, phosphorylcholine).

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Composition	*m/z*; Retention; Fraction; Intensity	Key MS/MS and Notes	Composition	*m/z*; Retention; Fraction; Intensity	Key MS/MS and Notes
$Hex_2HexNAc_2Fuc_1$	973.4 8.2 (Fr24) ++	446 as in *Pristionchus* α2/3Man sensitive (-1H)	$Hex_5HexNAc_2$	1313.5 7.2 (Fr18) ++	827 as in *Pristionchus*
$Hex_2HexNAc_2Fuc_1$	973.4 9.0 (Fr28) ++	446 as in *Pristionchus* α2/3Man insensitive α6Man (-1H)	$Hex_3HexNAc_3Fuc_1$	1338.5 8.8 (Fr27) ++	446,1176 as in *Pristionchus* and SFig. 2C α2/3Man insensitive FDL sensitive (-1N)
$Hex_3HexNAc_2$	989.4 7.2 (Fr18) +++	300,827 as in *Pristionchus*	$Hex_3HexNAc_3Fuc_1$	1338.5 12.0 (Fr35) ++	446,1135 as in *Pristionchus* and SFig. 2D α2/3Man sensitive (-1H)
$Hex_3HexNAc_2Fuc_1$	1135.4 5.2 (Fr8) +	446 small as in *Pristionchus* HF sensitive (-1F)	$Hex_3HexNAc_4$	1395.5 8.0 (Fr23) +	365,1030 as in *Pristionchus* and SFig. 2E α2/3Man insensitive
$Hex_3HexNAc_2Fuc_1$	1135.4 9.4 (Fr29) ++++	446 as in *Pristionchus* and SFig. 1A-D α2/3Man sensitive (-1H) αFuc sensitive (-1F) JBMan sensitive (-2H)	$Hex_3HexNAc_4$	1395.5 10.5 (Fr32) trace	407,989 late elution; SFig. 2F (indicative for upper arm)
$Hex_4HexNAc_2$	1151.4 7.4 (Fr19) ++	827 as in *Pristionchus* α2/3Man sensitive (-1H)	$Hex_6HexNAc_2$	1475.5 6.1 (Fr13) ++	827,1151 as in *Pristionchus*
$Hex_3HexNAc_3$	1192.5 6.7 (Fr15) ++	1030 as in *Pristionchus* and SFig. 2A FDL sensitive (-1N)	$Hex_6HexNAc_2$	1475.5 6.4 (Fr14) ++	989 as in *Pristionchus* JBMan sensitive (-5H) α2/3Man sensitive (-2H)
$Hex_3HexNAc_3$	1192.5 9.0 (Fr28) ++	989 as in *Pristionchus* and SFig. 2B α2/3Man sensitive (-1H)	$Hex_3HexNAc_4Fuc_1$	1541.6 10.5 (Fr32) ++	446,1176,1338 as in *Pristionchus* and SFig. 2G α2/3Man insensitive chitinase insensitive (SFig. 3B)
$Hex_3HexNAc_2Fuc_2$	1281.5 6.8 (Fr16) +++	446,592 as in *Pristionchus* and SFig. 1E-G α2/3Man (-1H) then α6Man sensitive; JBMan sensitive (-2H); αFuc/HF sensitive (-1F)	$Hex_3HexNAc_4Fuc_1$	1541.6 14 (Fr38) +	407,446,1135 (see SFig. 2H) α2/3Man sensitive (-1H) (after chitinase, -1N; SFig. 3D)

$Hex_3HexNAc_4PC_1$	1560.6 9.8 (Fr30) +	369,572,734 (see Fig. 4A) HF sensitive (-1PC) then chitinase sensitive (-1N) JBMan sensitive (-1H)	$Hex_8HexNAc_2$	1799.7 5.9 (Fr12) +	989 as in *Pristionchus*, Man 8A
$Hex_7HexNAc_2$	1637.6 5.5 (Fr10) ++	989 as in *Pristionchus*	$Hex_8HexNAc_2$	1799.7 6.7 (Fr15) trace	1151 Man 8C (retention of middle mannose correlates with late elution)
$Hex_7HexNAc_2$	1637.6 6.1 (Fr13) ++	1151 as in *Pristionchus*	$Hex_3HexNAc_4Fuc_3PC_1$	1852.7 6.8 (Fr16) +	369,572,718,880 loss of 445 (Fig. 4D)
$Hex_3HexNAc_4Fuc_2$	1687.7 8.0 (Fr23) ++	407,446,553,1484,1525 (SFig. 2I) α3Fuc then HEX-4 sensitive (SFig. 3), HF sensitive (-1F; Fig. 3D) α2/3Man insensitive, FDL sensitive after HF/chitinase (Fig. 3E/F)	$Hex_3HexNAc_4Fuc_2PC_1$	1852.7 7.5 (Fr20) +	369,515,572,718, 880, loss of 445 and 203 see Fig. 4E HF sensitive (-1PC/1F) then 407, 446, 1135 (SFig. 3I) α3Fuc sensitive, then HEX-4 sensitive
$Hex_3HexNAc_4Fuc_2$	1687.7 10.5 (Fr32) trace	407,446,553,1484 late elution (indicative for upper arm)	$Hex_3HexNAc_4Fuc_1PC_1$	1909.8 10.0 (Fr31) +	369, 572, 734; loss of 203 and 445 see Fig. 4F PC-modified version of 1744
$Hex_3HexNAc_4Fuc_1PC_1$	1706.7 8.8 (Fr27) +	369,531,572(high),734 loss of 445 and of 203 (Fig. 4B) HF sensitive (-1PC; then 407, 446, 1135, 1395); HEX-4 sensitive (-1N; then 369, 531; SFig. 4E)	$Hex_9HexNAc_2$	1961.7 5.5 (Fr10) +++	1151 as in *Pristionchus*
$Hex_3HexNAc_4Fuc_1PC_1$	1706.7 12.3 (Fr36) ++	369, 572, 734, loss of 445 (see Fig. 4C) HF sensitive (-1PC; Fig. 3H) then 407, 445, 1135 (SFig. 4G) then chitinase/α3Man or HEX-4 sensitive (Fig. 3I/J and SFig. 4I)	$Hex_3HexNAc_5Fuc_2PC_1$	2055.8 8.5 (Fr26) +	369,515,572,718,880, loss of 203 and 445 see Fig. 4G HF sensitive (-1PC/1F) then 407, 446, 1176, 1338
$Hex_3HexNAc_5Fuc_1$	1744.7 11.0 (Fr33) trace	407,446,1176,1338 (see SFig. 2J) 407/446 fragments (indicative of LacdiNAc and core Fuc)	$Hex_3HexNAc_6PC_2$	2131.9 8.2 (Fr24) trace	369, 572, 734 see Fig. 4H
$Hex_8HexNAc_2$	1799.7 5.2 (Fr8) +++	1151 as in *Pristionchus*,Man 8B	$Hex_3HexNAc_6Fuc_1PC_2$	2278.0 11.0 (Fr33) +	368,572, loss of 445 see Fig. 4I

Figure 2. Summary of N-glycan assignments

Predicted compositions in terms of hexose, *N*-acetylhexosamine, fucose and phosphorylcholine residues and CFG-type structures are presented together with (i) calculated *m/z* as $[M+H]^+$ for the pyridylaminated N-glycans, (ii) retention time in terms of glucose units and fraction number (RP-amide column), (iii) a qualitative indication of abundance (trace through to +++), (iv) important MS/MS fragments and (v) notes regarding identical properties as glycans from *Pristionchus*, sensitivity to chemical and enzymatic treatments and relevant figure (Fig) and supplementary figure (SFig) numbers. For reasons

of space, two variants of $Man_2GlcNAc_2$ (*m/z* 827) as well as a $Glc_1Man_9GlcNAc_2$ (*m/z* 2123) are not included. The MS/MS of the untreated glycans are also available as supplementary mzXML files.

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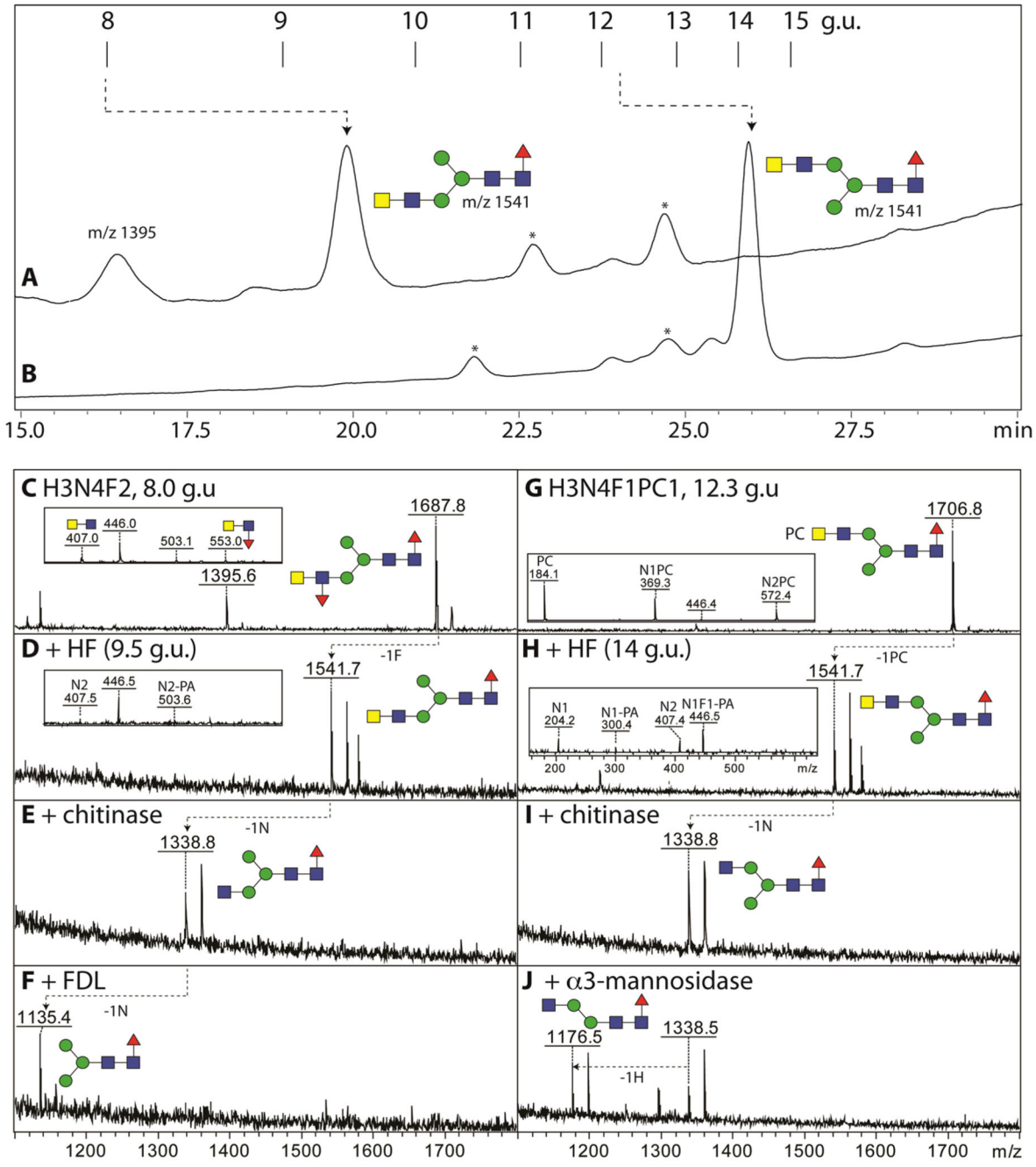

Figure 3. Analysis of antennal fucose and phosphorylcholine modifications on N-glycans from *T. suis*

(A, B) Two RP-HPLC fractions (8.0 and 12.3 g.u., respectively containing glycans of *m/z* 1687 and 1706) were treated with hydrofluoric acid prior to re-injection onto the HPLC column (collected fractions are annotated with the relevant m/z; those marked with an asterisk contained no detectable glycans). (C, D, G and H) The product fractions were analysed by MALDI-TOF MS showing the loss of either α1,3-fucose or phosphorylcholine as compared to the untreated glycans. (E, I) Both product fractions (i.e., those of 9.5 and 14 g.u.) were treated with β1,3/4/6-specific hexosaminidase (chitinase) prior to diagnostic

digests with either (F, J) FDL-hexosaminidase or α1,2/3-specific mannosidase (respectively specific for 'lower arm' GlcNAc or mannose residues). The effect of the hydrofluoric acid treatment is also obvious by the alteration of the fragment ions (insets in panels C, D, G and H); the loss of antennal fucose from the *m/z* 1687 glycan was also realized with almond α1,3-fucosidase (Supplementary Figure 3F). The contrasting HPLC retention times for the HF-treated glycans (*m/z* 1541) correlate with different arms being modified with the LacdiNAc motif. Partial shifts to sodiated adducts were observed; for full MS/MS of the two glycans, see Supplementary Figure 2I and 4F or the Supplementary mzXML files.

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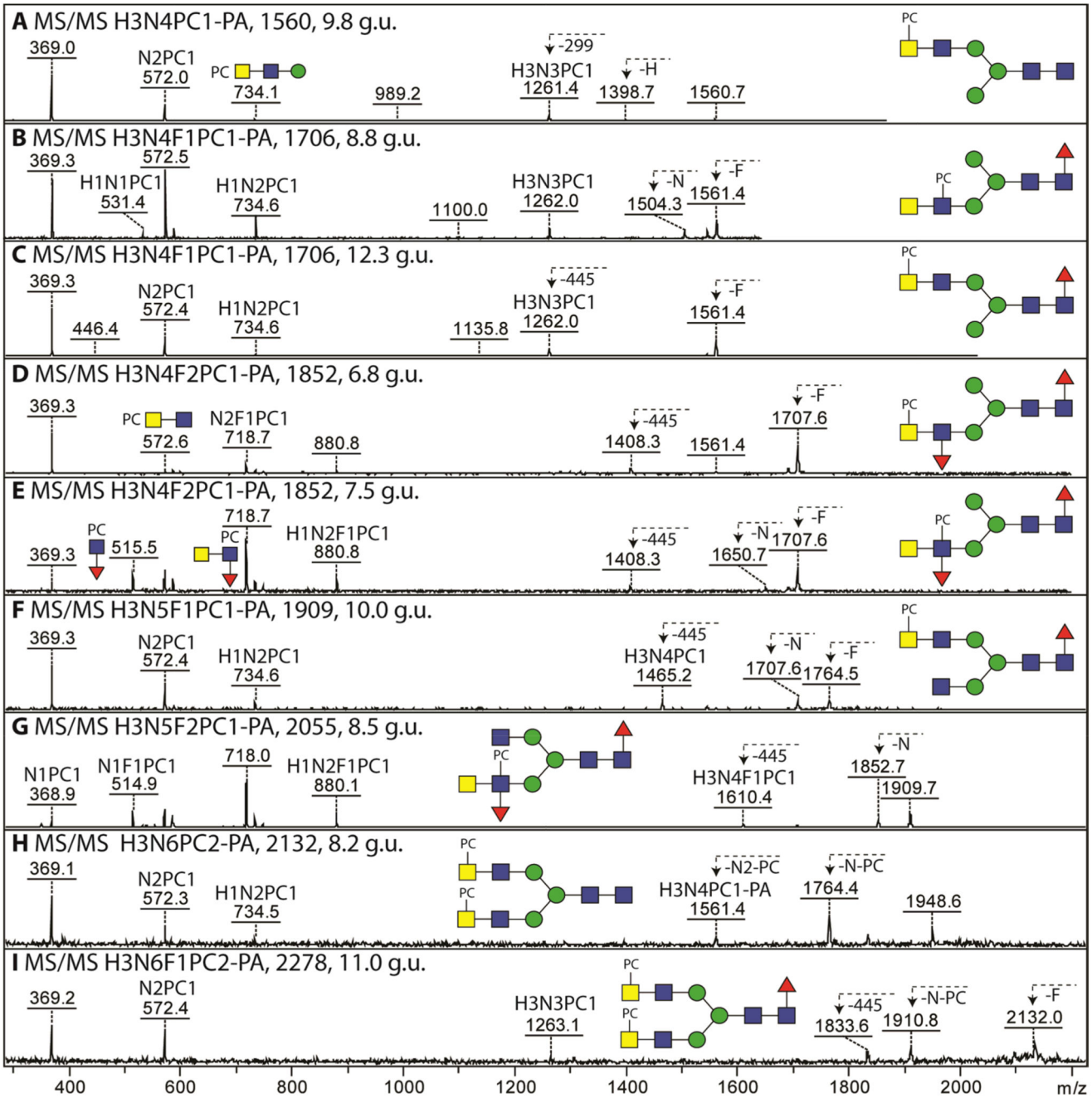

Figure 4. MS/MS analysis of phosphorylcholine-modified N-glycans from *T. suis*

(A-I) Positive ion mode MALDI-TOF MS/MS of phosphorylcholine-modified N-glycans in various RP-amide fractions (with the relevant glucose units given). Key fragments are annotated with abbreviated compositions: F, fucose; H, hexose; N, *N*-acetylhexosamine; PA, 2-aminopyridine; PC, phosphorylcholine. The *B*-fragment ions at *m/z* 369 ($HexNAc_1PC_1$) are typical for phosphorylcholine-modified N-glycans from this and other nematode species. Examples of ions resulting from loss of fucose (F), hexose (H), HexNAc (N), phosphorylcholine-modified HexNAc (N-PC) or of the reducing terminus (299 or 445) are

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also indicated. Further analyses of the 7.5 g.u. (*m/z* 1852) and 12.3 g.u. (*m/z* 1706) glycans are shown in Figure 3 and in Supplementary Figures 3 and 4. The terminal position for the phosphorylcholine in the case of *m/z* 1706 (12.3 g.u.; panel C) correlates with the resistance of this glycan (prior to hydrofluoric acid treatment) to the GalNAc-specific *C. elegans* HEX-4 (see Supplementary Figure 4 F-I), whereas the 8.8 g.u. isoform (panel B) is sensitive to this enzyme without removal of the zwitterionic moiety (see Supplementary Figure 4 D and E).