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Mass spectrometric analysis of the immunodominant glycan epitope of *Echinococcus granulosus* antigen Ag5

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ABSTRACT

In previous work we showed that Ag5, a major diagnostic antigen from the metacestode of *Echinococcus granulosus*, possesses a dominant sugar epitope that upon removal results in abolition of most of the antigen immunoreactivity with patient sera. Analysis of this glycan modification has now been performed by western blotting and mass spectrometry. Reactivity to both a specific monoclonal antibody (TEPC15) and human C-reactive protein as well as the presence of a modification of 165 mass units, as detected by mass spectrometry of both glycopeptides and released N-glycans, indicated that the immunodominant sugar epitope of the Ag5 38 kDa subunit is a biantennary structure modified by phosphorylcholine. We believe this is the first time that such a modification has been proven in cestodes and provides the structural basis for understanding the antigenicity of this major *E. granulosus* component.

1. Introduction

Cystic hydatid disease is a widespread zoonosis (i.e., a disease transmitted from the biological animal host to humans) that has the dog as a definitive host and different ungulates (typically sheep) as intermediate ones. Humans accidentally contract the disease by ingestion of parasite eggs released by the dog, which can develop as a larval cyst in liver, lung or, less frequently, other tissues (McManus et al., 2003). The infective agent, the (tapeworm) *Echinococcus granulosus*, belongs to the Cestoda class, a group of obligate parasitic platyhelminths (flatworms) closely related to trematodes, of which the best studied genus is the parasitic *Schistosoma* (Gryseels et al., 2006). A number of antigens, including many glycoproteins, are produced by the metacestode of *E. granulosus* and have been extensively used for the detection of antibodies in patients' sera. Among them, antigens AgB and Ag5 have been the most studied components of the parasite, due to their high concentration in hydatid cyst fluid and their immunoreactivity (Pozzuoli et al., 1975; Gonzalez-Sapienza et al., 2000; Lorenzo et al., 2005a; Carmena et al., 2007). While AgB is a 160 kDa thermostable lipoprotein, Ag5 is a dimeric protein composed of 22 and 38 kDa subunits linked by a disulphide bridge, with both subunits bearing an N-glycan modification (Lorenzo et al., 2003).

In previous work we analysed the immunogenicity of Ag5 and found that most patient antibodies were unreactive towards either the recombinant antigen produced in *Escherichia coli* or the deglycosylated native antigen. We also demonstrated that the immunorelevant epitope is a sugar moiety attached to the 38 kDa subunit of Ag5 (Lorenzo et al., 2003). This is in agreement with the immunodominant response against glycosidic epitopes of *E. granulosus* that has been reported in a model of secondary infection (Ferragut and Nieto, 1996) as well as with the diagnostic relevance of the Galβ1,6Gal-modified glycolipids and Galα1,4Gal-modified O-linked parasite glycans (Persat et al., 1992; Hülsmeier et al., 2002, 2010; Diaz et al., 2009; Yamano et al., 2009). These determinants are recognised by the sera of patients infected with other cestode species, including the causative agent of alveolar echinococcosis, *Echinococcus multilocularis*. We found a similar pattern of cross-reactivity associated with the immunodominant glycan epitope of *E. granulosus* antigen Ag5 (Lorenzo et al., 2005b).

To provide structural information on this major epitope of Ag5, in this study the N-glycans of the large subunit of Ag5 were examined. The mass spectrometric analysis of the glycopeptides and the released N-glycans showed that the 38 kDa subunit carries a bi antennary N-glycan modified with phosphorylcholine. Although this result could be predicted on the basis of antibody reactivity (Shepherd and McManus, 1987; Lightowlers et al., 1989; Lorenzo et al., 2005a), we believe this is the first structural proof for the modification of an N-glycan with this moiety in a species other than a nematode.

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2. Materials and methods

2.1. Materials

Ag5 was affinity purified from bovine hydatid cyst fluid using the agarose immobilized monoclonal antibody 1D1 as described previously (Lorenzo et al., 2003).

For screening the presence of either core fucose or phosphorylcholine modifications, 2 μg of untreated or PNGase F-treated Ag5 were applied to a standard SDS–PAGE gel. For PNGase F treatment, Ag5 (~8 μg) was first denatured in 10 μl of 0.5% SDS for 5 min at 95 °C, prior to addition of 3 μl of McIlvaine phosphate-citrate buffer, pH 7.5, and 2 μl of PNGase F (peptide:N-glycosidase F; Roche, Germany), and incubated for 2 days at 37 °C. After SDS–PAGE at 200 V for 50 min, proteins were subjected to western blotting (semi-dry blotting, 20 V, 50 min) and the nitrocellulose membrane was blocked with Tris-buffered saline supplemented with 0.05% Tween-20 and 0.5% BSA (TTBS/BSA) for 1 h. The membrane was then incubated for 1 h with either biotinylated *Aleuria aurantia* lectin (AAL; Vector Laboratories, USA; 1:1,000) or TEPC15 (anti-phosphorylcholine; Sigma–Aldrich, USA; 1:250), washed thrice with TTBS and incubated with alkaline phosphatase conjugates of either anti-biotin or anti-IgA (1:10,000; Sigma–Aldrich) prior to washing again and development using SigmaFAST™ 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium. In the case of detection with C-reactive protein (CRP; Sigma–Aldrich), the membrane was incubated with 50 μg/ml of human CRP, followed by 1:750 rabbit anti-CRP (DAKO, Denmark) and finally 1:2,000 alkaline phosphatase conjugated goat anti-rabbit (Vector Laboratories, USA) prior to colour development.

2.2. Tryptic peptide mapping

Ten micrograms of untreated Ag5 were applied to a SDS–PAGE gel, stained with Coomassie Brilliant Blue G-250 and the bands excised. The gel band pieces were washed serially with acetonitrile, twice with 50% acetonitrile in water, 1:1 0.1 M ammonium bicarbonate/acetonitrile and again with acetonitrile prior to drying, followed by reduction with 10 mM of DTT at 56 °C for 1 h, alkylation with 55 mM of iodoacetamide in the dark for 45 min and once again serially washing, with 50% acetonitrile in water (twice), 1:1 ammonium bicarbonate:acetonitrile and with 100% acetonitrile. The gel pieces were again dried and 15 μl of a 1:2 mixture of 50 ng/μl trypsin/0.1 M ammonium bicarbonate were applied; the gel pieces were then covered with 0.1 M ammonium bicarbonate and incubated overnight at 37 °C. The peptides were extracted with 660:330:1 acetonitrile/water/trifluoroacetic acid, dried and dissolved in the same. An aliquot (0.25 μl) was directly spotted onto a ground steel sample plate and analysed by MALDI-TOF MS with either α-cyanocinnamic acid (ACH), sinapinic acid (SA) or 6-aza-2-thiothymine (ATT) as matrices. The samples were analysed in positive ion mode using a Bruker Ultraflex I (Bruker Daltonics, Germany) equipped with a nitrogen laser (337 nm; laser frequency of 50 Hz and pulse length of 200 ns); typically 400–1000 shots were summed. Data were analysed and predicted using the ProteinProspector MS-Fit and MS-Digest on-line software (http://prospector.ucsf.edu).

2.3. Glycan release and analysis

Approximately one-half of the tryptic peptides were dried, dissolved in 15 μl of water and heat-treated for 5 min at 95 °C; 15 μl of 0.2 M ammonium acetate, pH 5, and 0.5 μl of PNGase A (Roche, Germany) were added and the sample incubated overnight at 37 °C; a portion was retained for direct analysis by MALDI-TOF MS. The rest of the sample was then acidified with 6 μl of 10% acetic acid and applied to a mini-column of 50 μl Dowex 50 W prewashed with 3 ml of 2% acetic acid; the column was then washed thrice with 300 μl of 2% acetic acid, the sample lyophilised and dissolved in 10 μl of water prior to application to another mini-column consisting of 1–2 mm of C18 LiChroprep (Merck) and 2–3 mm of porous graphitised carbon (with material taken from a Supelclean ENVI-Carb column; Supelco, USA), pre-washed with 2 ml of acetonitrile and 4 ml of water. After sample application, the column was washed six times with 250 μl of water and four times with 250 μl of 40% acetonitrile. The 40% acetonitrile fractions containing glycans were pooled, dried in a SpeedVac and analysed by MALDI-TOF MS (Bruker Ultraflex I as above) using ATT as a matrix.

In a separate experiment, 2 μg of purified Ag5 were incubated overnight with PNGase F in solution as described above (Section 2.1); the incubation was precipitated with a five volume excess of methanol and the supernatant was vacuum-dried prior to purification of the glycan on C18 Lichroprep and porous graphitised carbon (also as above). The glycan was then subject to pyridylamination as described (Paschinger et al., 2012), but on a tenth of the normal scale (i.e., with a tenth of the normal amounts of derivatisation reagents), prior to gel filtration and MALDI-TOF MS. For removal of phosphorylcholine, a portion of the glycan sample was dried and incubated overnight at 0 °C with 3 μl of 48% (v/v) hydrofluoric acid prior to evaporation; the sample was diluted in water and re-evaporated, prior to dissolving once again and analysis by MALDI-TOF MS.

3. Results and discussion

3.1. PNGase F sensitivity of Ag5 epitopes

Phosphorylcholine is a known component of a number of glycoconjugates including lipopolysaccharides from some bacteria, glycolipids from nematodes and earthworms and N-linked glycans from nematodes (Sugita et al., 1992; Lochnit et al., 1998; Pöltl et al., 2007; Fox et al., 2008). To confirm previous data suggesting the presence of phosphorylcholine on Ag5, blotting with the monoclonal IgA TEPC15 (Leon and Young, 1971) and with human CRP, a pentraxin produced as part of the inflammatory response and also known to bind phosphorylcholine, was performed on untreated and PNGase F-treated *E. granulosus* Ag5. The blots indicate that the reactivity of the 38 kDa subunit to the TEPC15 antibody and to CRP is PNGase F-sensitive (Fig. 1A and B). Similarly, the reactivity of Ag5 to the fucose-specific AAL (Iskratsch et al., 2009) is also PNGase F sensitive (Fig. 1C); this enzyme can release N-glycans modified with core α1,6-fucose, but not those with core α1,3-fucose (Tretter et al., 1991). Indeed, no reactivity to the core α1,3-fucose specific anti-horseradish peroxidase antiserum was observed (data not shown). The reduction in molecular mass accompanying PNGase F treatment of Ag5 was confirmed as judged by the shift observed on the Coomassie-stained gel (Fig. 1D). The sensitivity of TEPC15, CRP and AAL staining to treatment by this glycosidase was indicative that phosphorylcholine and core α1,6-fucose moieties are associated with N-glycans on the Ag5 38 kDa subunit.

3.2. Mass spectrometry of Ag5 (glyco)peptides

The tryptic peptides derived from the 38 kDa subunit of Ag5 were examined by MALDI-TOF MS using three different matrices: ACH, SA and ATT. Relative intensities varied, but a large number of peptides were observed using all three matrices (Table 1; Fig. 2). The peptide mapping data obtained was compatible with the Ag5 genome sequence from *E. granulosus* produced by the

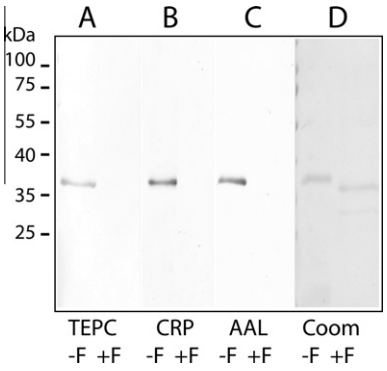

Fig. 1. Antibody and lectin reactivity of *Echinococcus granulosus* antigen Ag5. A sample of purified Ag5 (untreated or treated with PNGase F; −F or +F) was subject to western blotting with either (A) the anti-phosphorylcholine monoclonal antibody TEPC15, (B) human C-reactive protein or (C) *Aleuria aurantia* lectin. Coomassie Blue staining (D) demonstrates the shift resulting from PNGase F treatment; the faint lower band of around 30 kDa corresponds to PNGase F. Equal amounts of the native and deglycosylated forms of Ag5 were applied for both blotting and Coomassie staining.

Parasite Sequencing Group, Sanger Institute, UK (http://www.sanger.ac.uk/cgi-bin/submitblast/Echinococcus) as well as with the orthologous sequence from *E. multilocularis*. We therefore conclude that the correct sequence for residues 110–124 is DLVVIHPDWVAQRVDS and not the previously-published AWSSFIRIGLPNVLI (Lorenzo et al., 2003); furthermore, residue 221 is indeed Cys and not Arg. Additionally, during reviewing of this manuscript, a new Ag5 cDNA sequence from a Chinese isolate was published (Li et al., in press) which is also in accordance with our new mass spectrometric data. The overall sequence coverage is some 71%; however, one predicted tryptic peptide accounting for over 10% of the sequence (mass over 4 kDa) was not observed. Four molecular species of *m*/*z* 805, 841, 1847 and 1970 present in the spectra are not explained by the predicted N-glycosylated amino acid sequence of Ag5.

Striking, however, was the presence of three peptides (*m*/*z* 2711.1, 2876.2 and 3041.3) in the spectrum acquired when using ATT as matrix, none of which corresponded to a predicted peptide. The later data on the released N-glycans (see Section 3.3 below) enabled us to predict that these masses corresponded to glycopeptides. The difference in *m*/*z* of these three peptides (165 mass units) would be in agreement with the presence of phosphorylcholine on two of these glycopeptides and MS/MS of the glycopeptide of *m*/*z* 3041 showed the presence of a fragment of *m*/*z* 368, which corresponds to a phosphorylcholine-modified HexNAc residue. In contrast, the MS/MS spectrum of the *m*/*z* 2711 glycopeptide showed the presence of a fragment corresponding to unsubstituted terminal HexNAc (*m*/*z* 203.7). These data also indicate the utility of ATT not just as a matrix for glycans but also for glycopeptides (Lattová et al., 2007).

The peptides present after incubation with PNGase A were also analysed; whereas the aforementioned three putative glycopeptides were no longer detected, a new molecular ion of *m*/*z* 1267.8 was observed in the treated sample (Fig. 2, inset). This peptide is predicted to correspond to the deglycosylated peptide, AANYDASEGLTR, in which, as expected (Plummer et al., 1984), the glycosylated asparagine has been converted to aspartate. Thereby, a mass difference of +1 is observed as compared to the predicted mass of the non-glycosylated asparagine-containing peptide. This result is compatible with glycosylation of Asn_{25} of the 38 kDa subunit (i.e., Asn_{213} of the unprocessed Ag5 precursor); this is the only asparagine in the context of an Asn-Xaa-Ser/Thr sequon in the sequence of this subunit.

3.3. Analysis of the released Ag5 N-glycan

The nature of the N-glycan modification was studied in two independent experiments; in the first, tryptic peptides were treated with PNGase A and purified using two mini-columns, whereas in the second, denatured intact Ag5 was treated with PNGase F in solution. The results were similar in that a single major species (*m*/*z* 1793.6) was observed (Fig. 3A; only data for the PNGase A-released free glycan is shown). MS/MS (Fig. 3D) indicated the presence of fragments of *m*/*z* 368.9 and 530.9 compatible with these being $Hex_{0-1}HexNAc_1PC_1$; therefore, an overall composition of $Hex_3HexNAc_4dHex_1PC_2$ could be proposed for an $[M+H]^+$ ion, whereby the deoxyhexose (dHex) residue is presumed, due to the AAL staining, to be fucose. Upon treatment with hydrofluoric acid known to remove phosphorylcholine and core α1,3-fucose residues (Haslam et al., 1997; Pöltl et al., 2007), the *m*/*z* 1793.6

Table 1
Tryptic peptide mapping of the *Echinococcus granulosus* antigen Ag5 38 kDa subunit. The observed and calculated *m*/*z* values are listed, as well as the matrix with which the peptides were observed (α-cyanocinnamic acid, ACH; 6-aza-2-thiothymine, ATT; sinapinic acid SA) and the residues to which these species are predicted to correspond. For one detected peptide, the mass is compatible with an apparent incomplete carboxyamidomethylation ('CAM lacking'). The peptides with *m*/*z* 2163 and 2273 are derived from trypsin.

Observed *m*/*z* value	Predicted *m*/*z* value	Matrix	Corresponding residues	Comments
884.617	884.472	ATT	213–220	
940.627	940.568	ACH	173–180	
1018.555	1018.5164	ACH, ATT, SA	89–98	
1231.736	1231.668	ACH, ATT, SA	123–133	
1267.825	1266.607	ACH, ATT	21–32	Deglycosylated, Asp
1272.708	1272.652	ACH, SA	11–20	
1426.682	1426.620	ACH, ATT, SA	240–253	
1466.873	1466.819	ACH, ATT	201–212	
1771.952	1771.973	ACH, ATT	33–48	1 missed cleavage
1815.881	1815.912	ATT, SA	183–196	
1834.913	1834.960	ACH, ATT	279–293	
1898.907	1898.907	SA	134–152	CAM lacking
1956.220	1955.974	ATT	134–152	
1971.971	1971.958	SA	182–196	1 missed cleavage
2123.818	2123.818	ATT, SA	153–172	
2138.373	2138.979	ACH	260–277	1 missed cleavage
2585.214	2585.274	ACH, ATT, SA	101–122	
2711.167	2711.141	ATT	21–32	$+Hex_3HexNAc_4Fuc_1$
2842.183	2842.423	ACH, ATT, SA	99–122	1 missed cleavage
2876.242	2876.196	ATT	21–32	$+Hex_3HexNAc_4Fuc_1PC_1$
3041.324	3041.251	ATT	21–32	$+Hex_3HexNAc_4Fuc_1PC_2$

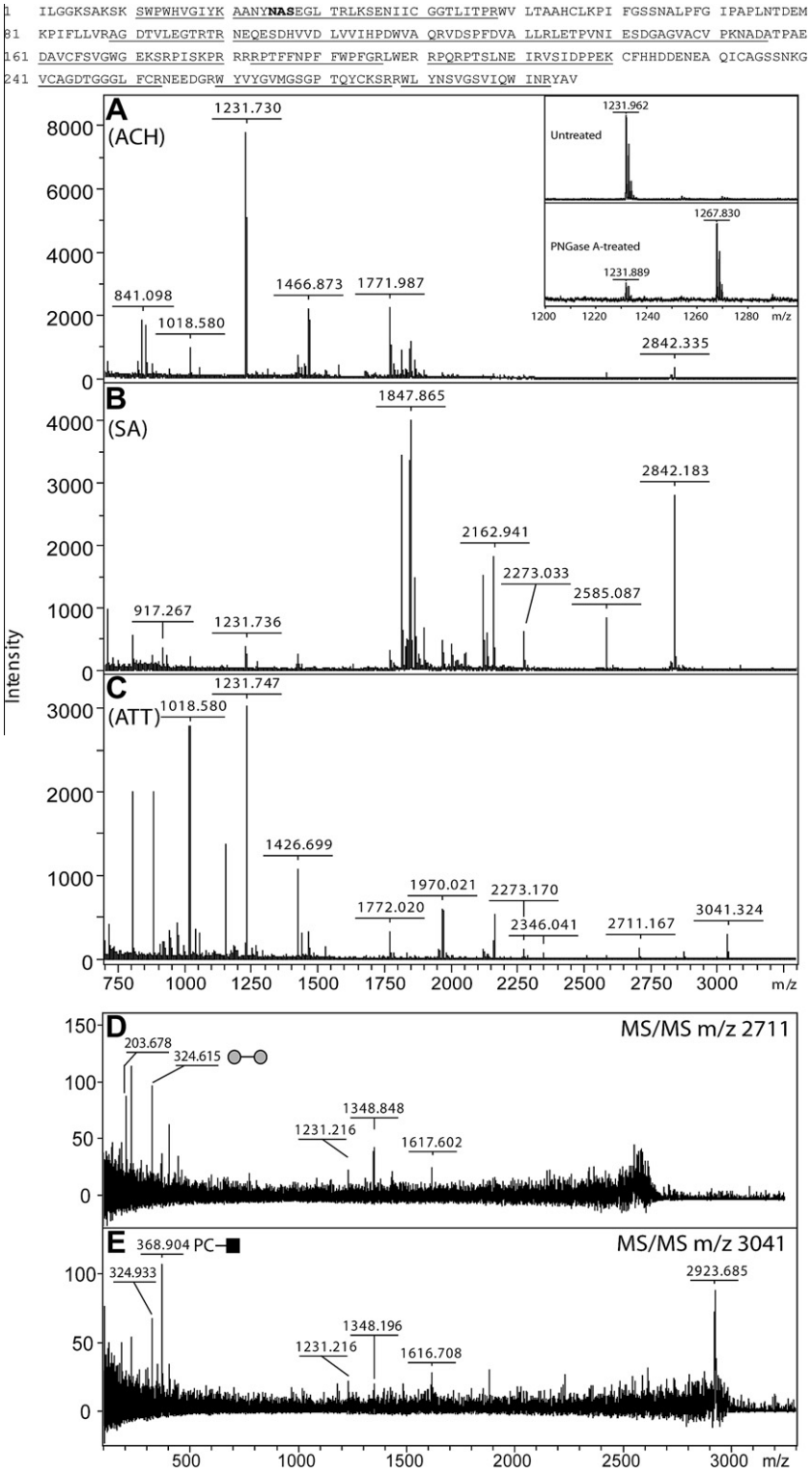

Fig. 2. Mass spectrometric analysis of *Echinococcus granulosus* antigen Ag5 tryptic (glyco)peptides. The revised sequence of the 38 kDa subunit of *E. granulosus* Ag5 is shown with the detected peptides underlined and the N-glycosylation site in bold. The MALDI-TOF MS spectra show the peptides analysed with either (A) α-cyanocinnamic acid, (B) sinapinic acid or (C) 6-aza-2-thiothymine as matrices. The inset in (A) shows the appearance of a peptide of *m*/*z* 1267.8 after PNGase A treatment of the tryptic peptides. (D and E) MALDI-TOF MS/MS of the *m*/*z* 2711 and 3041 molecular ions in (C) indicates the presence of fragments corresponding to unsubstituted terminal HexNAc (*m*/*z* 203.7) in the former spectrum and phosphorylcholine-HexNAc (*m*/*z* 368.9) in the latter.

molecular ion was replaced by 1485.1, compatible with an $[M+Na]^+$ form of $Hex_3HexNAc_4dHex_1$ resulting from the loss of two phosphorylcholine moieties (Fig. 3B); the dHex residue was resistant to this treatment. Thereby, the results of the glycopeptide analysis when using ATT as a matrix could be explained by the presence of a glycan conferring an increase in mass of 1775 to the peptide containing Asn_{25} (predicted mass 1265), thus resulting in a glycopeptide with *m*/*z* 3041.3.

The glycans released from the intact glycoprotein were also derivatised with pyridylamine. In the subsequent mass spectrum, two

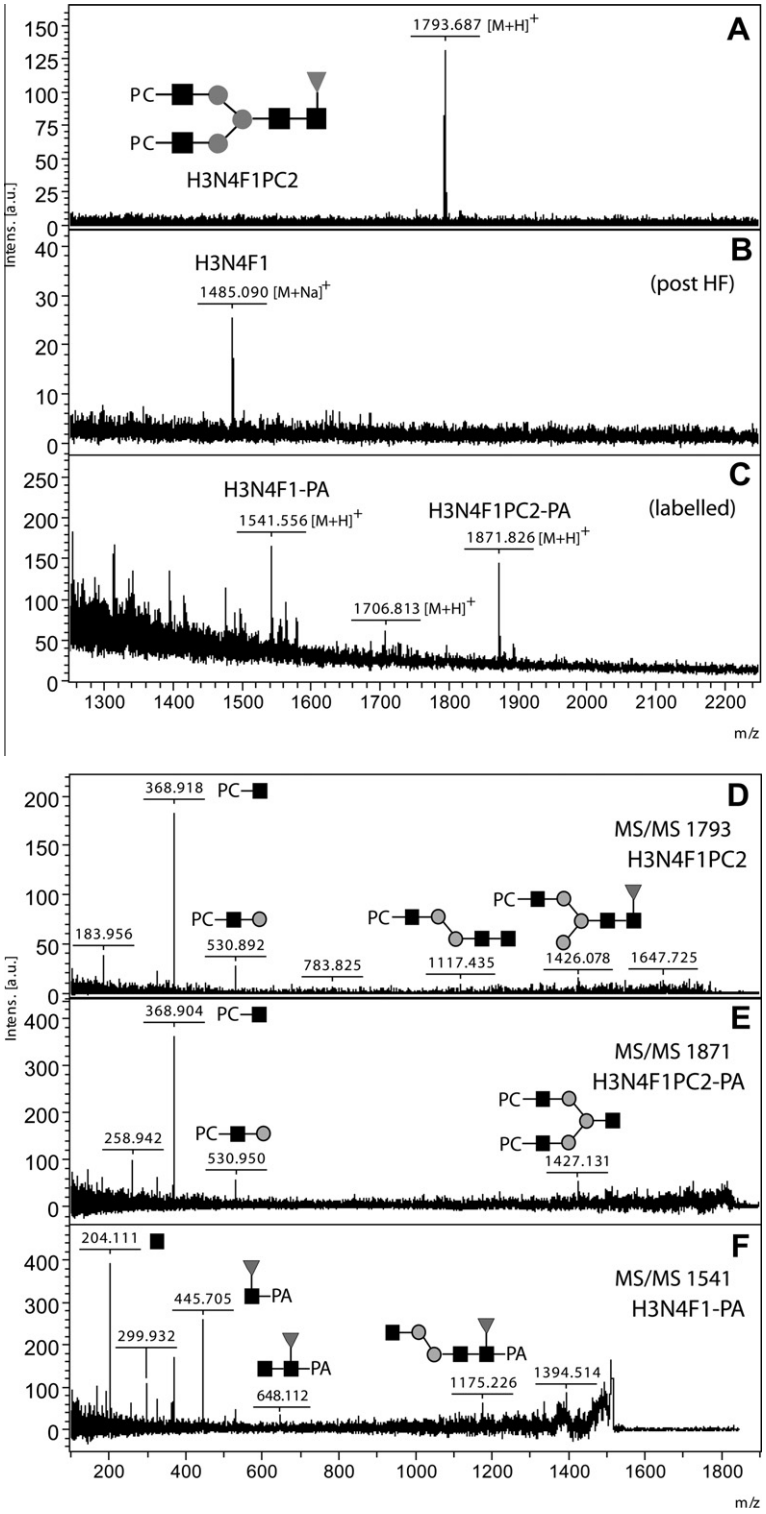

Fig. 3. Mass spectrometric analysis of *Echinococcus granulosus* antigen Ag5 N-glycans. MALDI-TOF MS spectra of N-glycans released from tryptic peptides of the 38 kDa subunit of Ag5 with PNGase A before (A) and after treatment with hydrofluoric acid (B) and of N-glycans released with PNGase F from intact Ag5 after pyridylamination (C). MALDI-TOF MS/MS spectra (D) of the major glycan in A and (E and F) of the two major pyridylaminated glycans in C, provide further compositional information. The predicted structures of the major glycan and selected fragments are depicted according to the nomenclature of the Consortium for Functional Glycomics as well as in an abbreviated code (H or circle, hexose; N or square, *N*-acetylhexosamine; F or triangle, fucose; PA, pyridylamine; PC, phosphorylcholine).

major peaks were observed with *m*/*z* values of 1541.6 and 1871.8 (Fig. 3C); the increase in mass of 78 as compared to the *m*/*z* value of the underivatised 1793.6 glycan is due to the addition of pyridylamine. Both species were subject to MS/MS (Fig. 3E and F), which showed the presence of dominant fragments indicative of core fucose ($HexNAc_1dHex_1PA$; *m*/*z* 445.7) and terminal HexNAc (*m*/*z* 204.1) in the former and phosphorylcholine ($HexNAc_1PC_1$; *m*/*z* 368.9) in the latter. Our experience is that phosphorylcholine-containing fragments dominate any MS/MS spectra (K. Paschinger, unpublished observations); thereby core modifications are 'hidden'

when analysing small amounts of such glycans. Compatible with this, hydrofluoric acid treatment of the pyridylaminated glycans resulted in a major product with *m*/*z* 1541 (data not shown) whose MS/MS spectrum lacked the *m*/*z* 368 fragment, whereas the core fucose fragment (*m*/*z* 446.2) was dominant (data similar to the MS/MS in Fig. 3F). Considering the binding to Ag5 by AAL, the lack of anti-horseradish peroxidase reactivity, the ability to cleave the glycan with PNGase F, the absence of antennal fucose and the insensitivity of the fucose residue to hydrofluoric acid (see above), the dHex residue is concluded to be a core α1,6-fucose linked to the reducing terminal GlcNAc residue.

The presence of pyridylaminated glycan species (*m*/*z* 1541.6 and 1706.8) lacking one or two phosphorylcholine moieties as compared to the $Hex_3HexNAc_4dHex_1PC_2$ glycan was not expected from the analyses of the underivatised glycans, but is rather compatible with the analyses of the glycopeptides which indicated a degree of heterogeneity not apparent in the spectra of the free glycans (see above). One may speculate that the presence of two phosphorylcholine moieties favourably alters the ionisation properties of underivatised glycans, whereas the presence of either arginine in the glycopeptide or pyridylamine label equalises ionisation of all glycan species present.

3.4. Conclusion

Using MALDI-TOF MS of glycans and (glyco)peptides, as well as confirmatory western blotting evidence, we show that the Ag5 antigen 38 kDa subunit from *E. granulosus* is modified on a single asparagine by a biantennary N-glycan decorated with up to two phosphorylcholine and one core α1,6-fucose residues; the presence of core fucose on biantennary glycans has been previously shown for both *E. granulosus* and *E. multilocularis* (Khoo et al., 1997; Hülsmeier et al., 2010). In light of the structural evidence presented here and our previous analysis of Ag5 antigenicity (Lorenzo et al., 2005b), we can conclude that the immunodominant N-glycan moiety of Ag5 is a complex antibody binding site formed by the sugar residues together with phosphorylcholine; indeed, while removal of the whole N-glycan caused a dramatic loss of immunoreactivity, inhibition experiments using monovalent phosphorylcholine only showed a modest decrease in reactivity.

Previous data have indicated Ag5-reactive sera cross-react with other cestode and nematode species (Shepherd and McManus, 1987); also, phosphorylcholine-dependent cross-reactivity towards cestode proteins of antibodies raised against nematode antigens has been reported (Romaris et al., 2001). In other studies, the presence of a phosphorylcholine-containing substance was indicated immunologically for a fish tapeworm and correlates with binding of cestode material to CRP (Fletcher et al., 1980; Taylor and Hoole, 1997).

In this context, the present study is important in showing for the first time that phosphorylcholine is an actual covalent modification of the N-glycans of a cestode antigen; to our knowledge, this is the first evidence for the presence of this moiety on N-glycans in a species other than nematodes such as *Caenorhabditis elegans* and *Ascaris suum* (Paschinger et al., 2006; Pöltl et al., 2007). As phosphorylcholine is known to possess immunomodulatory properties (Harnett and Harnett, 2001), its presence in a cestode suggests that this glycan modification may have been adopted during evolution, as a means of manipulating host immune systems, by a wider range of parasitic organisms than previously thought.

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