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Characterization of the acidic N-linked glycans of the zona pellucida of prepuberal pigs by a mass spectrometric approach

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Dedicated to Professor Dr. Hans Kamerling on the occasion of his 65th birthday

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ABSTRACT

Oocyte maturation is a prerequisite for successful fertilization. Growing evidence suggests that not only the oocyte but also the surrounding zona pellucida has to undergo maturational changes. In the pig, two-dimensional electrophoretic analysis demonstrated an acidic shift of the zona pellucida glycoproteins of about 1.5–2.0 pH units during the maturation process. These findings were corroborated by histological studies that indicated the synthesis of acidic glycoconjugates in the cumulus cells and an increased occurrence of acidic glycans in the zona pellucida after oocyte maturation. In order to provide structural data on prepuberal zona pellucida N-glycosylation, N-glycans were released from prepuberal zona pellucida glycoproteins by N-glycosidase F and studied by mass spectrometry before and after desialylation and treatment with *endo*-β-galactosidase. Our results verified the presence of high-mannose-type $Man_5GlcNAc_2$ compounds as well as diantennary N-glycans as major neutral species, whereas sialylated diantennary and triantennary species constituted the dominant non-sulfated acidic sugar chains. The major acidic N-glycans of prepuberal animals, however, represented mono-sulfated diantennary, triantennary and tetraantennary oligosaccharides carrying, in part, *N*-acetyllactosamine repeating units as well as additional Neu5Ac or Neu5Gc residues. Glycans comprising more than one sulfate residue were not detected. In contrast to the literature data on zona pellucida glycoprotein-N-glycans of cyclic animals, our data thus reveal a lower degree in glycan sulfation of the prepuberal zona pellucida.

1. Introduction

Fertilization is a fundamental event that involves a highly coordinated sequence of cellular interactions between the male and female gametes resulting in the formation of a completely new individual. The first contact between both gametes occurs at the outer face of the zona pellucida (ZP). This extracellular matrix surrounding the mammalian oocyte exerts important functions during gamete recognition, gamete binding, fertilization, and early embryonic development. Upon ZP binding the sperm acrosome reaction is induced, which enables the sperm to penetrate the matrix and to fertilize the egg. The fusion between both gametes induces modifications in the structure of the ZP resulting in the prevention of polyspermy and in the protection of the fertilized egg and pre-implantation embryo from physical and environmental damages.

The ZP is composed of three glycoproteins encoded by the genes ZPA, ZPB, and ZPC, which are largely orthologous throughout mammalian species.[1,2] In domestic animals the ZP glycoproteins are synthesized in an integrated and stage-specific pattern by both the oocyte and the granulosa cells forming the typical fibrogranular matrix between the oolemma and the innermost layer of the cumulus oophorus.[3–6] The ZP glycoproteins display a conserved structure, all sharing the so-called ZP domain, which spans about 260 amino acids[7] and has been implicated in the assembly of ZP

Abbreviations: AAA, *Anguilla anguilla* agglutinin; ACA, *Amaranthus caudatus* agglutinin; AB, Alcian Blue; CID, collision-induced dissociation; COCs, cumulus–oocyte-complexes; DSA, *Datura stramonium* agglutinin; DTT, dithiothreitol; FCS, fetal calf serum; IPG, immobilized pH gradient; IVF, in vitro fertilization; LacNAc, *N*-acetyllactosamine; MALDI-TOFMS, matrix-assisted laser-desorption ionisation time-of-flight mass spectrometry; MS, mass spectrometry; MS/MS, tandem mass spectrometry; Neu5Ac, *N*-acetylneuraminic acid; Neu5Gc, *N*-glycolylneuraminic acid; PAS, periodic acid Schiff's reagent; PBS, phosphate-buffered saline; PVDF, poly(vinylidene)difluoride; SDS, sodium dodecyl sulfate; SNA, *Sambucus niger* agglutinin; TBS, tris-buffered saline; THAP, 2,4,6-trihydroxyacetophenone; TCM, tissue culture medium; ZP, zona pellucida; ZPA (B, C), zona pellucida glycoprotein A (B, C).

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glycoproteins into filaments building up the three-dimensional architecture of the ZP.[8]

Differences in ZP glycoproteins between mammalian species reside mainly in their glycosylation and post-translational processing of the polypeptide backbone.[9,10] Their oligosaccharide structures have been first studied in the pig.[11–13] Recently a mass spectrometric approach has been used to characterize the N-glycans of porcine ZPA[14] as well as the corresponding bovine[15,16] and murine glycans.[17–20] Porcine ZP glycoproteins carry a complex pattern of N- and O-linked oligosaccharides that exhibit an enormous heterogeneity due to varying degrees of sialylation and sulfation.[11,12,21,22] N-Linked complex-type oligosaccharide structures are basically similar in the three species hitherto studied in that they are fucosylated at the chitobiose core and contain two, three, or four antennae carrying tandemly arranged *N*-acetyllactosamine (LacNAc) repeats. Bovine and murine zonae pellucidae are distinguished by the dominant expression of high-mannose-type N-glycans. Oligosaccharide structures of the studied species differ, however, in their degrees of sialylation and sulfation, in their terminal structures, and in the nature of their major neutral N-glycans.[12,17,23]

Carbohydrate-mediated primary and secondary binding events of both gametes, which occur at the oocyte ZP, are the essential prerequisites to enable the sequence of interactions leading to fertilization. It has been shown that the interaction of distinct carbohydrate structures of the ZP with surface-exposed receptor molecules of the sperm is involved in these binding events.[9,24–26] In this context, N-glycans have been shown to play a pivotal role in gamete recognition (primary binding) in the ungulate species. Neutral triantennary and tetraantennary complex-type N-glycans as well as high-mannose-type N-glycans have been found to be the determining sugar signals in the pig[23] and in cattle,[27] respectively. The anchoring of the sperm to the ZP by the carbohydrate side chains of ZP glycoproteins may facilitate the initiation of the acrosome reaction,[28,29] resulting in the exposure of acrosomal matrix proteins, which are required for the transient binding of the acrosome reacting/reacted sperm, and consequently, for penetration of the ZP by the sperm. Sulfated glycans of the ZP have been implicated in these secondary binding events.[30,31] Hence, binding events implicating the interaction of neutral and sulfated ZP glycans with carbohydrate-binding proteins exposed by the sperm appear to establish the contacts between both gametes.

Before fertilization, the immature oocytes have to resume meiosis, and the cytoplasm as well as the oocyte organelles have to complete the maturation process. Several lines of evidence suggest that the ZP also has to undergo structural and functional alterations, including changes in the post-translational modifications, such as, glycosylation.[24,32–35] An incomplete maturation process of the ZP under the conditions employed for in vitro culturing of the oocytes from prepuberal animals for porcine in vitro fertilization may contribute to the high incidence of polyspermic fertilization.[36] Preliminary studies have already pointed to an increased acidification of carbohydrate side chains during ZP maturation in the pig.[35] Therefore, as a first step of elucidating the biochemical basis of the maturation of ZP glycoproteins, the acidic N-linked glycans of the ZP of prepuberal animals were studied by mass spectrometry. Furthermore, changes in the expression of acidic glycoconjugates during oocyte development and maturation were elucidated by a histochemical approach.

2. Results and discussion

2.1. Histochemical localization of glycoconjugates in cumulus–oocyte-complexes (COCs) before and after in vitro maturation

As shown by periodic acid-Schiff's reagent (PAS) staining, glycoproteins are mainly synthesized in cells of the cumulus oophorus in both prepuberal COCs and in matured COCs in vitro (Fig. 1). In prepuberal COCs synthesis of glycoproteins is mainly carried out by the cumulus cells surrounding the ZP (the corona radiata) (Fig. 1A). Transportation of glycoproteins takes place in the processes of the cumulus cells reaching the ZP. Thus, the ZP is regularly strongly PAS-positive (Fig. 1A and B). Occasionally, the oocyte's cytoplasm is also weakly stained by PAS (Fig. 1A). After in vitro maturation, however, PAS-positive material is mainly produced in the other cells of the cumulus oophorus and is released into the intercellular matrix, which distinctly increases after maturation and widely separates the cumulus cells (Fig. 1B). PAS staining in conjunction with amylase digestion demonstrated that glycogen is neither synthesized in immature nor in matured COCs (data not shown). The cumulus cells of prepuberal COCs do not synthesize detectable amounts of acidic glycoconjugates as evidenced by Alcian Blue (AB) staining, which was used to detect acidic (AB-staining at pH 2.5) and specifically sulfated (AB-staining at pH 1.0) glycoconjugates in cumulus oocyte–complexes (COCs) during in vitro maturation (Fig. 2A). After maturation, however, AB-staining in the intercellular matrix is significantly increased (Fig. 2B). In contrast, the ZP in both immature and matured COCs is distinctly stained for acidic gly-

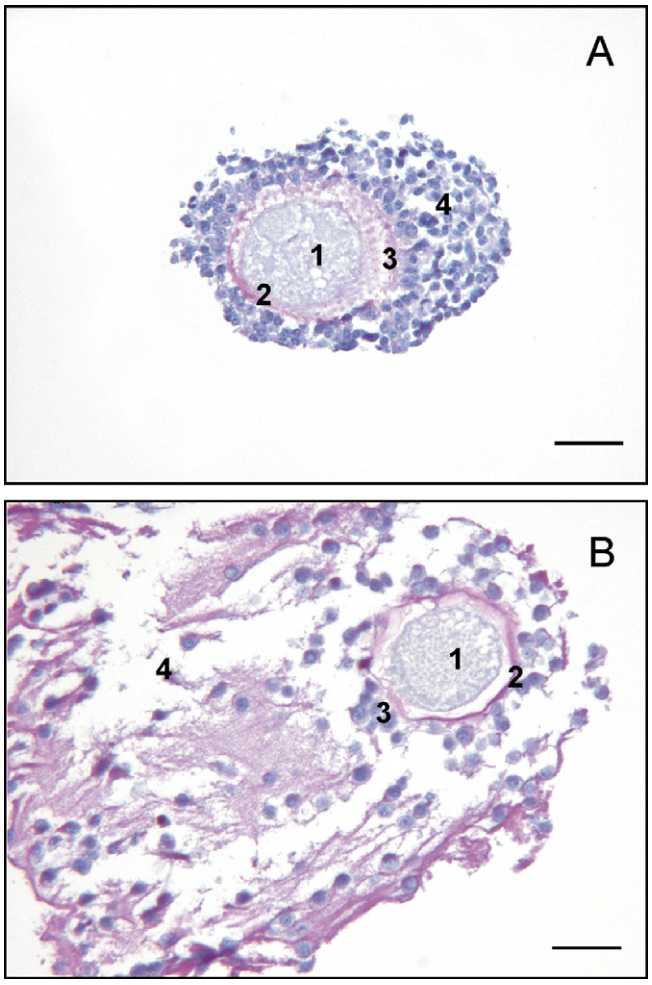

Figure 1. PAS-staining of immature and in vitro matured COCs. PAS-positive material stained violet; cells are counterstained with hematoxylin in blue. (A) PAS-staining of an immature COC. The ZP (2) and the cytoplasm of the corona radiata cells (3) are distinctly stained for glycoconjugates. The oocyte cytoplasm (1) is occasionally weakly stained. (B) PAS-staining of a COC after in vitro maturation. Glycoconjugates are mainly produced by the widely separated cells of the cumulus oophorus (4) and are released into the intercellular matrix. Bar = 90 μm.

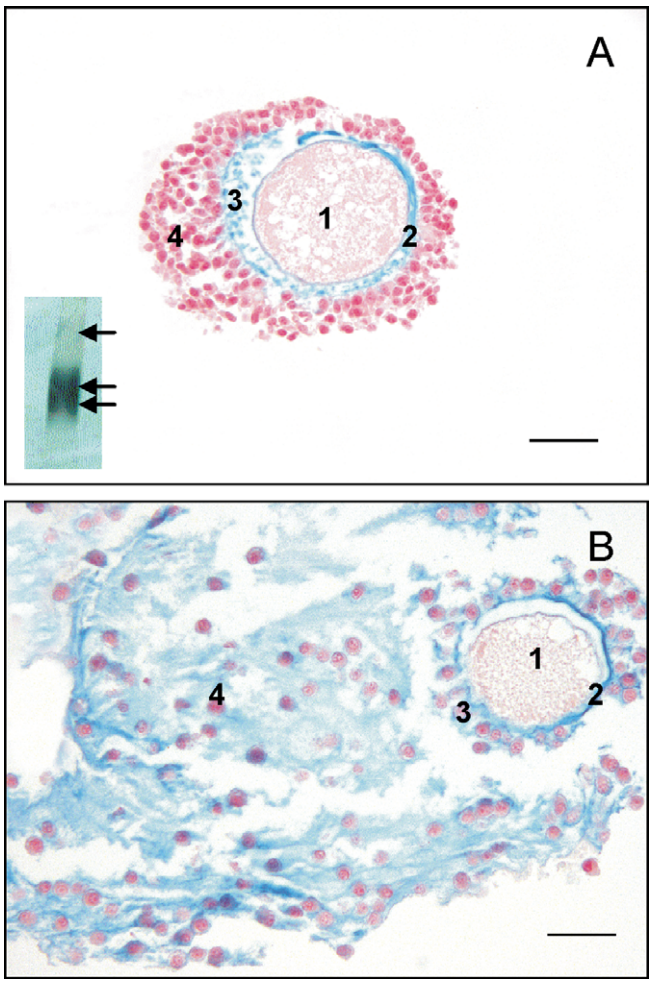

Figure 2. Alcian Blue staining of immature and in vitro matured COCs. Acidic glycoconjugates stained in azure; cells are counterstained with Fast Red in red. (A) AB staining (pH 2.5) of an immature COC. The ZP (2) and corona radiata cells (3) are distinctly stained. Inset: Solubilised ZP glycoproteins were labeled after SDS-PAGE for acidic glycoproteins by a combined AB–silver staining, indicating their sensitivity to Alcian Blue (arrow, ZPA and double arrow, ZPB/ZPC). (B) AB-staining (pH 2.5) of a COC after in vitro maturation. The cumulus cells (4) produce acidic glycoconjugates, which are secreted into the intercellular matrix. The zona pellucida (2) forms an intensively stained ring surrounding the oocyte. The oocyte cytoplasm (1) is regularly unstained. Bar = 90 μm.

coproteins. In matured COCs the ZP regularly appears as an intensively stained band surrounding the oocyte (Fig. 2B). The same—although moderate—changes were found for sulfated glycoproteins, as evidenced by AB-staining at pH 1.0 (data not shown), indicating an increased expression of acidic glycoproteins including sulfated species in the ZP during oocyte maturation. Combined AB–silver staining[37] of electrophoretically separated ZP glycoproteins showed that the acidic glycoproteins are, in fact, sensitive to AB. Particularly the ZPB/ZPC band revealed strong AB-labeling (Fig. 2A, inset). Hence, it can be concluded that AB-staining of the ZP in COCs is, to great extent, due to ZP glycoproteins. The increase in staining intensity after in vitro maturation of COCs points to an acidification of the ZP glycoproteins including sulfation (Fig. 2). Differences in the lectin binding pattern of the ZP during oocyte maturation have already been reported for pig and wild boar,[33] as well as for other species,[38] which support the idea that structural alteration of the ZP is part of the oocyte maturation process. In addition, after in vitro maturation the cumulus cells are separated by high amounts of intercellular material. This phenomenon is known as cumulus expansion and is the result of the production of hyaluronan by the cumulus cells and its accumulation in the intercellular space during oocyte maturation.[39] The strong staining of the intercellular matrix with Alcian Blue is in agreement with this observation. These results suggest that the time-specific and site-specific expression of acidic glycans in the ZP and in the intercellular space of the cumulus oophorus may play an important role in oocyte maturation, thus influencing the developmental capacity of the oocyte.

2.2. Electrophoretical analysis of ZP glycoproteins before and after in vitro maturation

The protein content of the ZPs collected from prepuberal animals (about 15 ng/ZP) was found to be about half of that collected from adult animals (about 30–35 ng/ZP). Because of the limited number of ZPs collected from in vitro matured oocytes, the protein content was not determined. For lectin blotting approximately 400 ZP equivalents of prepuberal animals and 200 ZP equivalents of in vitro matured oocytes were subjected to the analysis.

Two-dimensional gel electrophoresis of the solubilised ZP glycoproteins from prepuberal animals resolved as the known spot-like pattern of heterogeneous charge isomer families as described elsewhere.[14,40] The non-separated bands of the ZPB and ZPC proteins with molecular masses between 45 and 60 kDa extended over the pI range of 3.0–7.5, with the bulk of glycoisoforms accumulating between pI 4 and pI 6. The ZPA glycoproteins (95–120 kDa) ranged from pI 3 to pI 6.5, occurring preponderantly between pH 4.8 and pH 6.0 (Fig. 3A). ZP glycoproteins collected by mass isolation and after puncture of the follicles showed no difference in their electrophoretic pattern (data not shown). In order to amplify the ZP glycoprotein signals of immature and in vitro matured oocytes, lectin blotting with *Anguilla anguilla* agglutinin (AAA), *Amaranthus candatus* agglutinin (ACA), *Datura stramonium* agglutinin (DSA), and *Sambucus niger* agglutinin (SNA) was used in conjunction with chemiluminescent visualization. For immature oocytes all lectins revealed a similar distribution of the ZPB/ZPC band from 3.0 to 7.5 pH units as shown for the AAA-lectin blot (Fig. 3B). Labeling with DSA and AAA also visualized ZPA isoglycoproteins at pI 7.0 (Fig. 3B), whereas ACA and SNA (not shown) displayed a similar distribution of ZPA isoglycoproteins as demonstrated by the silver-stained gel (Fig. 3A). The sensitive AAA staining was therefore used to analyze the ZP glycoprotein pattern in in vitro matured oocytes, demonstrating an acidic shift of the ZPB/ZPC band of approximately 1.5–2.0 pH units (Fig. 3C). All lectin blots of in vitro matured oocytes showed a comparable acidic shift that indicated a significant acidification of the ZP glycoproteins, although acidification of ZPA isoglycoproteins appeared to be less pronounced.

2.3. Mass spectrometric studies of N-glycans from ZP glycoproteins of prepuberal animals

N-Glycan profiling was predominantly performed with a Kratos MALDI II Analytical Kompact mass spectrometer (Vs 5.2, 1996). Due to instrumental features, measured masses occasionally deviated from the calculated masses by 2500 ppm and varied also between different measurements. To facilitate the comparison of the glycan profiles, which were generated after different sample treatments, and in order to allow the detection of high-mass glycans, the data obtained under identical conditions with the Kratos instrument were used. However, the glycan profiles were controlled exemplarily by using a Bruker Ultraflex I mass spectrometer that exhibited masses with deviations of less than 300 ppm over the whole mass range.

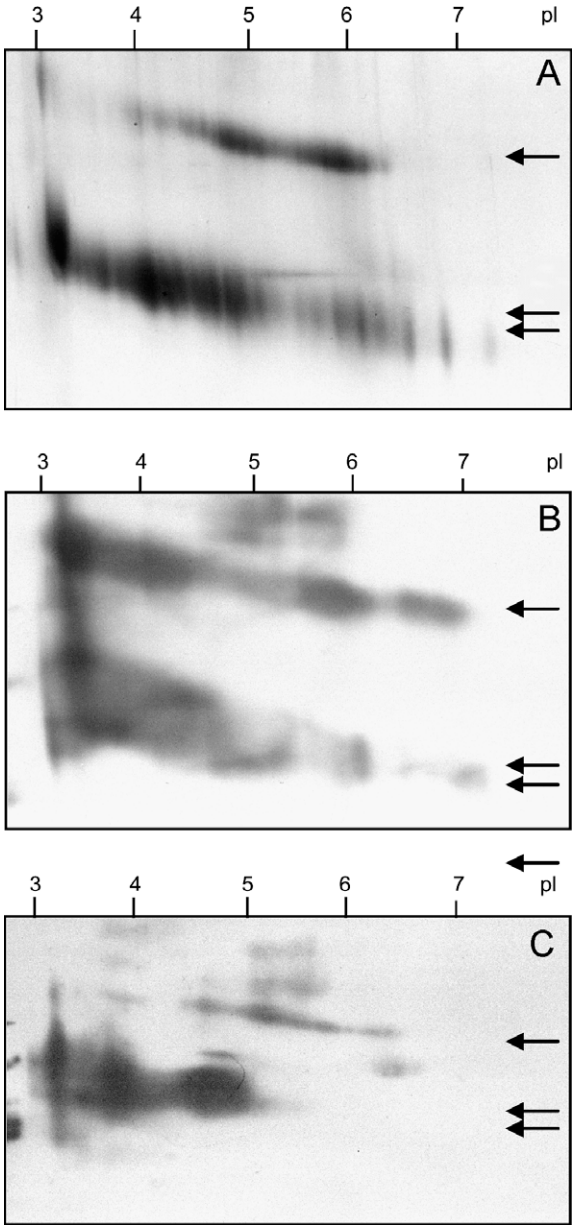

Figure 3. Two-dimensional electrophoresis of ZP glycoproteins from prepuberal pigs. (A) About 1000 zonae pellucidae equivalents of oocytes of prepuberal pigs were subjected to electrophoresis and then silver stained according to the method of Heukeshoven and Dernick.[47] (B and C) For lectin blotting, about 400 and 200 zonae pellucidae equivalents of immature (B) and in vitro matured (C) oocytes, respectively, were subjected to electrophoresis. The protein blots were labeled with biotinylated AAA agglutinin and visualized with streptavidin–peroxidase and chemiluminescent detection. (arrow, ZPA; double arrow, ZPB/ZPC).

2.4. Analysis of sialylated N-glycans of ZP glycoproteins from prepuberal pigs

Neutral and acidic N-glycan fractions derived from the native ZP glycoproteins of strongly selected (according to age and weight) prepuberal animals were investigated with MALDI-TOFMS. In agreement with previous studies on ZP glycoproteins from cyclic animals,[14,21] the dominant species of the separated neutral glycan fraction was the high-mannose-type N-glycan $Hex_5HexNAc_2$ (m/z_{exp} 1256.9 $[M+Na]^+$), followed by fucosylated diantennary complex-type N-glycans with one (m/z_{exp} 1647.0 $[M+Na]^+$) and two (m/z_{exp} 1808.8 $[M+Na]^+$) terminal galactosyl residues. Two additional signals with low intensities were observed at m/z_{exp} 2012.0 and 2174.8 $[M+Na]^+$ corresponding to complex-type $Hex_5HexNAc_5Fuc_1$ and $Hex_6HexNAc_5Fuc_1$ (data not shown).

The MS profile of the acidic fraction is shown in Figure 4A. Prominent peaks originated from monosialylated and disialylated glycans with *N*-acetylneuraminic (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc) (Table 1) in agreement with the compositions of the complex-type N-glycans in the neutral fraction. Corresponding asialo-glycan structures could be further verified by MALDI-TOFMS after on-target desialylation (data not shown).

In addition a number of peaks could be detected particularly in the higher mass region (Fig. 4A and Table 1), which corresponded either to mono-sulfated or to mono-sulfated, sialylated N-glycans

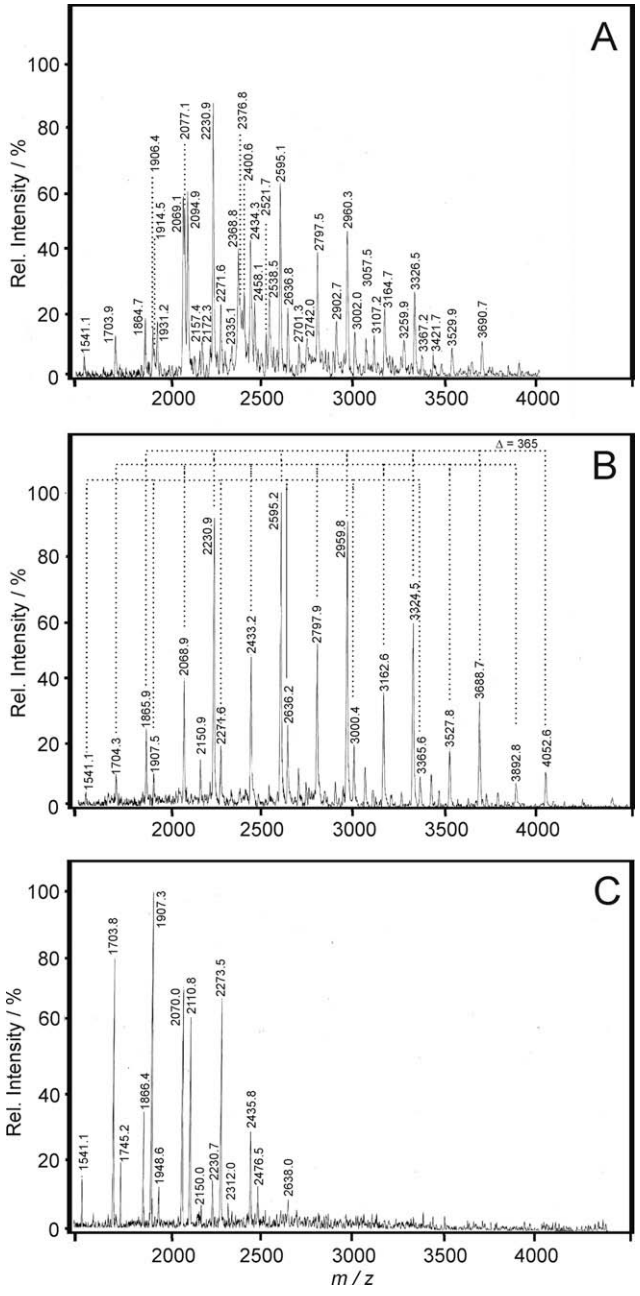

Figure 4. MALDI-TOFMS analysis of the N-glycans from ZP-glycoproteins of prepuberal pigs. (A) N-Glycan profile of the entire acidic N-glycan fraction of prepuberal ZP glycoproteins. (B) Profile of acidic, desialylated prepuberal ZP N-glycans (desZP). (C) Profile of the acidic, desialylated N-glycan fraction (desZP) after *endo*-β-galactosidase treatment (EβGalZP). The *m/z* values represent $[M-H]^-$ pseudomolecular ions.

Table 1
Acidic glycan species of unmodified ZP glycoproteins from prepuberal animals

m/z_{exp}[a] $[M-H]^-$	Composition of sialo-N-glycans	Composition of sulfated N-glycans	Composition of sulfated sialo-N-glycans	SI[b]
1541.1		$Hex_3HexNAc_4Fuc_1S_1$		+
1703.9		$Hex_4HexNAc_4Fuc_1S_1$		+
1864.7		$Hex_5HexNAc_4Fuc_1S_1$		+
1906.4		$Hex_4HexNAc_5Fuc_1S_1$		+
1914.5	$Hex_4HexNAc_4Fuc_1Neu5Ac_1$			+
1931.2	$Hex_4HexNAc_4Fuc_1Neu5Gc_1$			+
2069.1		$Hex_5HexNAc_5Fuc_1S_1$		+++
2077.1	$Hex_5HexNAc_4Fuc_1Neu5Ac_1$			+++
2094.9	$Hex_5HexNAc_4Fuc_1Neu5Gc_1$			+++
2157.4			$Hex_5HexNAc_4Fuc_1S_1Neu5Ac_1$	+
2172.3			$Hex_5HexNAc_4Fuc_1S_1Neu5Gc_1$	+
2230.9		$Hex_6HexNAc_5Fuc_1S_1$		+++++
2271.6		$Hex_5HexNAc_6Fuc_1S_1$		++
2335.1	$Hex_4HexNAc_6Fuc_1Neu5Gc_1$			+++
2368.8	$Hex_5HexNAc_4Fuc_1Neu5Ac_2$			+++
2376.8			$Hex_5HexNAc_5Fuc_1S_1Neu5Gc_1$	++
2400.6	$Hex_5HexNAc_4Fuc_1Neu5Gc_2$		$Hex_5HexNAc_6Fuc_1S_1Neu5Ac_1$	++
2434.3		$Hex_6HexNAc_6Fuc_1S_1$		+++
2458.1	$Hex_6HexNAc_5Fuc_1Neu5Gc_1$			++
2521.7			$Hex_6HexNAc_5Fuc_1S_1Neu5Ac_1$	+
2538.5	$Hex_4HexNAc_7Fuc_1Neu5Gc_1$		$Hex_6HexNAc_5Fuc_1S_1Neu5Gc_1$	++
2595.1		$Hex_7HexNAc_6Fuc_1S_1$		++++
2636.8		$Hex_6HexNAc_7Fuc_1S_1$		++
2701.3	$Hex_5HexNAc_7Fuc_1Neu5Gc_1$			+
2742.0	$Hex_4HexNAc_8Fuc_1Neu5Gc_1$		$Hex_6HexNAc_6Fuc_1S_1Neu5Gc_1$	+
2797.5		$Hex_7HexNAc_7Fuc_1S_1$		++
2902.7			$Hex_7HexNAc_6Fuc_1S_1Neu5Gc_1$	+
2960.3		$Hex_8HexNAc_7Fuc_1S_1$		+++
3002.0		$Hex_7HexNAc_8Fuc_1S_1$		+
3057.5			$Hex_5HexNAc_7Fuc_1S_1Neu5Ac_2$	+
3107.2	$Hex_5HexNAc_9Fuc_1Neu5Gc_1$		$Hex_7HexNAc_7Fuc_1S_1Neu5Gc_1$	+
3164.7		$Hex_8HexNAc_8Fuc_1S_1$		++
3259.9	$Hex_6HexNAc_6Fuc_1Neu5Gc_2Neu5Ac_1$		$Hex_5HexNAc_8Fuc_1S_1Neu5Ac_2$	+
3326.5		$Hex_9HexNAc_8Fuc_1S_1$	$Hex_6HexNAc_6Fuc_1S_1Neu5Gc_1Neu5Ac_2$	++
3367.2		$Hex_8HexNAc_9Fuc_1S_1$		+
3421.7	$Hex_7HexNAc_6Fuc_1Neu5Gc_2Neu5Ac_1$		$Hex_6HexNAc_8Fuc_1S_1Neu5Ac_2$	+
3529.9		$Hex_9HexNAc_9Fuc_1S_1$	$Hex_6HexNAc_7Fuc_1S_1Neu5Gc_1Neu5Ac_2$	+
3690.7		$Hex_{10}HexNAc_9Fuc_1S_1$	$Hex_7HexNAc_7Fuc_1S_1Neu5Gc_1Neu5Ac_2$	+

[a] Experimentally determined mass values. Pseudomolecular ions $[M-H]^-$ are given in monoisotopic masses.
[b] Relative abundance of species is roughly estimated from the respective signal intensities (SIs): (+), 2–5%; +, 5–20%; ++, 21–40%; +++, 41–60%; ++++, 61–80%; +++++, 81–100% signal intensity.

carrying one to three Neu5Ac or Neu5Gc residues. Respective asialo-core glycans were again corroborated by MALDI-TOFMS after on-target desialylation (data not shown). The compositions that were found suggested, in part, the presence of complex-type N-glycans carrying LacNAc repeating units. Glycans comprising more than one sulfate residue were not registered, although different MALDI-TOFMS instruments, matrices and experimental conditions have been employed. This is interesting insofar as structural studies on N- and O-glycans of ZP glycoproteins from cyclic animals documented the presence of linear, multiply sulfated oligo-*N*-acetyllactosamine chains.[11,21]

2.5. Analysis of sulfated N-glycans of ZP glycoproteins from prepuberal pigs

On-blot deglycosylation of the desialylated ZP glycoproteins and separation of the acidic glycans by fractionated elution from graphitized carbon[41] allowed a more detailed analysis of the sulfated N-glycans by MALDI-TOFMS (Fig. 4B and Table 2). Prominent signals corresponded to complex-type N-glycans with one sulfate group (S) and one deoxyhexose (Fuc), the latter of which could be assigned to the innermost HexNAc of the N-glycan core by MS/MS analysis (see Fig. 5). Masses of the observed peaks ranged from m/z 1541.1 $[M-H]^-$ to m/z 4052.6 $[M-H]^-$, corresponding to monosaccharide compositions of $Hex_{3-11}HexNAc_{4-10}Fuc_1S_1$. The profile spectrum comprised three series of signals with mass differences of m/z 365 (Fig. 4B) suggesting the existence of solely diantennary N-glycans elongated by a varying number of LacNAc repeats (m/z 1541.1 $Hex_{3+n}HexNAc_{4+n}Fuc_1S_1$, m/z 1704.3 $Hex_{4+n}HexNAc_{4+n}Fuc_1S_1$ and m/z 1865.9 $Hex_{5+n}HexNAc_{4+n}Fuc_1S_1$). After *endo*-β-galactosidase treatment of the desialylated ZP glycoproteins and release of the degraded N-glycans, however, the resulting MS profile was not reduced to the three expected peaks, but instead displayed 15 mass signals (Fig 4C, Table 2) that could be interpreted as being derived from diantennary, triantennary, and tetraantennary N-glycan structures.

The high molecular mass signal ($m/z > 2636.2$ $[M-H]^-$, $Hex_6HexNAc_7Fuc_1S_1$) and the signals at m/z 2230.9 $[M-H]^-$ and m/z 2595.2 $[M-H]^-$ corresponding to the compositions $Hex_6HexNAc_5Fuc_1S_1$ and $Hex_7HexNAc_6Fuc_1S_1$, respectively, were significantly reduced (m/z 2230.9) or completely absent after enzyme treatment. In addition, novel signals or an increase of signal intensities in the low-molecular-mass range (m/z 1541.1, 1703.8, 1745.2, 1907.3, 1948.6, 2070.0, 2110.8, 2273.5, and 2476.5) was observed (Fig. 4C). The dominant signal at m/z 2230.9 $[M-H]^-$ ($Hex_6HexNAc_5Fuc_1S_1$) can be, therefore, attributed to a fucosylated diantennary complex-type N-glycan carrying one LacNAc repeat and a single sulfate group. Treatment with *endo*-β-galactosidase gave rise to a signal corresponding to sulfated $Hex_4HexNAc_4Fuc_1S_1$ species (m/z 1703.8 $[M-H]^-$). The remaining signal at m/z 2230.7

Table 2
Compilation of sulfated N-glycans from ZP glycoproteins of prepuberal gilts after desialylation (desZP) and *endo*-β-galactosidase treatment (EβGalZP)

m/z [M−H][a]	Oligosaccharide composition	SI[b]	
		desZP	EβGalZP
1541.1 (1541.1)	$Hex_3HexNAc_4Fuc_1S_1$	(+)	+
1704.3 (1703.8)	$Hex_4HexNAc_4Fuc_1S_1$	(+)	++++
– (1745.2)	$Hex_3HexNAc_5Fuc_1S_1$	–	+
1865.9 (1866.4)	$Hex_5HexNAc_4Fuc_1S_1$	++	++
1907.5 (1907.3)	$Hex_4HexNAc_5Fuc_1S_1$	+	+++++
– (1948.6)	$Hex_3HexNAc_6Fuc_1S_1$	–	++
2068.9 (2070.0)	$Hex_5HexNAc_5Fuc_1S_1$	+++	++++
– (2110.8)	$Hex_4HexNAc_6Fuc_1S_1$	–	+++
2150.9 (2150)	$Hex_3HexNAc_7Fuc_1S_1$	+	+
2230.9 (2230)	$Hex_6HexNAc_5Fuc_1S_1$	++++	+
2271.6 (2273.5)	$Hex_5HexNAc_6Fuc_1S_1$	++	+++
– (2312)	$Hex_4HexNAc_7Fuc_1S_1$	–	+
2433.2 (2435.8)	$Hex_6HexNAc_6Fuc_1S_1$	+++	++
– (2476.5)	$Hex_5HexNAc_7Fuc_1S_1$	–	+
2595.2 (–)	$Hex_7HexNAc_6Fuc_1S_1$	++++	–
2636.2 (2638)	$Hex_6HexNAc_7Fuc_1S_1$	+	+
2798.9 (–)	$Hex_7HexNAc_7Fuc_1S_1$	++	–
2959.8 (–)	$Hex_8HexNAc_7Fuc_1S_1$	++++	–
3000.4 (–)	$Hex_7HexNAc_8Fuc_1S_1$	++	–
3162.6 (–)	$Hex_8HexNAc_8Fuc_1S_1$	++	–
3324.5 (–)	$Hex_9HexNAc_8Fuc_1S_1$	++++	–
3365.6 (–)	$Hex_8HexNAc_9Fuc_1S_1$	+	–
3527.8 (–)	$Hex_9HexNAc_9Fuc_1S_1$	++	–
3688.7 (–)	$Hex_{10}HexNAc_9Fuc_1S_1$	++	–
3892.8 (–)	$Hex_{10}HexNAc_{10}Fuc_1S_1$	+	–
4052.6 (–)	$Hex_{11}HexNAc_{10}Fuc_1S_1$	+	–

Relative abundance of species is roughly estimated from the respective signal intensities (SIs).

[a] Mass values determined after desialylation (desZP), values after *endo*-β-galactosidase treatment (EβGalZP) are given in parentheses.

[b] Relative abundance of species is roughly estimated from the respective signal intensities (SIs): (+), 2–5%; +, 5–20%; ++, 21–40%; +++, 41–60%; ++++, 61–80%; +++++, 81–100% signal intensity.

might reflect triantennary $Hex_6HexNAc_5Fuc_1S_1$ without a repeating unit. In a comparable manner, the major glycans at *m/z* 2595.2, 2797.9, and 2959.8 were obviously degraded to truncated triantennary complex-type N-glycans carrying zero to two terminal hexose residues (*m/z* 1745.2, 1907.3, 2070.0, i.e., $Hex_{3-5}HexNAc_5Fuc_1S_1$). Likewise, the appearance of the $[M-H]^-$ signals at *m/z* 1948.6 ($Hex_3HexNAc_6Fuc_1S_1$), 2110.8 ($Hex_4HexNAc_6Fuc_1S_1$) and 2273.5 ($Hex_5HexNAc_6Fuc_1S_1$) after *endo*-β-galactosidase treatment indicated the occurrence of sulfated tetraantennary structures with two to four LacNAc repeats. The signals at *m/z* 1865.9 (1866.4) and 2433.2 (2435.8) were nearly unchanged in both spectra and might argue for a complete diantennary N-glycan with two terminal galactose residues ($Hex_5HexNAc_4Fuc_1S_1$) and an incomplete tetra-antennary structure with three terminal galactose residues ($Hex_6HexNAc_6Fuc_1S_1$), respectively.

In earlier analyses the sulfate groups have been predominantly localized at the C-6 position of GlcNAc (SO_3-6GlcNAc) residues in the LacNAc repeats.[42] The dominant appearance of sulfated glycans after *endo*-β-galactosidase treatment observed in this study, however, argues for an alternative localization of the sulfate group at an inner HexNAc of one antenna or at the core GlcNAc residues. In order to assign the present sulfate group, three selected species were analyzed by MALDI-TOFMS/MS experiments. The spectra from diantennary $Hex_4HexNAc_4Fuc_1S_1$ (*m/z* 1703.4, Fig. 5A), predominantly occurring after *endo*-β-galactosidase treatment, from diantennary $Hex_5HexNAc_4Fuc_1S_1$ (*m/z* 1864.5, Fig. 5B), which is stable against *endo*-β-galactosidase treatment and from the species $Hex_6HexNAc_5Fuc_1S_1$ (*m/z* 2230.9, Fig. 5C), which mainly disappeared after enzyme digestion, provided similar results: in all three spectra signals at *m/z* 281 (SO_3-HexNAc) and *m/z* 443 (Hex-

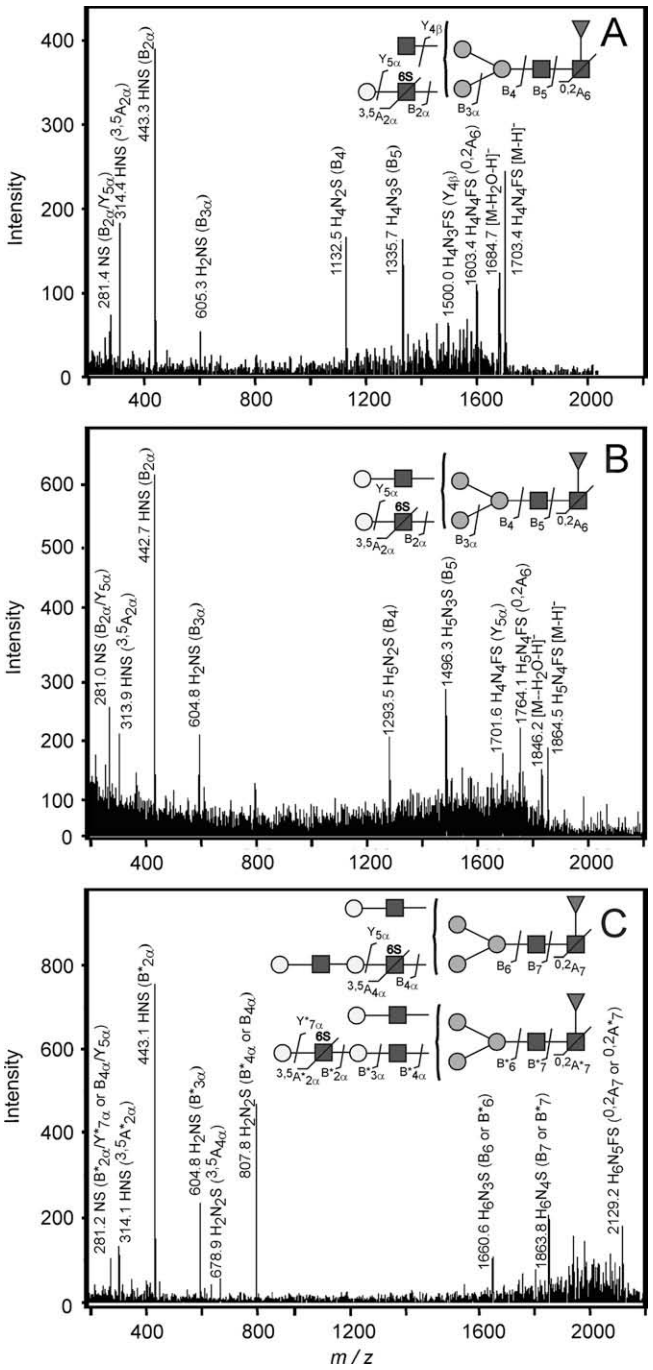

Figure 5. MALDI-TOFMS/MS (CID) spectra of sulfated N-glycans. (A) Fragment ion spectrum of diantennary $Hex_4HexNAc_4Fuc_1S_1$ (*m/z* 1703.4), (B) diantennary $Hex_5HexNAc_4Fuc_1S_1$ (*m/z* 1864.5), and (C) diantennary $Hex_6HexNAc_5Fuc_1S_1$ (*m/z* 2230.7) glycan species. Spectra were recorded in the negative-ion mode. Squares, *N*-acetylhexosamine; Circles, hexose; triangles, fucose; and S, sulfate. Assignment of fragment ions was performed according to Domon and Costello.[58]

(SO_3)HexNAc) clearly showed the localization of the sulfate group on the *N*-acetylhexosamine residue. Furthermore, fragments at *m/z* 314, formed by ^{3,5}A cross-ring fragmentation of HexNAc in Hex-(SO_3)HexNAc units, documented the linkage of sulfate in position 6 of the HexNAc. In the spectrum of the untreated species (*m/z* 2230), signals at *m/z* 604.8 ($Hex_2HexNAcS$) and 807.9 ($Hex_2HexNAc_2S$) allowed an assignment of sulfate to the antenna comprising a repeating unit (Fig. 5C). Moreover, the weak signal at *m/z* 678.9 (^{3,5}A cross-ring cleavage of HexNAc in Hex-HexNAc-Hex-(SO_3)

HexNAc) suggested, at least in part, the localization of sulfate also at the inner HexNAc residue. A similar cleavage has not been observed for unsubstituted HexNAc in our studies. Signals in the higher mass region of the three MS/MS spectra showed two prominent peaks, each of which represents the loss of fucosylated parts of the structure. The loss of HexNAcFuc and $HexNAc_2Fuc$ clearly demonstrated that all structures were core fucosylated. Taken together, our results suggest the preponderant occurrence of N-glycans carrying a single sulfate group at the C-6 position of repeated or non-repeated GlcNAc residues in the ZP glycoproteins of prepuberal animals. Hence, the low degree in glycan sulfation of the prepuberal ZP might be responsible for the distribution of the isoglycoproteins over a wide pI range. Combined data provide evidence that ZP glycoprotein–glycans become increasingly acidic during oocyte differentiation and maturation. In particular, domains with LacNAc repeating units might serve as targets for sulfation.

3. Experimental

3.1. Materials

Unless otherwise stated, highly purified chemicals were obtained from E. Merck (Darmstadt, Germany) and Sigma–Aldrich (Steinheim, Germany). The following enzymes were used: Neuraminidase EC 3.2.1.18 (Roche, Mannheim, Germany), N-glycosidase F EC 3.5.1.52 (Calbiochem, Bad Soden, Germany), and *endo*-β-galactosidase EC 3.2.1.103 (Calbiochem). For lectin blotting, the following biotinylated lectins were purchased from Vector/Alexis (Grünberg, Germany): *A. anguilla* agglutinin (AAA); *A. caudatus* agglutinin (ACA) and *S. niger* agglutinin (SNA), *D. stramonium* agglutinin (DSA). Streptavidin-peroxidase was obtained from Dianova (Hamburg, Germany). Luteinizing hormone (LH) was obtained from Harbor–UCLA Research and Education Institute, USA).

3.2. Collection and in vitro maturation of oocytes

Immature oocytes were collected from ovaries of prepuberal gilts (85–110 kg, mean age six months) obtained from a local abattoir and matured as described recently.[43] In brief, follicles (3–5 mm dia) were sliced and flushed with 0.9% saline supplemented with 1% heat-inactivated fetal calf serum (FCS; Sigma). Vital cumulus–oocyte-complexes (COCs) were identified using a stereo microscope. Only COCs with at least five compact cumulus cell layers were selected and washed with Dulbecco's phosphate-buffered saline (PBS) containing 10% (v/v) FCS. These COCs (25 COCs) were matured in 0.5-mL tissue culture medium (TCM 199, 20 μg/mL insulin, 80 μg/mL L-glutamine, 50 μg/mL gentamycin, 20% FCS (v/v), 2.5 μg/mL FSH, 5.0 μg/mL LH) at 39 °C in 5% CO_2 in air in a humidified atmosphere for 48 h.

Nuclear maturation stadium was controlled in an aliquot by the aceto-orcein staining according to the method of Eaton et al.[44] In general, oocytes analyzed immediately after collection were in the germinal vesicle I stage. After in vitro maturation about 80% of the oocytes were in the metaphase II.

3.3. Histochemistry

Immature COCs obtained by follicular puncture as well as COCs after in vitro maturation were investigated by PAS-staining to elucidate the amount and localization of glycoconjugates in prepuberal as well as in vitro matured COCs. The COCs were washed three times in PBS and fixed in Bouin's fluid for 5 min. Specimens were dehydrated in graded series of EtOH (50–100%), treated with xylene, and embedded in paraffin. Sections (6 μm) were cut with a microtome (Leica, Wetzlar, Germany). After treatment with xylene and hydration, the slides were washed in distilled water and were incubated in $NaHSO_3$ solution (1:10 dilution of 10% $NaHSO_3$ mixed with 1 N HCl in a ratio of 1:1) for 10 min. After washing in distilled water, the COCs were incubated in Schiff's reagent (E. Merck) for 10 min. Counterstaining was performed with hematoxylin for 5–10 s. After dehydration, specimens were treated with xylene and mounted with Eukitt.

To distinguish between glycogen and other PAS-positive polysaccharides and glycoproteins, digestion of glycogen using the enzyme amylase was applied. Before starting the PAS reaction, COCs were incubated in 0.5% amylase solution at 37 °C for 10 min. Adult liver, known to be rich in glycogen, was used as a control for the PAS reaction and enzyme digestion.

In order to compare the expression of sulfated versus generally acidic glycoconjugates in immature as well as in matured COCs, AB (Sigma) staining at pH 1 and pH 2.5 was applied. AB- staining at pH 1 detects specifically sulfated glycoconjugates, whereas AB staining at pH 2.5 marks acidic glycoconjugates in general. For AB staining at pH 1, the hydrated COCs were washed in distilled water, treated with 0.1 N HCl for 3 min, and incubated in AB for 30 min. After treatment with 0.1 N HCl and washing in distilled water, slides were counterstained with 0.1% Fast Red (Roche) for 5 min. For AB staining at pH 2.5, the COCs were incubated in 3% HOAc for 3 min and stained with 3% AB in HOAc. After washing steps in HOAc and distilled water, counterstaining was performed with 0.1% Fast Red. After dehydration specimens were treated with xylene and mounted with Eukitt.

3.4. Isolation of zonae pellucidae

Oocytes were collected from prepuberal gilts (85–110 kg, mean age six months), according to Dunbar et al.[45] Frozen/thawed ovaries were shredded in a meat grinder and then passed through nylon screens of decreasing pore size (200–2000 μm). Zona pellucida-encased oocytes of 120–180 μm were collected from an 80 μm nylon screen and further purified by Percoll (Amersham Biosciences, Freiburg, Germany) density centrifugation using a discontinuous gradient (10%, 20%, 40% Percoll in 0.9% NaCl, 1900*g*, 20 min). The oocytes were collected from the 10% Percoll interface. The oocyte fraction was homogenized, and the zonae pellucidae were collected from a 42.5-μm nylon screen in water, lyophilized, and stored at −20 °C until use. The protein content of the ZP of prepuberal animals was determined by amino acid analysis after employing hydrolysis of exactly 1000 ZPs in 6 N HCl for 24 h at 110 °C in evacuated and sealed ampules.

Alternatively, immature and in vitro-matured oocytes were repeatedly washed first in PBS containing 2% polyvinylalcohol and then in water. The oocytes were mechanically denuded. The ZPs of the denuded oocytes were obtained by repeated processing through a micropipette (diameter <120 μm) and manually collected with a micropipette. The ZPs were solubilised in water at 72 °C, lyophilized, and stored at −80 °C until they were used.

3.5. AB–silver staining after SDS–PAGE

The solubilised ZP glycoproteins (13 μg/lane) of unselected oocytes were subjected to SDS–PAGE at 12% polyacrylamide gels.[46] The gels were stained with the combined AB–silver staining procedure as described.[37]

3.6. Two-dimensional electrophoresis and lectin blotting

Solubilised ZP glycoproteins were analyzed by two-dimensional electrophoresis as recently described.[40] About 150–1000 zonae pellucidae equivalents (3–30 μg) were dissolved in 130 μL

rehydration buffer and applied to 7-cm long Immobiline dry strips (pH 3–10; Amersham Biosciences). Isoelectric focussing was carried out on an IPGphor Unit (Amersham Biosciences) at 20 °C and 20,000 Vh (1 h 150 V; 1 h 500 V; 1 h 1000 V). For the second dimension the strips were equilibrated for 20 min in 0.05 M Tris–HCl, pH 6.8, containing 2% SDS, 30% glycerol, and 6 M urea and placed onto 15% SDS–polyacrylamide gels. Electrophoresis was carried out according to LaemmLi.[46] The gels were either stained by silver[47] or blotted (2 h, 1 mA/cm^2) onto poly(vinylidene)difluoride (PVDF; Millipore, Schwalbach, Germany) membranes[48] for lectin staining. Lectin binding was visualized by chemiluminescence (Super Signal West Pico, Pierce, Rockford, IL, USA). For subsequent analysis membranes were stripped after two washings in TBS/1% Tween 20 with 3 M KSCN. Stripping was controlled using streptavidin–peroxidase as described before. Controls were performed by incubation of biotinylated lectins with the hapten sugars (L-fucose for AAA; sialyllactose for SNA and lactose for ACA). Sugar concentrations of 0.5 M completely inhibited lectin binding.

3.7. On-blot deglycosylation

On-blot deglycosylation was carried out according to Zhou et al.[49] and Küster et al.[50] In brief, aliquots (15–45 μg/15 μL) of unmodified (native), desialylated (des ZP), and *endo*-β-galactosidase-treated ZP glycoproteins (EβGal ZP) were dotted on PVDF membranes and subsequently carbamidomethylated. The dot blots were cut into small pieces and washed. Deglycosylation was performed by incubation with 20 U N-glycosidase F in 300 μL of aqueous $NaHCO_3$ solution, pH 7.0, at 37 °C for 20 h. The supernatant was collected, and the membrane pieces were additionally extracted with water under sonication for 10 min. The supernatants were combined and dried in a SpeedVac. The oligosaccharides were resolved in water and separated into neutral and acidic fractions using small-tip columns of graphitized carbon (20 μL; Alltech, Germany)[41,51] by sequential elution with water and 25% acetonitrile eluting the neutral oligosaccharides followed by 25% acetonitrile, and 0.05% trifluoroacetic acid for release of acidic glycans.

3.8. MALDI-TOFMS analysis

Neutral glycans were co-crystallized with 2,5-dihydroxybenzoic acid (Sigma) on stainless steel targets. Acidic glycans were dissolved in 0.2 M NH_4OH solution, applied to the target and air dried. The spots were rehydrated with water and 2,4,6-trihydroxy-acetophenone (THAP; Sigma; 1 mg/mL) in 20 mM ammonium citrate/acetonitrile (1:1, v/v) was added and allowed to crystallize. Mass spectra were recorded with a Kratos MALDI-II Analytical Kompact (V5.2, Kratos Analytical, Manchester, UK) in the positive-ion mode (neutral glycans) or negative-ion mode (acidic glycans) at linear high power. About 20–80 laser shots were added per spectrum.

Alternatively, the acidic glycan fraction was on-target desialylated according to Geyer et al.[52] After co-crystallization with 6-aza-2-thiothymine (Aldrich) and MS, the crystals were rehydrated in 3 μL 25 mM NH_4OAc buffer, pH 5, containing 6 mU of neuraminidase and incubated for 16 h at 37 °C in a humid chamber. After recrystallization mass spectra were recorded as described before.

In parallel, the acidic glycans were co-crystallized with D-arabinose phenylosazone ("D-arabinosazone", 3–6 mg/mL) in EtOH–H_2O (3:1, v/v) according to Chen et al.[53] MS and MS/MS spectra were recorded on an Ultraflex-MALDI-TOF/TOF mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) in the negative-ion mode with an acceleration voltage of 20 kV. Parent ions were detected with a standard reflector setup, and about 200 laser shots were collected. After switching into the LIFT mode, fragment ions were measured after post-source decay or collision-induced dissociation (CID); data obtained after up to 1500 laser shots were collected for one LIFT spectrum. Mass spectra were calibrated using a standard peptide mixture (Bruker).

3.9. Evaluation of glycopeptide and glycan structures

Compositions of glycans were calculated by the EXPASYGLYCOMOD tool[54] and the GLYCO-PEAKFINDER tool.[55] Composition-derived proposals for structure candidates of the glycans are essentially based upon the main structures of the complex-type N-glycans of the porcine ZP-glycoproteins described.[14,21,42,56] The final matching of the structure candidates with the experimentally retrieved peak lists was performed using the GLYCOWORKBENCH software suite.[57]

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