2008

Analysis of the N-glycans of recombinant human Factor IX purified from transgenic pig milk

Geun-Cheol Gil
University of Nebraska-Lincoln

William H. Velander
University of Nebraska-Lincoln, wvelander2@unl.edu

Kevin E. Van Cott
University of Nebraska-Lincoln, kvancott2@unl.edu

Glycobiology vol. 18 no. 7 pp. 526–539, 2008
doi:10.1093/glycob/cwn035
Advance Access publication on May 2, 2008

Analysis of the *N*-glycans of recombinant human Factor IX purified from transgenic pig milk

Geun-Cheol Gil[2], William H Velander[2,3], and Kevin E Van Cott[2,1]

[2]Department of Chemical and Biomolecular Engineering, University of Nebraska-Lincoln, Lincoln, NE 68588, USA; and [3]Progenetics LLC, 1872 Pratt Drive, Suite 1400, Blacksburg, VA 24060, USA

Received on October 10, 2007; revised on April 26, 2008; accepted on April 27, 2008

Glycosylation of recombinant proteins is of particular importance because it can play significant roles in the clinical properties of the glycoprotein. In this work, the *N*-glycan structures of recombinant human Factor IX (tg-FIX) produced in the transgenic pig mammary gland were determined. The majority of the *N*-glycans of transgenic pig-derived Factor IX (tg-FIX) are complex, bi-antennary with one or two terminal *N*-acetylneuraminic acid (Neu5Ac) moieties. We also found that the *N*-glycan structures of tg-FIX produced in the porcine mammary epithelial cells differed with respect to *N*-glycans from glycoproteins produced in other porcine tissues. tg-FIX contains no detectable Neu5Gc, the sialic acid commonly found in porcine glycoproteins produced in other tissues. Additionally, we were unable to detect glycans in tg-FIX that have a terminal Galα(1,3)Gal disaccharide sequence, which is strongly antigenic in humans. The *N*-glycan structures of tg-FIX are also compared to the published *N*-glycan structures of recombinant human glycoproteins produced in other transgenic animal species. While tg-FIX contains only complex structures, antithrombin III (goat), C1 inhibitor (rabbit), and lactoferrin (cow) have both high mannose and complex structures. Collectively, these data represent a beginning point for the future investigation of species-specific and tissue/cell-specific differences in *N*-glycan structures among animals used for transgenic animal bioreactors.

Keywords: glycoprotein/Factor IX/glycosylation/mammary gland/transgenic animal

Introduction

Factor IX (FIX) is a vitamin K-dependent plasma glycoprotein that plays an essential role in the blood clotting pathway. A deficiency of FIX activity leads to the bleeding disorder hemophilia B, and is currently treated by replacement therapy. A number of posttranslational modifications are made to Factor IX, including γ-carboxylation of 12 glutamates near the *N*-terminus, β-hydroxylation of Asp64, phosphorylation of Ser158, sulfation of Tyr155, and both *N*- and *O*-glycosylation. These complex posttranslational modifications require production of recombinant Factor IX (r-FIX) in mammalian cell expression systems. Factor IX for replacement therapy is currently purified from donor plasma or produced recombinantly in Chinese Hamster Ovary (CHO) cell culture (BeneFIX®).

Glycosylation of recombinant proteins is of particular importance because it can affect properties including enzyme activity, protein stability, pharmacokinetics, and immunogenicity (Varki et al. 1999). Human Factor IX zymogen has two potential *N*-glycosylation sites at Asn157 and Asn167 in the activation peptide (amino acids 146–180), which is proteolytically removed by Factor XIa or Factor VIIa-Tissue Factor (reviewed in Kurachi et al. 1982). The *N*-glycans of human plasma-derived Factor IX (pd-FIX) are reported to be complex, tri- and tetra-antennary, and sialylated with *N*-acetylneuraminic acid (Neu5Ac) (Makino et al. 2000). The only other pd-FIX *N*-glycans that have been structurally analyzed are those from bovine Factor IX, which has three *N*-glycan sites at Asn158, Asn168, and Asn173 in the activation peptide. Bovine Factor IX *N*-glycan structures are also of the complex classification (Mizuochi et al. 1983). There are currently no published reports that relate the structure of Factor IX *N*-glycans to specific in vivo biological or pharmacokinetic properties.

It is well established that glycoproteins exist as a population of glycoforms due to macro- and microheterogeneity, and that a recombinant protein will likely have different *N*-glycan structures compared to the endogenous protein. The challenge for the production of a recombinant therapeutic glycoprotein is to ensure that there is consistency in the glycoforms that are produced and that any differences in the glycan structure do not result in detrimental clinical properties. The *N*-glycans of BeneFIX® are reported to be of a more complex structure and contain different linkages, more fucosylation, and more poly-*N*-acetyllactosamine repeat structures than plasma-derived FIX (Bond et al. 1998). These differences were not linked to any adverse effects, and the manufacturing process of BeneFIX® results in a highly consistent *N*-glycan fingerprint (Harris et al. 1998). Thus, despite differences in the *N*-glycan structure and other posttranslational modifications (e.g., the extent of serine phosphorylation and tyrosine sulfation), BeneFIX® was licensed by the US and European regulatory agencies and is currently used to treat hemophilia B patients.

Transgenic animal bioreactors are another potential source of recombinant human glycoproteins (Lubon et al. 1996), but there are only a few published reports on the *N*-glycan structures that are present on transgenic-derived glycoproteins. To date, the most complete analyses to be published are that of recombinant human antithrombin III produced in transgenic goats (Edmunds et al. 1998), recombinant human

[1]To whom correspondence should be addressed: Tel: +1-402-472-1743; Fax: +1-402-472-6989; e-mail: kvancott2@unl.edu

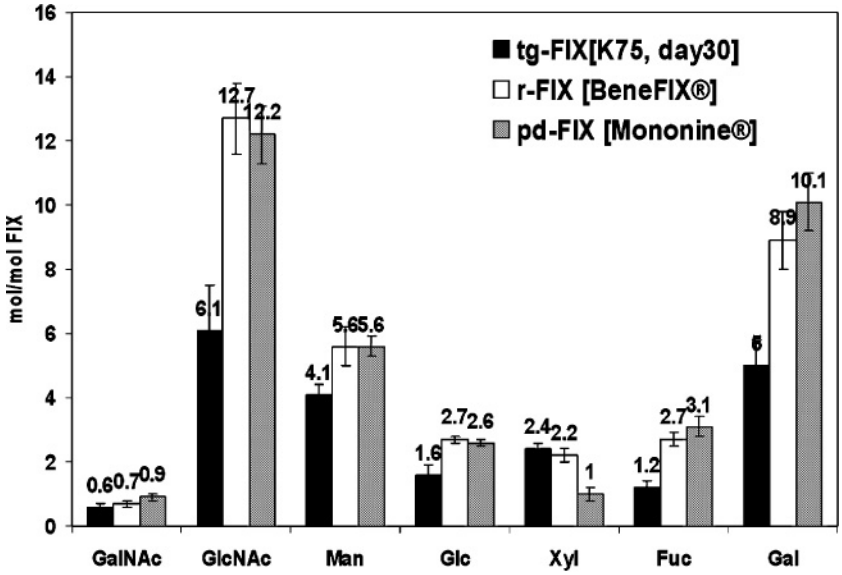


Fig. 1. Monosaccharide composition of tg-FIX from pig K75 day 30 of lactation, r-FIX from CHO cells (BeneFIX®), and pd-FIX (Mononine®).

lactoferrin produced in transgenic cows (Van Berkel et al. 2002), and the recombinant human C1 inhibitor produced in transgenic rabbits (Koles et al. 2004a). In this work, we determined the structure of the *N*-glycans of recombinant human Factor IX purified from transgenic pig milk. To our knowledge, this is the first report of detailed structural analysis of *N*-glycans from a recombinant protein produced in the porcine mammary epithelial cells. Our purpose in doing this was 3-fold: (1) to determine how *N*-glycan structures of transgenic-derived Factor IX differ from *N*-glycans of pd-FIX so as to provide a foundation for future structure/function investigations; (2) to investigate tissue-specific differences in the *N*-glycans of glycoproteins produced in the pig; and (3) to contribute toward the future investigation of species-specific differences of *N*-glycans produced in transgenic animal bioreactors. Using a combination of capillary electrophoresis, high performance liquid chromatography (HPLC), and mass spectrometry, we have found that the majority of the *N*-glycans of tg-FIX are complex, bi-antennary, and with one or two terminal sialic acid groups.

Results

Transgenic FIX (tg-FIX) products purified from two daily milk samples of two animals (K45 and K75) were used as representative samples for this study. The purified tg-FIX contains many subpopulations that differ with respect to the extent of posttranslational modifications such as γ-carboxylation (Lindsay et al. 2004). For this work the tg-FIX was purified by heparin affinity and anion exchange chromatography, and then polished by reverse phase HPLC. The heparin binding site in FIX is in the heavy chain (Yang et al. 2002), a region of the protein devoid of posttranslational modifications, and the product of heparin affinity chromatography represents the entire population of Factor IX molecules produced by the bioreactor. The biologically active tg-FIX subpopulations were further purified by anion exchange chromatography, a purification step that separates the subpopulations based on the γ-carboxyglutamate (Gla) content (Gillis et al. 1997). We have found that this final purification step does not fractionate the tg-FIX with respect to glycoforms; the Gla content determines the protein–column interactions. The tg-FIX was purified from mid-lactation milk samples of K75 (day 30) and K45 (day 45), and we have determined that the *N*-glycan features described here are representative of those from all the transgenic pigs analyzed so far.

Asn157 and Asn167, which are located in the FIX activation peptide, are the only asparagine residues in an Asn-Xxx-Ser/Thr sequon. We have not found any other *N*-glycosylation sites by LC-MS/MS analysis in tg-FIX and in plasma-derived FIX (data not shown). We also confirmed that the *N*-glycan site occupancy at Asn157 and Asn167 is essentially complete by analysis of the deglycosylated activation peptide by LC-ESI-TOF mass spectrometry: removal of the *N*-glycans with PNGaseF results in the conversion of occupied asparagine residues to aspartic acid, and a +1 Da change in molecular weight for each occupied Asn (supplementary Figure 1). These experiments were also performed in the presence of $H_2{}^{18}O$ to confirm that nonspecific deamidation was not occurring prior to or during this analysis. We could find no significant evidence for partial *N*-glycosylation in purified tg-FIX.

Monosaccharide and sialic acid analysis

Glycan composition was obtained using the monosaccharide analysis method developed by Chen and Evangelista (1995). Bovine fetuin run as a standard resulted in a monosaccharide composition similar to that reported in Chen and Evangelista and was repeatable with less than 5% of relative standard deviation (RSD) (data not shown). It should be noted that this method gives a quantitation of monosaccharides from both *O*- and *N*-glycans. Human Factor IX contains two *O*-glycosylation sites in the light chain at Ser53 and Ser61: the glycan at Ser53 is a unique Xyl-Xyl-Glc and Xyl-Glc structure (Nishimura et al. 1989) found in epidermal growth factor domains, and the glycan at Ser61 is a NeuAcα(2–6)Galβ(1–4)GlcNAcβ(1–3)Fucα1-tetrasaccharide (Nishimura et al. 1992; Harris et al. 1993). The four potential

Table I. Sialic acid contents of tg-FIX, r-FIX (BeneFIX®), pd-FIX (Mononine®), porcine thyroglobulin, and pig IgG (ND: not detected)

Sample	Neu5Ac (mol/mol protein)	Neu5Gc (mol/mol protein)
tg-FIX [pig K45, lactation day 45]	5.4 ± 0.4	ND
tg-FIX [pig K75, lactation day 30]	3.2 ± 0.2	ND
rFIX [BeneFIX®]	6.5 ± 0.9	ND
pd-FIX [Mononine®]	8.8 ± 0.3	ND
Porcine thyroglobulin	25.2 ± 0.1	2.2 ± 0.2
Pig IgG	ND	0.7 ± 0.1

O-glycan sites in the activation peptide of FIX are sparsely occupied, and do not contribute significantly to the monosaccharide composition. A representative analysis of a tg-FIX sample purified from a mid-lactation milk sample (pig K75, lactation day 30) is shown in Figure 1. BeneFIX® and Mononine® had significantly higher amounts of *N*-acetylglucosamine (GlcNAc), mannose (Man), fucose (Fuc), and galactose (Gal) than tg-FIX. As expected, glucose (Glc) and xylose (Xyl) were also detected in all FIX samples, and glycopeptide analysis has shown that the Xyl-Xyl-Glc glycan at Ser53 and the tetrasaccharide at Ser61 are present in tg-FIX (data not shown).

Sialic acids were analyzed by reverse phase HPLC using the method described in Anumula (1995). This method accounts for sialic acids released from both *N*- and *O*-glycans. Results from the two representative tg-FIX samples are compared with BeneFIX® and Mononine® in Table I. Our results show no Neu5Gc on BeneFIX® and Mononine® (limit of quantitation was 0.05 mol/mol protein), which is consistent with previous reports (Bond et al. 1998; Harris et al. 1998; Makino et al. 2000). The tg-FIX also had no quantifiable Neu5Gc, as have all other tg-FIX samples we have analyzed throughout the course of lactation for multiple animals to date. The Neu5Ac levels in tg-FIX (~3–5 mol/mol protein) were lower than that found in Mononine® (8.8 mol/mol protein) and BeneFIX® (6.5 mol/mol protein). For comparison, two endogenous porcine proteins were also analyzed; Neu5Gc was detected in both thyroglobulin (2.2 mol/mol protein) and IgG (0.7 mol/mol protein).

N-Glycan profiling using normal phase high performance liquid chromatography (NP-HPLC)

HPLC *N*-glycan profiling separates released oligosaccharides by size, charge, linkage, and overall structure (Anumula and Dhume 1998). The complete digestion of *N*-glycans from glycoproteins by PNGase F was confirmed by SDS–PAGE analysis of the undigested and digested proteins. We obtained a single band on the gels after PNGase F treatment (data not shown). The well-characterized glycoprotein, bovine fetuin, was used as a standard in assignment of *N*-glycan separation on the basis of the number of sialic acid moieties (Figure 2A). The separated *N*-glycans of bovine fetuin are grouped and designated as 0S for neutral glycans, 1S for mono-, 2S for di-, 3S for tri-, and 4S for tetrasialylated oligosaccharides. In agreement with the literature, tri- and tetrasialylated glycans are predominant in Mononine® (Figure 2B) and BeneFIX® (Figure 2C). Representative profiles of tg-FIX *N*-glycans are presented in Figure 2D, E. The *N*-glycans of tg-FIX from both animals are primarily mono- and disialylated. We have found that the *N*-glycan profiles have been consistent from animal to animal with respect to the identity of the peaks present. There is variation, however, in the relative amounts of each peak between animals and throughout the course of an animal's lactation, as evidenced by the profiles given for K45 and K75. The *N*-glycan profiles of two endogenous porcine glycoproteins are significantly different from tg-FIX. Porcine thyroglobulin (Figure 2F), a glycoprotein synthesized in the thyroid, contains a mix of neutral and acidic oligosaccharides. Porcine IgG (Figure 2G), a glycoprotein synthesized in B cells, contains primarily neutral *N*-glycans.

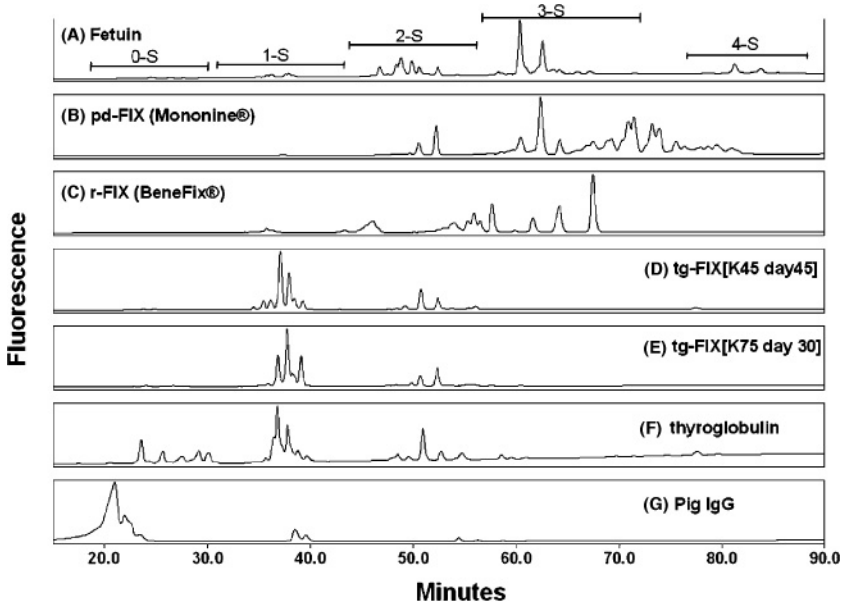


Fig. 2. HPLC *N*-glycan profiles of (**A**) Bovine Fetuin, (**B**) pd-FIX (Mononine®), (**C**) r-FIX (BeneFIX®), (**D**) tg-FIX [K45 day 45], (**E**) tg-FIX [K75 day 30], (**F**) porcine thyroglobulin, and (**G**) pig IgG. The labels *0-S*, *1-S*, *2-S*, *3-S*, and *4-S* indicate mono-, di-, tri-, and tetrasialylated oligosaccharide groups, respectively.

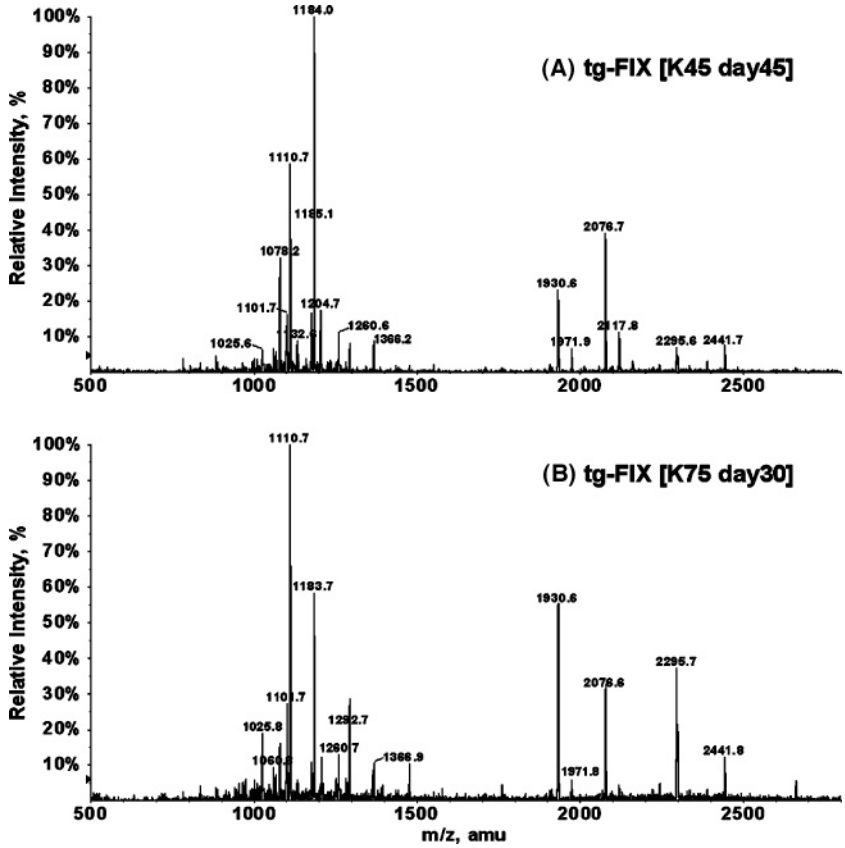


Fig. 3. MS spectra of underivatized *N*-glycans of tg-FIX from (**A**) K45 day 45 and (**B**) K75 day 30.

Underivatized N-glycan analysis using ESI-MS/MS

While the NP-HPLC profiling method gives information about *N*-glycan heterogeneity and sialic acid content, it does not give detailed structural information. *N*-Glycans were enzymatically released from tg-FIX and structures were determined by ESI-MS and ESI-MS/MS analysis in the negative ion mode on a triple-quadrupole/ion trap instrument. As described in Harvey (2005), ammonium nitrate was used to enhance ionization of the acidic oligosaccharides. In the ion trap scanning mode, precursor ions were a mixture of the nitrate adducts and deprotonated ions. By increasing the collision energy (CE) in Q2 of the instrument (applied to all ions), the nitrate adducts were converted to deprotonated ions, which simplified the spectra. A CE = 40 V was chosen as the optimal setting for obtaining simpler spectra without fragmenting the oligosaccharides. Figure 3 shows the MS spectra of *N*-glycans from the two tg-FIX samples. Possible candidate structures of *N*-glycans were obtained from the molecular masses from the ion trap survey scan (by searching theoretical precursor ion mass on web-based Glycomod: [http://ca.expasy.org/tools/glycomod/]) and by determining if the corresponding MS/MS spectra were consistent with the precursor ion definition. We present detailed MS/MS data from the tg-FIX from pig K45/day 45 to illustrate the data analysis process.

The MS/MS structural characterization of *N*-glycans in this work was performed based on the *N*-glycan biosynthesis scheme. On the core region (Man α1–3/Man α1–6 Man β1–4 GlcNAc β1–4 GlcNAc), GlcNAc is linked to Man α1–3 Man (which is called "the 3-antenna") or Man α1–6 Man (which is "the 6-antenna") via β1–2, 4, and 6 to make various branches. The linkage between Gal (or *N*-acetylgalactosamine (GalNAc)) and GlcNAc is likely the β1–4 linkage. Sialic acid can be linked to Gal (or GalNAc) through α2–3, or 6. Fuc on the reducing GlcNAc is attached by the α1–3 or 1–6 linkage. This *N*-glycan biosynthetic scheme was used as the basis for determining the proposed structures with sequences and linkages.

Figure 4 presents the MS/MS spectra of the precursor ions *m/z* 1183 $[M\text{-}2H]^{2-}$ (panel A) and *m/z* 1110 $[M\text{-}2H]^{2-}$ (panel B) from the *N*-glycans of tg-FIX from pig K45/day 45. The precursor ion *m/z* 1183 $[M\text{-}2H]^{2-}$ is proposed as a fucosylated disialylated biantennary structure, and the precursor ion *m/z* 1110 $[M\text{-}2H]^{2-}$ is similar but without fucosylation. The MS/MS spectrum of these precursor ions has a B_3 ion (*m/z* 655), but no *m/z* 696 ion, so both antennae are consistent with Neu5Ac-Gal-GlcNAc, not Neu5Ac-GalNAc-GlcNAc. The abundant B_1 ion (*m/z* 290) is consistent with Neu5Ac termination of this glycan, which is also confirmed by B_3 (*m/z* 655) and B_4 (*m/z* 817) ions. We found no results consistent with Neu5Gc incorporation. The coexistence of the fragment ions $^{2,4}A_7$ (*m/z* 1030 $[M\text{-}2H]^{2-}$), $^{2,4}A_7/Y_6$ (*m/z* 1769 $[M\text{-}H]^{-}$), $^{0,2}A_7$ (*m/z* 1133 $[M\text{-}2H]^{2-}$), and $^{0,2}A_7/Y_6$ (*m/z* 1975 $[M\text{-}H]^{-}$) in the MS/MS spectrum of the *m/z* 1183 precursor ion indicates α(1–6) linked core fucosylation. In the MS/MS spectrum of the precursor ion *m/z* 1110 $[M\text{-}2H]^{2-}$, the fragment ions $^{0,2}A_7$ (*m/z* 1060 $[M\text{-}2H]^{2-}$) and $^{0,2}A_7/Y_6$ (*m/z* 1829 $[M\text{-}H]^{-}$) indicate a lack of fucosylation.

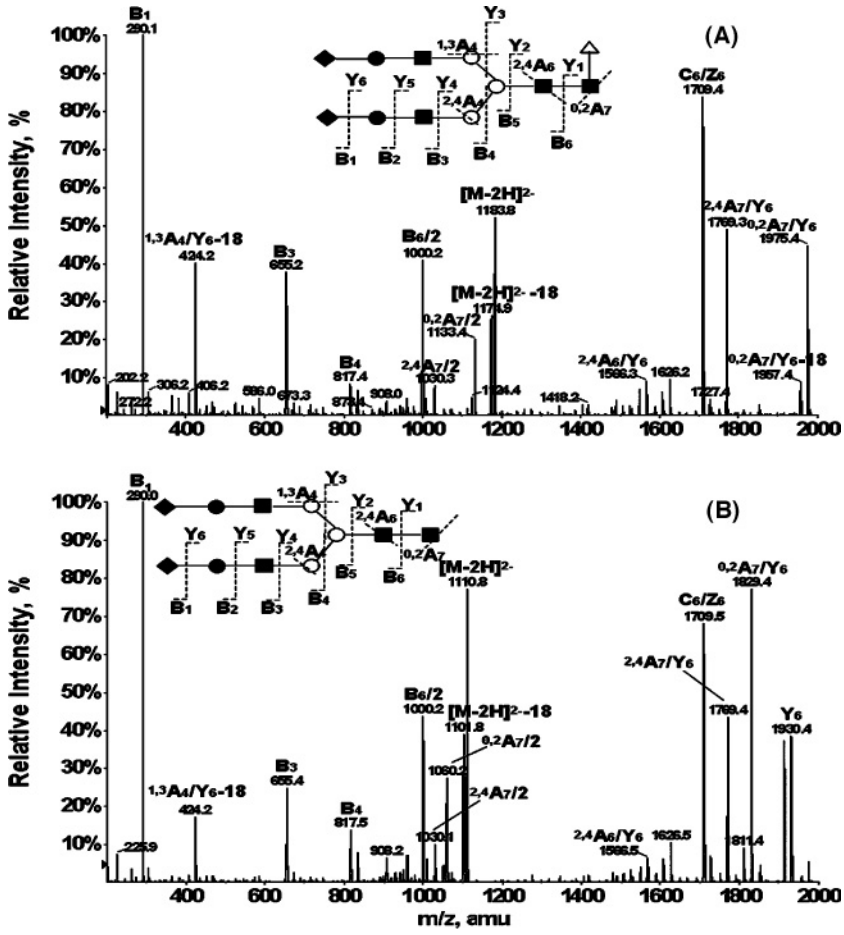


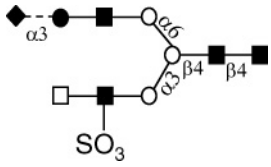
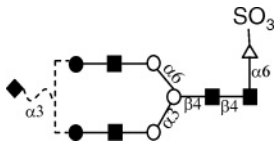
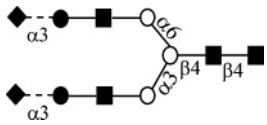
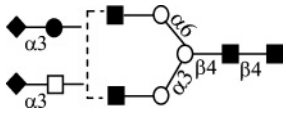
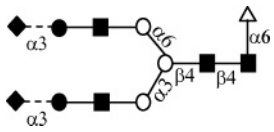
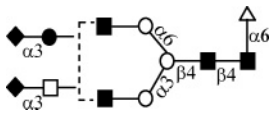
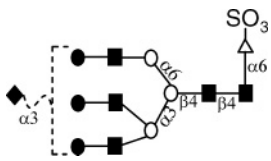
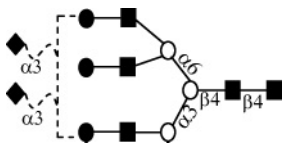
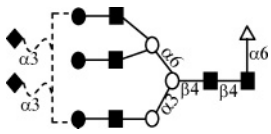
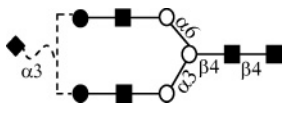
Fig. 4. MS/MS spectra of underivatized *N*-glycans from tg-FIX (K45 day 45) illustrating how structural assignment is made: (**A**) $m/z = 1184$ $[M\text{-}2H]^{2-}$ and (**B**) $m/z = 1110$ $[M\text{-}2H]^{2-}$ (△, fucose; ○, mannose; ●, galactose; ■, GlcNAc; ◆, Neu5Ac).

The structures of the other precursor ions in Figure 3 were also identified by analyzing MS/MS spectra (see supplementary Figure 2), and these structures are summarized in Table II. All precursor ions were identified as deprotonated adducts. We identified monosialylated triantennary (*m/z* 2441 and 2295), monosialylated biantennary (*m/z* 2117, 2076, 1971, and 1930), disialylated triantennary (*m/z* 1366), and disialylated biantennary glycans (*m/z* 1203, 1183, 1110, and 1130). We found evidence for isomers containing Gal-GlcNAc and GalNAc-GlcNAc sequences on the 3- and 6-antennae for the precursor ions *m/z* 1130 $[M\text{-}2H]^{2-}$, 1203 $[M\text{-}2H]^{2-}$, 1971 $[M\text{-}H]^{-}$, and 1058 $[M\text{-}2H]^{2-}$ (supplementary Figure 2a, b, e, and g). The ion $^{0,4}A_5/Y_6$ (*m/z* 586) indicates that the 6-antenna contains a Gal-GlcNAc sequence, which also denotes that the corresponding GalNAc-GlcNAc is located on the 3-antenna. However, the MS/MS spectra show a weak signal of the fragment ion, $^{0,4}A_5/Y_6$ (*m/z* 627), which reflects a GalNAc-GlcNAc sequence on the 6-antenna. Based on this observation, we conclude that the structures bearing GalNAc-GlcNAc on the 3-antenna are more abundant ion than those bearing GalNAc-GlcNAc on the 6-antenna. The two possible isomers are expressed as a dotted line between Gal (or GalNAc) and GlcNAc in Table II.

In addition to these more common structures, we were also able to identify *N*-glycans that appear to be sulfated. The structure of *m/z* 1078 $[M\text{-}2H]^{2-}$ is proposed to be monosialylated, biantennary, and with sulfation on the core Fuc. As shown in Figure 5A, the presence of $^{0,4}A_7$ (*m/z* 285), $^{2,4}A_7$ (*m/z* 1769), and B_6 (*m/z* 1709) are consistent with a sulfated α(1–6) linked core Fuc. To confirm the presence of sulfate, and not phosphate on the glycans, the ion-pairing method using the peptide trilysine (K3) was employed, as described in Zhang et al. (2006). During MS/MS fragmentation in the presence of K3, sulfated glycans tend to undergo sulfur–oxygen cleavage, resulting in $[M\text{-}SO_3\text{+}H]^{+}$ and $[K3\text{+}SO_3\text{+}H]^{+}$ ions, while phosphorylated glycans produce fragment ions from the dissociation of the non-covalent bond between the glycan and K3. The MS/MS spectra of this proposed sulfated *N*-glycan ($[M\text{+}K3\text{+}2H]^{2+}$ *m/z* 1281) in this work has the $[K3\text{+}SO_3\text{+}H]^{+}$ (*m/z* 483) ion and the $[M\text{-}SO_3\text{+}H]^{+}$ type ions, which are consistent with a sulfated, not phosphorylated, glycan (supplementary Figure 3a). The abundant B_1 ion (*m/z* 290) indicates Neu5Ac termination of this glycan, which is also confirmed by B_3 (*m/z* 655) and B_4 (*m/z* 817) ions. The presence of the *m/z* 655 ion also indicates that the composition of an antenna is Neu5Ac+Gal+GlcNAc.

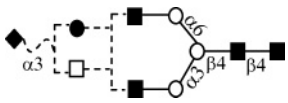
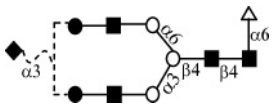
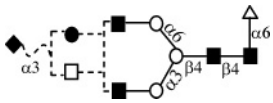
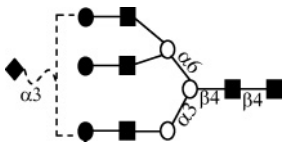
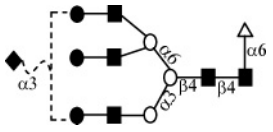
The structure of the *m/z* 1260 $[M\text{-}2H]^{2-}$ ion is proposed to be a monosialylated triantennary with a sulfation on the core Fuc (Figure 5B). The fragment ions $^{0,4}A_7$ (*m/z* 285) indicate a sulfation on the core α(1–6) linked Fuc, and Z_1 (*m/z* 428) also confirms a sulfated Fuc linked to a GlcNAc. B_1 (*m/z* 290) and

Table II. Proposed *N*-glycan structures of tg-FIX and their relative percentage (based on HPLC profiling and MS analysis) from K45/day 45 and K75/day 30 (△, fucose; ○, mannose; ●, galactose; ■, GlcNAc; □, GalNAc; ◆, Neu5Ac; ND, not detected)

Experimental *m*/*z*	Theoretical *m*/*z*	Adduct	Structure	Amount (%) K45/day 45	Amount (%) K75/day 30
1025.2	1025.3	$[M\text{-}2H]^{2-}$		ND	ND
1078.3	1077.8	$[M\text{-}2H]^{2-}$		ND	ND
1110.7	1110.4	$[M\text{-}2H]^{2-}$		4.6	11.2
1130.8	1130.9	$[M\text{-}2H]^{2-}$		1.1	2.5
1183.8	1183.4	$[M\text{-}2H]^{2-}$		10.1	5.3
1203.8	1203.9	$[M\text{-}2H]^{2-}$		3.9	0.9
1260.9	1260.4	$[M\text{-}2H]^{2-}$		ND	ND
1293.0	1292.9	$[M\text{-}2H]^{2-}$		0.9	ND
1366.2	1366.0	$[M\text{-}2H]^{2-}$		1.7	0.5
1930.6 965.1	1930.7 964.8	$[M\text{-}H]^{-}$ $[M\text{-}2H]^{2-}$		25.2	31.1

Continued

Table II. *Continued*

Experimental *m/z*	Theoretical *m/z*	Adduct	Structure	Amount (%) K45/day 45	Amount (%) K75/day 30
1971.9 985.6	1971.7 985.3	$[M-H]^-$ $M-2H]^{2-}$		5.7	2.0
2076.7 1038.2	2076.7 1037.9	$[M-H]^-$ $[M-2H]^{2-}$		30.7	24.6
2117.8 1057.8	2117.8 1058.4	$[M-H]^-$ $[M-2H]^{2-}$		4.4	1.1
2295.6 1147.2	2295.8 1147.4	$[M-H]^-$ $[M-2H]^{2-}$		5.8	5.4
2441.7 1220.8	2441.9 1220.4	$[M-H]^-$ $[M-2H]^{2-}$		5.3	11.3

B_3 (*m/z* 655) ions indicate Neu5Ac termination of this glycan. As described in Harvey 2005, the ^{0,4}A type ring fragment ions can provide information about the sequences and elongations at the 6-antenna. For example, the ion, $^{0,4}A_5/Y_6 - 18$ (*m/z* 586) in Figure 5B indicates that the oligosaccharide consists of one branch of Gal-GlcNAc on the 6-antenna. This information may also suggest two branches of Gal-GlcNAc on the 3-antenna. The ^{0,4}A ions generally do not bear sialic acid, because sialic acid is readily lost during cross-ring fragmentation. Therefore, the position of sialic acid termination on the branch was not assigned. The presence of sulfation on this structure was also examined by the MS/MS fragmentation with K3 ($[M+K3+2H]^{2+}$ *m/z* 1463). The $[K3+SO_3+H]^+$ ion was observed, indicating a sulfated structure (supplementary Figure 3b).

The MS/MS spectrum of *m/z* 1025 $[M-2H]^{2-}$ (Figure 6) shows fragment ions indicating a sulfated biantennary structure with a Gal or GalNAc residue on each antenna. The fragment ion *m/z* 282 (HexNAc+80) and *m/z* 485 ($HexNAc_2$+80) indicate a sulfation on GlcNAc or GalNAc of the antenna having GalNAc+GlcNAc composition. The fragment ion $^{1,4}A_5/Z_{5\beta}$ (*m/z* 534) indicates that the sulfation is on the GlcNAc residue of the 3-antenna. These results are consistent with a composition of the 3-antenna being SO_3+GalNAc+GlcNAc. We were unable to detect a fragment ion indicating Neu5Ac+SO_3+GalNAc+GlcNAc (*m/z* 776). Therefore, it is deduced that Neu5Ac termination may be only positioned on the 6-antenna composed of Gal+GlcNAc composition. The $^{2,4}A_7$ type ions ($^{2,4}A_7$ *m/z* 944 $[M-2H]^{2-}$ and $^{2,4}A_7/Y_{6\alpha}$ *m/z* 1599 $[M-H]^-$) and $^{0,2}A_7$ ion (*m/z* 1659 $[M-H]^-$) in the MS/MS spectrum indicate no core fucosylation. The MS/MS fragmentation of this structure with K3 ($[M+K3+2H]^{2+}$ *m/z* 1228) also showed the $[K3+SO_3+H]^+$ ion, indicating the presence of sulfation (supplementary Figure 3c). In summary, the precursor ion *m/z* 1025 is a biantennary structure with SO_3+GalNAc+GlcNAc composition of the 3-antenna and Neu5Ac+Gal+GlcNAc composition of the 6-antenna. The identified sulfated structures above were found from tg-FIX *N*-glycans of both animals.

To get a more quantitative understanding the distribution of *N*-glycans, we combined the NP-HPLC profiling with off-line ESI-MS/MS analysis. Derivatized oligosaccharides were collected, dried and reconstituted with 10 mM ammonium hydroxide in 50% methanol, and analyzed in the negative ion mode. The results are summarized in Figure 7 and the corresponding relative percentages of each identified *N*-glycan for pig K45/day 45 and K75/day 30 are listed in Table II. The elution times of the derivatized glycans were consistent with the sialic acid content assigned by comparison with bovine fetuin glycans. The structures from 33.9 to 38.6 min retention time are monosialylated structures, while the structures from 47.5 to 51.4 min are disialylated. In the MS/MS spectra of the collected major peaks from the *N*-glycan profile, most of the fragment ions from the MS/MS spectra were the results of glycosidic linkage fragmentation (B, C, Y, and Z type), consistent with results reported by Harvey (2005) (data not shown). We observed that fucosylated glycans (e.g., the peak 1S-4 and 2S-3 in Figure 7) elute earlier than their corresponding nonfucosylated glycan (e.g., peaks 1S-5 and 2S-4 in Figure 7). We confirmed that derivatized *N*-glycan peaks

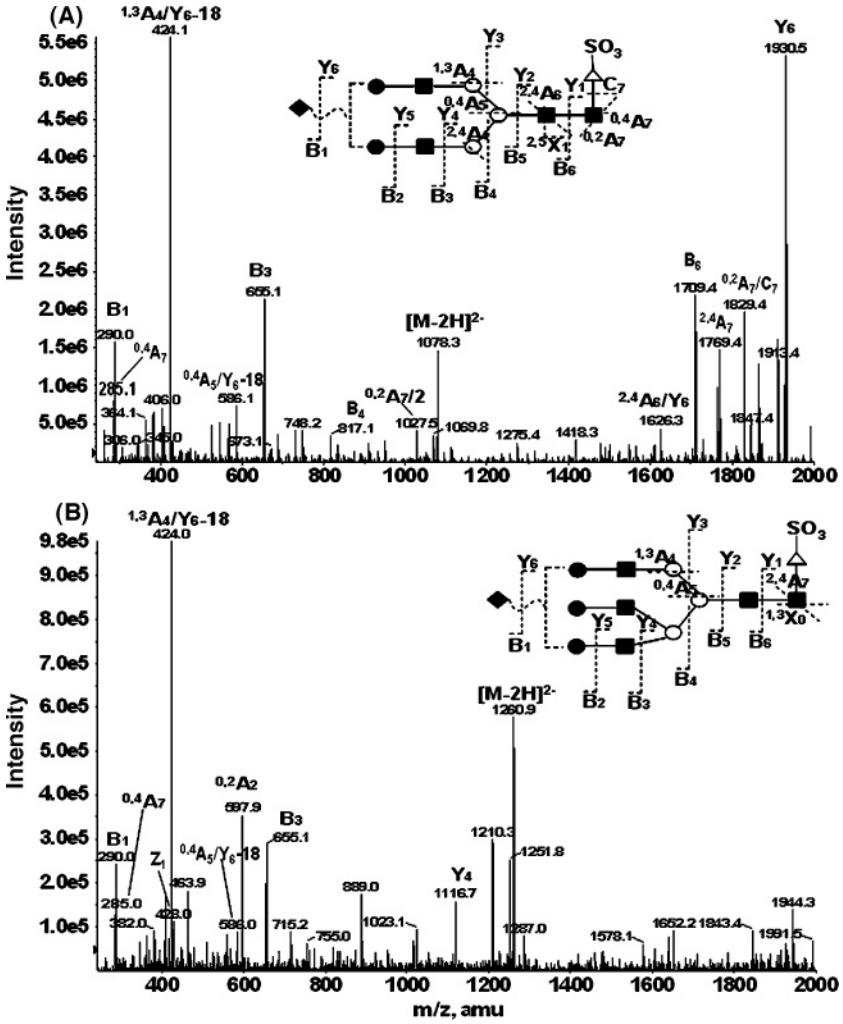


Fig. 5. MS/MS spectra of the putative sulfated *N*-glycans (**A**) $m/z = 1078$ $[M\text{-}2H]^{2-}$ and (**B**) $m/z = 1260$ $[M\text{-}2H]^{2-}$ from tg-FIX (K45 day 45) (△, fucose; ○, mannose; ●, galactose; ■, GlcNAc; ◆, Neu5Ac).

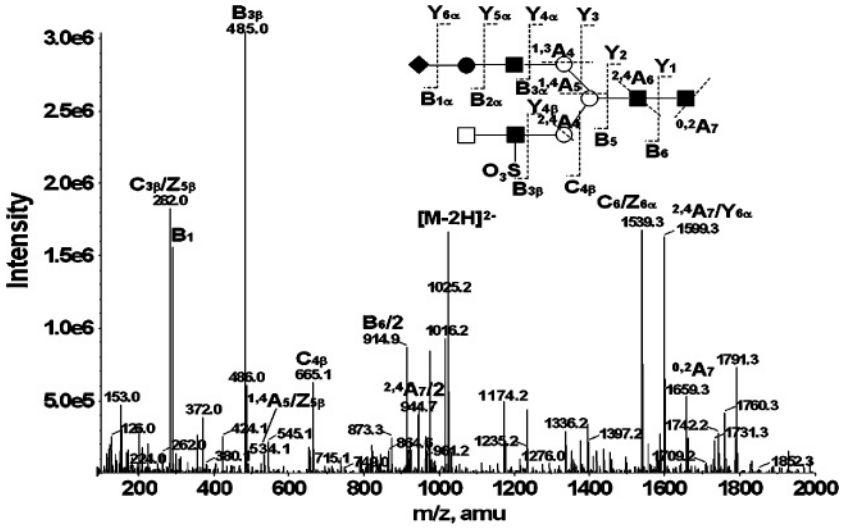


Fig. 6. MS/MS spectrum of the putative sulfated *N*-glycan ($m/z = 1025$ $[M\text{-}2H]^{2-}$) from tg-FIX (K45 day 45) (△, fucose; ○, mannose; ●, galactose; ■, GlcNAc; □, GalNAc; ◆, Neu5Ac).

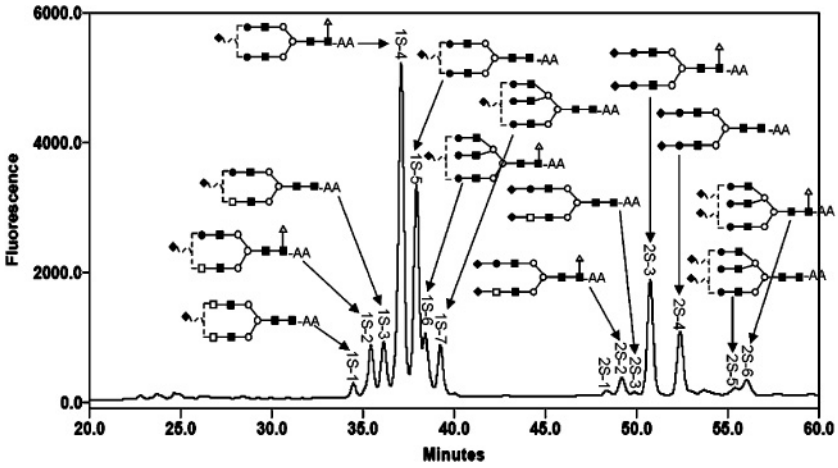


Fig. 7. Summary of identified structures of AA-derivatized *N*-glycans of tg-FIX (K45 day 45) from HPLC profiling and ESI-MS/MS analysis (△, fucose; ○, mannose; ●, galactose; ■, GlcNAc; □, GalNAc; ◆, Neu5Ac; AA, 2-aminobenzoic acid).

having the same retention time from two different transgenic pigs were the same structures. The most abundant *N*-glycan from tg-FIX [K45 day 45] is the monosialylated core-fucosylated biantennary (*m*/*z* 2076 $[M\text{-}H]^-$ underivatized mass), while that from tg-FIX [K75 day 30] is the monosialylated biantennary without fucosylation (*m*/*z* 1930 $[M\text{-}H]^-$ underivatized mass). Sialylated *N*-glycans comprise over 95% of the total *N*-glycan population. The linkage of Neu5Ac-Gal (or GalNAc) is confirmed to be α(2–3) by comparing NP-HPLC *N*-glycan profiles for samples treated with a sialidase specific for the α(2–3) linkage and another sialidase that cleaves at both α(2–3) and α(2–6) linkages. The acidic *N*-glycans are completely converted to neutral species after the treatment with both the α(2–3) sialidase and the α(2–3,6) sialidase (supplementary Figure 4a), indicating that Neu5Ac is linked via α(2–3) linkage. Fully sialylated *N*-glycans (with no terminal Hex or HexNAc residues) account for about 20% of the total population; the majority of the *N*-glycans are partially sialylated. We were not able to detect sulfated glycans using the NP-HPLC profiling-MS method.

The structures determined by MS/MS analysis did not have any *N*-glycans with Galα(1–3)Gal terminal disaccharides, and we found evidence for only α(1–6) linked core Fuc structures. The absence of α-Gal moiety was also investigated by α-galactosidase digestion and the resulting effect on the *N*-glycan profiles. As shown in supplementary Figure 4b, no peak shifts were observed on NP-HPLC *N*-glycan profiles treated with α-galactosidase. We also confirmed the Fuc linkage by exoglycosidase treatment. To confirm the absence of α(1–3) linked fucosylation, α(1–3) fucosidase digestion was employed and the resulting *N*-glycan profiles did not show any peak shift, indicating the absence of α(1–3) linked fucosylation (supplementary Figure 4c).

Discussion

The objective of this work is to report on the *N*-glycan structures of tg-FIX and how they compare with *N*-glycans found in plasma-derived FIX, other porcine glycoproteins, and in recombinant glycoproteins produced in other transgenic animal species. The only potential *N*-glycosylation sites of FIX (Asn-Xxx-Thr/Ser sequons) are located at Asn157 and Asn167 in the activation peptide of the protein. We have confirmed by LC-MS/MS and LC-MS analysis that tg-FIX is not *N*-glycosylated at any other Asn residues, and that glycosylation at Asn157 and Asn167 is essentially complete. The site occupancy by particular *N*-glycan structures at either Asn157 or Asn167 was not determined as part of this work; this aspect of site occupancy of tg-FIX and other recombinant glycoproteins produced in the transgenic animal bioreactor will be the subject of future studies.

Differences in the *N*-glycan structure between a recombinant protein and its human version are to be expected, and transgenic animal-derived proteins are no exception. Human plasma-derived FIX *N*-glycans are complex, highly branched (tri- and tetra-antennary), and highly sialylated (Makino et al. 2000). Approximately 35% of human FIX *N*-glycans are fucosylated, and 80% of the antennae are terminated with sialic acid. Multiple analytical methods were used in this study to ensure that the results for tg-FIX analysis were consistent. Monosaccharide analysis showed that tg-FIX has lower amounts of Neu5Ac, Gal, GlcNAc, and Man compared with Mononine® and BeneFIX®. This indicates that the *N*-glycans of tg-FIX are less branched and less sialylated. Results from HPLC profiling and ESI-MS analysis confirmed this: tg-FIX *N*-glycans are complex, bi- and tri-antennary, partially sialylated with Neu5Ac (no Neu5Gc), and partially core-fucosylated.

The two animals studied in this work had different tg-FIX expression levels during their lactations: K45 ranged from 3 to 4 mg/mL, and K75 ranged from 1 to 2 mg/mL. For both animals, approximately 95% of the *N*-glycans of tg-FIX detected by HPLC profiling are sialylated, but only about 20% of the glycans are fully sialylated. However, there appears to be significant differences for core fucosylation of tg-FIX with expression level, as pig K45/day 45 had 56%, but K75/day 30 contained 37% of fucosylated *N*-glycans. The variations of tg-FIX *N*-glycans from other animals and different days of lactation are under investigation. So far, the *N*-glycan profiles of tg-FIX K45 showed that the relative proportions of the disialylated structures decreased, but the total sialylated structures and the overall sialic acid content remained constant during lactation (unpublished observations). In comparison to our data, Koles et al. (2004b) reported that disialylated structures and the

overall sialic acid content decreased on recombinant C1 inhibitor *N*-glycan produced in transgenic rabbit milk. Plus, fucosylation of tg-FIX K45 *N*-glycans increased, while that of the recombinant C1 inhibitor *N*-glycans decreased, as lactation progressed. These interanimal and daily variations in the relative amounts of tg-FIX *N*-glycans will be further explored and presented in a future publication, as an established production herd becomes available.

The effects of the tg-FIX *N*-glycans on protein function are yet to be determined. Very little has been published about how *N*-glycan structures affect FIX function. Bharadwaj et al. (1995) showed that enzymatic removal of sialic acid from Factor IX had no effect on in vitro procoagulant activity, but it is not known how fucosylation or partial sialylation affects in vivo properties. It is likely that enzymatic activity (proteolysis of Factor X to Factor Xa) of FIX is not affected by the *N*-glycan structures since they are not present in the active enzyme. This does not rule out other important in vivo functions, however, as the *N*-glycans are present in the circulating zymogen. Glycans are known to mediate protein–protein and protein–cell interactions, and the degree of sialylation of a glycoprotein is thought to be an important determinant of its pharmacokinetic properties (Varki et al. 1999; Sinclair and Elliott 2005). Glycoproteins with desialylated glycans generally can bind to the hepatic asialoglycoprotein receptors, so that they are cleared rapidly from a blood circulation (Hoermann et al. 1993; Joziasse et al. 2000; Jones et al. 2007). It remains to be seen how partial sialylation affects the in vivo function of tg-FIX.

Significant efforts have been made to improve the sialylation of recombinant glycoproteins so as to improve initial recovery and circulation half-life. Hamilton et al. (2006) have engineered yeast for human-like *N*-glycosylation and sialylation with Neu5Ac. Several strategies have been explored to improve sialylation of recombinant glycoproteins in CHO cells, including media optimization and coexpression of glycosyltransferases (Baker et al. 2001; Bobrowicz et al. 2004; Li et al. 2006). Additionally, in vitro methods have been developed to sialylate recombinant glycoproteins after purification (Raju et al. 2001; Wrotnowski 2001; Zopf and Vergis 2002). Similar strategies of nutritional optimization, coexpression of glycosyltransferases, or in vitro processing could also be used for tg-FIX. We also found evidence for sulfated *N*-glycans in tg-FIX, which has not been reported for pd-FIX. It was reported that liver cells have receptors which can bind to sulfated glycans (Szkudlinski et al. 1995; Leteux et al. 2002). Therefore, glycoproteins having sulfated glycan may be rapidly cleared from circulation. It remains to be seen if this effect will be significant. Future experiments that compare tg-FIX with pd-FIX and CHO cell-derived FIX pharmacokinetics will be instructive toward resolving these questions.

The *N*-glycans of tg-FIX were also compared to what is known about other porcine glycoprotein *N*-glycans. To our knowledge, there are no published reports specifically analyzing glycosyltransferases/glycosidase expression in the porcine mammary epithelial cells, but inferences into cell-specific *N*-glycosylation can be made by comparing our data with that of previously published data for porcine lactoferrin and other porcine glycoproteins. The two points of comparison that we will highlight are (1) Gal(α1,3)Gal at the nonreducing termini of the antennae and (2) incorporation of Neu5Gc. The Gal(α1,3)Gal moiety is strongly antigenic in humans (Sandrin and McKenzie 1994; Konakci et al. 2005). It is reported that the Gal(α1,3)Gal antigen is present in glycoproteins from porcine kidney, liver, pancreas, and lung (Oriol et al. 1993; Kim et al. 2006). However, Spik et al. (1994) reported the absence of Gal(α1,3)Gal in the *N*-glycans of porcine lactoferrin, an endogenous glycoprotein synthesized in the porcine mammary epithelial cells and secreted into the milk. Animal glycoproteins also have Neu5Gc, which is synthesized by hydroxylation of Neu5Ac by CMP-Neu5Ac hydroxylase. Humans do not produce Neu5Gc due to a point mutation in the gene encoding CMP-Neu5Ac hydroxylase (Varki 2001; Irie and Suzuki 1998). Since Neu5Gc is not expressed in humans it is thought to be antigenic (Malykh et al. 2001; Zhu and Hurst 2002; Miwa et al. 2004). Malykh et al. (1998) analyzed several porcine tissues for the Neu5Gc content and CMP-Neu5Ac hydroxylase activity. They found that the amount of CMP-Neu5Ac hydroxylase activity was proportional to the Neu5Gc content, and that the amount of Neu5Gc per glycoprotein is predominant in the porcine submandibular gland, lymph node, small intestine, spleen, and thymus. Neu5Ac is more prevalent in glycoproteins from the lung, liver, kidney, and heart. They did not analyze mammary gland tissue. Neu5Gc has not been detected in porcine lactoferrin as reported by Spik et al. (1994). The absence of Neu5Gc and Gal(α1,3)Gal in tg-FIX supports the hypothesis that the porcine mammary epithelial cells glycosylate proteins in a cell-specific manner that is different from other porcine tissues, and that glycosylation of tg-FIX is similar to other milk glycoproteins produced in the pig.

Although there are no other reported cases of *N*-glycan characterization for r-FIX produced in other transgenic animal species for direct comparison, our data can be compared with other glycoproteins produced in the mammary glands of the transgenic goat, cow, rabbit, and mouse as a beginning point for future studies that will more thoroughly investigate differences in *N*-glycosylation machinery in the different species. Recombinant antithrombin III produced in transgenic goat milk had *N*-glycan structures that included oligomannose glycans on Asn155, and complex fucosylated, mono- and disialylated glycans at Asn96, Asn135, and Asn192 (Edmunds et al. 1998). In addition, the complex *N*-glycans detected in transgenic goat-produced antithrombin III were sialylated with both Neu5Gc and Neu5Ac. In contrast, human plasma-derived antithrombin III *N*-glycans are complex, nonfucosylated, and sialylated with Neu5Ac (Franzen et al. 1980). The *N*-glycans of recombinant human lactoferrin produced in transgenic cows contain oligomannose, hybrid, and complex structures, whereas endogenous human lactoferrin contains only complex *N*-glycans (Van Berkel et al. 2002). The *N*-glycans of the human plasma-derived C1 inhibitor are reported to be complex, biantennary, and disialylated with Neu5Ac (Strecker et al. 1985), while *N*-glycans from the recombinant C1 inhibitor produced in transgenic rabbit milk were found to be of all three major classes of *N*-glycans: oligomannose, hybrid, and complex (Koles et al. 2004a). The complex glycans of the transgenic rabbit-derived C1 inhibitor accounted for approximately 50–55% of the *N*-glycans and were mono- and bi-antennary, and sialylated with Neu5Ac (no Neu5Gc detected). Transgenic mouse-derived recombinant human interferon-γ (James et al. 1995) showed a similar *N*-glycosylation pattern to endogenous human interferon-γ (Sareneva et al. 1996). Asn25-linked glycans were of complex and fucosylated structures, but

Asn97-linked glycans were of oligomannose and hybrid structures for both the recombinant and endogenous glycoproteins. In contrast to the above-mentioned examples in the goat, cow, rabbit, and mouse, we have found only complex glycans in the tg-FIX from the pig. Additionally, in contrast to the goat, we could detect no Neu5Gc in the tg-FIX *N*-glycans. As other glycoproteins are produced in the transgenic pig and other species, it will be interesting to see if the currently observed features of *N*-glycosylation are maintained, and whether any particular species glycosylate recombinant proteins with more human-like structures.

In summary, we have presented data showing that the *N*-glycans of tg-FIX are complex, bi- and tri-antennary, sialylated with Neu5Ac, and partially fucosylated. These structures differ from pd-FIX in the degree of branching and the level of sialylation. We were unable to detect high Man *N*-glycans, glycans with Gal(α1,3)Gal termination, or glycans with Neu5Gc, but we did find evidence for sulfated glycans. The identified *N*-glycans from tg-FIX are consistent with structures found in the KEGG (Kyoto Encyclopedia of Genes and Genomes) glycan structure composite map of humans, and similar to that reported earlier for porcine lactoferrin. These data provide evidence that the porcine mammary epithelial cells glycosylate recombinant proteins in a manner that is different from other transgenic animal species and from other cells within the transgenic pig bioreactor. The different *N*-glycan structures found on tg-FIX versus pd-FIX will also be useful in studying the relationship between the FIX *N*-glycan structure and in vivo function.

Experimental

Materials

All reagents, standard sugars, reference proteins, and trilysine (K3) were purchased from Sigma (St. Louis, MO), unless otherwise noted. 9-Aminopyrene-1,4,6 trisulfonate (APTS) was obtained from Beckman-Coulter (Fullerton, CA). Tetrahydrofuran (0.025% BHT inhibited) and phosphoric acid (85% HPLC grade) were from J. T. Baker (Phillipsburg, NJ). Acetonitrile (Burdick and Jackson, HPLC grade) was purchased from VWR (Chicago, IL). pd-FIX (Mononine®) and r-FIX (BeneFIX®) were gifts from Dr. Paul E. Monahan (University of North Carolina, Chapel Hill, NC). PNGase F, α2–3, and α(2–3, 6) sialidase were purchased from New England Biolabs (Beverly, MA). α(1–3, 4) fucosidase and α-galactosidase were obtained from Prozyme (San Leandro, CA) and QA-Bio (Palm Desert, CA), respectively. *N*-Glycan standards were purchased from V-labs (Covington, CA).

Two transgenic pigs-derived FIXs (tg-FIX) expressed in two transgenic pigs (K75 and K45) were used in this study. Pig K75 and K45 are animals that contain a cDNA human Factor IX transgene construct with a 4.2 kb mouse whey acid protein (mWAP) promoter (Van Cott et al. 1999). The average expression levels over the course of lactation were approximately 1000–2000 μg/mL for pig K75, and 3000–4000 μg/mL for pig K45. The lactating pigs were milked by hand and the milk was immediately frozen at −50°C.

Purification of transgenic-Factor IX

Transgenic-FIX was purified from transgenic pig milk by heparin affinity chromatography as described previously (Lindsay et al. 2004). A BioCAD Vision chromatography system (Applied Biosystems, Foster City, CA) was used for all purification steps below with 280 nm detection. Heparin-Sepharose FF (Amersham Biosciences, Piscataway, NJ) was packed in a Poros HP glass column (PerSeptive Biosystems, Framingham, MA). The milk was thawed and mixed 1:1 (v:v) with 200 mM EDTA pH 7.4 and centrifuged for 15 min at 5000 rpm at 4°C, and the solidified milk fat was separated from the skim milk/EDTA. The skim milk/EDTA was diluted 1:5 (v:v) with loading buffer (20 mM Tris, 50 mM NaCl, 0.1% Tween 20, pH 7.4), and loaded onto the column (25 mm ID × 90 mm L) at 0.5 cm/min. The column was washed with the loading buffer at 1 cm/min, and then washed with 20 mM Tris, 200 mM NaCl, 0.1% Tween 20, pH 7.4 at 2 cm/min. Transgenic-FIX was eluted with 20 mM Tris, 500 mM NaCl, 0.1% Tween 20, pH 7.4 at 2 cm/min.

Transgenic-FIX purified through the Heparin Sepharose column was further purified by an anion exchange column to separate biologically active populations. Source 15Q (Amersham Biosciences, Piscataway, NJ) was packed in a Poros HP glass column (25 mm ID × 120 mm L). One part of the Heparin Sepharose product was diluted with two parts of a loading buffer (20 mM Tris, pH 9), and loaded onto the column at 1 cm/min. The column was washed with 90% : 10% loading buffer : elution buffer (20 mM Tris, 1 M ammonium acetate, pH 9) for 5 CV (column volume) at 1 cm/min. Inactive tg-FIX was eluted with 54% : 46% (loading buffer : elution buffer), and active FIX with a 100% elution buffer at 1 cm/min.

The active fraction from the anion exchange column was purified further by reversed phase on a Supecosil LC318 HPLC column (4.6 mm ID × 250 mm L, 5 μm particles, Supelco, St. Louis, MO). The solvents were A: 0.1% (w/v) TFA in water and B: 0.1% (w/v) TFA in acetonitrile. The solvent program was as follows: 5% B for 3 CV, followed by 5–35% B over 6 min, and then 35–65% B for 15 min, followed by 6 min of 65–95% B. The flow rate was constant at 1 mL/min.

Monosaccharide analysis using capillary electrophoresis

The monosaccharide content of glycoproteins was analyzed using the method as described by Chen and Evangelista (1995). Glycoprotein samples (∼5 μg) were dried in a speed-vac (Labconco CentriVap, Kansas City, MO), and then mixed with 20 μL of 2.0 N TFA in a 0.5 mL Eppendorf Biopur Safe-lock tube. The samples were heated at 100°C for 5 h. After cooling down to room temperature, samples were dried by speed-vac and then redissolved with 5 μL of 25 mM carbonate (pH 9.5). The hydrolyzed sugars were reacetylated by adding 2 μL of acetic anhydride and incubating for 30 min at room temperature. The mixture was concentrated to dryness for derivatization with APTS. The dried samples were mixed with 2 μL of APTS (100 mM in 0.9 M citric acid) and 1 μL of $NaBH_3CN$ (1 M in THF). The mixture was vortexed and then heated at 55°C for 2 h. The reaction mixture was diluted with 197 μL of the borate buffer (120 mM, pH 10.2). CE was performed using Beckman P/ACE 5000 with a laser-induced fluorescent detector (Fullerton, CA). A 20 μm × 27 cm eCAP fused silica capillary was used with a 120 mM borate buffer (pH 10.2). Separation conditions were as follows: pressure injection (0.5 psi) for 2 s, followed by 50 min electrophoresis at 25 kV. Detector settings were 488 nm excitation and 520 nm emission, and the cathode was at the capillary outlet. A monosaccharide standard mixture

was also treated and derivatized with every batch analysis of glycoproteins using the same method. The mixture was GalNAc, GlcNAc, Man, Glc, Xyl, Fuc, and Gal (purchased from Sigma, St. Louis, MO). A 5-point calibration was employed (0.1, 0.5, 1, 5, and 10 nmol of each sugar). Linearity of calibration curves was validated with criteria of correlation coefficient better than 0.99. Precision and repeatability of the assay were confirmed by analyzing a test sample (bovine fetuin) with standards three times. The stability of samples prepared prior to analysis was tested analyzing bovine fetuin and standards both after preparation and 48 h later. Protein concentrations were determined by the bicinchoninic acid (BCA) assay and used for calculation of the monosaccharide to protein ratio (mol/mol).

Sialic acid analysis using reversed phase HPLC

Sialic acids were analyzed by reverse phase HPLC using the method described in Anumula (1995). Glycoprotein samples (5–10 μg in 50 μL) were mixed with 50 μL of 0.5 M sodium bisulfate ($NaHSO_4$) in 1.6 mL screw cap microcentrifuge tubes, and then hydrolyzed at 80°C for 20 min. The hydrolyzed samples were derivatized by adding with 100 μL of *O*-phenylenediamine-2HCl, OPD (20 mg/mL in 0.25 M $NaHSO_4$). The tubes were heated at 80°C for 40 min. Solvent A consisted of 0.15% (v/v) 1-butylamine, 0.5% (v/v) phosphoric acid, and 1% (v/v) tetrahydrofuran in water. Solvent B was 50% of solvent A in acetonitrile. After samples were cooled down to room temperature, 0.8 mL of solvent A was added and vortexed vigorously. Proteins and any other particles were precipitated by centrifugation and the supernatant (100 μL) was analyzed by reverse phase HPLC (Supelcosil LC318 HPLC column; 4.6 mm ID × 250 mm L, 5 μm particles, Supelco, St. Louis, MO). The column was equilibrated with 5% B for 10 min at 1 mL/min. The OPD-derivatized sialic acids were eluted with 13% B for 20 min and the column was washed for 10 min with 100% solvent B. Waters 2695 Separations module and 2475 fluorescent detector (Milford, MA) were used to detect OPD-derivatized sialic acids. The detector settings were 230 nm excitation, 425 nm emission, and 40 nm bandwidth. The sialic acid standard mixture (Neu5Ac and Neu5Gc) was analyzed each time with the analysis of glycoproteins. Calibration curves were determined with six different concentrations of each sialic acid standard (100, 200, 400, 600, 800, and 1000 pmol). Linearity of calibration curves were accepted if the correlation coefficient was better than 0.99. Reproducibility and repeatability were evaluated by analyzing a test sample (pd-FIX, Mononine) with standards three times. The stability of prepared samples was tested by analyzing Mononine and standards right after preparation and 3 d after preparation. Protein concentrations were obtained from the BCA assay.

PNGase F digestion and purification of N-glycans from glycoproteins

Purified tg-FIX, pd-FIX (Mononine®), r-FIX (BeneFIX®), porcine thyroglobulin, pig IgG, and bovine fetuin were incubated with PNGase F (substrate: enzyme = 250:1 mass ratio) in 50 mM sodium phosphate at 37°C for 24 h. Released *N*-glycans were separated from proteins using C18 Extract Clean columns (100 mg, 1.5 mL Alltech, Deerfield, IL). The cartridges were equilibrated with 6 mL of 5% acetic acid in water, and then the sample was applied to the column. The flow through, along with the wash of 1 mL of 5% acetic acid, contained the *N*-glycans.

The *N*-glycan fraction was further desalted by using Carbograph Extract Clean columns (150 mg, 4 mL, Alltech, Deerfield, IL). The solvent system was as follows: A: 0.1 (w/v)% TFA in 50% acetonitrile/50% water, B: 0.1 (w/v)% TFA in 5% acetonitrile/95% water. The cartridge was washed with 30% acetic acid in water first and then HPLC grade water, and primed with 3 mL of solvent A followed by 6 mL of solvent B. The glycan solution was applied to the column, and then washed with water and solvent B. Glycans were eluted with 2 × 0.5 mL of solvent A, and dried by speed-vac.

For MS analysis, the glycan samples were further purified by a Stylus Protip 5–50 μL HILIC (Hydrophilic Interaction Chromatography) needle (The Nest Group Inc. Southboro, MA). The dried glycan was dissolved in 50 μL of 90% acetonitrile and then aspirated in and out of the tip to allow maximum binding to the media. The adsorbed sample was washed with 90% acetonitrile. Glycans were eluted with 50 μL of water.

Exoglycosidase digestions of N-glycans

The purified *N*-glycans were further digested with exoglycosidase enzymes prior to HPLC profiling. For sialidase digestion, the *N*-glycans were incubated with α(2–3) sialidase or with α(2–3, 6) sialidase in 50 mM sodium citrate, pH 6 at 37°C for 12 h. The *N*-glycans were also reacted with α-galactosidase in 50 mM sodium phosphate, pH 6.5 or with α(1–3, 4) fucosidase in 50 mM sodium citrate, pH 5 at 37°C for 12 h. The digested *N*-glycans were centrifuged on the Millipore EZ filtration cartridge (Millipore, Billerica, MA) and purified from the enzymes by collecting the filtrate.

Derivatization of N-glycan with 2-aminobenzoic acid (2-AA)

N-Glycan profiles were determined based on the HPLC method of Anumula and Dhume (1998). Dried *N*-glycans were reconstituted with 100 μL water prior to derivatization. The derivatization reagent was prepared fresh by dissolving 30 mg of 2-AA and 20 mg of sodium cyanoborohydride in 1 mL of 4% sodium acetate trihydrate (w/v) and 2% boric acid (w/v) in methanol. Purified *N*-glycans (20 μL) were mixed with 100 μL of the derivatization reagent in 1.5 mL screw cap centrifuge tubes, and then reacted at 80°C for 40 min. After cooling, the sample was diluted with 1 mL 95% (v/v) acetonitrile/water. The excess reagent was removed by the Waters Oasis HLB cartridge (1 mL, Milford, MA). The cartridge was rinsed with 2 mL of 95% (v/v) acetonitrile/water. The sample was applied to the cartridge, followed by washing with 2 mL of 95% (v/v) acetonitrile/water. AA derivatized *N*-glycans were eluted by 1 mL of 20% (v/v) acetonitrile/water. The eluted *N*-glycans were stored at −80°C for NP-HPLC profiling analysis. The viability of the AA-derivatized samples can be maintained up to 3 years (Anumula and Dhume, 1998). The reagent and solvent are stable for several months.

NP-HPLC profiling of 2-AA derivatized N-glycan

Profiling of AA-derivatized *N*-glycan was performed on an amine bonded polymeric column (Polymer-NH_2, 5 μm, 4.6 mm ID × 250 mm L, Astec, Whippany, NJ) with a CPF10 prefilter (Vydac, Hesperia, CA). Solvent A was 2% acetic acid

and 1% tetrahydrofuran (inhibited) in acetonitrile, and Solvent B 5% acetic acid, 3% triethylamine and 1% tetrahydrofuran (inhibited) in water. 100 μL of the derivatized sample was injected by the autosampler. The gradient program started 30% B for 2 min, followed by an increase to 95% B over 80 min. The column was isocratic with 95% B for 15 min and equilibrated with initial conditions for 15 min prior to next injection. The column temperature was 50°C and flow rate constant 1 mL/min. The HPLC system consisted of the Waters 2695 Separations module and 2475 fluorescent detector (Milford, MA). The detector settings were 360 nm excitation, 425 nm emission, and 20 nm bandwidth. Fraction collect started at 20 min every 30 s for 60 min. The collected fractions were dried by speed-vac for electrospray ionization ion trap mass spectrometry (ESI-Ion Trap MS) analysis.

ESI-Ion trap MS analysis of N-glycans

MS analysis was performed on a 4000 Q-Trap hybrid triple quadruple/ion trap system (Applied Biosystems, Foster City, CA) with a MicroIon Spray II ion source. Approximately 30–50 pmol/μL of *N*-glycans were prepared in 50% methanol with 10 mM ammonium hydroxide or ammonium nitrate for the negative ion mode. The sample solution was infused using a nanoflow Picotip emitter (uncoated SilicaTip, 360 μm OD/15 μm ID, New Objectives, Woburn, MA) at 0.5 μL/min. The source settings were as follows: curtain gas = 20, collision gas = high, ion spray voltage = −2400 V, Gas 1 = 5, interface heater temperature = 150°C, and the declustering potential was set at −90 V. Collision energy (CE) in Q2 was dependent on the analyte. The best MS/MS spectra for the precursor ions above *m*/*z* 1500 were generally obtained at CE approximately −130 to −80 V; smaller precursor ions were fragmented at CE of approximately −100 to −50 V. The scan rate was set to 1000 amu/s for enhanced MS (EMS) (ion trap mode) and enhanced product ion (EPI) scans (MS/MS), and 250 amu/s for enhanced resolution (ER) scans. For EMS, the linear ion trap (LIT) fill time was 100 ms with Q0 trapping activated. In EPI, Q1 was set to low resolution, with Q0 trapping activated, and an LIT fill time of up to 400 ms.

IDA (information-dependent acquisition) was also used for data collection from underivatized *N*-glycan samples. Survey scans (MS) were performed in EMS, followed by ER of the four most intense peaks. Then MS/MS spectra were taken on these four peaks. IDA criteria were set as follows: select 1–4 most intense peak, include charge states 1–4 including unknown, ER was used to confirm the charge state, and former target ions were excluded after one occurrence for 60 s.

AA-derivatized *N*-glycans separated using HPLC were dried under vacuum and reconstituted with 10 μL of 50% methanol/0.1% ammonium hydroxide. The sample solution was infused using a discrete Picotip emitter (GlassTip, 1.2 mm OD/0.94 mm ID, New Objectives, Woburn, MA). For EMS scans, the declustering potential was −30 V, the ion spray voltage was −1700 V, the curtain gas was set at 20, the interface heater temperature was set at 150°C, the trap scan speed was set to 1000 amu/s, and the LIT fill time was 20 ms with Q0 trapping activated. In the EPI mode, collision energy was applied at −80 V and Q1 resolution was set to low resolution, and the LIT fill time was 200 ms with Q0 trapping.

Funding

The National Heart, Lung, and Blood Institute (R01 HL078944-01) and the University of Nebraska.

Acknowledgements

The authors wish to acknowledge helpful discussions with Dr. Stephan B. Abramson (LifeSci Partners LLC), and Prof. Paul Monahan (UNC-Chapel Hill) during the preparation of this manuscript.

Supplementary Data

Supplementary data for this article is available online at http://glycob.oxfordjournals.org/.

Conflict of interest statement.

W.H.Velander is a co-founder and stockholder in ProGenetics LLC, a company that is commercializing the production of recombinant proteins in transgenic animal milk.

Abbreviations

2-AA, 2-aminobenzoic acid; APTS, 9-aminopyrene-1,4,6 trisulfonate; BCA, bicinchoninic acid; ESI-Ion Trap MS, electrospray ionization ion trap mass spectrometry; Fuc, fucose; Gal, galactose; GalNAc, *N*-acetylgalactosamine; Glc, glucose; GlcNAc, *N*-acetylglucosamine; IDA, information-dependent acquisition; Man, mannose; Neu5Ac, *N*-acetylneuraminic acid; Neu5Gc, *N*-glycolylneuraminic acid; NP-HPLC, normal phase high performance liquid chromatography; pd-FIX, plasma-derived Factor IX; r-FIX, recombinant Factor IX; tg-FIX, transgenic pig-derived Factor IX; Xyl, xylose.

References

Anumula KR. 1995. Rapid quantitative determination of sialic acids in glycoproteins by high-performance liquid chromatography with a sensitive fluorescence detection. *Anal Biochem*. 230:24–30.

Anumula KR, Dhume ST. 1998. High resolution and high sensitivity methods for oligosaccharide mapping and characterization by normal phase high performance liquid chromatography following derivatization with highly fluorescent anthranilic acid. *Glycobiology* 8(7):685–694.

Baker KN, Rendall MH, Hills AE, Hoare M, Freedman RB, James DC. 2001. Metabolic control of recombinant protein *N*-glycan processing in NS0 and CHO cells. *Biotechnol Bioeng*. 73:188–202.

Bharadwaj D, Harris RJ, Kisiel W, Smith KJ. 1995. Enzymatic removal of sialic acid from human Factor IX and Factor X has no effect on their coagulant activity. *J Biol Chem*. 270(12):6537–6542.

Bobrowicz P, Davidson RC, Li H, Potgieter TI, Nett JH, Hamilton SR, Stadheim TA, Miele RG, Bobrowicz B, Mitchell T, et al. 2004. Engineering of an artificial glycosylation pathway blocked in core oligosaccharide assembly in the yeast *Pichia pastoris*: Production of complex humanized glycoproteins with terminal galactose. *Glycobiology*. 14(9):757–766.

Bond M, Jankowski M, Patel H, Karnik S, Strang A, Xu B, Rouse J, Koza S, Letwin B, Steckert J, et al. 1998. Biochemical characterization of recombinant Factor IX. *Semin Hematol*. 35(2):11–17.

Chen FA, Evangelista RA. 1995. Analysis of mono-and oligosaccharide isomers derivatized with 9-aminopyrene-1,4,6-trisulfonate by capillary electrophoresis with laser-induced fluorescence. *Anal Biochem*. 230:273–280.

Edmunds T, Van Patten SM, Pollock J, Hanson E, Bernasconi R, Higgins E, Manavalan P, Ziomek C, Meade H, McPherson JM, et al. 1998.

Transgenically produced human antithrombin: Structural and functional comparison to human plasma-derived antithrombin. *Blood*. 91(12):4561–4571.

Franzen LE, Svensson S, Larm O. 1980. Structural studies on the carbohydrate portion of human antithrombin III. *J Biol Chem*. 255(11):5090–5093.

Gillis S, Furie BC, Furie B, Patel H, Huberty MC, Switzer M, Foster WB, Scoble HA, Bond MD. 1997. Gamma-carboxyglutamic acids 36 and 40 do not contribute to human factor IX function. *Protein Science*. 6:185–196.

Hamilton SR, Davidson RC, Sethuraman N, Nett JH, Jiang Y, Rios S, Bobrowicz P, Stadheim TA, Li H, Choi BK, et al. 2006. Humanization of yeast to produce complex terminally sialylated glycoproteins. *Science*. 313:1441–1443.

Harris RJ, van Halbeek H, Glushka J, Basa LJ, Ling VT, Smith KJ, Spellman MW. 1993. Identification and structural analysis of the tetrasaccharide NeuAc alpha(2→6)Gal beta(1→4)GlcNAc beta(1→3)Fuc alpha1→*O*-linked to serine 61 of human factor IX. *Biochemistry*. 32(26):6539–6547.

Harris DP, Andrews AT, Wright G, Pyle DL, Asenjo JA. 1998. The application of aqueous two-phase systems to the purification of pharmaceutical proteins from transgenic sheep milk. *Bioseparation*. 7(1):31–37.

Harvey DJ. 2005. Fragmentation of negative ions from carbohydrates: Part 3. Fragmentation of hybrid and complex *N*-linked glycans. *J Am Soc Mass Spectrom*. 16:647–659.

Hoermann R, Kubota K, Amir SM. 1993. Role of subunit sialic acid in hepatic binding, plasma survival rate, and in vivo thyrotropic activity of human chorionic gonadotropin. *Thyroid*. 3(1):41–47.

Irie A, Suzuki A. 1998. CMP-*N*-Acetylneuraminic acid hydroxylase is exclusively inactive in humans. *Biochem Biophys Res Commun*. 248(2):330–333.

James DC, Freedman RB, Hoare M, Ogonah OW, Rooney BC, Larionov OA, Dobrovolsky VN, Lagutin OV, Jenkins N. 1995. *N*-Glycosylation of recombinant human interferon-g produced in different animal expression systems. *Bio/Technology*. 13:592–596.

Jones AJS, Papac DI, Chin EH, Keck R, Baughman SA, Lin YS, Kneer J, Battersby JE. 2007. Selective clearance of glycoforms of a complex glycoprotein pharmaceutical caused by terminal *N*-acetylglucosamine is similar in humans and cynomolgus monkeys. *Glycobiology*. 17(5):529–540

Joziasse DH, Lee RT, Lee YC, Biessen EAL, Schiphorst WECM, Koeleman CAM, Van Den Eijinden DH. 2000. α3-Galactosylated glycoproteins can bind to the hepatic asialoglycoprotein receptor. *Eur J Biochem*. 267:6501–6508.

Kim Y-G, Kim S-Y, Hur Y-M, Joo H-S, Chung J, Lee D-S, Royle L, Rudd PM, Dwek RA, Harvey DJ, et al. 2006. The identification and characterization of xenoantigenic nonhuman carbohydrate sequences in membrane proteins from porcine kidney. *Proteomics*. 6:1133–1142.

Koles K, Van Berkel PHC, Pieper FR, Nuijens JH, Mannesse MLM, Vliegenthart JFG, Kamerling JP. 2004a. *N*- and *O*-glycans of recombinant human C1 inhibitor expressed in the milk of transgenic rabbits. *Glycobiology*. 14(1):51–64.

Koles K, Van Berkel PHCJH, Mannesse MLM, Zoetemelk R, Vliegenthart JFG, Kamerling JP. 2004b. Influence of lactation parameters on the *N*-glycosylation of recombinant human C1 inhibitor isolated from the milk of transgenic rabbits. *Glycobiology*. 14(11):979–986.

Konakci KZ, Bohle B, Blumer R, Hoetzenecker W, Roth G, Moser B, Boltz-Nitulescu G, Gorlitzer M, Klepetko W, Wolner E, et al. 2005. Alpha-Gal on bioprostheses: Xenograft immune response in cardiac surgery. *Euro J Clin Invest*. 35:17–23.

Kurachi K, Davie EW. 1982. Isolation and characterization of a cDNA coding for human Factor IX. *Proc Natl Acad Sci USA* 79:6461–6464.

Leteux C, Chai W, Loveless W, Yuen C-T, Uhlin-Hansen L, Combarnous Y, Jankovic M, Maric SC, Misulovin Z, Nussenzweig MC, et al. 2002. The cystein-rich domain of the macrophage mannose receptor is a multispecific lectin that recognizes chondrointin sulfated A and B and sulfated oligosaccharides of blood group lewis and lewis types in addition to the sulfated *N*-glycans of Lutropin. *J Exp Med*. 191(7):1117–1126.

Li H, Sethuraman N, Stadheim TA, Zha D, Prinz B, Ballew N, Bobrowicz P, Choi B-K, Cook WJ, Cukan M, et al. 2006. Optimization of humanized IgGs in glycoengineered *Pichia pastoris*. *Nature Biotechnol*. 24(2):210–215.

Lindsay M, Gil G, Cadiz A, Velander WH, Zhang C, Van Cott KE. 2004. Purification of recombinant Factor IX produced in transgenic pig milk and fractionation of active and inactive subpopulations. *J Chromatogr A*. 1026(1-2):149–157.

Lubon H, Paleyanda RK, Velander WH, Drohan WN. 1996. Blood proteins from transgenic animal bioreactors. *Transfus Med Rev*. 5(2):131–143.

Makino Y, Omichi K, Kuraya N, Ogawa H, Nishimura H, Iwanaga S, Hase S. 2000. Structural analysis of *N*-linked sugar chains of human blood clotting Factor IX. *J Biochem*. 128:175–180.

Malykh YN, Shaw L, Schauer R. 1998. The role of CMP-*N*-acetylneuraminic acid hydroxylase in determining the level of *N*-glycolylneuraminic acid in porcine tissues. *Glycoconjugate J*. 15:885–893.

Malykh YN, Schauer R, Shaw L. 2001. *N*-Glycolylneuraminic acid in human tumors. *Biochimie*. 83:623–634.

Miwa Y, Kobayashi T, Nagasaka T, Liu D, Yu M, Yokoyama I, Suzuki A, Nakao A. 2004. Are *N*-glycolylneuraminic acid (Hanganutziu-Deicher) antigens important in pig-to-human xenotransplantation? *Xenotransplantation*. 11:247–253.

Mizuochi T, Taniguchi T, Fujikawa K, Titani K, Kobata A. 1983. The structures of the carbohydrate moieties of bovine blood coagulation Factor IX (Christmas Factor). *J Biol Chem*. 258(10):6020–6024.

Nishimura H, Kawabata S, Kisiel W, Hase S, Ikenaka T, Takao T, Shimonishi Y, Iwanaga S. 1989. Identification of a disaccharide (Xyl-Glc) and a trisaccharide(Xyl_2-Glc) *O*-glycosidically linked to a serine residue in the first epidermal growth factor-like domain of human Factors VII and IX and protein Z and bovine protein Z. *J Biol Chem*. 264(34):20320–20325.

Nishimura H, Takao T, Hase S, Shimonishi Y, Iwanaga S. 1992. Human factor IX has a tetrasaccharide *O*-glycosidically linked to serine 61 through the fucose residue. *J Biol Chem*. 267(25):17520–17525.

Oriol R, Ye Y, Koren E, Cooper DKC. 1993. Carbohydrate antigens of pig tissues reacting with human natural antibodies as potential targets for hyperacute vascular rejection in pig-to-man organ xenotransplantation. *Transplantation*. 56(6):1433–1442.

Raju TS, Briggs JB, Chamow SM, Winkler ME, Jones AJS. 2001. Glycoengineering of therapeutic glycoproteins: In vitro galactosylation and sialylation of glycoproteins with terminal *N*-acetylglucosamine and galactose residues. *Biochemistry*. 40(30):8868–8876.

Sandrin MS, McKenzie IFC. 1994. Galα(1–3)gal, the major xenoantigen recognized in pig by human natural antibodies. *Immunol Rev*. 141:169–190.

Sareneva T, Mortz E, Tolo H, Roepstorff P, Julkunen I. 1996. Biosynthesis and *N*-glycosylation of human interferon-γ Asn25 and Asn97 differ markedly in how efficiently they are glycosylated and in their oligosaccharide composition. *Eur J Biochem*. 242:191–200.

Sinclair AM, Elliott S. 2005. Glycoengineering: the effect of glycosylation on the properties of therapeutic proteins. *J Pharm Sci*. 94(8):1626–1635.

Spik G, Coddevilee B, Mazurier J, Bourne Y, Carnbillaut C, Montreuil J. 1994. Primary and three-dimensional structure of lactotransferrin (lactoferrin) glycans. *Adv Exp Med Biol*. 357:21–32.

Strecker G, Ollier-Hartmann M-P, van Halbeek H, Vliegenthart JFG, Montreuil J, Hartmann L. 1985. Primary structure elucidation of carbohydrate chains of normal C1-esterase inhibitor (C1-INH) by 400-MHz 1H-NMR study. *CR Acad Sci Paris*. 301:571–576.

Szkudlinski MW, Thotakura R, Tropea JE, Grossmann M, Wienthraub BD. 1995. Asparagine-linked oligosaccharide structures determine clearance and organ distribution of pituitary and recombinant thyrotropin. *Endo*. 136(8):3325–3333.

Varki A, Cummings R, Esko J, Freeze H, Hart G, Marth J. 1999. Exploring the biological roles of glycans. *Essentials of Glycobiology*. New York: Cold Spring Harbor Laboratory Press. p. 57–68.

Varki A. 2001. Loss of *N*-glycolylneuraminic acid in humans: Mechanisms, consequences, and implications for Hominid evolution. *Yrbk Phys Anthropol*. 44:54–69.

Van Berkel PHC, Welling MM, Geerts M, van Veen HA, Ravensbergen B, Salaheddine M, Pauwels EKJ, Pieper F, Nuijens JH, Nibbering PH. 2002. Large scale production of recombinant human lactoferrin in the milk of transgenic cows. *Nature Biotechnol*. 20:484–487.

Van Cott KE, Butler SP, Russel CG, Subbramanian A, Lubon H, Gwazdauskas FC, Knight J, Drohan WN, Velander WH. 1999. Transgenic pigs as bioreactors: A comparison of gamma-carboxylation of glutamic acid in recombinant human protein C and Factor IX by the mammary gland. *Biomol Engineering*. 15:155–160.

Wrotnowski C. 2001. Neose targets complex carbohydrate products. *Genetic Eng. News*. 21:6.

Yang L, Manithody C, Rezaie AR. 2002. Localization of the heparin binding exosite of factor IXa. *J Biol Chem*. 277(52):50756–50760.

Zhang Y, Jiang H, Go EP, Desaire H. 2006. Distinguishing phosphorylation and sulfation in carbohydrates and glycoproteins using ion-pairing and mass spectrometry. *J Am Soc Mass Spectrom*. 17:1282–1288.

Zhu A, Hurst R. 2002. Anti-*N*-glycolyneuraminic acid antibodies identified in healthy human serum. *Xenotransplantation*. 9:376–381.

Zopf D, Vergis G. 2002. Glycosylation: A critical issue in protein development and manufacturing. *Pharmaceutical Visions*. Spring, 10–14.