Differential Susceptibility of Transferrin Glycoforms to Chymotrypsin: A Proteomics Approach to the Detection of Carbohydrate-Deficient Transferrin

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ABSTRACT: Transferrin exhibits heterogeneity in glycosylation characteristic of pathological changes in alcohol abuse and congenital disorders in glycosylation. This study investigated an alternative approach in the detection of carbohydrate-deficient transferrin based on the premise that glycosylation may afford some degree of protection to proteolytic action. Differential susceptibility to proteolysis by chymotrypsin was demonstrated for normal glycosylated and nonglycosylated recombinant human transferrin, using reverse-phase (RP) HPLC, matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry, and LC-tandem mass spectrometry (MS/MS). Peptide fragmentation profiles were consistent with a predominantly high-specificity cleavage pattern of chymotrypsin. The observed peptide fragmentation profile showed that the C-lobe of recombinant full-length nonglycosylated transferrin (rhTf-NG) appeared to be preferentially cleaved, while cleavage of the N-lobe was restricted to the N-terminal and link sequence regions. Although chymotryptic cleavage sites abound in the N-lobe, their resistance to cleavage was independent of glycosylation. Compared to previous studies of lactoferrin, our data suggest disparity in the role by which glycosylation exerts a protective effect in the siderophilin family. It was clear from the transferrin digestions analyzed by HPLC that N-linked glycosylation did confer protection from proteolysis by chymotrypsin. After fragmentation, a range of peptides representing previously cryptic epitopes were identified as potential candidates for an immunological approach to differentiate between the different transferrin glycoforms. Based on its proximity to the Asn413 glycosylation site, a 15-mer peptide, *m*/*z* 1690.472 (NKSDNCEDTPEAGYF), was identified as a suitable candidate for raising anti-peptide antibodies for subsequent immunological detection. This novel approach could form the basis for an alternative assay or reference method for the detection of carbohydrate-deficient transferrin.

Transferrin, a vital iron-transport protein, is a member of the iron-binding siderophilin family that includes ovotransferrin, lactoferrin, and melanotransferrin (*1*). Transferrin is a single polypeptide glycoprotein with a molecular mass of 79.57 kDa. It comprises 679 amino acids divided into two homologous regions, the N-terminal domain or N-lobe (residues 1−336) and the C-terminal domain or C-lobe (residues 337−679). Both of these regions have a metal-binding site, each of which is capable of capturing a single iron atom (Fe^{3+}). The C-lobe also has two N-linked glycosylation sites at residues 413 and 611 (*2*). Under normal physiological conditions these sites contain complex oligosaccharide chains with variable degrees of branching and heterogeneity in the extent of sialylation. This microheterogeneity allows for nine different transferrin glycoforms containing zero to eight terminal sialic acid residues. The predominant form of transferrin is that which comprises two biantennary structures, also referred to as tetrasialotransferrin (*3*). Changes in the pattern or profile of these glycoforms are known to occur in certain pathological conditions whereby glycosylation is disrupted, such as congenital disorders of glycosylation (CDG),[1] and in alcohol abuse (*4*, *5*).

The abuse or misuse of ethanol is a universal but remarkably underestimated clinical problem. It has a profound social impact and adversely contributes to a great many medical conditions and treatment complications. In a French study by Reynaud et al. (*6*), it was estimated that around 20% of patients seen in clinical practice had an underlying alcohol-use disorder. While there is no consensus on the amount of alcohol required to effect changes in transferrin glycosylation, it is generally accepted that prolonged expo-

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[1] Abbreviations used: MALDI-TOF, matrix-assisted laser desorption ionisation time-of-flight; ESI, electrospray ionization; CDG, congenital disorders of glycosylation; CDT, carbohydrate-deficient transferrin; TFA, trifluoroacetic acid; TCEP, tris(2-carboxyethyl)phosphine; rhTf-NG, nonglycosylated recombinant human transferrin; hTf, human transferrin; RP-HPLC, reverse-phase HPLC; MS/MS, tandem mass spectrometry; Q-TOF, quadrupole/time-of-flight hybrid mass spectrometer.

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sure to around 60 g of alcohol per day for 2 weeks will elevate the specific transferrin glycoforms known as CDT or carbohydrate-deficient transferrin (*7*). CDT is generally considered to comprise the asialo-, monosialo-, and disialotransferrin glycoforms, although the proportions do vary among individuals and between sexes. The transferrin glycoforms were originally thought to represent deficiencies in the terminal sialylation (hence the nomenclature). However, these modifications are now known to be more extensive such that entire glycan chains are absent reflecting a more profound effect of alcohol upon liver glycosylation mechanisms (*8*, *9*).

Traditionally interview techniques have been used to monitor the treatment progress of known or suspected alcohol abusers, but these approaches are prone to under-reporting of alcohol consumption. As a result, laboratory markers have had an increasingly important role in the diagnosis of alcohol abuse. CDT is one of the most promising markers of alcohol abuse with high sensitivity and specificity in differentiating between chronic, heavy-drinking subjects and abstainers or light social drinkers (*5*, *7*, *10*).

There have been many previous reports in the literature citing the use of lectins for the detection of the different transferrin glycoforms. Although some approaches have met with a degree of success (*11*), they have yet to be developed. Furthermore, microheterogeneity in the transferrin and CDT isoforms, together with intellectual property obstacles, has complicated the development of reliable antibodies for immunological detection of CDT. Consequently, laboratory methods for the detection of CDT have focused on more reliable separation methods based on the variation in charge among the different transferrin isoforms (e.g., %CDT, HPLC, capillary electrophoresis). However, these approaches are not easily automated and currently require a degree of technical skill.

The aim of the present study was to investigate the difference in susceptibility of transferrin to proteolysis to determine whether this approach could be used as an alternative method in CDT detection.

MATERIALS AND METHODS

Human Transferrin. Human holo-transferrin (hTf) preparation was obtained from Sigma Chemicals (Poole, Dorset, U.K.). Recombinant full-length nonglycosylated transferrin (rhTf-NG) was obtained from Dr. A. Mason (University of Vermont). This rhTf-NG was produced in BHK cells, and its purity was verified by electrospray ionization (ESI) mass spectrometry, anion exchange chromatography, and urea gel analysis, which showed a singular molecular species consistent with the expected theoretical mass. Furthermore, immunoreactivity and spectral properties (indicative of correct folding and iron-binding) also showed that the rhTf-NG was identical to three commercial normal human serum preparations (*12*).

Theoretical Cleavage Maps. Theoretical cleavage maps were identified using the on-line "Peptide Cutter" (Protein Identification and Analysis Tools on the ExPASy (expert protein analysis system) server (*13*, *14*) provided by the Swiss Institute of Bioinformatics). The theoretical cleavage maps were compared with MALDI-TOF mass spectrometric data and some sequences further verified by LC-MS/MS sequencing.

Chymotrypsin Digestion of Transferrin. Iron-saturated hTf and rhTf-NG samples were prepared to 1 mg/mL solution in 100 mM Tris buffer containing 10 mM $CaCl_2$ at pH 7.8. The amount of protein in these preparations was verified by a BCA protein assay kit (Pierce, Rockford, IL). Two nanomoles of transferrin (150 uL) of both preparations was subjected to digestion, where sequencing grade chymotrypsin (C-6423, Sigma) was added at 10% w/w dilution. Controls without transferrin were also included in the digestion protocol. Samples were incubated at 30 °C. Incubation times varied from 15 min to 16 h. The reaction was stopped by the addition of 15 μL of 0.1% TFA followed by 50 μL of 100 mM TCEP, and samples were placed on ice prior to further analysis by HPLC or MALDI-TOF.

Chromatography. Peptides from digested transferrin were separated by reverse-phase chromatography on 0.1 × 15 cm Vydac C18 column (300 Å, 5 μm, LC-Packings) using a SMART system (Amersham Pharmacia Biotech, Uppsala, Sweden). Elution was performed using a linear gradient of acetonitrile (0−60% in 100 min) in 0.1% TFA at a flow rate of 50 μL/min. Chromatography was monitored for absorbency at 214 nm, and the peptide containing fractions were collected.

For the LC-MS/MS analysis, the peptides were separated by microbore RP-HPLC on a 0.075 mm × 150 mm PepMap column (LC packings, Amsterdam, Netherlands) by elution with a linear gradient of acetonitrile (5−50% in 30 min) in 0.1% formic acid. Chromatography was performed at a flow rate of 0.25 μL/min, and the eluent was directly injected into a quadrupole/time-of-flight hybrid (Q-TOF) mass spectrometer (Micromass, Manchester, U.K.).

Mass Spectrometry. Before MALDI-TOF mass spectrometric analysis, chymotrypsin-digested transferrin peptides were desalted using Millipore μC18 ZipTip. The peptides were analyzed with a Biflex MALDI-TOF mass spectrometer (Bruker-Daltonics, Bremen, Germany) equipped with a nitrogen laser operating at 337 nm in a positive ion reflector mode using α-cyano-4-hydroxycinnamic acid as the matrix. Samples were prepared by mixing 1 μL of peptide with 1 μL of the matrix on the target plate and dried under a gentle stream of warm air. All mass spectra were calibrated externally with the standard peptides, angiotensin II and adrenocorticotropin-18−39.

ESI mass spectrometric analyses of chymotrypsin-digested transferrin peptides were performed using a Micromass Q-TOF (Micromass, Manchester, United Kingdom) equipped with an ESI source. The peptides were injected into the mass spectrometer via a nanoflow interface either using a Hamilton-syringe pump with a flow rate of 0.5 μL/min or from the nanoscale HPLC in a process of LC-MS/MS. In both cases, the mass spectrometer was calibrated using 5 pmol/μL glufibrinogenic peptide B fragmentation pattern as a standard. MS/MS fragmentation spectra of the peptides were acquired by colliding the doubly charged precursor ions with argon collision gas with accelerating voltages of 30−45 eV.

NH_2-Terminal Sequence Analysis. NH_2-terminal sequence analyses were performed by Edman degradation using a Procise 494A sequencer (PerkinElmer Applied Biosystems Division).

Table 1: Monoisotopic Masses of Peptides Derived from rhTf-NG Digested by Chymotrypsin for 30 min and Analyzed by MALDI-TOF[a]

obsd monoisotopic mass $[M + H]^+$	position (amino acids)	calcd monoisotpic mass $[M + H]^+$	peptide sequence
821.363	632−638	821.383	(F)RDDTVCL
918.570			
956.506	602−608	956.507	(L)RQQQHLF
994.497	663−671	994.536	(L)RKCSTSSLL
1000.550	1−8	1000.558	(−)VPDKTVRW
1030.422	648−655	1030.473	(Y)EKYLGEEY
1081.438			
1108.529	623−631	1108.600	(F)RSETKDLLF
1236.571	522−532	1236.641	(F)RCLVEKGDVAF
1310.599			
1409.527	563−574	1409.674	(L)CLDGTRKPVEEY
1422.509	469−479	1422.659	(Y)NKINHCRFDEF
1439.535	348−358	1439.649	(L)SHHERLKCDEW
1443.370	504−515	1443.622	(L)NLCEPNNKEGYY
1448.634	303−314	1448.776	(F)LKVPPRMDAKMY
1455.523			
1464.239	609−622	1464.578	(F)GS**D**VTDCSGNFCLF
1546.544	398−412	1546.830	(Y)IAGKCGLVPVLAENY
1569.585	469−480	1569.727	(Y)NKINHCRFDEFF
1690.472	413−427	1690.654	(Y)**D**KSDNCEDTPEAGYF
1726.756	345−358	1726.779	(W)CALSHHERLKCDEW
1732.623	468−480	1732.791	(L)YNKINHCRFDEFF
1910.787	623−638	1910.965	(F)RSETKDLLFRDDTVCL
1984.777			
1991.498	498−515	1991.830	(L)CMGSGLNLCEPNNKEGYY
2002.660	533−550	2003.010	(F)VKHQTVPQNTGGKNPDPW
2028.932	442−460	2029.040	(W)DNLKGKKSCHTAVGRTAGW
2315.883			
2508.647			
2526.933	359−382	2527.209	(W)SVNSVGKIECVSAETTEDCIAKIM
3260.167			
3393.517	580−608	3393.856	(L)ARAPNHAVVTRKDKEACVHKILRQQQHLF
3433.166			
3506.290			
3682.046	481−515	3682.632	(F)SEGCAPGSKKDSSLCKLCMGSGLNLCEPNNKEGYY
3931.687	575−608	3932.088	(Y)ANCHLARAPNHAVVTRKDKEACVHKILRQQQHLF

[a] The masses of autolytic chymotryptic peptides were removed from the mass map. Probable peptide sequences derived according to the chymotrypsin cleavage rules that were possible to assign for each observed mass are shown. The D−N substitutions at the 413 and 611 glycosylation sites in the nonglycosylated recombinant human transferrin are highlighted in bold typeface.

RESULTS

Human Nonglycosylated Transferrin Susceptibility to Chymotryptic Proteolysis. Human recombinant transferrin, rendered nonglycosylated by mutating glycosylated asparagine residues at 413 and 611 into aspartic acid residues, was digested with chymotrypsin. This enzyme was chosen to visualize the differential proteolysis pattern of nonglycosylated and glycosylated protein forms since the putative chymotrypsin high-specificity cleavage site was immediately adjacent to N-glycosylation site 413 in transferrin. The observed peptide fragmentation map from MALDI-TOF mass analysis was largely consistent with the theoretical proteolytic cleavage pattern as determined using the ExPASy "Peptide Cutter" (*13*, *14*) (Tables 1 and 2). Since these observations were based only on peptide monoisotopic masses, the identities of some of these fragments were subsequently confirmed by sequence analysis on LC-MS/MS (data not shown). In these MS/MS sequencing analyses, some nonspecific peptides that were not cleaved under chymotryptic cleavage rules were also observed, but their amount was not significant at 30 min digestion. The two fragments containing the unoccupied 413 and 611 glycosylation sites were also visible on MALDI-TOF peptide mass maps, but both retained an internal cleavage site and were thus consistently miscleaved (Tables 1 and 2). The digestion was repeated four times under similar conditions giving exactly the same miscleaved pattern. From the digestion and subsequent MALDI analyses of the nonglycosylated recombinant transferrin, there was 40.8% peptide sequence coverage. Interestingly the C-lobe of transferrin appeared to be preferentially cleaved with N-lobe cleavage restricted to the N-terminal and link sequence regions (Table 2).

Differential Chymotryptic Cleavage of Nonglycosylated and Glycosylated Transferrin. Preliminary analysis by reverse-phase chromatography using a C8 column already showed a clear difference in the extent of proteolysis in the glycosylated and nonglycosylated recombinant transferrin with 2 h digests (data not shown). Since shorter incubation times were found to be sufficient for reproducible chymotryptic cleavage (Table 1), further analyses were performed at 30 min incubations with separation on a capillary scale reverse-phase HPLC system. Subsequently, 1 pmol of either hTf or rhTf-NG digested with chymotrypsin was applied to a 0.75 μm × 150 mm C18 column. There was more than a 10-fold difference between the peptides produced from the rhTf-NG sample compared to the hTf digest (Figure 1). The existing N-glycan structures at amino acids 413 and 611 protect transferrin against chymotryptic proteolysis thus leaving the majority of the protein intact or as relatively large protein fragments that are not eluted from the hydrophobic

Table 2: Human Transferrin Sequence Showing the Peptide Fragments (Red) Observed after Digestion of rhTf-NG with Chymotrypsin for 30 min[a]

1	VPDKTVRW | CAVSEHEATKCQSFRDHMKSVIPSDGPSVACVKKASYLDCIRAIAANEADAV	60
61	TLDAGLVYDAYLAPNNLKPVVAEFYGSKEDPQTFYYAVAVVKKDSGFQMNQLRGKKSCHT	120
121	GLGRSAGWNIPIGLLYCDLPEPRKPLEKAVANFFSGSCAPCADGTDFPQLCQLCPGCGCS	180
181	TLNQYFGYSGAFKCLKDGAGDVAFVKHSTIFENLANKADRDQYELLCLDNTRKPVDEYKD	240
241	CHLAQVPSHTVVARSMGGKEDLIWELLNQAQEHFGKDKSKEFQLFSSPHGKDLLFKDSAH	300
301	GF | LKVPPRMDAKMY | LGYEYVTAIRNLREGTCPEAPTDECKPVKW | CAL | SHHERLKCDEW | SV	360
361	NSVGKIECVSAETTEDCIAKIM | NGEADAMSLDGGFVY | IAGKCGLVPVLAENY | **NKS**DNCED	420
421	TPEAGYF | AVAVVKKSASDLTW | DNLKGKKSCHTAVGRTAGW | NIPMGLL | Y | NKINHCRFDEF | F |	480
481	SEGCAPGSKKDSSLCKL | CMGSGL | NLCEPNNKEGYY | GYTGAF | RCLVEKGDVAF | VKHQTVPQ	540
541	NTGGKNPDPW | AKNLNEKDYELL | CLDGTRKPVEEY | ANCHL | ARAPNHAVVTRKDKEACVHKI	600
601	L | RQQQHLF | GS**NVT**DCSGNFCLF | RSETKDLLF | RDDTVCL | AKLHDRNTY | EKYLGEEY | VKAVG	660
661	NL | RKCSTSSLL | EACTFRRP	679

[a] The experimentally observed cleavage sites are indicated by blue vertical lines. The N-lobe (1−330) remains largely uncleaved despite the abundance of theoretical cleavage sites. A predominantly high-specificity cleavage pattern was observed, C-terminal to FYW but not before P, with additional fragments consistent with a lower specificity cleavage C-terminal also to ML. The two N-linked glycosylation sites in the C-lobe at 413 and 611 are highlighted in bold (for clarity these are noted as NKS and NVT rather than the D−N substitutions in the recombinant form). The underlined sequence shows the short connecting region (331−349) separating the N- and C-lobes.

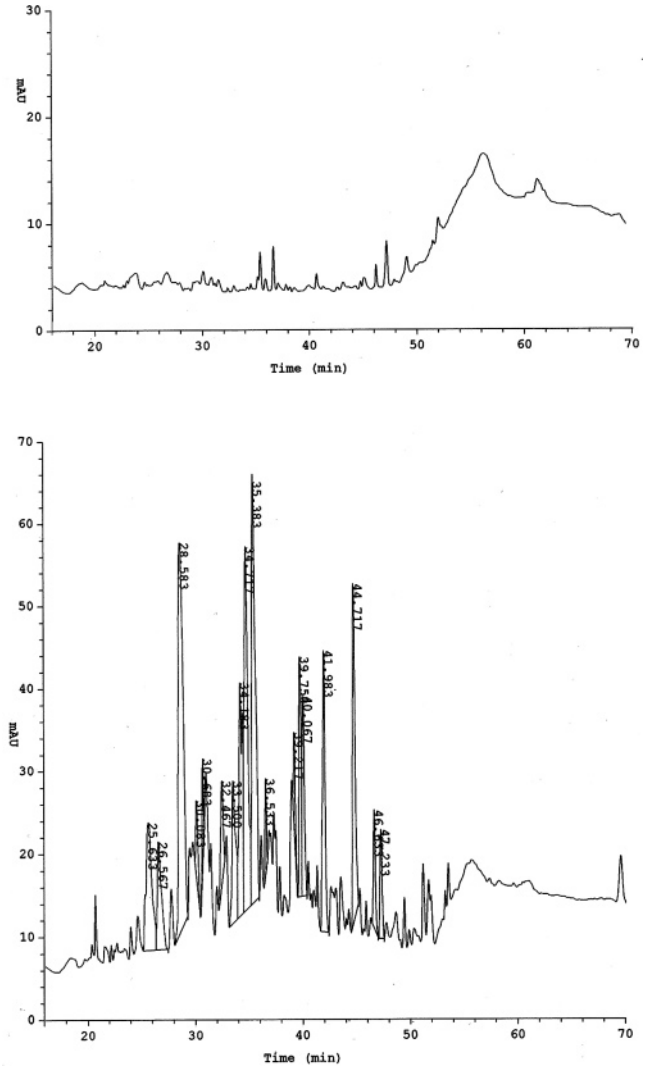

FIGURE 1: Reverse-phase chromatographic analyses of human transferrin digested with chymotrypsin for 30 min. One picomole of both glycosylated hTf (upper trace) and nonglycosylated rhTf-NG digests (lower trace) was subjected to RP C18 chromatographic separation.

C18 reverse-phase column. Differential susceptibility of transferrin C- and N-lobes to proteolysis even in rhTf-NG

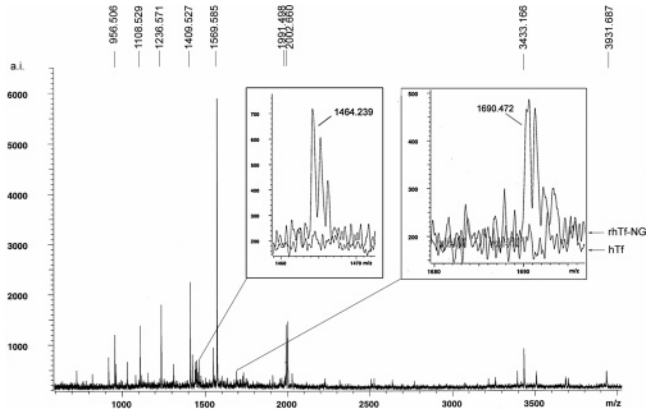

FIGURE 2: Overlay of MALDI-TOF mass spectrometric analyses of rhTf-NG and hTf digested with chymotrypsin for 30 min. Major monoisotopic masses visible in the spectra are annotated. Inserts show the overlays for the peptides of *m*/*z* 1464.239 and 1690.472, which correspond in mass to amino acids 609−622 (with Asn611−Asp611 substitution) and 413−427 (with Asn413−Asp413 substitution) in rhTf-NG, respectively.

(Table 2) suggests that the N-lobe is inherently more stable even without the protective effects of the glycosylated C-lobe.

Identification of a Chymotryptic Peptide from rhTf-NG Absent in hTf. The MALDI-TOF mass map differences were consistent with the differences observed by HPLC. Whereas the MALDI spectrum of rhTf-NG peptides was easily obtained, the glycosylated hTf digest had to be subjected to much higher laser power and more laser shots to obtain any mass spectrometric signal. This was probably due to the dramatically lower amount of peptides produced in that sample. The two MALDI-TOF peptide spectra from rhTf-NG and hTf digests were overlayed and contained mainly the same monoisotopic peptide masses (Figure 2). However, peak overlays from 30 min chymotryptic digests revealed 10 minor peaks that were only visible in the nonglycosylated rhTf-NG. One of these peaks specific to the digested rhTf-NG had a *m*/*z* of 1690.472 (Figure 2), which corresponded to the $[M + H]^+$ weight of the peptide located between amino acid 413 and 427 (this fragment also contained the Asn413, Asp413 substitution). In addition, the peak having a $[M + H]^+$ weight of 1464.239, which corresponded to the normally

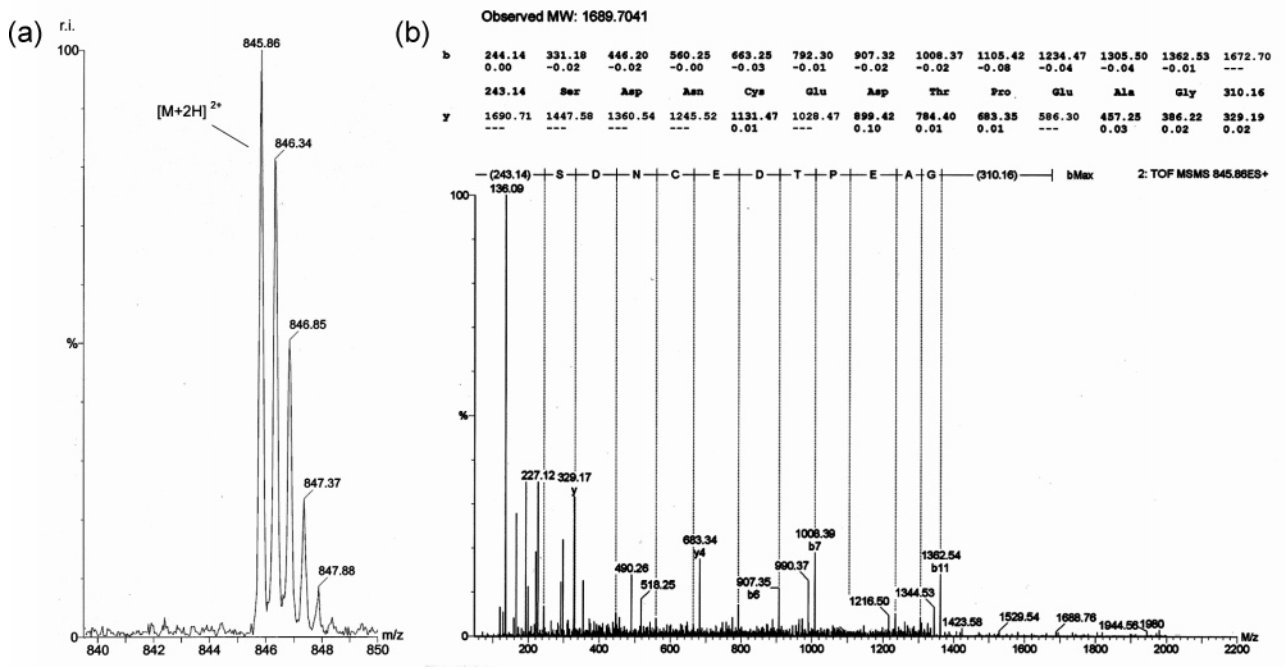

FIGURE 3: LC-MS analysis and LC-MS/MS fragmentation spectra of the peptide M_w 1689.704 corresponding to amino acids 413−427 in rhTf-NG: (A) ESI mass spectrum of the peptide of *m*/*z* 845.86 representing the $[M + 2H]^{2+}$ precursor ion selected for fragmentation; (B) CID fragmentation spectrum for the precursor ion *m*/*z* 845.86 with acceleration voltage 32 eV. The peptide sequence derived from fragmented b-ions is annotated on the spectrum. Mass errors (Da) from the theoretical residual amino acid masses are shown.

glycosylated peptide 609−622, was absent in the glycosylated hTf digestion (this fragment also contained the Asn611, Asp611 substitution). Both peptides were consistently miscleaved, maintaining an internal tyrosine or phenylalanine residue, respectively.

The S/N ratios for the peptides *m*/*z* 1690.5 and *m*/*z* 1464.2 in the rhTf-NG digests varied from 3 to 5 (mean 3.87, SD 0.85) and from 4 to 6 (mean 5.15, SD 0.87), respectively. For both peptide peaks in the hTf digestions, there was no difference from the noise.

The 10 peptides observed specifically in nonglycosylated rhTf-NG were further subjected to LC-MS/MS sequence analysis to confirm their nature. The majority of these minor peaks were of typical background proteins often observed in mass spectrometric analysis of protein digests. The peptide having $[M + H]^+$ of 1464.239 in MALDI-TOF could be visualized in ESI mass spectrum and was subjected to further fragmentation. However, the fragmentation pattern was not clear since the peptide, if it corresponded to the transferrin sequence 609−622, did not contain any lysine or arginine residues that would have greatly improved the ionization and fragmentation of the peptide. Nevertheless, the peptide having $[M + H]^+$ of 1690.472 in MALDI spectrum was clearly visible as a *m*/*z* 845.86 representing the $[M + 2H]^{2+}$ of the peptide (Figure 3A) and gave a clear fragmentation spectra at collision energy of 32 eV (Figure 3B). The clear fragmentation of the peptide is most probably due to the existence of a lysine residue in a second N-terminal position of the peptide. Because of this same lysine residue, the major fragmentation masses in the MS/MS spectrum are composed of b-ions. From the b-ion fragmentation pattern, the sequence of SDNCEDTPEAG is easily readable (Figure 3B) with the flanking mass (310.16 residual mass) at the C-terminal end covered by a dipeptide of tyrosine and phenylalanine, while at the N-terminal end the mass of 243.14 is attributable to a dipeptide of aspartic acid and lysine. Thus, this rhTf-NG peptide sequence (amino acids 413−427) is unambiguously the peptide *m*/*z* 1690.472 in the MALDI-TOF spectrum of the nonglycosylated rhTf digest that is absent in the glycosylated hTf digest. The existence of aspartic acid in the N-terminal position of the peptide was further verified by fractionating the chymotryptic rhTf-NG peptides in a microbore C18 column and collecting the peptide-containing fractions. The fraction containing the *m*/*z* 1690.502 was identified by MALDI-TOF and further subjected to N-terminal sequence analyses, which confirmed the first five amino acids of the peptide to be DKSDN (data not shown).

DISCUSSION

One of the many and diverse roles attributed to glycosylation is that of protection from proteolysis in the cellular environment. While there are many clear examples of this in the literature, the rule is not absolute and the degree of protection offered, if any, may vary depending on the glycoprotein in question, the biological context, and specific evolutionary selection pressures (*15*, *16*).

Although the susceptibility of glycoforms of transferrin to chymotrypsin does not appear to have been previously documented, the proteolysis of lactoferrin has been investigated (*17*−*19*). Lactoferrin, like transferrin, is a member of the siderophilin family of iron-binding proteins. However, while they have 59% homology, they are products of different genes and have quite different patterns of glycosylation: lactoferrin is glycosylated in both the N- and C-lobes and contains three potential sites for N-linked glycosylation at Asn138, -479, and -624 (*20*). One of these previous studies concluded that a major function of glycosylation in human lactoferrin was to protect it from trypsin activity (*17*). However, a later study by the same group comparing human and bovine lactoferrin showed that while

this was still true for bovine lactoferrin, it was clear that N-linked glycosylation was not involved in the protection of human lactoferrin from trypsin (*18*).

From our own studies with hTf, it was clear from HPLC that N-linked glycosylation did confer protection from proteolysis by chymotrypsin (Figure 1). However, this protection was not absolute since the peptide preceding the glycosylated Asn413 (amino acids 398−412) was also visualized, but to a much lesser extent, in the chymotryptic digestion of normal glycosylated hTf. While our own data and the lactoferrin studies are not directly comparable (different enzyme and different proteins), there are some general features that would indicate some disparity in the role and manner in which glycosylation exerts a protective effect in the siderophilin family. Indeed, it may also reflect diversity in the role of siderophilins that extends beyond that of iron binding and transport.

Interestingly, this disparity was also observed between the two lobes of transferrin. The observed peptide fragmentation profile showed that the C-lobe of rhTf-NG appeared to be preferentially cleaved, while cleavage of the N-lobe was restricted to the N-terminal and link sequence regions. Although theoretical chymotryptic cleavage sites abound in the N-lobe, their resistance to cleavage appears to be independent of any protective role of glycosylation. Indeed the extensive amino acid sequence homology between the two lobes of transferrin (around 42%) has led to the suggestion that the modern-day protein has arisen by a process of gene duplication from an ancestral gene coding for only one lobe comprising 350 amino acids (40 000 kDa), which is about half the present-day protein (*21*). Perhaps somewhere in evolutionary development, which included multiple independent mutations, both endogenous and exogenous selection pressures (e.g., microbial/parasitic competition for iron) determined that only the C-lobe evolved or maintained a requirement for glycosylation to retain functional stability. This would in part explain the observed peptide fragmentation profile.

Based on the detailed structural and functional analyses previously performed on rhTf-NG, it is reasonable to assume that the different susceptibility to proteolysis by chymotrypsin is due to the effect of glycosylation and not to any synthetic effects of the recombinant protein (*12*). Furthermore, the aspartic acid substitution for the two asparagine N-glycosylation sites in the rhTf-NG had no influence on the theoretical cleavage by chymotrypsin. MALDI-TOF analysis confirmed the presence of the 15-mer peptide fragment (mass 1689.5) from rhTf-NG but not hTf digests. Q-TOF analysis confirmed the peptide to be the transferrin sequence 413−427 with the Asn413−Asp413 substitution. Based on its proximity to the Asn413 glycosylation site the 15-mer peptide *m*/*z* 1690.472 (NKSDNCEDTPEAGYF) was identified as a suitable candidate for raising anti-peptide antibodies for subsequent immunological detection.

Carbohydrate-deficient transferrin (CDT) has for many years been associated with alcohol-induced changes (*5*). The CDT transferrin glycoforms, previously thought to comprise only sialic acid-deficient versions of their glycan side chains, are now known to be characterized by a complete loss of glycans at one or both glycosylation sites in the C-lobe (*8*, *9*). The disialo glycoforms have yet to be characterized to determine which glycan site, if any, is predominantly occupied. More recent studies have suggested that the completely glycan-free version (also known as asialotransferrin) is perhaps the more clinically relevant target for measuring alcohol abuse by offering the highest degree of discrimination between moderate and excessive drinking habits (*22*, *23*). However, other studies using capillary electrophoresis suggest that this may not be the case (*24*).

The development of a lectin or antibody assay for detection of the transferrin glycoforms has been the focus of many groups over several years. However, the microheterogeneity associated with CDT, together with intellectual property issues, has raised significant obstacles slowing the progress for immunological differentiation between the glycoforms. In the present study, the different susceptibility of glycan-free transferrin to chymotrypsin provides a novel approach for the detection of specific glycoforms that currently represent clinically relevant components of CDT. The chymotryptic cleavage generates a novel panel of peptide fragments representing previously cryptic epitopes that, under the constraints of digestion, are unique to specific nonglycosylated transferrin forms. In addition to exposing new epitopes, there is also the advantage of using alternative technological approaches, such as fluorescent polarization, for the detection of smaller antigens (<2000 kDa). Chymotryptic proteolysis has been performed in complex mixtures such as serum but the detection of the nonglycosylated novel epitope (i.e., the transferrin peptide fragment) has been limited to the use of mass spectrometric technology without substantial prepurification of the sample. Consequently, a sensitive immunological approach is currently being developed for the detection of the peptide from complex serum mixtures. The exposure of cryptic peptide epitopes in this manner provides new immunological targets that could be used for the detection of the transferrin glycoforms associated with alcohol abuse and CDG. The fractionation of transferrin samples in this manner and subsequent mass spectrometric identification of the two peptide fragments containing the glycosylation sites could also form the basis of a nonimmunological reference method for CDT.

It remains to be seen whether this susceptibility to proteolysis could be applied in the detection of other glycosylated proteins wherein multiple glycoforms are associated with a specific pathology.

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REFERENCES

1. Aisen, P., and Listowsky, I. (1980) Iron transport and storage proteins, *Annu. Rev. Biochem. 49*, 357−393.
2. MacGillivray, R. T., Mendez, E., Shewale, J. G., Sinha, S. K., Lineback-Zins, J., and Brew, K. (1983) The primary structure of human serum transferrin. The structures of seven cyanogen bromide fragments and the assembly of the complete structure, *J. Biol. Chem. 258*, 3543−3553.
3. de Jong, G., van Dijk, J. P., and van Eijk, H. G. (1990) The biology of transferrin, *Clin. Chim. Acta 190*, 1−46.
4. Stibler, H., and Kjellin, K. G. (1976) Isoelectric focusing and electrophoresis of the CSF proteins in tremor of different origins, *J. Neurol. Sci. 30*, 269−285.

5. Arndt, T. (2001) Carbohydrate-deficient transferrin as a marker of chronic alcohol abuse: a critical review of preanalysis, analysis, and interpretation, *Clin. Chem. 47*, 13−27.
6. Reynaud, M., Malet, L., Facy, F., and Glanddier, P. (2000) Hospital morbidity of alcohol use disorders in the center of France, *Alcohol Clin. Exp. Res. 24*, 1057−1062.
7. Stibler, H. (1991) Carbohydrate-deficient transferrin in serum: a new marker of potentially harmful alcohol consumption reviewed, *Clin. Chem. 37*, 2029−2037.
8. Inoue, T., Yamauchi, M., and Ohkawa, K. (1999) Structural studies on sugar chains of carbohydrate-deficient transferrin from patients with alcoholic liver disease using lectin affinity electrophoresis, *Electrophoresis 20*, 452−457.
9. Flahaut, C., Michalski, J. C., Danel, T., Humbert, M. H., and Klein, A. (2003) The effects of ethanol on the glycosylation of human transferrin, *Glycobiology 13*, 191−198.
10. Meerkerk, G. J., Njoo, K. H., Bongers, I. M., Trienekens, P., and van Oers, J. A. (1999) Comparing the diagnostic accuracy of carbohydrate-deficient transferrin, gamma-glutamyltransferase, and mean cell volume in a general practice population, *Alcohol Clin. Exp. Res*. *23*, 1052−1059.
11. Yoshikawa, K., Umetsu, K., Shinzawa, H., Yuasa, I., Maruyama, K., Ohkura, T., Yamashita, K., and Suzuki, T. (1999) Determination of carbohydrate-deficient transferrin separated by lectin affinity chromatography for detecting chronic alcohol abuse, *FEBS Lett. 458*, 112−116.
12. Mason, A. B., Miller, M. K., Funk, W. D., Banfield, D. K., Savage, K. J., Oliver, R. W., Green, B. N., MacGillivray, R. T., and Woodworth, R. C. (1993) Expression of glycosylated and non-glycosylated human transferrin in mammalian cells. Characterization of the recombinant proteins with comparison to three commercially available transferrins, *Biochemistry 32*, 5472−5479.
13. Gasteiger, E., Hoogland, C., Gattiker, A., Duvaud, S., Wilkins, M., Appel, R. D., and Bairoch, A. (2005) Protein Identification and Analysis Tools on the ExPASy Server, in *Proteomics Protocols Handbook* (Walker, J. M., Ed.), Humana Press, Totowa, NJ.
14. Gasteiger, E., Gattiker, A., Hoogland, C., Ivanyi, I., Appel, R. D., and Bairoch, A. (2003) ExPASy: the proteomics server for in-depth protein knowledge and analysis, *Nucleic Acids Res. 31*, 3784−3788.
15. Varki, A. (1993) Biological roles of oligosaccharides: all of the theories are correct, *Glycobiology 3*, 97−130.
16. Gagneux, P., and Varki, A. (1999) Evolutionary considerations in relating oligosaccharide diversity to biological function, *Glycobiology 9*, 747−755.
17. van Berkel, P. H., Geerts, M. E., van Veen, H. A., Kooiman, P. M., Pieper, F. R., de Boer, H. A., and Nuijens, J. H. (1995) Glycosylated and unglycosylated human lactoferrins both bind iron and show identical affinities towards human lysozyme and bacterial lipopolysaccharide, but differ in their susceptibilities towards tryptic proteolysis, *Biochem. J. 312*, 107−114.
18. van Veen, H. A., Geerts, M. E., van Berkel, P. H., and Nuijens, J. H. (2004) The role of N-linked glycosylation in the protection of human and bovine lactoferrin against tryptic proteolysis, *Eur. J. Biochem. 271*, 678−684.
19. Brines, R. D., and Brock, J. H. (1983) The effect of trypsin and chymotrypsin on the in vitro antimicrobial and iron-binding properties of lactoferrin in human milk and bovine colostrum. Unusual resistance of human apolactoferrin to proteolytic digestion, *Biochim. Biophys. Acta 759*, 229−235.
20. van Berkel, P. H., van Veen, H. A., Geerts, M. E., de Boer, H. A., and Nuijens, J. H. (1996) Heterogeneity in utilization of N-glycosylation sites Asn624 and Asn138 in human lactoferrin: a study with glycosylation-site mutants, *Biochem. J. 319*, 117−122.
21. Welch, S. (1992) The transferrin gene − structure, regulation, and tissue expression, in *Transferrin: the iron carrier* (Welch, S., Ed.), pp 130−157, CRC Press, Boca Raton, FL.
22. Legros, F. J., Nuyens, V., Baudoux, M., Zouaoui Boudjeltia, K., Ruelle, J. L., Colicis, J., Cantraine, F., and Henry, J. P. (2003) Use of capillary zone electrophoresis for differentiating excessive from moderate alcohol consumption, *Clin. Chem. 49*, 440−449.
23. Arndt, T. (2003) Asialotransferrin − an alternative to carbohydrate-deficient transferrin? *Clin. Chem. 49*, 1022−1023.
24. Daeppen, J.-B., Anex, F., Favrat, B., Bissery, A., Leutwyler, J., Gammeter, R., Mangin, P., and Augsburger, M. (2005) Carbohydrate-deficient transferrin measured by capillary zone electrophoresis and by turbidimetric immunoassay for identification of young heavy drinkers, *Clin. Chem. 51*, 1046−1048.

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