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Site-Specific *N*-Glycosylation of Endothelial Cell Receptor Tyrosine Kinase VEGFR-2

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Abstract

Vascular endothelial growth factor receptor-2 (VEGFR-2) is an important receptor tyrosine kinase (RTK) that plays critical roles in both physiologic and pathologic angiogenesis. The extracellular domain of VEGFR-2 is composed of seven immunoglobulin-like domains, each with multiple potential *N*-glycosylation sites (sequons). *N*-glycosylation plays a central role in RTK ligand binding, trafficking and stability. However, despite its importance, the functional role of *N*-glycosylation of VEGFR-2 remains poorly understood. The objectives of the present study were to characterize *N*-glycosylation sites in VEGFR-2, via enzymatic release of the glycans and concomitant incorporation of ¹⁸O into formerly *N*-glycosylated sites followed by tandem mass spectrometry (MS/MS) analysis to determine *N*-glycosylation site occupancy and the site-specific *N*-glycan heterogeneity of VEGFR-2 glycopeptides. The data demonstrated that all seven VEGFR-2 immunoglobulin-like domains have at least one occupied *N*-glycosylation site. MS/MS analyses of glycopeptides and deamidated, deglycosylated (PNGase F-treated) peptides from ectopically expressed VEGFR-2 in porcine aortic endothelial (PAE) cells identified *N*-glycans at the majority of the 17 potential *N*-glycosylation sites on VEGFR-2 in a site-specific manner. The data presented here provide direct evidence for site-specific, heterogeneous *N*-glycosylation and *N*-glycosylation site occupancy on VEGFR-2. The study has important implications for therapeutic targeting of VEGFR-2, ligand binding, trafficking and signaling.

Graphical Abstract

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Keywords

VEGFR-2; *N*-glycosylation; RTK; receptor tyrosine kinase; vascular endothelial growth factor receptor-2

Introduction

Receptor tyrosine kinases (RTKs) are a family of cell surface receptors that regulate essential cellular processes and serve as key mediators of cellular signaling. The extracellular domains of RTKs bind soluble growth factors and initiate intracellular signal transduction via their cytoplasmic tyrosine kinase domains. Dysregulation of RTK signaling is associated with the development and progression of cancer.¹ Vascular endothelial growth factor receptor-2 (VEGFR-2) is an endothelial cell RTK that plays a central role in physiologic and pathologic angiogenesis.^{2–6} Upon VEGF binding to the extracellular domain, VEGFR-2 undergoes dimerization and trans-phosphorylation, leading to activation of diverse angiogenic signaling pathways that orchestrate endothelial cell migration, proliferation, capillary tube formation and permeability.^{5, 6} In addition to ligand binding, the extracellular domain of VEGFR-2 plays a significant role in VEGFR-2 angiogenic signaling through the formation of lateral heterodimers of VEGFR-2 with other cell surface molecules such as the neuropilin receptors.⁷

Both the extracellular and cytoplasmic regions of VEGFR-2 can undergo post-translational modification.^{8, 9} The extracellular portion of VEGFR-2 is composed of seven immunoglobulin (Ig)-like domains and is highly *N*-glycosylated. In line with its established significance in the regulation of cell surface receptors, *N*-glycosylation could play several important roles in VEGFR-2 function. First, the co-translational addition of *N*-glycans to immature VEGFR-2 should aid its appropriate folding, as is well-established for proteins that pass through the secretory apparatus. Whereas properly folded proteins pass through the secretory pathway and are sorted to their final destination, misfolded proteins are targeted for endoplasmic reticulum-associated degradation (ERAD), which usually operates in a glycan-dependent manner, although during times of cellular stress misfolded glycoproteins may also be degraded in a glycan-independent manner.^{10, 11}

Maturation of VEGFR-2 is also regulated by RNF121 in the ER; higher levels of RNF121 lead to ubiquitination and degradation of its immature forms.¹² Second, metabolic flux

through the hexosamine pathway could increase *N*-glycan branching during glycan remodeling steps and ultimately influence the surface residency of glycosylated VEGFR-2 via the formation of galectin lattices.^{13, 14} Third, *N*-glycosylation of VEGFR-2 could lead to ligand-independent activation of VEGFR-2 by galectins, as demonstrated for other RTKs in drug-resistant tumor cells.^{15, 16} Finally, glycosylation has the potential both to regulate the interactions of VEGFR-2 with other proteins by introducing new recognition epitopes on the surface of VEGFR-2 and/or to block recognition of previously accessible polypeptide epitopes.¹⁷

Despite the critical importance of *N*-glycosylation in RTK biology, to date the occupancy of specific glycosylation sites and potential functional role(s) of glycosylation in VEGFR-2 remain poorly understood. In this study, we aimed to characterize the *N*-glycosylation sites of VEGFR-2 derived from an endothelial cell line, using MS/MS analysis. Our study is the first to comprehensively determine site-specific *N*-glycosylation of VEGFR-2. Given the importance of RTKs in signaling and the role of RTK extracellular domains in ligand binding and interaction with other proteins, these findings are critical to advance the understanding of the role *N*-glycans play in RTK function, and, specifically, the role of VEGFR-2 *N*-glycosylation in angiogenic signaling.

Experimental Section

Reagents

PNGase F was obtained from New England Biolabs (Ipswich, MA). MS Grade proteases, including Trypsin (TPCK treated), Glu-C, and Chymotrypsin (TLCK treated), Recombinant Protein A Agarose, NuPAGE Novex 4–12% Bis-Tris Protein Gels (1.5 mm, 10-well), and GelCode Blue Staining Reagent were purchased from Thermo Scientific (Waltham, MA). Anti-VEGFR-2/FLK1 antibody against the kinase insert region (cytoplasmic) was generated in-house.¹⁸

Immunoprecipitation

Porcine aortic endothelial (PAE) cells with moderate ectopic expression of murine VEGFR-2 (Flk-1)¹⁹ were grown to confluence, washed twice with H/S buffer and lysed (lysis buffer, pH 7.4: 1% Triton X-100, 10 mM Tris-HCl, 5 mM EDTA, 50 mM NaCl, 50 mM NaF, 2 mM sodium orthovanadate, 1 μ M leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 20 μ g/ml aprotinin). VEGFR-2 was immunoprecipitated from PAE cell lysate (VEGFR-2) using a rabbit polyclonal anti-VEGFR-2/FLK1 antibody. Immunoprecipitated VEGFR-2 was eluted from Protein A beads in Pierce LDS Sample Buffer (Thermo Scientific, Waltham, MA), and run on a 4–12% Bis-Tris gel for 2 hours at 150 V (constant). Gels were stained with GelCode Blue stain, followed by overnight destaining in water. Bands from 170 kDa to >250 kDa were excised from the gels and stored at –20 °C.

Proteolysis

Excised gel bands were cut into ~1 mm pieces and destained using 50% acetonitrile/50% 50 mM ammonium bicarbonate, then reduced with 10 mM DTT at 60 °C for 30 min, and alkylated with 15 mM iodoacetamide at room temperature in the dark for 45 min, then

flushed three times, with alternating water and 1:1 acetonitrile/water washes. Gel pieces were dried under vacuum. For all enzymatic digestions, 0.2 µg of enzyme was added per band. Trypsin digestions were buffered with 50 mM ammonium bicarbonate. Chymotrypsin digestions were buffered with 100 mM Tris-HCl, pH 8, and 2 mM CaCl₂ was added to enhance enzyme specificity. Glu-C digestions were performed in 50 mM phosphate buffer, pH 7.4. RapiGest™ (Waters, Milford, MA) was added to a concentration of 0.1% (w/v). All digestions were performed at 37 °C in a Thermomixer (Eppendorf, Hamburg, Germany), with repeated 15-second mixing intervals at 300 RPM followed by 5 min at rest. Glu-C/trypsin digests were performed by adding Glu-C buffered with phosphate, as above, allowing overnight digestion, followed by addition of Tris-HCl, pH 8 (100 mM), addition of trypsin, and a 12-hour digestion step. Formic acid was added to a final concentration of 0.5% (v/v) to halt enzymatic activity and destroy RapiGest™. Samples were dried under vacuum, dissolved in HPLC-grade water with 0.1% formic acid, and peptides were extracted using C18 tips (Thermo) eluted with 1:1 water/acetonitrile with 0.1% TFA.

PNGase F/H₂¹⁸O

Each of the VEGFR-2 peptide mixtures obtained by treatment with one of the proteases was split into two equal amounts and taken to dryness in a centrifugal evaporator. GlycoBuffer 2 (NEB, 50 mM Sodium Phosphate, pH 7.5 @ 25°C) was aliquoted and dried under vacuum and then 20 µL of H₂¹⁸O (99%, Cambridge Isotopes, Tewksbury, MA) was added to each tube of dried buffer to control the pH during PNGase F treatment. After mixing, each of the buffer solutions was transferred to a tube containing one of the dried aliquots of VEGFR-2 peptides. Next, 1 µL of PNGase F was added to one tube of peptides in each pair of aliquots; the second tube was not treated with the glycosidase and served as the control. The contents were gently mixed by vortexing, and the tubes were placed on the Thermomixer at 37 °C overnight (16 h). Samples were dried under vacuum, and cleaned via C18 ZipTip™ (Millipore, Billerica, MA) according to the manufacturer's protocol.

LC-MS/MS of Peptides

VEGFR-2 peptide samples were analyzed on a Q Exactive Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Scientific) equipped with a nanoAcquity UPLC system (Waters) and a Triversa Nanomate (Advion, Ithaca, NY). For chromatographic separation, a nanoACQUITY UPLC Symmetry™ C18 Trap Column (100Å, 5 µm, 180 µm × 20 mm, Waters) column was used for trapping and an ACQUITY UPLC Peptide BEH C18 nanoACQUITY Column (130Å, 1.7 µm, 150 µm × 100 mm, Waters) column was used for separation. The peptide trapping step was performed at 4 µL/min for 4 min with 1% acetonitrile and 0.1% formic acid (Solvent A). Following the trapping step, peptides were separated on the analytical column according to the following conditions: 0–1 min: 2% B, 1–3 min: 2–5%B, 3–43 min: 5–40%B (Solvent A: 1% acetonitrile and 0.1% formic acid in water; Solvent B: 99% acetonitrile and 0.1% formic acid). MS scans were acquired with the following settings: 70,000 resolution @ *m/z* 400, scan range *m/z* 370–1880, 1 µscan/MS, AGC target 1e⁶, and a maximum injection time of 100 ms. MS2 scans were acquired with the following settings: 17,500 resolution @ *m/z* 400, AGC target of 5e⁵, maximum injection time of 60 ms, isolation window of 2.0 *m/z*, isolation offset of 0.4 *m/z*, normalized collision energy (NCE) of 27%, exclusion of charge states 1 and >8, underfill ratio of 1.2%, and

dynamic exclusion of 8 seconds. Profile data were recorded for MS and MS2 scans. To calculate the total areas of the peaks corresponding to the $[M + nH]^{n+}$ selected peptides, ion chromatograms of all detected charge states were extracted, and the areas were summed.

Glycopeptide LC-MS/MS

VEGFR-2 glycopeptides were enriched/separated/analyzed using a 6550 Q-TOF MS with a 1200 series nanoflow HPLC-Chip-ESI source fitted with a custom HPLC-Chip with a 360 nL TSK Gel Amide-80 5 μ m trapping/enrichment column and a 150 mM $\times$ 75 μ m Polaris C18-A 3- μ m analytical column (all from Agilent Corp., Santa Clara, CA). VEGFR-2 digests were initially dissolved in 50% acetonitrile with 0.1% TFA. Immediately prior to injection, the concentration of acetonitrile was adjusted to 80% acetonitrile/0.1% TFA. After injection of the sample onto the Amide-80 enrichment column, the column was washed at a flow rate of 1.5 μ l/min for 4 min using 80% acetonitrile/0.1% TFA, followed by elution of the sample onto the C18 analytical column with 1% acetonitrile/0.1% formic acid. Finally, glycopeptides were separated on the analytical column using a gradient from 1% to 40% acetonitrile with 0.1% formic acid at a flow rate of 0.2 μ l/min. The 6550 Q-TOF mass spectrometer was operated in positive mode using the High Resolution, Extended Dynamic Range (2 GHz) setting. MS spectra were collected from m/z 150–2000 and MS2 spectra were collected from m/z 50–3000. The source gas temperature was set to 225 $^{\circ}$ C and the flow was set at 11 l/min, with a capillary voltage of 1900 V. Precursors 1200 counts and charge states 2 were selected for fragmentation, and the collision energy was set according to the equation $y = mx + b$, with y being the collision energy, slope $m = 5$, x representing the charge state, and the offset $b = -4.8$. Spectra were collected in profile mode. The error for all peaks with S/N ratio >10 is within 5 ppm.

Analysis of Peptide LC-MS/MS Data

LC-MS/MS data were processed using Proteome Discoverer 1.4.1.14 with the SEQUEST search engine (Thermo). For all datasets, cleavage rules were applied for each specific protease (trypsin: K, R, P1' P; endoproteinase Glu-C: D, E; chymotrypsin: Y, W, F, L; or endoproteinase Glu-C/trypsin: K, R, D, E) and peptides with up to two missed cleavages were considered. The following peptide modifications were considered: methionine oxidation (variable), deamidation (variable), deamidation with 18 O (variable) and carbamidomethylation (fixed). A target FDR of $<5\%$ for 'Medium' confidence peptides and $<1\%$ for 'High' confidence peptides was applied. *Sequence Alignment*. Clustal Omega (European Molecular Biology Laboratory – European Bioinformatics Institute, Hinxton, Cambridgeshire, UK) was used for alignment of protein sequences.²⁰

Analysis of Glycopeptide LC-MS/MS Data

Data files were converted to mzXML format using MSConvert, and peak picking was performed. GlycoPeptideSearch²¹ was used to process glycopeptide data, with the following parameters: for the glycan database, the human 'Glyco' subset of glycan structures from GlycomeDB (version 1.0) database was selected. Peptides settings included the specific protease used (trypsin, Glu-C, chymotrypsin, Glu-C/trypsin), a molecular weight between 400–5000 Da, up to two missed cleavages, fixed carbamidomethylation of cysteine residues, variable oxidation, and a maximum of one variable modification per peptide.²¹ Spectra were

filtered based on the following criteria: at least one oxonium ion with intensity > 50%, two peptide fragments, and a peptide intensity threshold of 2%. Precursor mass tolerance was set to 0.1 Da and fragment m/z tolerance of 0.2 Da. Glycan compositions were restricted to those consistent with *N*-linked glycans.

Results

Purification of VEGFR-2 and Enzymatic Removal of *N*-linked Glycans

To study VEGFR-2 *N*-glycosylation, we first immunoprecipitated VEGFR-2 from porcine aortic endothelial (PAE) cells with ectopic expression of murine VEGFR-2 and performed a Western blot using an anti-VEGFR-2 antibody, targeting the extracellular domain of the receptor. Two distinct VEGFR-2 bands were visible in the Western blot, at approximately 200 and 230 kDa (Figure 1A). We analyzed the glycopeptides and deglycosylated peptides via tandem mass spectrometry (Figure 1B) to determine which glycosylation sites are occupied by *N*-linked glycans. Both bands shifted after treatment with PNGase F to enzymatically remove *N*-linked glycans (Figure 1A, far right lane), whereas treating lysate with heat-denatured PNGase F had no effect on VEGFR-2 molecular weight. The decrease in molecular weight of VEGFR-2 after treatment with PNGase F suggests that *N*-glycans constitute a significant portion of the molecular weight of mature VEGFR-2. The presence of two bands both before and after removal of *N*-glycans suggests that additional modifications may impact the electrophoretic mobility of VEGFR-2 independently of *N*-glycosylation. Next, VEGFR-2 was immunoprecipitated and separated via SDS-PAGE. Tandem mass spectrometry (MS/MS) analysis of peptides from excised gel bands after treatment with PNGase F (to remove *N*-glycans and facilitate detection of formerly-glycosylated peptides) confirmed the presence of murine VEGFR-2 (40.7% sequence coverage with tryptic peptides alone, and 58.7% combined sequence coverage with multiple proteases; see Supporting Figures S1–2).

VEGFR-2 *N*-Glycosylation Site Occupancy

The extracellular domains of murine and human VEGFR-2 share 17 of 18 *N*-glycosylation sequons (Supporting Figure S3). A schema of murine VEGFR-2, indicating the positions of the *N*-glycosylation sequons, is shown in Figure 1C. To determine if the 17 murine VEGFR-2 sequons are *N*-glycosylated, we digested VEGFR-2 with trypsin, Glu-C, chymotrypsin, or a combination of trypsin and Glu-C, followed by treatment of the resulting peptides with PNGase F in the presence of heavy water ($H_2^{18}O$). PNGase F is both a glycosidase and an amidase. Therefore, in the presence of heavy water, glycosylated asparagine residues are de-glycosylated and converted to aspartic acid residues containing one ^{18}O atom, while non-glycosylated asparagine residues do not incorporate ^{18}O but can be partially hydrolyzed to aspartic acid prior to or during sample handling. To distinguish between peptides with non-enzymatic deamidation and formerly *N*-glycosylated peptides, controls with identical buffer conditions but not exposed to PNGase F were analyzed in parallel with PNGase F-treated samples.^{22, 23} Tandem mass spectrometry (MS/MS) analysis of VEGFR-2 peptides after treatment with PNGase F/ $H_2^{18}O$ confirmed that at least 15 of the 17 receptor sequons are *N*-glycosylated (Table 1). Peptides in Table 1 were assigned based on the accurate mass of the precursor ion and the confirmation of the peptide sequence in the

product ion spectra. The values measured for all precursors are within 3 ppm of the theoretical peptide mass. We did not detect any peptides containing N320 or N629 in our proteolytic digests, but x-ray crystallography studies suggest that N320 is also glycosylated.²⁴ At sites N98, N160, N247, N521, N578 and N719, both unmodified and deglycosylated forms of the peptide were detected. The extracted ion chromatograms (EICs) of the unmodified and deglycosylated peptides containing these sites are shown in Figure 2. The intensities of the signals for the formerly glycosylated (deamidated, ¹⁸O labeled) peptides accounted for greater than 95% of the summed areas of the unmodified and formerly glycosylated peptides in the extracted ion chromatograms (EIC), indicating that almost all the detected sites are highly glycosylated, with the exception of N578 and N719, for which the signals for the modified peptides correspond to 23.5% and 79.1%. Detection of peptides TVSTLVIQAAN⁵²¹/D⁵²¹(¹⁸O)VSALYK and VIQAAN⁵²¹/D⁵²¹(¹⁸O)VSALY in tryptic and chymotryptic analyses, respectively, enabled comparison of relative site occupancy at N521. The intensity of the signal corresponding to TVSTLVIQAAD⁵²¹(¹⁸O)VSALYK accounted for 99.3% of the summed area of peptides TVSTLVIQAAN⁵²¹/D⁵²¹(¹⁸O)VSALYK in the tryptic peptide analysis, and VIQAAD⁵²¹(¹⁸O)VSALY accounted for 99.4% of the summed area of chymotryptic peptides VIQAAN⁵²¹/D⁵²¹(¹⁸O)VSALY in the EIC. These values are largely in agreement, despite differences in the lengths of the peptide backbone.

VEGFR-2 *N*-Glycosylation on Domains 2 and 3

We were particularly interested to determine if *N*-glycosylation sequons on Domain 2 (N145, N160) and Domain 3 (N247, N320) are glycosylated, because VEGFR-2 ligand binding occurs at the interface of Ig-like domains 2 and 3.²⁴ As shown in Table 1, N145 and N160 in Domain 2 and N247 in Domain 3 are high-occupancy *N*-glycosylation sites. An EIC for the *m/z* value corresponding to the deamidated tryptic peptide with one missed cleavage SPFIASVSDQHGVITYTENKD¹⁴⁵(¹⁸O)K (817.4203, 3+) was observed, but no EIC for the corresponding value appropriate for the Asn-containing peptide SPFIASVSDQHGVITYTENKN¹⁴⁵K (816.4237, 3+), was detected in LC-MS/MS analyses, indicating that the glycosylated peptide exists in significant excess over the unmodified form. MS2 spectra of SPFIASVSDQHGVITYTENKD¹⁴⁵(¹⁸O)K confirm the peptide sequence and location of ¹⁸O incorporation. The intensity of the signal corresponding to GSIENLD¹⁶⁰(¹⁸O)VSCAR accounted for 99.7% of the summed areas of peptides GSIENLN¹⁶⁰/D¹⁶⁰(¹⁸O)VSCAR, indicating that N160 is also a high-occupancy *N*-glycosylation site. Detection of peptides LVLN²⁴⁷/D²⁴⁷(¹⁸O)CTAR and VLN²⁴⁷/D²⁴⁷(¹⁸O)CTARTEL in tryptic and chymotryptic analyses, respectively, enabled comparison of relative quantification of N247 site occupancy using two independent peptides. The intensity of the signal for LVLN²⁴⁷(¹⁸O)CTAR accounted for 95.9% of the summed areas of peptides LVLN²⁴⁷/D²⁴⁷(¹⁸O)CTAR in the tryptic peptide analysis and VLN²⁴⁷(¹⁸O)CTARTEL accounted for 96.5% of the summed areas of peptides VLN²⁴⁷/D²⁴⁷(¹⁸O)CTARTEL in the chymotryptic peptide analysis.

Product ion spectra of peptides LVLN²⁴⁷CTAR (not glycosylated) and LVLN²⁴⁷(¹⁸O)CTAR (formerly glycosylated) are shown in Figure 3(A, B). Note that the *m/z* values of the precursor ions LVLN²⁴⁷CTAR (*m/z* 473.7601, 2+) and LVLN²⁴⁷(¹⁸O)CTAR (*m/z* 475.2549,

2⁺) differ by 2.988 Da, and that the y₁–y₄ ions are identical in the spectra of both peptides, while the y₅–y₇ ions containing the *N*-glycosylation sequon differ by 2.988 Da between the two peptides, confirming that the 2.988 Da shift is localized to the glycosylation site at residue N²⁴⁷/D²⁴⁷(¹⁸O). Figure 2C shows the relative intensities of LVLN²⁴⁷CTAR (*m/z* 473.7601, 2⁺) and LVLD²⁴⁷(¹⁸O)CTAR (*m/z* 475.2549, 2⁺). The intensity of the signal corresponding to the formerly glycosylated peptide LVLD²⁴⁷(¹⁸O)CTAR accounts for 95.9% of the summed intensities of the unmodified and formerly glycosylated peptides. The detection of chymotryptic peptides containing site N/D²⁴⁷(¹⁸O) shows a similar pattern (%D = 96.5%). However, as noted above, we did not observe any peptides containing site N320, also on Domain 3. Our inability to observe site N320 may be due to experimental limitations. Multiple lysine and arginine residues surround N320. Therefore, tryptic peptides containing N320 would be short and thus may be lost during C18 desalting steps. However, we also failed to detect N320 peptides in preparations treated with chymotrypsin and Glu-C, despite the fact that the predicted peptides are longer and therefore more amenable to LC-MS/MS analysis.

VEGFR-2 *N*-Glycosylation on Domains 4 and 7

VEGFR-2 Ig-like Domains 4 and 7 are involved in homodimer stabilization after ligand binding, receptor dimerization, and allosteric regulation of the receptor.²⁵ Therefore, it was also important to characterize *N*-glycosylation site occupancy in Ig-like domains 4 and 7. After treatment with PNGase F in H₂¹⁸O, only deamidated and ¹⁸O-labeled peptides containing sites N376 and N397 were detected. The chymotryptic peptide YRNGRPIESD³⁷⁶(¹⁸O)Y (*m/z* 457.8894, 3⁺) and the Glu-C peptide SD³⁷⁶(¹⁸O)YTMIVGDE (*m/z* 574.2401, 2⁺) were detected in EICs and confirmed based on the presence of *b*- and *y*-ion series in MS2 spectra, but the equivalent unmodified peptides were not detected, providing strong evidence that this *N*-linked site is fully glycosylated. Similarly, for site 397, only the deamidated and isotopically labeled tryptic peptide DAGD³⁹⁷(¹⁸O)YTVILTNPISME (*m/z* 934.9639, 2⁺) and the Glu-C peptide AGD³⁹⁷(¹⁸O)YTVILTNPISME (*m/z* 821.3996, 2⁺) were detected. All peptides were assigned based on accurate precursor mass (within 2 ppm error) and the detection of sequence-specific peptide *b*- and *y*-ions in the spectra of the HCD-fragmented peptides (within 10 ppm error). Formerly glycosylated peptides containing labeled aspartic acid D(¹⁸O) at sites 673, 702 and 719 were also detected (see Table 1). Formerly glycosylated peptides D⁶⁷³(¹⁸O)QTTTIGETIE and RMAPMITGNLED⁶⁷³(¹⁸O)QTTTIGE were detected in the Glu-C VEGFR-2 peptide digest mixtures. Formerly glycosylated peptides confirming the presence of glycosylation at site 702 were detected after all four protease digestions (trypsin, chymotrypsin, Glu-C and trypsin/Glu-C), including tryptic peptide DD⁷⁰²(¹⁸O)ETLVEDSGIVLR (*m/z* 781.8919, 2⁺). The equivalent unmodified peptides were not detected. The tryptic peptides DGNRN⁷¹⁹LTIR (*m/z* 529.7888, 2⁺) and DGNRD⁷¹⁹(¹⁸O)LTIR (*m/z* 531.2835, 2⁺) with one missed trypsin cleavage were detected, and the formerly glycosylated peptide accounted for 79.1% of the summed peptide intensities (Table 1, and Figure 3). Therefore, there is evidence for full glycan site occupancy at sites N376 and N397 in Domain 4 and Domain 7 sites N673 and N702 and substantial but not total *N*-glycosylation at site N719 on Domain 7.

Site-Specific *N*-Glycosylation of VEGFR-2

To provide orthogonal, site-specific evidence of *N*-glycosylation at the VEGFR-2 *N*-glycosylation sites, we digested VEGFR-2 with trypsin, chymotrypsin, Glu-C or trypsin-GluC (as above), enriched the glycopeptides using hydrophilic interaction chromatography (HILIC), separated the glycopeptides using reversed phase chromatography, and fragmented the glycopeptides using collision-induced dissociation (CID). Table 2 contains the glycopeptides assigned on the basis of results from the four experiments using different proteases. Glycopeptides precursor *m/z* values of peaks with S/N ratio > 10 were within 5 ppm of theoretical masses, and assignments were based on the presence of both carbohydrate oxonium ions resulting from glycan fragmentation and sequence-specific peptide *b*- and *y*-ions. Most (but not all) of the VEGFR-2 glycopeptides from the cell line contained glycan compositions consistent with high mannose *N*-linked glycans. The two most common glycan compositions were HexNAc₂Hex₈ and HexNAc₂Hex₉, corresponding to minimally processed high mannose *N*-linked glycans (Table 2 and Figure 4). Released glycan analyses support the assignment high mannose *N*-linked glycans (Figures S4–5). We also detected several distinct VEGFR-2 glycopeptides with glycan compositions that indicated the presence of hybrid and complex glycans, *i. e.*, containing fucose, HexNAc_{>2} and, in some cases, *N*-acetylneuraminic acid (sialic acid) at sites N145, N397 and N521 (Figure 4A, C, and D). Glycopeptide fragment ions and released glycan analyses support the assignment of hybrid and complex *N*-linked glycans (Figure S6). No evidence of glycans with bisecting HexNAc was found. Most of the amino acid sequences of detected glycopeptides correspond to those assigned to the formerly-glycosylated peptides observed in the PNGase F/H₂¹⁸O experiments performed previously, corroborating these observations.

The CID spectrum of glycopeptide GSISNLN¹⁶⁰VSLCAR+HexNAc₂Hex₉ (Figure 5A) corresponds to VEGFR-2 Domain 2 site N160, and, as is typical of glycopeptide MS2 spectra, it contains oxonium ions at *m/z* 204.086 (HexNAc) and *m/z* 366.139 (Hex-HexNAc) resulting from fragmentation of the glycan moiety. The x-axis in the region from *m/z* 400 to 1800 has been expanded to better display the peptide fragment (*b*-, *y*-) ions. The ions corresponding to the intact peptide (*m/z* 1390.712) and peptide+HexNAc fragment (*m/z* 1593.786) are also prominent. Exclusively high mannose glycopeptides were assigned at sites 46, 98, 160, 376, 509, 578, and 702 (Figure 4B and 4E–H, Table 2), indicating low *N*-glycan processing at these sites. The HCD spectrum of the corresponding formerly-glycosylated peptide GSISNLD¹⁶⁰(¹⁸O)VSLCAR (Figure 5B) contains peptide *b*- and *y*-ion fragments that corroborate the glycopeptide assignment and confirm residue 160 as the site of glycosylation based on the localization of the 2.988 Da shift (from conversion of N to D¹⁸O). The CID spectrum of glycopeptide DN⁷⁰²ETLVEDSGIVLR+HexNAc₂Hex₈ (Figure 5C) and the corresponding HCD spectrum from the formerly-glycosylated peptide DD⁷⁰²(¹⁸O)ETLVEDSGIVLR (Figure 5D) on VEGFR-2 Domain 7 offer definitive evidence of *N*-glycosylation at site N702. The glycan composition is also consistent with a high mannose *N*-linked glycan.

Discussion

VEGFR-2 activation plays a fundamental role in health and disease.^{2, 26} Targeting VEGFR-2 signaling is the mainstay of many anti-angiogenic therapeutics used to treat cancer, retinopathy and advanced macular degeneration. Ramucirumab, a monoclonal antibody, targets the extracellular domain of VEGFR-2, while other therapeutic agents such as bevacizumab (Avastin) and Sorafenib target the VEGF-A ligand or the intracellular kinase domain of the receptor, respectively.²⁷ Despite evidence of promising clinical responses to anti-angiogenesis drugs, their successes are restricted by insufficient efficacy and development of refractory tumors and resistance.²⁸ Understanding the *N*-glycosylation of VEGFR-2 may offer insight into the role of *N*-linked glycans in VEGFR-2 function and possible links between insufficient efficacy and refractoriness of anti-angiogenesis therapeutics. A variety of techniques have been used to study VEGFR-2 function and structure, ranging from molecular techniques to x-ray crystallography.^{24, 25, 29} However, while the extracellular domain of VEGFR-2 is highly *N*-glycosylated, site-specific characterization of VEGFR-2 *N*-glycosylation has not previously been performed. Here, we have used tandem mass spectrometry of glycopeptides and enzymatically deglycosylated, deamidated peptides to site-specifically characterize VEGFR-2 *N*-glycosylation. Using LC-MS/MS, we have demonstrated that at least 15 of the 17 *N*-glycosylation motifs in the VEGFR-2 extracellular domain are glycosylated (Figure 6A, B). Glycopeptide analyses indicate the presence of high-mannose, hybrid and complex glycans, and most complex glycans are not sialylated. This appears to be consistent with findings that porcine aortic endothelial cells have low expression of sialyltransferases.³⁰ According to a recent analysis, removal of *N*-linked glycans from proteins with *N*-linked glycans increases structural dynamics while *N*-glycosylated proteins are less dynamic in comparison, suggesting that glycosylation stabilizes protein structure and reduces protein dynamics.³¹ Therefore, based on the high number of *N*-linked glycans present on the VEGFR-2 extracellular domain, *N*-linked glycans likely could play a significant role in stabilizing the VEGFR-2 extracellular domain and may also influence ligand and drug binding to VEGFR2.

The impact of VEGFR-2 *N*-glycosylation should be considered comprehensively with respect to the VEGFR-2 extracellular domain as a whole and also in relation to the impact of *N*-glycosylation locally on each of the seven immunoglobulin-like subdomains of the extracellular domain. The potential of *N*-linked glycans to modulate and/or interfere with protein interactions via steric hindrance must be considered in the context of (1) ligand binding, which occurs at the interface of Ig-like domains 2 and 3 of the receptor, and (2) receptor dimerization, in domains 4 to 7 where homotypic interactions occur that stabilize receptor dimers. The crystal structures of Ig-like domains 2, 3 and 7 have been determined, enabling the merging of protein structure and glycosylation information.

VEGFR-2 *N*-Glycosylation on Domains 2 and 3

VEGF ligand binding occurs at the interface of Ig-like domains 2 and 3 of the VEGFR-2 homodimer.²⁴ Our observations indicate that *N*-glycosylation sequons on Ig-like domains 2 (sites N145, N160) and 3 (sites N247) are glycosylated. The crystal structure of the ligand-bound homodimer containing VEGFR-2 domains 2 and 3 (PDB structure 2X1W)

demonstrates that N145 is positioned at a β -turn, while no structure was assigned in the region of N160, and N247 and N320 both occur in β -strands (Supporting Figures S7 and S8).²⁴ Previous studies suggest that residues within β -turns are highly accessible to glycosidase and glycosyltransferase enzymes during the passage of the protein through the secretory pathway, potentially leading to higher glycan processing compared to more shielded glycosylation sites.³² Consistent with this conclusion, we observed higher *N*-glycan heterogeneity at site N145 compared to other sites in the extracellular domain, including the presence of more highly processed (fucosylated and sialylated) glycans (Figure 4A). While crystallography is not an ideal method for studying *N*-glycosylated proteins, the same study also shows evidence of glycosylation at N247, which is consistent with our results. We were unable to detect any peptides spanning residue N320, and therefore lack evidence to conclude whether this site is glycosylated. Our inability to observe peptides spanning residue N320 is likely due to the presence of multiple lysine and arginine residues (trypsin cleavage sites) on both sides of the N320 sequon leading to the generation of very short tryptic peptides ill-suited to our analytical workflow. However, there is evidence that this site is glycosylated based on crystallography studies.²⁴ The impact of these glycosylation sites on ligand binding has not been explored. However, both fucosylation and sialylation of EGFR *N*-linked glycans has been shown to impact ligand binding and signaling.³³ Therefore, based on the presence of four confirmed glycosylation sites on Ig-like domains 2 and 3 of VEGFR-2, the impact of glycosylation on ligand binding requires further study.

VEGFR-2 N-Glycosylation on Domains 4 and 7. VEGFR-2 Ig-like Domains 4 – 7 are involved in homodimer stabilization after ligand binding, receptor dimerization, and allosteric regulation of the receptor.^{2, 34} The current study provides strong evidence of *N*-glycosylation on Ig-like domains 4 – 7. Indeed, we have evidence of *N*-glycosylation at every sequon on domains 4 – 7 with the exception of site N629. We lack evidence for or against *N*-glycosylation at the site. The crystal structure of the dimeric Ig-like Domain 7 (expressed in *E. coli*) is available.³⁵ N673 occurs in a region with no assigned secondary structure, while N702 occurs in a bend and N719 occurs in a β -strand. Based on the dimeric Ig Domain 7 crystal structure, site N720 appears to be oriented inward toward the dimerization interface, and therefore glycosylation at this site may impact dimerization based on steric considerations and deserves further study (Supporting Figures S9 and S10).

Conclusion

Based on our analyses, it is clear that all seven Ig-like domains of VEGFR-2 are *N*-glycosylated, and that glycosylation site heterogeneity exists both within and between sites of glycosylation. The majority of the 15 *N*-glycosylation sites that we have identified appear to be high-occupancy sites. VEGFR-2 dimerization depends on both ligand binding and the precise orientation of two receptor monomers; homotypic interactions between receptor monomers contribute to allosteric regulation of receptor activation³⁴. In addition, recent studies demonstrate that glycosylation impacts RTK dimerization as was demonstrated for EGFR.³³ Given that *N*-glycosylation is thought to reduce protein dynamics and impact protein interactions based on steric considerations, the impact of *N*-glycosylation on receptor dimerization and activation and necessitates further investigation, as differences in occupancy and heterogeneity at each site may influence RTK dimerization and receptor

signaling potential. Furthermore, characterization of VEGFR-2 *N*-glycosylation could provide new insight into therapeutic targeting of VEGFR-2, which aims to interrupt ligand-receptor interaction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ERAD	endoplasmic reticulum-associated degradation
PAE	Porcine aortic endothelial cells
RTK	Receptor tyrosine kinase
VEGF	Vascular endothelial growth factor
VEGFR-2	Vascular endothelial growth factor receptor-2

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Figure 1. Murine VEGFR-2 (FLK-1) is Highly Glycosylated

A. Lysate from porcine aortic endothelial (PAE) cells with ectopic expression of murine VEGFR-2 (FLK-1) without treatment (–), treated with heat denatured PNGase F (Denat.), or treated with 500 units of PNGase F (+). **B.** Schematic of experimental workflow for the analysis of VEGFR-2 N-glycosylation. **C.** Schematic of murine VEGFR-2, with the seven immunoglobulin (Ig)-like domains in the ectodomain, the transmembrane (TM) domain, and the cytoplasmic domain (not to scale). N-glycosylation sequons are marked with circles and labeled according to their location in the sequence.

Figure 2. Unmodified and Deglycosylated Peptides at High Occupancy VEGFR-2 N-Glycosylation Sites

The Extracted Ion Chromatograms (EICs) of the unmodified (asparagine, 'N' form) and ^{18}O -labeled, deglycosylated (aspartic acid, 'D(^{18}O)' form) are shown. The unmodified 'N' form is shown in the upper panel and the formerly-glycosylated 'D' form is shown in the lower panel for six selected N-glycosylation sites of interest: **A.** Site N/D98, **B.** Site N/D160, **C.** Site N/D247, **D.** Site N/D521, **E.** Site N/D578, and **F.** Site N/D719 are shown. All peptide assignments were confirmed based on charge state and peptide fragment ions.

Figure 3. High Glycosylation Site Occupancy at Site N247 on Domain 3 Demonstrated by Unmodified Peptide LVLN²⁴⁷CTAR and Formerly-Glycosylated Peptide LVLD²⁴⁷(¹⁸O)CTAR
A. MS2 Spectrum of the unmodified peptide LVLN²⁴⁷CTAR with peptide *b*- and *y*-ions labeled. **B.** MS2 Spectrum of formerly-glycosylated peptide LVLD²⁴⁷(¹⁸O)CTAR with peptide *b*- and *y*-ions labeled. **C.** Extracted ion chromatograms (EICs) of the unmodified (left) and formerly-glycosylated (right) peptides containing the unmodified and formerly-glycosylated *N*-glycosylation site, respectively, at residue 247 on Domain 3 of VEGFR-2.

Figure 4. Relative Areas of VEGFR-2 N-Glycopeptides

The relative areas of *N*-glycopeptides with 2 assigned glycoforms/site are shown. **A.** Site N145, **B.** Site N702, **C.** Site N521, **D.** Site N397, **E.** Site N46, **F.** Site N98, **G.** Site N160 and **H.** Site N578, are shown. For each site, glycoforms were normalized to the glycoform with the greatest area. The y-axis indicates the relative area (%), and the x-axis lists the glycoform composition (Hex = hexose; HexNAc = N-acetylhexosamine; dHex = deoxy-hexose; NeuAc = N-acetylneuraminic acid). The peptide sequences are given at the top of each plot, with the glycosylated residue indicated.

Figure 5. Glycopeptide Spectra Provide Additional Evidence of VEGFR-2 N-Glycosylation
A. MS2 spectrum (CID) of glycopeptide GSISNLN¹⁶⁰VSLCAR+HexNAc₂Hex₉. **B.** MS2 spectrum (HCD) of the formerly-glycosylated peptide GSISNLD¹⁶⁰(¹⁸O)VSLCAR. **C.** MS2 spectrum (CID) of glycopeptide DN⁷⁰²ETLVEDSGIVLR+HexNAc₂Hex₈. **D.** MS2 spectrum (HCD) of the formerly-glycosylated peptide DD⁷⁰²(¹⁸O)ETLVEDSGIVLR. For all spectra, peptide b- and y- fragment ions are labeled. The location of the N-glycosylation sequon is denoted with an asterisk (*) in the glycopeptide. Squares represent N-acetylhexosamine (HexNAc) and circles represent Hexose (Hex). The site of the ¹⁸O label is denoted by (°) on formerly glycosylated peptides.

Figure 6. Mapping VEGFR-2 *N*-Glycosylation Sites to the Immunoglobulin (Ig)-like Domains in the Extracellular Domain

A. Tabulation of assigned glycopeptides and formerly-glycosylated (D¹⁸O) peptides. Grey boxes indicated peptides/glycopeptides assigned in trypsin (T), Glu-C (G), Glu-C and trypsin (G-T), and chymotrypsin (C) digests. **B.** A schematic of the dimerized VEGFR-2 extracellular domain (light grey), with dimerized ligand (dark grey) bound in the middle. Domains 1–7 (D1–D7) are labeled, with *N*-glycosylation sequons labeled with circles. Black circles indicate sites that have been observed to be glycosylated in this study. Empty circles indicate sites that we were not able to observe. The sites are labeled on the right with the site of each predicted *N*-glycosylation sequon.

Table 1
Site-Specific Evidence for VEGFR-2 N-Glycosylation Site Occupancy Based on MS and MS/MS Analyses of Peptides Obtained After Protease Digestions Followed by PNGase F Release of N-linked Glycans

The table lists unmodified and formerly-glycosylated ($N>D^{18}O$) VEGFR-2 peptides detected in trypsin (T), chymotrypsin (C), Glu-C and trypsin (G-T), and Glu-C (C) digests of immunoprecipitated murine VEGFR-2. For all detected sites, the domain (D1–7), site, peptide sequence, and enzyme used for proteolysis (Enz) are listed. For unmodified (asparagine, N) peptides containing N-glycosylation sequons and labeled deglycosylated peptides (aspartic acid, $D^{18}O$), the mass to charge ratio (m/z), charge (z), error (Delta ppm), area (Area), and retention time (Ret. Time) are given. For each peptide, the area of the labeled deglycosylated peptide divided by the sum of the unmodified and labeled deglycosylated peptide areas, expressed as a percent (%D), is also shown. The area of each peak was calculated according to the formula described in the Methods section. Abbreviations: n.a (not available), n.d. (not detected). All cysteines are carbamidomethylated; M(ox) indicates methionine oxidation.

				Unmodified N Peptide				Labeled D(¹⁸ O) Peptide (D*)						
Site	Peptide	Enz	[M+nH] ^{u+} m/z	z (+)	(ppm)	Area	Ret. Time (min)	[M+nH] ^{u+} m/z	z (+)	(ppm)	Area	Ret. Time (min)	%D	
D1	N46	LSTQKDILTILANTTLQTCR	T	-	-	n.a.	n.d.	n.a.	802.7766	3	-0.27	9.30e ⁶	43.6–43.8	N<D*
	N98	VVGNDTGAYK	T	512.2592	2	0.39	2.52e ⁶	15.9–16.1	513.7530	2	-0.08	4.92e ⁷	16.6–16.9	95.1%
		TIPRVVGNDTGAY	C	-	-	n.a.	n.d.	n.a.	683.3490	2	0.88	1.65e ⁸	24.9–25.1	N<D*
D2	N145	SPFIASVSDQHGIVYTENKKNK	T	-	-	n.a.	n.d.	n.a.	817.4203	3	0.59	4.90e ⁸	28.7–28.9	N<D*
	N160	GSISNLNVSLCAR	G–T	695.8591	2	0.14	2.02e ⁶	27.5–27.7	697.3538	2	0.95	7.90e ⁸	28.1–28.4	99.7%
D3	N247	LVLNCTAR	T	473.7601	2	-1.03	1.57e ⁷	20.6–20.8	475.2549	2	0.28	3.71e ⁸	21.5 – 21.8	95.9%
		VLNCTARTEL	C	588.8059	2	0.20	1.46e ⁷	22.4–22.6	590.3001	2	0.42	3.97e ⁸	23.2–23.5	96.5%
D4	N376	YRNGRPESNY	C	-	-	n.a.	n.d.	n.a.	457.8894	3	-0.16	2.39e ⁶	18.3–18.5	N<D*
		SNYTMIVGDE	G	-	-	n.a.	n.d.	n.a.	574.2401	2	1.60	5.01e ⁶	25.0–25.3	N<D*
	N397	DAGNYTVILTNPISMEK	T	-	-	n.a.	n.d.	n.a.	934.9639	2	0.48	7.90e ⁶	34.7 – 35.0	N<D*
D5	N509	AGNYTVILTNPISME	G	-	-	n.a.	n.d.	n.a.	821.3996	2	-0.46	1.56e ⁷	33.8–34.1	N<D*
		NQYALIEGKNK	T	-	-	n.a.	n.d.	n.a.	640.8406	2	0.68	9.01e ⁶	20.8 – 21.1	N<D*
		ALIEGKNKTVSTL	C	-	-	n.a.	n.d.	n.a.	688.8971	2	-0.92	7.52e ⁸	23.3–23.5	N<D*
D6	N521	TVSTLVIQAANVSALYK	T	889.5056	2	-1.12	6.57e ⁶	34.9–35.0	891.0015	2	0.84	9.72e ⁸	35.6–35.9	99.3%
		VIQAANVSALY	C	574.8201	2	1.57	5.05e ⁶	30.3–30.5	576.3134	2	0.22	8.33e ⁸	31.1–31.4	99.4%
		VIQAANVSAL	C	-	-	n.a.	n.d.	n.a.	494.7817	2	0.22	2.11e ⁷	28.8–29.1	N<D*
	N578	NTFENLTWYK	T	658.3200	2	0.65	3.45e ⁸	30.7–30.9	659.8132	2	-0.63	1.06e ⁸	31.8–31.9	23.5%

				Unmodified N Peptide						Labeled D(¹⁸ O) Peptide (D*)						
Site	Peptide	Enz		[M+nH] ^{nt} m/z	z (+)	(ppm)	Area	Ret. Time (min)	[M+nH] ^{nt} m/z	z (+)	(ppm)	Area	Ret. Time (min)	%D		
N611	KLNGTMF	C		-	-	n.a.	n.d.	n.a.	407.2069	2	0.57	6.12e ⁷	27.1–27.4	N<D*		
	KLNGTM(ox)F	C		-	-	n.a.	n.d.	n.a.	415.2037	2	-1.19	2.32e ⁷	22.2–22.5	N<D*		
N611 + 617	LNGTM(ox)FSNSTND	G–T		-	-	n.a.	n.d.	n.a.	661.7631	2	-0.04	2.31e ⁷	22.0–22.3	N<D*		
N673	RMAPMITGNLENQTTIGE	G		-	-	n.a.	n.d.	n.a.	694.0033	3	0.82	9.16e ⁷	29.9–30.1	N<D*		
	NQTTTIGETIE	G		-	-	n.a.	n.d.	n.a.	605.2904	2	0.32	4.88e ⁷	24.8–25.1	N<D*		
N702	DNETLVEDSGIVLR	T		-	-	n.a.	n.d.	n.a.	781.8919	2	-1.88	6.21e ⁶	31.7–32.1	N<D*		
	KDNETLVEDSGIVL	C		-	-	n.a.	n.d.	n.a.	767.8911	2	1.05	1.64e ⁷	33.2–33.5	N<D*		
	FKDNETLVEDSGIVL	C		-	-	n.a.	n.d.	n.a.	841.4255	2	1.22	9.34e ⁶	34.7–35.0	N<D*		
N719	VTCPASGNPTPHITWFKDNE	G		-	-	n.a.	n.d.	n.a.	758.6848	3	0.64	8.87e ⁷	29.4–29.8	N<D*		
	DGNRNLTR	T		529.7888	2	1.22	4.87e ⁶	17.5–17.7	531.2835	2	1.22	1.84e ⁷	18.2–18.5	79.1%		

Table 2

Site-Specific N-Glycosylation of Murine VEGFR-2

The table shows a list of assigned CID-fragmented glycopeptides, assigned based on accurate mass (within 10 ppm), peptide fragment ions, and oxonium ions. The domain (D), N-glycosylation site (Site), peptide sequence, enzyme used for proteolysis (Enz), mass-to-charge ratio (m/z), charge (z), retention time (RT), error in daltons (Delta (Da)), error in parts per million (Delta(ppm)), and the carbohydrate composition associated with the peptide. N-acetylhexosamine: HexNAc; Hexose: Hex, deoxyhexose: dHex; N-acetylneuraminic acid (NeuAc). All cysteines are carbamidomethylated; M(ox) indicates methionine oxidation.

Domain	Site	Peptide	Enz	m/z	z	RT (min)	(ppm)	Area	Composition		
D1	N46	K.DILTILANTTLQITCR.G	T	1183.534	3	32.51	-2.0	8.62e ⁴	HexNAc2Hex8		
				887.904	4	32.54	0.0	8.37e ⁴			
		K.DILTILANTTLQITCR.G	T	1237.551	3	32.50	-2.0	7.93e ⁴	HexNAc2Hex9		
				928.419	4	32.55	1.3	5.91e ⁴			
	N98	K.LSTQKDILTILANTTLQITCR.G	T	1027.234	4	30.68	0.5	1.82e ⁵	HexNAc2Hex8		
		K.LSTQKDILTILANTTLQITCR.G	T	1067.743	4	30.05	-3.5	8.01e ⁵	HexNAc2Hex9		
		L.TIPRVVGNDTGAY.K	C	1022.431	3	23.21	-1.7	3.32e ³	HexNAc2Hex8		
		L.TIPRVVGNDTGAY.K	C	1076.449	3	22.63	-2.0	2.37e ⁴	HexNAc2Hex9		
		R.SPFIASVSDQHGIVYITENKNK.T	T	973.702	4	25.04	-1.3	7.26e ⁴	HexNAc4Hex3dHex1		
		R.SPFIASVSDQHGIVYITENKNK.T	T	1038.226	4	25.81	2.8	2.84e ⁴	HexNAc2Hex8		
D2	N145	R.SPFIASVSDQHGIVYITENKNK.T	T	1078.733	4	25.78	1.0	3.89e ⁴	HexNAc2Hex9		
		R.SPFIASVSDQHGIVYITENKNK.T	T	1095.246	4	25.11	0.8	6.29e ⁴	HexNAc4Hex6dHex1		
		R.SPFIASVSDQHGIVYITENKNK.T	T	1119.240	4	25.16	-0.5	9.51e ⁴	HexNAc2Hex10		
		R.SPFIASVSDQHGIVYITENKNK.T	T	1135.759	4	25.74	0.8	8.29e ⁴	HexNAc4Hex7dHex1		
		R.SPFIASVSDQHGIVYITENKNK.T	T	1172.017	4	26.11	0.3	1.42e ⁵	HexNAc4Hex7NeuAc1		
		R.GSISNLNVSLCAR.Y	T	1031.768	3	24.56	-1.3	9.96e ⁴	HexNAc2Hex8		
	N160	R.GSISNLNVSLCAR.Y	T	1085.785	3	24.50	-2.3	1.24e ⁵	HexNAc2Hex9		
		R.NGRPIESNYTM(ox)IVGDELTIMEVTER.D	T	1191.758	4	26.76	-9.3	1.54e ⁴	HexNAc2Hex9		
		E.ATVGSQVRIPVKYLSYPAPDIKWYRNGRPIESNYTM(ox)IVGDEL	G	1092.509	6	27.64	-4.0	8.51e ⁴	HexNAc2Hex9		
		E.RDAGNYTVILTNPISM(ox)E.K	G-T	944.395	4	26.01	-3.0	2.07e ⁴	HexNAc2Hex9		
N397							1258.862	3		25.93	0.7

Domain	Site	Peptide	Enz	m/z	z	RT (min)	(ppm)	Area	Composition
D5	N521	R.DAGNYTVILTNPIISMEK.Q	T	990.430	4	27.74	2.3	5.07e ⁴	HexNAc4Hex7dHex1
		R.DAGNYTVILTNPIISM(ox)JEK.Q	T	994.425	4	27.64	0.3	3.66e ⁴	HexNAc4Hex7dHex1
		R.DAGNYTVILTNPIISMEK.Q	T	1026.689	4	28.18	1.3	3.67e ⁴	HexNAc4Hex7NeuAc1
		R.DAGNYTVILTNPIISM(ox)JEK.Q	T	1030.685	4	27.08	-3.2	4.31e ⁴	HexNAc4Hex7NeuAc1
		R.DAGNYTVILTNPIISM(ox)JEK.Q	T	1195.511	3	26.12	-1.0	1.62e ⁴	HexNAc2Hex8
		R.DAGNYTVILTNPIISMEK.Q	T	1244.192	3	27.29	-1.7	7.73e ⁴	HexNAc2Hex9
		R.DAGNYTVILTNPIISM(ox)JEK.Q	T	1249.526	3	26.11	-0.3	4.80e ⁴	HexNAc2Hex9
		Y.ALIEGKNTVSTL.V	C	1080.148	3	22.14	-2.0	3.02e ⁴	HexNAc2Hex9
		L.VIQAAANVSALYK	C	1005.093	3	25.86	-1.0	1.18e ⁴	HexNAc2Hex9
		K.TVSTLVIQAAANVSALYK.C	T	998.814	3	28.05	-0.7	4.01e ⁵	HexNAc2Hex5
		K.TVSTLVIQAAANVSALYK.C	T	1160.867	3	28.02	-1.0	3.07e ⁵	HexNAc2Hex8
		K.TVSTLVIQAAANVSALYK.C	T	870.903	4	28.06	1.0	3.24e ⁵	HexNAc2Hex8
		K.TVSTLVIQAAANVSALYK.C	T	1214.886	3	27.89	0.0	6.85e ⁵	HexNAc2Hex9
		K.TVSTLVIQAAANVSALYK.C	T	911.418	4	27.98	2.0	6.65e ⁵	HexNAc2Hex9
		K.TVSTLVIQAAANVSALYK.C	T	927.932	4	27.64	-1.5	1.47e ⁵	HexNAc4Hex6dHex1
D6	N578	K.TVSTLVIQAAANVSALYK.C	T	964.186	4	29.27	-1	6.89e ⁴	HexNAc4Hex6NeuAc1
		K.TVSTLVIQAAANVSALYK.C	T	968.446	4	28.47	0.5	2.80e ⁵	HexNAc4Hex7dHex1
		K.TVSTLVIQAAANVSALYK.C	T	1000.708	4	29.71	1.0	1.12e ⁵	HexNAc4Hex6dHex1NeuAc1
		K.TVSTLVIQAAANVSALYK.C	T	1004.704	4	28.98	0.3	3.29e ⁵	HexNAc4Hex7NeuAc1
		R.NTFENLTWYK.L	T	1006.743	3	25.61	0.0	7.72e ⁴	HexNAc2Hex8
D7	N578	R.NTFENLTWYK.L	T	1060.759	3	25.48	-2.0	1.77e ⁵	HexNAc2Hex9
		K.DNETLVEDSGIVLR.D	T	980.095	3	26.26	0.3	2.41e ⁴	HexNAc2Hex6
	N702	K.DNETLVEDSGIVLR.D	T	1034.115	3	25.74	3.3	2.87e ⁴	HexNAc2Hex7
		K.DNETLVEDSGIVLR.D	T	1088.125	3	25.64	-1.3	4.61e ⁴	HexNAc2Hex8
		K.DNETLVEDSGIVLR.D	T	1142.146	3	25.62	0.3	6.73e ⁴	HexNAc2Hex9
		E.VTCPASGNPTPHITWFKDNE.T	G	994.162	4	24.76	-2.5	4.81e ⁴	HexNAc2Hex8
		E.VTCPASGNPTPHITWFKDNE.T	G	1034.679	4	24.71	1.8	4.60e ⁴	HexNAc2Hex9
		R.DGNRNLTR.R	G-T	975.068	3	2.69	-6.0	2.26e ⁴	HexNAc2Hex9

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Domain	Site	Peptide	Enz	<i>m/z</i>	<i>z</i>	RT (min)	(ppm)	Area	Composition
		L.VEDSGIVLRDGNRNLTIIRRYRKEDGGL.Y	C	948.850	5	26.90	-5.2	5.70e ³	HexNAc2Hex8