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Fourier transform mass spectrometry to monitor hyaluronan–protein interactions: use of hydrogen/deuterium amide exchange

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Abstract

The use of Fourier transform mass spectrometry (FTMS) to monitor noncovalent complex formation in the gas phase under native conditions between the Link module from human tumor necrosis factor stimulated gene-6 (Link_TSG6) and hyaluronan (HA) oligosaccharides is reported. In particular, a titration experiment with increasing concentrations of octasaccharide (HA₈) to protein produced a noncovalent complex with 1:1 stoichiometry when the oligosaccharide was in molar excess. However, in the presence of a molar excess of tetrasaccharide (HA₄) nearly all proteins and oligosaccharides were observed in their unbound charge states. These results are consistent with solution-phase properties for this interaction in which HA₈, but not HA₄, supports high affinity Link_TSG6 binding. Hydrogen/deuterium amide exchange mass spectrometry (H/D-EX MS) was also utilized to investigate the level of global deuterium incorporation, over time, for Link_TSG6 in both the absence and presence of HA₈. After dilution into quenching conditions, deuterium incorporation reached limiting asymptotic values of 37 and 26 deuterons for the free and bound protein at 240 and 480 min, respectively, indicating that the oligosaccharide interferes with amide exchange on binding. To detect sequence-specific deuterium incorporation, pepsin digestion of Link_TSG6 in both the absence and presence of HA₈ was performed. A level of deuterium incorporation of 10–30% was observed for peptides analyzed in free Link_TSG6. Interestingly, HA₈ blocked some sites of proteolysis in Link_TSG6 compared to the free protein. Molecular modeling indicated that amino acids proximal to the ligand correlated with regions of the protein that were resistant to enzymatic digestion. Of the peptides that could be analyzed by H/D-EX MS in the presence of the ligand, a 30–60% reduction in deuterium incorporation, relative to the free protein, was observed, even for those sequences not directly involved in HA binding. These results support the utility of FTMS as a method for the characterization of protein–carbohydrate interactions.

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SUPPLEMENTARY MATERIAL

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Protein–carbohydrate interactions are of profound importance in diverse biological phenomena such as extracellular matrix assembly, cell adhesion, tumor metastasis and the immune response.^{1,2} When possible, high-resolution methods such as nuclear magnetic resonance (NMR) spectroscopy and X-ray crystallography are the preferred ways to study the three-dimensional structure of complexes between proteins and carbohydrates. However, complexes involving oligosaccharides are frequently not suitable for analysis by these techniques because they are difficult to purify in sufficient quantities and do not readily crystallize due to the hydrophilicity and dynamics of the carbohydrate component in solution.^{3,4} Thus, sensitive techniques that minimize the amounts of material analyzed are needed to investigate protein–carbohydrate structure and function. One such technique, hydrogen/deuterium amide exchange mass spectrometry (H/D-EX MS), has proven useful in the study of protein structure, dynamics and ligand interactions.^{5–9} This method capitalizes on the ability of labile amide hydrogen atoms in a protein backbone to exchange with solvent deuterium, resulting in a mass increase of 1 Da for each amide. This mass shift can be readily detected by high-resolution MS. Moreover, the high sensitivity of MS allows relatively small quantities (<1 µg) of protein and ligand to be analyzed.

Only those labile hydrogen atoms located on the amide backbone of a protein have exchange rates that can currently be reasonably measured by MS, since labile side-chain hydrogens (bound to N, O, and S atoms) exchange too rapidly.^{5,9} The exchange rate for amide hydrogen atoms in folded proteins under native conditions depends both on their participation in intramolecular hydrogen bonding and their degree of solvent accessibility.^{9,10} Protein stability and structure is typically characterized by slow exchanging amide hydrogen atoms found within hydrogen bonds in secondary structural elements, such as α -helices and β -sheets.¹¹ The mechanism of amide hydrogen exchange for proteins near physiological pH will often follow the EX2 regime, in which base-catalyzed exchange occurs from the unfolded (open), solvent-exposed protein that exists in equilibrium with the normally well-folded (closed) protein.^{5,12} Amide exchange rates are also temperature- and pH-dependent; being minimal near approximately pH 2.5 and 0°C. Under these conditions the half-life of exchange can be significantly extended from seconds to 40–50 min, which facilitates experimental measurement.^{10,13}

Both the global amide exchange rate for an entire protein and the exchange rate within particular sequences can be investigated by H/D-EX MS. These studies usually involve exposing the protein to D₂O for a given period of time (on-exchange) followed by quenching in a buffer at low pH and temperature. Quenching prevents both further deuterium incorporation and minimizes undesirable back-exchange. At this point the protein can be directly analyzed by MS and deuterium uptake measured from the increase in isotopic distribution (m/z values) for the deuterated protein, compared to the undeuterated control. To obtain site-specific exchange information, the deuterated protein is rapidly digested after quenching with an acid-stable protease, such as pepsin, and analyzed by electrospray ionization (ESI) or matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS.^{14,15} The H/D-EX MS approach can also be used to probe protein–ligand interactions. In this case, the difference in deuterium uptake between the free and bound complexes could, in principle, reveal certain solvent-accessible amides involved in

the interaction that are shielded from exchange in the presence of the ligand.¹⁶ However, amides distal to the binding site that are influenced may also be prevented from exchange in the presence of the ligand.^{7,17} Therefore, the ability to differentiate between true protection and allosteric effects, caused by stabilization of dynamics or remote protein conformational changes, on ligand binding, are essential for data interpretation.

To date, few examples of protein–carbohydrate interactions employing H/D-EX MS have been reported in the literature.^{18,19} In the present study ESI Fourier transform mass spectrometry (FTMS) was used to monitor an interaction involving human tumor necrosis factor stimulated gene-6 (TSG-6) and hyaluronan oligosaccharides. Hyaluronan (HA) is a linear glycosaminoglycan comprised entirely of repeating disaccharide units of $\beta(1 \rightarrow 3)$ -*N*-acetyl-D-glucosamine- $\beta(1 \rightarrow 4)$ -D-glucuronic acid. Unlike other glycosaminoglycans (GAGs), such as chondroitin sulfate and heparin, HA is not sulfated and is not found covalently attached to a core protein making it structurally the simplest GAG. In spite of its simplicity, HA has significant roles in embryonic development, ovulation and inflammation mainly through its interactions with HA-binding proteins, which differ in tissue expression, regulation, specificity, and affinity.^{20–22} The majority of HA-binding proteins interact with HA via a common structural domain of approximately 100 amino acids termed the Link module.²⁰ Previously, the Link module from human TSG-6 was expressed in *E. coli*²³ and this material (referred to Link_TSG6) was used to determine its tertiary structure by NMR spectroscopy in both the presence and absence of an HA octasaccharide (HA₈).²⁴ Additionally, the binding properties of Link_TSG6 have been extensively characterized, wherein the critical amino acids involved in HA binding have been located by site-directed mutagenesis and the oligosaccharide-binding groove has been mapped by NMR methods.^{24,25} Therefore, the interaction with Link_TSG6 and HA oligosaccharides provides a model protein–carbohydrate system to probe binding interactions by H/D-EX MS.

Prior to H/D-EX MS exchange experiments, noncovalent complexes between Link_TSG6 and HA oligosaccharides of defined length (HA₄ and HA₈) were detected in the gas phase by FTMS. Subsequent global and sequence-specific deuterium incorporation in Link_TSG6 was measured by H/D-EX MS in both the absence and presence of HA₈. A three-dimensional interpretation for these results was provided by mapping the MS data onto the NMR structure of Link_TSG6. Amide-specific deuterium uptake was also assessed by tandem mass spectrometry (MS/MS) using collision-induced dissociation (CID).

EXPERIMENTAL

Materials

The 98 amino acid Link module of human TSG-6 (Link_TSG-6) was expressed, refolded, and purified by high-performance liquid chromatography (HPLC) to homogeneity as described previously.^{23,25} HA oligosaccharides, derived from testicular hyaluronidase digestion, were produced from polymeric HA and purified as described.²⁶ Deuterium oxide (99.9% D atom), acetic acid-*d* and tris(2-carboxyethyl)phosphine hydrochloride (TCEP) were purchased from Sigma Aldrich (St. Louis, MO, USA). Immobilized pepsin (6% cross-linked agarose) beads were purchased from Pierce Chemicals (Rockford, IL, USA).

Identification of pepsin fragments by LC/MS/MS

Since pepsin cleavage is nonspecific, yet reproducible, it cannot be predicted in advance. Therefore, prior to H/D-EX MS experiments, Link_TSG6 peptic fragments were identified using MS/MS. Equal volumes (2.5 μ L) of Link_TSG6 (0.2 mg/mL in water) and 10 mM TCEP were mixed and incubated at room temperature for 10 min. To this, porcine pepsin (2.5 μ L; 1 mg/mL in 2.5% (v/v) acetic acid) was added and the sample incubated for 1 h at room temperature. The sample was filtered through a 10 kDa molecular weight cut-off membrane (Millipore, Bedford, MA, USA) at 6000 g for 5 min to remove pepsin and residual undigested protein. The filtered peptides were analyzed independently on an Agilent 1100 capillary liquid chromatograph (Palo Alto, CA, USA) interfaced directly to a hybrid LTQ-FT linear ion trap/7-Tesla Fourier transform ion cyclotron resonance (FTICR) mass spectrometer (Thermo Electron, San Jose, CA, USA). Mobile phases A and B were H₂O/0.1% (v/v) formic acid and acetonitrile/0.1% (v/v) formic acid, respectively. Each fraction was loaded onto the C18 column (15 cm $\times$ 150 μ m, Micro-tech Scientific, Vista, CA, USA) at a flow rate of 1 μ L/min and peptides were eluted into the mass spectrometer during a 70 min linear gradient from 5–55% (v/v) B. The instrument was set to acquire MS/MS spectra on the nine most abundant precursor ions from each MS scan with a repeat count of 3 and repeat duration of 15 s. Dynamic exclusion was enabled for 160 s. Raw MS/MS spectra were converted into mzXML format then into a peak list using RedAW followed by mzXML2Other.²⁷ The peak lists were then searched using Mascot version 1.9 (Matrix Science, Boston, MA, USA) against a database in the FASTA format containing the 98 amino acid Link_TSG6 sequence.²⁴ Searches were performed against the Link_TSG6 database using the following parameters: no enzyme specificity, peptide tolerance of 10 parts-per-million, and a fragment ion tolerance of 0.6 Da.

Complex formation and H/D amide exchange

To assess noncovalent complex formation under native conditions, a volume of 2.5 μ L of HPLC-purified Link_TSG6 (0.2 μ g/ μ L in water) was diluted to a final concentration of 1.6 μ M protein in 10 mM ammonium acetate, pH 5.8, containing either 0.0, 0.06, 0.6, 6.0 or 12.0 μ M of HA₈, or alternatively 6.0 μ M HA₄ (final concentrations). Samples were independently infused directly into the LTQ-FT at 1 μ L/min using a syringe pump (Harvard Apparatus, Holliston, MA, USA) and passed into the ICR cell for FTMS analysis. The spray voltage was set at 1.9–2.2 kV, capillary temperature 220°C, capillary voltage 50 V, and tube lens 90 V.

For H/D-EX MS experiments, lyophilized samples of Link_TSG6 aliquots (5 μ g) were resuspended into 20 μ L of 10 mM ammonium acetate (prepared in 99.9% D₂O and pH adjusted to 5.8 with acetic acid-*d*) with or without 3.5-fold excess of HA₈ (2.5 μ g added from a 1 mg/mL (w/v) stock in 99.9% D₂O) to give final protein and oligosaccharide concentrations of 23 and 81 μ M, respectively. Samples were allowed to exchange at room temperature for 30, 60, 120, 240, 480 and 960 min, respectively. Exchange was quenched by dilution (1:100) into ice-cold 2.5% (v/v) acetic acid, followed by immediate infusion into the LTQ-FT at 2 μ L/min with the syringe pump housed within a refrigerator (2–4°C). For pepsin digestion, 0.75 μ L from a fully deuterated Link_TSG6 sample with or without HA₈ was added to 1 μ L of 10 mM TCEP-HCl for 3 min on ice to both quench exchange (pH 2.0–

3.0; checked with pH paper) and reduce disulfide bonds. To this a volume of 20 μL of immobilized pepsin slurry in ice-cold 2.5% (v/v) acetic acid (1:1 v/v) was added and samples incubated for 12 min on ice. The final volume was brought up to 32 μL by addition of ice-cold 2.5% (v/v) acetic acid and immobilized pepsin removed by filtering over chilled silica barrier pipette tips (CLP, San Diego, CA, USA). The samples were directly infused as above. The average number of deuterons present on each peptide was determined by calculating the centroid of the isotopic cluster as reported previously.²⁸ For MS/MS analysis, precursor ions were selected using zoom scan profile mode in the ion trap with a 5 m/z window and fragmented using CID with a normalized energy of 35.

Molecular modeling

The NMR structure of the Link module of human TSG-6 (Link_TSG6) acquired in the presence of HA₈ ligand (pdbid: 1O7C)²⁴ was used to generate the model for the HA₈-Link_TSG6 complex. Of the 20 model structures deposited in the pdb file, model 1 was selected on the basis of favorable orientations of the side chains of some key residues, namely: Y12, Y59, and Y78, which are involved in HA₈ binding.²⁴ To generate the starting ligand structure employed in the binding studies, explicit solvent energy minimizations and molecular dynamics (MD) simulations of HA₈ were performed following protocols described previously.²⁹ A model for HA₈ was generated by setting the glycosidic (ϕ, ψ) angles for each linkage (Supplemental Figure 1, see Supplementary material) to the most representative values from the MD data (Supplemental Table 1, see Supplementary material). For ligand docking, a fragment-based docking protocol previously described³⁰ was modified by employing the rigid whole-molecule docking approach. This modification was adopted because of the large numbers of degrees of freedom present in highly flexible molecules, and the associated potential energy convergence problems.³¹ The binding groove of Link_TSG6 was embedded in an affinity grid box having dimensions of $100 \times 34 \times 110 \text{ \AA}$, with a $0.375\text{-\AA}$ grid point spacing. At the start of the docking simulation, HA₈ was placed at the center of the grid. The complex which best reproduced the contact analyses of the reported NMR-derived complex³² was selected and refined by energy minimization.

RESULTS

Complex formation between Link_TSG6 and HA₈

Unlike other members of the Link module superfamily, the interaction of TSG-6 with HA is highly pH-dependent, with maximal binding near pH 6, and a dramatic reduction in activity as the pH is increased.³³ Therefore, interactions between Link_TSG6 and HA₈, which is the minimum sized even oligosaccharide that supports high affinity binding,^{24,25} were incubated under associative pH conditions (10 mM ammonium acetate, pH 5.8) and analyzed by high-resolution FTMS. Link_TSG6 (1.6 μM) was electrosprayed in the absence of HA₈ and the 6+, 7+, 8+ and 9+ charge states for the protein were observed (Fig. 1(A)). The narrow, low charge-state distribution for Link_TSG6 under native conditions, compared to the protein sprayed from acidic denaturing conditions (8+ to 14+ charge states), indicates that the protein has retained its native folded state.³⁴ The average deconvoluted molecular mass for the protein was measured at 10 921.9 Da, which is in agreement with the average molecular mass (10 922.5 Da) reported previously for Link_TSG6.²³ To assess noncovalent complex

formation, titration studies were performed in which an increasing concentration of HA₈ (0.06, 0.6, 6.0 and 12.0 μM, respectively) was added to a constant concentration of protein (1.6 μM). When Link_TSG6 was placed in molar excess (~27-fold) to HA₈ (0.06 μM) the mass spectrum appears similar to that of Link_TSG6 alone indicating that no interaction has occurred (Figs. 1(A) and 1(B)). However, when the concentration of oligosaccharide was increased 10-fold (0.6 μM), a 7+ charge state (average $m/z = 1780.58$) was observed (Fig. 1(C)), having a deconvoluted average mass of 12 457.1 Da, which is in agreement with the average mass calculated for a complex between Link_TSG6 and HA₈ having 1:1 stoichiometry ($M_r \text{ Link_TSG6} + M_r \text{ HA}_8 = 10921.9 + 1534.5 = 12\,456.4 \text{ Da}$). Interestingly, only a single 7+ charge state was observed for the complex. At the concentrations analyzed here, a majority of Link_TSG6 was observed in its unbound/free charge states because the protein was in molar excess over the oligosaccharide (Fig. 1(C)). However, as the concentration of HA₈ (6 μM) was increased, placing the sugar in molar excess to protein (~3.8-fold), the most abundant ion within the mass spectra was the 7+ noncovalent complex indicating that nearly all Link_TSG6 was bound to the oligosaccharide (Fig. 1(D)). Under these conditions, a 2+ charge state at m/z 768.25, representing the free/unbound HA₈ oligosaccharide, was also observed having a deconvoluted monoisotopic molecular mass at 1534.5 Da (Figs. 1(D) and Fig. 2). Salt adducts (+Na, +K and +NH₄) of the oligosaccharide are also denoted in Fig. 2, as observed previously by MALDI-TOF MS.^{26,35} No smaller hexasaccharides (HA₆) or larger decasaccharides (HA₁₀) were observed in the spectrum, confirming the purity and homogeneity of the HA₈ sample. To completely saturate the residual free protein (6+ and 7+ charge states) observed at 6.0 μM oligosaccharide (Fig. 1(D)), Link_TSG6 was sprayed in the presence of 12.0 μM HA₈ (Fig. 1(E)). However, even at this higher concentration of oligosaccharide, some free protein was observed indicating that complete binding was not achieved or that a percentage (<10%) of the complex was becoming dissociated after introduction into the mass spectrometer.³⁶ Alternatively, this may also represent a percentage of denatured Link_TSG6 that is incapable of binding the ligand. To assess the specificity of this interaction Link_TSG6 was incubated in the presence of a molar excess of tetrasaccharide (HA₄), an oligosaccharide unable to support high affinity binding, and previously shown to bind with an ~250-fold lower affinity than HA₈.²⁴ As seen in Figs. 3(A) and 3(B), nearly all protein and oligosaccharide were observed in the free/unbound charge state. Only under closer inspection was a low abundant 7+ ion (average $m/z = 1672.25$) detected representing a complex between Link_TSG6 and HA₄ having a deconvoluted average mass of 11 698.8 Da (Figs. 3(A) and 3(C)). This experimental mass is in agreement with the average calculated mass for a 1:1 stoichiometric complex between Link_TSG6 and HA₄ ($M_r \text{ Link_TSG6} + M_r \text{ HA}_4 = 10921.9 + 776.25 = 11\,698.2 \text{ Da}$) indicating the presence of a weak association between the protein and the tetrasaccharide. Similar to the interaction between Link_TSG6 and HA₈, the 7+ charge state was the only ion observed for the complex. Therefore, the above results indicate that a single charge state noncovalent complex in the gas phase between HA oligosaccharides and Link_TSG6 can be detected by FTMS.

H/D-EX MS of intact Link_TSG6

To assess the effect of ligand binding on Link_TSG6 deuterium uptake, global deuterium incorporation over time for the protein was measured in both the absence and presence of

HA₈. After on-exchange in D₂O the protein was diluted into ice-cold 2.5% (v/v) acetic acid to quench exchange. Deuterium incorporation at each time point was determined by taking the difference between the centroid of the isotopic peak cluster for the deuterated Link_TSG6 sample (alone or with HA₈) and the centroid of the control from water (Figs. 4(A)-4(C)). It must be noted that the 12+ charge state was selected for deuterium assessment because it was the most intense ion in the spectra (Fig. 1(A), inset). After 30 min of on-exchange, assessment of the 12+ charge state of Link_TSG6 reveals incorporation of approximately 15 and 12 deuterons for the free and HA₈-bound Link_TSG6, respectively (Fig. 4(D)). Again an increase in deuterium incorporation was observed between 30 and 60 min for both free and bound Link_TSG6, having incorporation values of 19 and 16 deuterons, respectively. After 120 min of on-exchange, HA₈ has significantly reduced deuterium incorporation within the protein by preventing approximately 12 protons from exchange in the bound relative to the free protein (Fig. 4(D)). The level of deuterium incorporation reached asymptotic values of approximately 37 and 26 deuterons for the free and HA₈-bound Link_TSG6 after 240 and 480 min, respectively (Fig. 4(D)). Therefore, in the presence of HA₈, deuterium incorporation in Link_TSG6 takes twice as long to reach its asymptotic limit than the free protein while also preventing approximately 11 amides from undergoing exchange.

Sequence-specific H/D-EX MS analysis of Link_TSG6

To localize sequence-specific deuterium incorporation, Link_TSG6, in both the absence and presence of HA₈, was digested with pepsin on ice at low pH. Samples were incubated in D₂O for at least 8 h prior to digestion to ensure complete exchange (Fig. 4(D)). Nine peptides were identified in the free protein corresponding to 80% coverage (Fig. 5). Although the protein was digested in the presence of reducing agent (TCEP) only the disulfide bond between Cys⁴⁷ and Cys⁶⁸ was found reduced. The second disulfide (Cys²³ and Cys⁹²) remained intact and, therefore, information regarding amino acids 21–24 and 79–98 was not obtained (Fig. 5). The inability of TCEP to break this disulfide bond results in part from the short protein digestion time (15 min) necessary for H/D-EX MS studies, since complete protein coverage was observed after digestion for 1 h at room temperature. An example of sequence-specific deuterium incorporation is shown for a peptide representing amino acids 1–19 in Link_TSG6, as determined by MS/MS fragmentation (Fig. 6(A)). In the presence of D₂O the center of the isotopic distribution for this peptide displays an average increase in approximately 4 deuterons compared to this peptide from a Link_TSG6 digest incubated in H₂O. This corresponds to 21.2% deuterium incorporation (Figs. 6(A) and 6(B) and Table 1). However, in the presence of excess HA₈, a shift of the isotopic envelope representing an average of 2 deuterons was observed compared to the control (Fig. 6). This corresponds to a 43.5% reduction in deuterium incorporation (Table 1). Therefore, the HA₈ oligosaccharide prevented two amides from exchange within this peptide when bound to Link_TSG6.

For free Link_TSG6 a mass corresponding to an increase of approximately 20–30% deuteration was seen for most peptides analyzed relative to the free nondeuterated protein. The highest degree of incorporation (~32% increase for each peptide) was observed for peptides representing amino acids 33–44 and 45–69, respectively (Table 1). Both the β 3 and

β 4 sheets of Link_TSG6 are covered in the latter peptide containing 26 amino acids. The former sequence containing 12 amino acids covers a significant portion of the α 2 helix in the protein.^{24,32} The least amount of deuterium incorporation for free Link_TSG6, with values of 10.6 and 14.9%, was seen for sequences representing amino acids 45–51 and 25–32 covering a majority of the loop regions prior to the β 3 and β 2 sheets, respectively.^{24,32}

In the presence of HA₈, the percentage of deuterium incorporated, relative to the unbound protein, was reduced approximately 30–60% for nearly all peptides analyzed. However, protein coverage was limited because three peptides covering amino acids 45–69 were not observed in the presence of the oligosaccharide (Fig. 5 and Table 1). Although proteolytic digestion was performed under reducing conditions at low pH, it appears that the oligosaccharide inhibits Link_TSG6 digestion. Of the peptides available for interpretation the most reduction in deuterium incorporation relative to the free protein was seen in amino acids 33–38 at 58.5% (Table 1). This sequence lies within the α 2 helix of Link_TSG6 and is not directly involved in HA binding.^{24,32} The difference in deuterium uptake for this peptide with or without HA₈ is 1 deuteron indicating that approximately one amide is prevented from exchange when Link_TSG6 is in the presence of HA₈ (Table 1). To localize the site of protection within the peptide, MS/MS was performed using CID in the ion trap (Fig. 7). Only b-fragment ions were analyzed since deuterium rearrangement in the gas phase within these ions is less likely to occur compared to their complementary y-fragment ion pairs.^{37,38} In the presence of D₂O the b₅ ion shows a shift in isotopic distribution, compared to the control from H₂O, indicating that deuterium incorporation has occurred (Figs. 7(A) and 7(B)). An average increase of approximately 1–2 deuterons is seen for the sample from D₂O, which corresponds well with the amount of deuterium calculated on the precursor peptide (Table 1). In comparison, the b₅ fragment ion derived from a peptide from Link_TSG6 in the presence of HA₈ shows an average increase of slightly less than 1 deuteron. This also corresponds well with that of the precursor ion (Table 1). The mass difference of approximately 1 deuteron was observed on consecutive fragment ions (b₄, b₃ and b₂). Thus, if deuterium rearrangement has not occurred, then it appears that amide protection in the presence of HA₈ was occurring on either one of two amino acids (Y33 or K34) within peptide 33–38.

DISCUSSION

In this study noncovalent gas-phase interactions between Link_TSG6 and HA oligosaccharides of defined length (HA₄ and HA₈) recapitulate solution-phase properties in which HA₈, but not HA₄, was found to display high affinity Link_TSG6 binding.^{24,25} It is not surprising that complexes between Link_TSG6 and HA oligosaccharides were detected by ESI-MS since various gas-phase interactions, such as those between proteins and small ligands, carbohydrates or DNA, have been previously reported.^{39,40} Additionally, recent studies have also characterized noncovalent gas-phase interactions between chemokines and the GAG heparan sulfate.^{41,42} Interestingly, in this study only a single 7+ charge state for the noncovalent complexes with either HA₈ or HA₄ was observed (Figs. 1 and 3). This may result from a change in the structure for the complex compared to the free protein. For example, individual proteins sprayed from native conditions show low and narrow charge-state distributions compared to the broad, high charge-state distributions observed for

proteins sprayed from denaturing solvents,^{34,39} as was the case for Link_TSG6 (Fig. 1(A)). It is thought that compact folded proteins are less likely to have available sites for protonation than unfolded proteins, which would correlate the total charge on a protein with its structure. This same rationale might also apply to noncovalent HA–protein complexes. Thus, the single 7+ charge state for the complex may represent a higher-order more compact structure compared to the free protein alone. Alternatively, the charge state for the complex may be evidence for stable salt bridges between Link_TSG6 and HA₈. Previous isothermal calorimetry (ITC) and NMR experiments indicate that there are 1 or 2 salt bridges in the Link_TSG6–HA₈ complex; possibly formed between the basic amino acids Lys11 or Arg81 and the carboxylate groups of glucuronic acid.²⁴ Thus, these stable salt bridges could block potential sites of protonation on Link_TSG6, resulting in a uniform charge state.

Key advantages for the characterization of protein–carbohydrate interactions by ESI-MS are both the speed and high sensitivity for analysis in which typically less than 1 µg is needed.³⁹ In addition, unlike nonequilibrium techniques that are sensitive to the shape of macromolecules, such as size-exclusion chromatography and gel electrophoresis, the stoichiometry of interactions are attainable by ESI-MS because the molecular weight of these complexes can be directly measured. Analysis of protein–ligand interactions by ESI-MS can also be used to measure binding affinities as shown previously between protein–carbohydrate^{39,43} and protein–GAG systems.⁴⁴ However, it must be assumed that the gas-phase ion intensities (i.e., spectral amplitudes) for the complex, free protein and ligand are directly related to their equilibrium concentrations in solution, which is not always the case with ESI-MS, as it can discriminate in this regard.⁴⁵ Alternatively, methods that utilize H/D amide exchange, such as PLIMSTEX (Protein-Ligand Interactions by Mass Spectrometry, Titration, and H/D EXchange),⁴⁵ can correlate protein-binding affinity with its deuterium incorporation (D) in the presence of various ligand concentrations.

In this study deuterium incorporation was analyzed on intact Link_TSG6 in both the absence and presence of HA₈ (Fig. 4). These experiments revealed an increase of approximately 3 deuterons in the free protein relative to the bound after 30 and 60 min of on-exchange, respectively. However, an asymptotic difference of approximately 11 deuterons is seen between the free and bound protein. This may result from a dynamic change in EX2 exchange for Link_TSG6 in the presence of the ligand. For example, in free Link_TSG6, EX2 exchange occurs occasionally from the unfolded (open), solvent-exposed protein that exists in equilibrium with the normally well-folded (closed) protein;¹² therefore, deuterium incorporation will gradually increase overtime until a limiting asymptote is reached (Fig. 4(D)). However, in the presence of HA₈, the ligand can stabilize the folded (closed) form of the protein, thereby preventing additional deuterium uptake in the complex relative to the free protein.⁴⁶ It is also this phenomenon which allows binding affinities to be determined by PLIMSTEX studies.⁴⁷

Previous work has identified five critical amino acids necessary for Link_TSG6 HA-binding by site-directed mutagenesis (Lys11, Tyr12, Tyr59, Phe70 and Tyr78), and confirmed with NMR studies wherein eleven amino acids (Lys11, Tyr12, Val57, Tyr59, Pro60, Ile61, Phe70, Ill76, Tyr78, Arg81 and Trp88) were found to interact with the ligand.^{24,25} Therefore, the Link_TSG6–HA₈ interaction provides a model system to assess deuterium

uptake in specific peptides within the binding groove. In this study, however, analysis of sequence-specific deuterium incorporation was limited by incomplete digestion (Fig. 5), arising from the experimental requirements of the short digest times near 0°C or the persistence of partially folded protein.⁴⁸ One drawback of the longer digestion time needed for complete protein coverage in H/D-EX MS studies is the increase in back-exchange, which compromises deuterium measurement. Interestingly, in the presence of HA₈, three peptides covering amino acids 45–69 were not observed (Fig. 5 and Table 1). Molecular modeling of peptide coverage for the free, and HA₈-docked, Link_TSG6 (Fig. 8) reveals a correlation between the undigested regions of the protein with proximity to the ligand. Whether HA₈ specifically blocks proteolytic digestion or the reduction of the disulfide bond between Cys47 and Cys68 remains to be determined. However, from the model (Fig. 8), it appears that the presence of the HA₈ facilitated proteolytic ‘foot-printing’ of the protein regions close to the oligosaccharide.

Of those peptides that could be analyzed by H/D-EX MS in the presence of HA₈, a percentage of deuterium incorporation relative to the unbound protein was reduced by approximately 30–60%. In peptides 1–19 and 70–78, both having critical amino acids involved in HA binding (K11, Y12, and Y78), percent reductions of 43.5 and 34.0% were observed, respectively. However, the biggest reduction was seen between amino acids 33–38 at 58.5% (Table 1), which does not have amino acids directly involved in HA binding from NMR or site-directed mutagenesis studies. Interestingly, K34 is also involved in the interaction of Link_TSG6 with heparin.⁴⁹ Thus, Link_TSG6–HA binding may affect this distal site and therefore potentially modulate heparin binding, as postulated previously.⁴⁹ The reduced incorporation of deuterium at allosteric sites distant from the binding interfaces upon ligand binding has been attributed to changes in protein dynamics or hydrogen bonding.¹⁷ Therefore, it appears that H/D-EX MS can be used to investigate protein–HA interactions. However, results should be interpreted with caution when attempting to map sites of interactions. More extensive experimental approaches such as molecular modeling and MD simulations might be necessary to fully understand the precise origin of the observed amide protection. That is, MD simulations could in principle discriminate between true protection and allosteric effects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Titration analysis of Link_TSG6 with increasing concentrations of HA₈. (A) Charge-state distributions for Link_TSG6 (1.6 μM) sprayed from either native (10 mM ammonium acetate pH 5.8) or denaturing (2.5% (v/v) acetic acid) conditions (*inset*) in the absence of oligosaccharide. Link_TSG6 (1.6 μM) sprayed from native conditions in the presence of 0.06, 0.6, 6.0 and 12.0 μM HA₈ (B, C, D, and E, respectively). A 7+ charge-state ($m/z = 1780.58$) complex between Link_TSG6 and HA₈ having a 1:1 stoichiometry at or above 0.6 μM oligosaccharide is denoted (C, D and E).

Figure 2.

HA_8 oligosaccharide analyzed by FTMS. The 2+ charge state (monoisotopic $m/z=768.25$) for the octasaccharide was observed. The high resolution of FTMS (100 000) provides clear identification of a wide distribution of 2+ HA_8 adducts.

Figure 3.

Link_TSG6 in the presence of a molar excess of HA_4 . Link_TSG6 ($1.6\mu\text{M}$) was sprayed from native conditions in the presence of $6.0\mu\text{M}$ HA_4 (A). The charge states for Link_TSG6 ($6+$, $7+$, $8+$, and $9+$) and singly charged HA_4 ($m/z=777.25$) are denoted (A and B). Upon closer inspection, a low abundant $7+$ charge state (average $m/z=1672.25$) corresponding to a 1:1 complex ($M_r=11698.8$) between the Link_TSG6 and HA_4 is observed (C).

Figure 4.

Global amide exchange monitored on intact Link_TSG6 in the absence and presence of HA_8 . The 12+ charge state of Link_TSG6 incubated in H_2O (A), or incubated in D_2O in the absence (B) or presence of molar excess HA_8 (C) after 240 min of on-exchange, respectively. The y-axis is reported as the difference in deuterium incorporated between Link_TSG6 samples incubated in D_2O (alone or with HA_8) and the control H_2O (D). The spectra at each time point are summed between 2–3 min post-quench $\pm$ the standard deviation of three measurements.

Figure 5.

Sequence coverage of Link_TSG6 following digestion with immobilized pepsin.

Approximately 80% coverage is observed for the protein in the absence of HA₈. Disulfide bonds between Cys⁴⁷ and Cys⁶⁸ and between Cys²³ and Cys⁹² are denoted. In the presence of HA₈ peptides between amino acids 45–69 were not observed (dashed lines).

Figure 6.

Hydrogen/deuterium amide exchange FTMS monitored on pepsin-digested Link_TSG6 in the absence and presence of HA_8 . A 4+ charge-state ($m/z = 550.55$) peptic fragment of Link_TSG6 from H_2O (A) corresponding to amino acids 1–19 as determined by collision-induced dissociation (CID) with a Mascot database search (see Experimental section). Link_TSG6 was incubated in D_2O in the absence (B) or presence of excess HA_8 (C). Spectra are summed between 18–21 min post-quenching.

Figure 7.

Observations of site-specific deuterium incorporation by MS/MS. Zoom scan MS/MS was performed on peptide 33–38 using CID from a Link_TSG6 digest in either H_2O (A), D_2O (B), or D_2O with HA_8 (C). The summed average of seven spectra between 18.3 and 18.5 min post-quench (3.3–3.5 min post-acquisition) are reported.

Figure 8. Molecular modeling. Link_TSG6 alone (A) and docked to HA₈ (B). Observed peptide fragments in the free protein, compared to the protein, in the presence of HA₈ are illustrated in (C) and (D), respectively. Alternating contiguous peptides are represented by blue and purple coloring in the protein. The amino acids that come within 10Å of the oligosaccharide are represented in red and the four cysteines in yellow.

Table 1

Sequence-specific deuterium incorporation in Link_TSG6 by H/D-EX MS*

Sequence	Control (H ₂ O) ^a	Free (D ₂ O) ^a	± SD	(Free- Control) ^a	% D Incorporated	Complex (D ₂ O) ^a	± SD	(Complex- Control) ^a	% D Incorporated	(Free- Complex) ^a	% Reduction
1-19	2199.28	2203.3	0.53	4.02	21.2	2201.55	0.03	2.27	11.9	1.75	43.5
25-32	830.82	832.01	0.39	1.19	14.9	831.37	0.02	0.55	6.9	0.64	53.8
33-38	750.76	752.4	0.28	1.64	27.3	751.44	0.02	0.68	11.3	0.96	58.5
39-44	690.66	692.22	0.48	1.56	26.0	691.69	0.06	1.03	17.2	0.53	34.0
33-44	1423.53	1427.38	0.58	3.85	32.1	1426.28	0.08	2.75	22.9	1.1	28.6
45-51	742.62	743.36	0.35	0.74	10.6	n/a	n/a	n/a	n/a	n/a	n/a
52-69	1843.95	1849.48	0.37	5.53	30.7	n/a	n/a	n/a	n/a	n/a	n/a
45-69	2568.84	2576.83	0.31	7.99	32.0	n/a	n/a	n/a	n/a	n/a	n/a
70-78	1013.03	1014.99	0.51	1.96	21.8	1014.33	0.18	1.3	14.4	0.66	33.7

* Link_TSG6 samples were incubated in D₂O in both the absence and presence of HA8. Samples were analyzed in duplicate (spectra summed between 18–21 min post-quench) and the errors reported as ± standard deviation (SD). Peptides not available (n/a) in the presence of HA8 are also denoted in Fig. 5.

^a Average mass in Daltons (Da).