The Link Module from Ovulation- and Inflammation-associated Protein TSG-6 Changes Conformation on Hyluronan Binding*§

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The solution structure of the Link module from human TSG-6, a hyaladherin with important roles in inflammation and ovulation, has been determined in both its free and hyluronan-bound conformations. This reveals a well defined hyluronan-binding groove on one face of the Link module that is closed in the absence of ligand. The groove is lined with amino acids that have been implicated in mediating the interaction with hyluronan, including two tyrosine residues that appear to form essential intermolecular hydrogen bonds and two basic residues capable of supporting ionic interactions. This is the first structure of a non-enzymic hyaladherin in its active state, and identifies a ligand-induced conformational change that is likely to be conserved across the Link module superfamily. NMR and isothermal titration calorimetry experiments with defined oligosaccharides have allowed us to infer the minimum length of hyluronan that can be accommodated within the binding site and its polarity in the groove; these data have been used to generate a model of the complex formed between the Link module and a hyluronan octasaccharide.

Hyaluronan (HA), a high molecular weight polysaccharide with a central role in extracellular matrix organization and cell adhesion in mammals (1), is essential to a wide range of normal physiological processes including development, immunology, and reproduction (2–4). Alterations in the metabolism and localization of this molecule underlie the progression of many diseases, for instance arthritis, pulmonary/vascular disorders, and cancer (5, 6). These diverse biological activities may seem surprising for a linear polymer composed entirely of a repeating disaccharide (i.e. -glucuronic acid-β-1,3-N-acetylgalacosamine-β-1,4-; up to 10^7 Da) that, unlike other glycosaminoglycans, is neither attached to a core protein nor sulfated. This functional complexity is thought to arise from the interaction of HA with a large number of specific HA-binding proteins (7), which can form structurally diverse complexes (see Ref. 8). The majority of these “hyaladherins” belong to a superfamily of proteins that share a common ~100 amino acid domain, termed a Link module, that mediates the interaction with HA.

Previously we have determined the solution structure of the Link module from human TSG-6 (the protein product of the tumor necrosis factor-stimulated gene-6 (9)), thereby defining the consensus fold for this superfamily (10). In TSG-6, a 35-kDa secreted protein composed mainly of contiguous Link and CUB modules, the Link module is sufficient to mediate a high affinity interaction with HA (10, 11); this has been termed a “type A” HA-binding domain (7). The HA receptor CD44, which has an important role in mediating lymphocyte migration, however, requires N- and C-terminal extensions to its Link module for correct folding and functional activity of its type B interaction domain. Most other members of the superfamily, such as link proteins and chondroitin-sulfate proteoglycans (critical for extracellular matrix organization; see Ref. 12), have larger HA-binding domains containing two tandem Link modules (7).

TSG-6 is not constitutively expressed in normal adult tissues but is produced during inflammatory disease (13), e.g. in the joint tissues of arthritis patients (14, 15). Recently it has been found that TSG-6 protects against cartilage matrix destruction (16, 17) and has anti-inflammatory activities (18) in mouse models of arthritis; the Link module alone is a potent inhibitor of neutrophil migration in vivo (19). These studies suggest that TSG-6 is an endogenous component of a negative feedback loop capable of down-regulating the inflammatory response (13). TSG-6 is also expressed in inflammation-like processes such as ovulation (20) and deletion of the TSG-6 gene (4), or decreased TSG-6 expression (21) cause female infertility in mice.

Significant progress has been made in the characterization of the HA-binding properties of the TSG-6 Link module (termed Link_TSG6). Thermodynamic studies of the interaction between Link_TSG6 and defined oligomers of HA by isothermal titration calorimetry (ITC) indicated that an octasaccharide (HA8) was close to the minimum length that bound optimally to HA, in the absence and presence of HA2 (11), and site-directed mutagenesis (22) have been used to identify the position of the HA-binding site. However, to date it has only been possible to map these...
data on the structure of the free Link module (10), which does not provide a clear picture of how HA is recognized by the protein.

Here we have determined the structure of the TSG-6 Link module in its HA<sub>8</sub>-bound conformation. This has revealed a well defined HA-binding groove containing all the amino acids implicated previously in binding. Comparison with a de novo calculated structure of the free protein demonstrates that a small but significant ligand-induced conformational change occurs on interaction with HA, switching the Link module from a closed to an open state. The minimum length of HA that can be accommodated within the binding site and its polarity in the groove have been identified. These structural studies provide valuable new insights into the function of TSG-6 and the Link module superfamily in general.

**EXPERIMENTAL PROCEDURES**

Sample Preparation—Unlabeled and uniformly <sup>15</sup>N- and <sup>13</sup>C-labeled Link<sub>TSG6</sub> were expressed in *Escherichia coli* and purified as described previously (see Ref. 11). HA oligosaccharides of defined length (including HA<sub>6</sub> and HA<sub>8</sub>, which have GlcUA at either end) were purified from high molecular weight HA (unlabeled or <sup>15</sup>N-enriched) following digestion with testicular hyaluronidase as before (23). Uniformly labeled <sup>15</sup>N-HA polysaccharide was produced by fermentation (3 days at 30 °C) of *E. coli* K5 transfected with recombinant HA synthase from *Pasteurella multocida* (24) in M9 minimal media with <sup>15</sup>N<sub>2</sub>CH<sub>3</sub>C<sub>2</sub>H<sub>4</sub>O (99 atom %; Spectra Stable Isotopes) as the nitrogen source. The polymer in the media was purified by cetylpyridinium chloride precipitation, DNAase/ribonuclease treatment, chloroform extraction, and reverse phase extraction as described previously (25). NMR samples were prepared from lyophilized material reconstituted in 10% (v/v) D<sub>2</sub>O, 0.02% (w/v) NaN<sub>3</sub> (or 99.98 atom % D<sub>2</sub>O) and adjusted to pH 6.0; oligosaccharides or protein were added, as required, to a 1:1 stoichiometry (unless stated otherwise).

**NMR Data Collection**—All NMR experiments were performed at 25 °C on spectrometers operating at 500, 600, or 750 MHz. Assignment used the spectra recorded previously (21, 22). Oligosaccharide solutions (ranging from 10.0 to 58.6 μM) were added to buffer (5 mM, 15N-HA<sub>8</sub> 0.3 mM) were performed (with 15N offset at 122.5 ppm) in the absence and presence of equimolar concentrations of HA oligomers of different lengths were investigated on a Microcal VP-ITC instrument at 25 °C in 5 mM MES, pH 6.0, as described previously (19, 22). Oligosaccharide solutions (ranging from 180 to 870 μM, determined on the basis of the accurately determined protein concentration and known stoichiometry as described previously (11)) were added in N-μl injections (28 in total) to protein (ranging from 10.0 to 58.6 μM). Data were fitted to a one-site model by nonlinear least squares regression with the Origin software package, after subtracting the heats resulting from the addition of oligosaccharide into buffer alone. Affinities for the interaction with HA<sub>8</sub> and HA<sub>5</sub> were determined by averaging results from 5 or 10 experiments, respectively, whereas all other sugars were analyzed at least twice.

**RESULTS**

**Resonance Assignments and Structure Determination**—Assignments were determined for nearly all <sup>1</sup>H, <sup>15</sup>N, and <sup>13</sup>C nuclei in both free and HA<sub>8</sub>-bound Link<sub>TSG6</sub> (21, 22). Oligosaccharide solutions (ranging from 10.0 to 58.6 μM) were added to buffer (5 mM, 15N-HA<sub>8</sub> 0.3 mM) were performed (with 15N offset at 122.5 ppm) in the absence and presence of equimolar concentrations of HA oligosaccharides (HA<sub>5</sub> and HA<sub>7</sub> which have GlcUA at either end) were purified from high molecular weight HA (unlabeled or <sup>15</sup>N-enriched) following digestion with testicular hyaluronidase as before (23). Uniformly labeled Link<sub>TSG6</sub> were expressed in *E. coli* K5 transfected with recombinant HA synthase from *Pasteurella multocida* (24) in M9 minimal media with <sup>15</sup>N<sub>2</sub>CH<sub>3</sub>C<sub>2</sub>H<sub>4</sub>O (99 atom %; Spectra Stable Isotopes) as the nitrogen source. The polymer in the media was purified by cetylpyridinium chloride precipitation, DNAase/ribonuclease treatment, chloroform extraction, and reverse phase extraction as described previously (25). NMR samples were prepared from lyophilized material reconstituted in 10% (v/v) D<sub>2</sub>O, 0.02% (w/v) NaN<sub>3</sub> (or 99.98 atom % D<sub>2</sub>O) and adjusted to pH 6.0; oligosaccharides or protein were added, as required, to a 1:1 stoichiometry (unless stated otherwise).

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Solution structures of Link<sub>TSG6</sub> in both its free and HA<sub>8</sub>-bound states were generated completely independently using NOE restraints, <sup>13</sup>C<sup>15</sup>N<sup>13</sup>C chemical shifts, and H-bonds identified on the basis of hydrogen exchange data (Fig. 1 and Table I). As can be seen from Fig. 2 (and Table I) the lowest energy structure families are well defined, with backbone r.m.s.d. values over amino acids 2–94 (i.e. excluding N- and C-terminal "tails") of 0.49 and 0.53 Å for free and HA<sub>8</sub>-bound protein, respectively.

Redefining the Link Module Structure—A much larger number of NOEs were used here (3 free 1234, bound 1445; Table I) than in the previous structure determinations of the free protein (875 NOEs), which was based primarily on homonuclear NMR data (10). This, in conjunction with the additional <sup>13</sup>C chemical shift assignments and hydrogen-exchange data, has enabled the determination of a much more accurate structure for free Link<sub>TSG6</sub>. It should be noted that whereas the newly determined structure has a very similar fold to that described in Ref. 10, there is considerably improved reliability in the definition of secondary structure elements (predicted previ-
The Link module fold, which is identical in both free and HA₈-bound forms of Link_TSG6 determined here (see Fig. 2, B and D), is composed of two triple-stranded anti-parallel β-sheets (SI (β1, β2, and β6) and SII (β3, β4, and β5)) and two α-helices (α1 and α2); disulfide bridges connect α1 to β6 (Cys⁴³–Cys⁵⁵), and the irregular loop following α2 to the long loop between β4 and β5 (Cys⁴⁵–Cys⁶⁵). As can be seen from Fig. 3 the SI β-sheet and α1-helix are identical to those described before (10). There are, however, subtle differences in the definition of the SII sheet (i.e., β3, β4, and β5 correspond to residues 49–52, 56–61, and 74–77, respectively, rather than 49–51, 56–60, and 75–81 (with a bulge at 77–79) reported previously (10)). In addition, the α2-helix was incorrectly orientated in our original structure and corresponds to residues 33–42 (instead of 36–41); slowly exchanging amides support the presence of caps at both N (Thr⁵²–Gln³⁵) and C termini (Schellman motif (29), Gly⁴⁵). The proposed N-cap on α1 (Thr¹⁵–Glu¹⁸ (10)) has been confirmed, but the C-terminal Schellman motif does not appear to be present. Consistent with this, these three cap motifs are highly conserved across the Link module superfamily, whereas an α1 Schellman motif is not (see Fig. 3). Slowly exchanging amide protons were also observed for Trp⁴⁸ and Leu¹⁴, which H-bond to each other in an antiparallel arrangement. Leu¹⁴ packs behind β1 and against the side of the α2-helix; a large hydrophobic residue is conserved at this position across the Link module superfamily (Fig. 3). These interactions orientate the β1/α1 loop (which contains the HA-binding residues Lys¹¹ and Tyr¹² (22)) and could constitute an additional, short β-element denoted here as “β1a” (see Fig. 3 and Supplemental Material Fig. S1).

Comparison of the TSG-6 Link Module in Its Free and HA₈-bound Forms—The structure determination of Link_TSG6 in its free and HA₈-bound states was performed using identical data sets recorded on samples of the same concentration and with the same methodology. The almost total assignment of all ¹⁵N, ¹³C, and ¹H atoms within free and bound forms (see above) led to high confidence in the assignment of NOEs. Consequently, the differences observed in NOE connectivities and intensities (see Supplemental Fig. S2) correspond to real differences between the calculated free and bound conformations.

As shown in Fig. 4A, the majority of residues within Link_TSG6 has the same structure in free and bound states. The backbone of the secondary elements (defined above) overlay between the 20 free and 20 bound structures with a r.m.s.d. of 0.54 Å, barely greater than that of the families individually (see Table I), and critical core residues such as Tyr⁴¹ and Tyr⁵⁰ (Fig. 4A) occupy identical positions in the overlaid 40 structures. Clearly, there is no gross alteration to the Link module structure on its interaction with HA.

However, significant differences in both chemical shift and NOE networks (and hence calculated structures) were observed in the region of Link_TSG6 where the five critical HA-binding residues, established previously by site-directed mutagenesis (22), are located. As can be seen from Fig. 5D, these residues (red) are brought together from different parts of the structure on the basis of NOE connectivities alone), loop geometry, and side chain orientations.

FIG. 1. Structural characteristics for the free and HA₈-bound Link module from human TSG-6. Histograms showing the number of NOEs (per residue) used in the structure calculations (A and B) compared with the average backbone atomic r.m.s.d. (C and D) for the free (A and C) and HA₈-bound (B and D) Link module structures. The corresponding secondary structure organization is depicted at the top of the figure. The important core residues Trp⁴¹, Trp⁴⁶, Tyr⁴¹, and Tyr⁵⁰, which together account for ~10% of the total non-intraresidual NOEs (in both structures), are denoted by *. The long loop between β4 and β5 strands (→ , residues 62–73), which has a lower than average number of NOEs per residue (due to its high percentage of glycines i.e. amino acids 65, 69, and 71) and its protrusion from the rest of the structure), exhibits the highest local backbone r.m.s.d. In the free protein this region displays significant flexibility as determined from a ¹⁵N-(¹H) NOE experiment, whereas in the complex it is considerably less dynamic. The C-terminal tail (residues 95–86) is more flexible than the family of structures and local backbone r.m.s.d. would suggest because only the major resonance assignment gave rise to NOEs, and so only this conformation is represented.
primary sequence (Fig. 3) to form a surface patch on one face of the Link module; Lys\(^{11}\) and Tyr\(^{12}\) are located in the loop between the \(\beta\)-1-strand and \(\alpha\)-helix (Fig. 4C), whereas Tyr\(^{29}\), Phe\(^{70}\), and Tyr\(^{78}\) are in an adjacent region comprising the \(\beta\)4 and \(\beta\)5 strands and the long loop connecting them (Fig. 4B). This face also contains Trp\(^{88}\), which has a different side chain orientation in the free and bound structures (Fig. 4A).

**Definition of the HA-binding Groove**—The near-complete assignment of the free and bound proteins described here has allowed us to extend previous mapping of the HA-binding surface (11). Significant shift alterations caused by binding HA\(_8\) were determined using the same conservative cut-off values for \(^1\)H, \(^15\)N, and \(^13\)C\(_{\text{caliphasic}}\) as before (11), and a threshold of \(\delta \pm 0.25\) ppm was introduced for the aromatic carbons. When these shift perturbations are mapped onto the structure of Link_TSG6 (in its HA\(_8\)-bound conformation), it can be seen clearly in rapid exchange in the free protein. Given their apparent solvent-exposed position in the bound structure, it is likely that these hydroxyl protons are stabilized by making direct hydrogen bonds to the HA. This is consistent with the observation that mutation of Tyr\(^{12}\) or Tyr\(^{78}\) to phenylalanine reduces binding affinity by \(-100-\) and 16-fold, respectively (19). Replacement of Tyr\(^{29}\) with Phe also results in a large reduction in the binding constant (\(-25\)-fold (22)). This residue, however, may be acting as a hydrogen bond acceptor as the hydroxyl proton has not been observed in the Link_TSG6-HA\(_8\) complex.

Discounting amino acids that appear to have chemical shift perturbations arising mainly from changes in backbone conformation (see below), rather than direct contact with the ligand, the HA-interaction surface is thus generated by Lys\(^{11}\), Tyr\(^{12}\), Val\(^{27}\), Tyr\(^{29}\), Pro\(^{66}\), Ile\(^{67}\), Phe\(^{70}\), Ile\(^{76}\), Tyr\(^{78}\), Arg\(^{81}\), and Trp\(^{88}\) (i.e. the residues that form the binding groove).

**Evidence of HA-induced Conformational Change**—The extensive nature of the perturbations throughout the \(\beta\)-4-\(\beta\)-5 loop (i.e. the lobe with Phe\(^{70}\) at the top; see Fig. 5) is consistent with an HA-induced conformational change in this region (see Fig. 4, A and B). Although this loop (amino acids 62–73) is the least well defined part of the structures (see Fig. 1), there are sufficient distance restraints to define confidently the conforma-
tional change (i.e. an average of 7.3 and 9.3 NOEs per loop residue in the free and HA8-bound forms, respectively, with a total of 120 NOE differences between them in this region). The α1-β1 loop also undergoes a subtle but significant rearrangement on HA binding (Fig. 4C).

The side chains of the key functional residues assume differ-

Fig. 2. Solution structures of the TSG-6 Link module in its free (A and B) and HA8-bound states (C and D). A and C, stereoviews of backbone traces for the family of 20 structures superimposed on the backbone heavy atoms in the secondary structure elements. B and D, secondary structure organization of the Link module, shown on the lowest energy structure of each family. The fold consists of two antiparallel β-sheets SI (light blue; residues 2–6 (β1), 29–31 (β2), and 89–93 (β6)) and SII (dark blue; residues 49–52 (β3), 56–61 (β4), and 74–77 (β5)), connected in a parallel arrangement by two H-bonds between strands β3 and β6 (see Supplemental Material Fig. S1) and two helices (residues 16–25 (α1) and 33–42 (α2)) shown in red.

Fig. 3. Link module sequence alignment. In the sequence of Link, TSG6, HA-binding residues determined by site-directed mutagenesis or NMR are indicated in red and pink, respectively. The secondary structure elements determined in this study (New) are compared with those proposed previously by Kohda et al. (10); in the latter the bulge in the β5 strand is shown as a white box. The structure determined here has allowed the identification of helix capping boxes (yellow) and residues that form the hydrophobic core (blue); non-consensus cysteines present in KIA0527 (34) are shown in purple.

### Kohda 2* elements

**TSG-6**

| Residue | Amino Acid |
|---------|------------|
| α1      | GYV       |
| α2      | A           |
| β1      | L           |
| β2      | A           |
| β3      | T           |
| β4      | P           |

**New 2* elements**

| Residue | Amino Acid |
|---------|------------|
| α1      | GYV       |
| α2      | A           |
| β1      | L           |
| β2      | A           |
| β3      | T           |
| β4      | P           |

COOH

| Residue | Amino Acid |
|---------|------------|
| α1      | GYV       |
| α2      | A           |
| β1      | L           |
| β2      | A           |
| β3      | T           |
| β4      | P           |

Lyne-1

| Residue | Amino Acid |
|---------|------------|
| α1      | GYV       |
| α2      | A           |
| β1      | L           |
| β2      | A           |
| β3      | T           |
| β4      | P           |

KIA0256

| Residue | Amino Acid |
|---------|------------|
| α1      | GYV       |
| α2      | A           |
| β1      | L           |
| β2      | A           |
| β3      | T           |
| β4      | P           |

CAB1558

| Residue | Amino Acid |
|---------|------------|
| α1      | GYV       |
| α2      | A           |
| β1      | L           |
| β2      | A           |
| β3      | T           |
| β4      | P           |

KIA0572

| Residue | Amino Acid |
|---------|------------|
| α1      | GYV       |
| α2      | A           |
| β1      | L           |
| β2      | A           |
| β3      | T           |
| β4      | P           |

HAPI-1

| Residue | Amino Acid |
|---------|------------|
| α1      | GYV       |
| α2      | A           |
| β1      | L           |
| β2      | A           |
| β3      | T           |
| β4      | P           |

HAPI-2

| Residue | Amino Acid |
|---------|------------|
| α1      | GYV       |
| α2      | A           |
| β1      | L           |
| β2      | A           |
| β3      | T           |
| β4      | P           |

Free and Hyaluronan-bound Conformations of TSG-6

The α1-β1 loop also undergoes a subtle but significant rearrangement on HA binding (Fig. 4C). The side chains of the key functional residues assume differ-
Free and Hyaluronan-bound Conformations of TSG-6

Fig. 4. HA<sub>8</sub> binding to TSG-6 does not cause a gross conformational change in the Link module. A, stereoviews of backbone traces for the overlaid families of 20 structures for free (blue) and bound (green) proteins, superimposed on the backbone heavy atoms in the secondary structure elements; side chains of three important core residues (from top to bottom: Trp<sup>70</sup>, Tyr<sup>78</sup>, and Tyr<sup>80</sup>) are shown in yellow (free) and red (bound). B and C, families of side chain positions in the free (yellow) and bound (red) protein for the key HA-binding residues (determined from mutagenesis) and the local backbone trace of the lowest energy free (blue) and complexed (red) structures aligned by superposition of both families on the backbone heavy atoms of residues 56–59, 74–77 (B) and 2–5, 14–19 (C). The regions in B and C are shown in orientations different from those in A that best illustrate the change in side chain position and ordering on HA<sub>8</sub> binding.

is observed for each of the H<sup>1</sup> (6.54 ppm) and H<sup>5</sup> (6.45 ppm) pairs of ring protons, due to rapid rotation of the ring averaging their chemical environments. On binding, however, both H<sup>3</sup> and H<sup>5</sup> chemical shifts are significantly perturbed, and the H<sup>8</sup> protons become distinguishable (H<sup>1</sup> 6.26 ppm, H<sup>2</sup> 6.16 ppm), implying that the ring is no longer able to rotate (at least on the NMR time scale). This could be accounted for by a stacking interaction of Tyr<sup>59</sup> against a sugar ring in HA, as has been observed in the crystal structures of hyaluronate lyases in complex with HA oligosaccharides (31, 32). This is also likely to be the case for Tyr<sup>78</sup>, which becomes significantly ordered on binding (Fig. 4B), lies flatter against the protein, and exhibits distinct shifts for its H<sup>5</sup> protons (6.35 and 5.91 ppm).

Movement of the β4-β5 Loop Opens the HA-binding Groove—As the aromatic rings of Tyr<sup>59</sup> and Tyr<sup>78</sup> become flat against the protein surface on HA<sub>8</sub> binding, the β4-β5 loop (containing Phe<sup>70</sup>) retracts away from them (see Fig. 4, A and B). These, and other rearrangements, such as the movement of Trp<sup>60</sup> and ordering of Lys<sup>11</sup>, combine to open a previously closed groove on the surface of the protein (Fig. 6). The loop is effectively hinged at either end (Pro<sup>60</sup> and Gly<sup>74</sup>) and is opened by a change in the geometry of the disulfide bridge between Cys<sup>47</sup> and Cys<sup>68</sup> (Fig. 6, C and D). Rotation occurs around the Cys<sup>47</sup> 6 and 4<sup>1</sup> bonds, and the side chain chemical shifts of this amino acid exhibit large differences in the free and bound states (C<sup>6</sup> 3.14 ppm, H<sup>5</sup> 0.27 ppm, and H<sup>1</sup> 0.31 ppm). Dynamics experiments clearly indicate that the β4-β5 loop is stabilized significantly on HA binding<sup>2</sup>; for example, the side chain of Asn<sup>67</sup> becomes much less dynamic upon binding (11) even though it does not appear to be directly involved in the interaction with HA (22). Fig. 6 (E and F) shows a simple docking

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*a* C. D. Blundell and A. J. Day, manuscript in preparation.
Fig. 6. The interaction of HA with the TSG-6 Link module induces the opening of the binding groove. A and B, atomic spheres depiction of the lowest energy free (closed) and HA-bound (open) structures, in the same orientation, with the bottom portion of each structure are shown in a ribbon representation. The conformational change of the β4-β5 loop opens a groove, exposing the key HA-binding residues (red); the binding site can be extended by mutation of Glu6 (green) to Lys, resulting in a higher affinity interaction with HA. The closed (A) and open (B) states differ principally in the geometry of the disulfide bridge (sulfur atoms in yellow) linking the β4-β5 loop (Cys47) to the rigid connection between α2 and β4 (Cys68), as shown by sticks in C and D. E and F, the open groove, which is lined with atoms that experience significant shift perturbations on ligand binding (red), can accommodate an HA octasaccharide (blue sticks and green atomic spheres) in a favorable geometry without serious steric clashes; one possible conformation of HA is shown. The polarity and register were determined as described in text (see Fig. 7). F is rotated 90° toward the reader around the horizontal axis relative to E.

model illustrating that the open groove is of a size and shape (~20 Å long, ~10 Å wide, and ~10 Å deep) that would allow good intermolecular van der Waals contacts and favorable glycosidic ϕ,ψ angles in a bound HA molecule.

What Size of HA Fits into the Binding Groove?—Previously we performed ITC experiments to determine the optimal size of HA oligosaccharide to be used in our structural studies (11). For this, we concluded that an octasaccharide is likely to be the minimal length of HA that could make a full interaction network with Link_TSG6. However, in this previous study we only examined HA oligomers with even numbers of sugar rings (i.e. HA6, HA8, HA10, etc.; see Ref. 11). Here we have used ITC to re-examine this question including HA4 and HA2 (prepared as described in Ref. 23); representative titration plots are shown in Supplemental Material Fig. S3. From Table II it can be seen that HA7, HA8, and HA9 all have very similar binding constants (ranging from 52.2 to 58.7 × 10^5 M^-1), whereas the interactions with HA4 and HA5 are weaker (about 15- and 40-fold, respectively, compared with HA8). HA4 was estimated from NMR experiments to bind with a much lower affinity than HA8 (greater than 200-fold). Therefore, it seems likely that HA8 is the minimum size of oligosaccharide that binds with maximum affinity and can make the complete interaction network with the protein. In this regard, the slowly exchanging H4 protons of Tyr12 and Tyr78 are visible in the Link_TSG6-HA8 complex but are not seen with the shorter oligomers.

Determination of the HA Orientation Within the Binding Groove—HA is a molecule with inherent directionality (e.g. it has non-reducing and reducing termini; the latter can undergo α/β anomerization). Because there is good evidence that several highly specific contacts are made between HA and Link_TSG6 (see above), it is likely that this glycosaminoglycan will only be able to bind in one orientation relative to the protein. Therefore, a series of NMR experiments was performed to determine the polarity of HA within the binding groove.

1H,15N-HSQC spectra were collected for 15N-labeled Link_TSG6 in the absence and presence of unlabeled HA oligosaccharides of different length (i.e. HA4, HA5, HA6, HA7, HA8, and HA10). The pattern of chemical shift perturbations caused by the interaction of HA and Link_TSG6 (i.e. the “shift map”) is extremely similar for all of these oligosaccharides (see Supplemental Material Fig. S4A). This indicates that the various HA oligomers all bind into the same site on the protein surface and cause a similar conformational change in the β4-β5 loop. However, there are some discrete differences in the perturbations seen for particular residues (see Fig. 7A) that are likely to be due to differences in the structures of the oligosaccharides or their register within the binding groove.

The shift maps of HA4 and HA10 are almost identical, except that the side chain NH group of Arg81 has two resonances in the presence of HA10 (one at the HA4 position; see Supplemental Material Fig. S4B); this “split” population probably arises from HA10 binding in two different registers (see schematic model in Fig. 7B), where differential end effects (e.g. in dynamic mobility) may account for the observed differences in chemical shift. Thus, it seems likely that HA4 is able to completely fill the binding site, in agreement with the ITC data. Therefore, the HA4 shift map was used as a reference against which the other oligomers were compared.

The only difference seen between the 7- and 8-mers (which have similar binding affinities; Table II) is a larger perturbation of the side chain HN group of Arg81 (R81sc) in the pres-
Fig. 7. Determination of the HA polarity within the Link module binding groove. A, overlays of portions of $^{15}$N-HSQC spectra of $^{15}$N-labeled Link_TSG6 (panels 1–3) and $^{15}$N-labeled HA oligosaccharides (panels 4 and 5). Panels 1–3, comparison of resonance positions (panel 1, NH side chain group of Arg$^{81}$ (R81sc); panel 2, backbone amide of Tyr$^{78}$ (Y78); panel 3, backbone amide of Lys$^{81}$ (R63)) between Link_TSG6 in its free state (black, F) and in complex with different lengths of unlabeled HA oligosaccharide (HA$_n$, purple; HA$_a$, blue; HA$_b$, green; HA$_c$, yellow; HA$_d$, red)). Panels 4 and 5, comparison of resonance positions between $^{15}$N-HA$_4$ (panel 4) and $^{15}$N-HA$_5$ (panel 5) in their free states (F, black) and in complex with unlabeled Link_TSG6 (green and red, respectively). The resonances corresponding to the amide groups from the α- and β-anomers (in the free sugars) are indicated. B, model of how the HA oligosaccharides of various lengths fit into the Link_TSG6 binding groove (based on analysis of NMR spectra in A); the reducing terminus of each oligomer is denoted by a free hydroxyl group (HO), and the sugar rings of HA$_n$ are numbered 1–8 from the non-reducing terminus. The protein surface is represented as a strip with pockets at which only GlcUA (yellow) or GlcNac (red) can fit. Two of these binding subsites correspond to the key residues Tyr$^{78}$ (Y78) and Arg$^{81}$ (R81), which experience differential shift perturbations with different lengths of HA, and a third site represents the likely position of Lys$^{81}$ (R11). The register and polarity shown for the different oligosaccharides with respect to this surface is the only reasonable model that can account for the $^{15}$N-$^{15}$N-HSQC shift map data. HA$_n$ is shown in two possible registers within the groove.

As described above, the differential perturbations seen for Tyr$^{78}$ and Arg$^{81}$ with the different lengths of HA oligomer can all be explained on the basis of the positions of the reducing terminal sugar rings. These amino acids are located at one end of the HA-binding groove (see Fig. 5) providing clear evidence for the polarity of HA relative to the Link module. This is illustrated in the schematic model shown in Fig. 7B, which also shows the registers of the various oligosaccharides. In this model Lys$^{81}$, which is at the other end of the binding groove from Arg$^{81}$, is positioned so that it can interact with ring 3 of HA$_n$ (i.e. a glucuronic acid), which is reasonable given the separation of these residues (17.4 Å from Lys$^{81}$ N$^\alpha$ to Arg$^{81}$ C$^\beta$, respectively, in the lowest energy bound structure) and the likely distance between the carbonylates on rings 3 and 7 (20 Å).

It has been noted previously that mutation of Glu$^6$ (colored green on Fig. 6) to lysine causes ~4-fold increase in the Link_TSG6 binding affinity for HA$_n$ (19). This observation can now be explained in light of our alignment model (Fig. 7B); a lysine at this position could form an additional ionic interaction with the GlcUA at ring 1, effectively extending the binding site.

Based on the similarity of the shift maps, all the oligosaccharides tested can be concluded to cause a conformational alteration on binding to Link_TSG6 (Supplemental Material Fig. 4A). However, the extent of this ligand-induced conformational change may differ with the size of HA. As can be seen from Fig. 7A (panel 3), HA$_a$, HA$_b$, and HA$_c$ all cause an identical large perturbation of the backbone amide resonance for Lys$^{81}$, whereas HA$_d$ and HA$_e$ have a smaller effect; this is also apparent for other residues on the β4-β5 loop (data not shown). Therefore, a 6-mer is the minimum size of HA that can induce...
a full conformational change in the protein, and it is likely that the smaller oligomers probably generate intermediate conformational states.

The Link Module Is Related to the C-type Lectin Domain—An automated search of the DALI data base (33) with the free Link_TSG6 coordinates identified 15 other structures with similar folds (Z score > 2.0); the greatest similarities were seen for human eosinophil granule major basic protein (Protein Data Bank code 1h8u, Z = 5.0), invasin (Protein Data Bank 1cwy, Z = 4.5), intimin (Protein Data Bank 1f00, Z = 4.1), E-selectin (Protein Data Bank 1esl, Z = 3.9), and macrophage mannose receptor (Protein Data Bank 1egg, Z = 3.7). The matching regions of these 15 structures all correspond to C-type lectin-like folds (in most cases from proteins known to interact with carbohydrate), confirming its structural similarity to the Link module noted previously (10, 34). The highest scoring match is with eosinophil granule major basic protein (EMBP), which is clearly a member of the C-type lectin superfamily (on the basis of sequence), but does not have a typical Ca\(^{2+}\)/carbohydrate-binding site (35, 36). Interestingly, EMBP has been shown to interact with the sulfated glycosaminoglycan heparin, and the basic residues implicated in binding (35) are found on an equivalent face of the protein as the HA-binding site in the TSG-6 Link module. It is possible therefore that EMBP and TSG-6 constitute a subgroup of C-type lectins that interact with glycosaminoglycans in a Ca\(^{2+}\)-independent manner. As noted previously (10), the Link module lacks the long Ca\(^{2+}\)-binding loop found in classical C-type lectins, and this is also absent in invasin and intimin, cell adhesion molecules from enteropathogenic bacteria (37, 38).

**DISCUSSION**

Significant recent progress has been made in our understanding of HA-protein interactions with the determination of the structures of glycosaminoglycan-digesting enzymes in complex with HA oligosaccharides (31, 32, 39, 40). Here we have determined the solution structure for the Link module from human TSG-6 in the presence of an HA octasaccharide, the first tertiary structure of an HA-binding domain from a non-enzymatic hyaladherin in its HA-bound conformation. Determination of a high resolution structure for the free Link module, in parallel with this, has revealed that a groove on the surface of the protein opens on interaction with HA; molecular modeling demonstrates that an HA\(_6\) molecule can be accommodated in the “open” conformation in an orientation consistent with the experimentally derived polarity. This binding groove is bounded at either end by Lys\(^{11}\) and Arg\(^{81}\); basic amino acids have long been implicated as major determinants in the interaction of HA with other Link module-containing proteins, e.g. aggrecan, link protein, and CD44 (reviewed in Ref. 11). This probably represents a general feature of HA binding in these proteins because basic residues, which are likely to form salt bridges with the GlcUA sugars, are highly conserved at these sequence positions across the Link module superfamily. In the case of TSG-6, we have estimated previously that only ~25% of the free energy of HA\(_6\) binding to Link_TSG6 comes from ionic interactions at physiological salt strengths (30). This is consistent with our data from mutagenesis suggesting that Tyr\(^{12}\), Tyr\(^{14}\), and Tyr\(^{78}\) have important roles in mediating HA binding (19, 22), and the finding here is that these highly conserved aromatic residues line the binding groove. Our NMR data provide evidence that Tyr\(^{59}\) and Tyr\(^{78}\) are likely to be involved in stacking interactions, in which the flat plane of the aromatic ring aligns with a hydrophobic face of a saccharide, as commonly seen in protein-carbohydrate complexes (41, 42). Such interactions could contribute to the precise positioning of the HA molecule within the binding groove as has been noted in the structures of the streptococcal hyaluronate lyases (31, 32, 40). Phe\(^{77}\), which is on the top of the long β4-β5 loop that changes conformation on interaction with ligand, may also stack against a sugar ring, closing over the bound HA molecule and clamping it in place. However, not all of the contacts with aromatic residues are likely to be ring-stacking interactions because the orientation of Tyr\(^{77}\) appears to preclude this, and this residue (as well as Tyr\(^{59}\) and Tyr\(^{78}\)) appears to hydrogen-bond to the sugar. NMR and calorimetric studies are consistent with a 7-mer (with terminal GlcUA sugars) being the minimum size of HA oligosaccharide that can make a complete interaction network with the protein.

As noted above, the TSG-6 Link module has a distinct conformation in its free state compared with that of the Link_TSG6-HA\(_6\) complex, and these are interchanged by rotation around the Cys\(^{47}\)-Cys\(^{68}\) disulfide bridge (with the concomitant movement of the β4-β5 loop). This disulfide is found in all Link modules except KIA0527 (34), and the residues that provide the hinges on which the loop moves (Pro\(^{60}\) and Gly\(^{74}\)) are very highly conserved (Fig. 3), indicating that the conformational change seen for TSG-6 is likely to occur in most members of the superfamily. This could provide a mechanism for regulation of HA binding and may be relevant in CD44, because this receptor can clearly exist in different activation states (7).

Recently it has become apparent that TSG-6 has a crucial role in mammalian ovulation and fertilization via its stabilization of the nascent HA-rich matrix formed during the cumulus-oocyte complex (COC) expansion (4, 21). One of the mechanisms underlying this stabilization appears to be the formation of covalent complexes between TSG-6 and the heavy chains (HC) of inter-α-inhibitor (20). These HC-TSG-6 complexes can become firmly associated with HA and may function as matrix cross-links through HA binding to TSG-6. Furthermore, HC-TSG-6 complexes also act as intermediates in the covalent transfer of the HC\(_6\)s to HA (4), which become associated via an ester linkage between the carboxylic acid group of C-terminal aspartic acids in the HC and a C-6 hydroxyl of GlcNAc residues in HA (43); it seems likely that HA binding to the TSG-6 Link module, in the context of the HC-TSG-6 complex, serves to orientate the HA in the correct position relative to the HC and may activate the sugar, thus facilitating the transfer reaction (i.e., formation of the ester bond). Mice lacking these HC-HA or HC-TSG-6 complexes, due to impairment of either TSG-6 (4) or inter-α-inhibitor genes (44), are infertile because they are unable to incorporate HA into the COC extracellular matrix; the covalently linked HC may act as cross-links between HA chains. In addition to being produced during ovulation, HC-HA (and HC-TSG-6 (14)) complexes are also a feature of inflammation as they have been detected in the synovial fluids of patients with arthritis and may correlate with disease severity (45). It is clear therefore that the interaction of TSG-6 with HA has a fundamental role in both normal physiological and pathological processes.

The determination here of the structure of the TSG-6 Link module in its HA-bound state provides important new insights into the molecular basis of HA binding and will greatly facilitate further studies to determine the mechanism underlying the HC transfer reaction. This structure will also allow homology modeling of other Link module-containing proteins in their active conformations, thus aiding identification of important functional residues in their HA-binding sites.

Not all of the functions of TSG-6 are dependent on its interaction with HA; for example, its inhibition of neutrophil migra-

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tion, an activity encoded in the Link module domain, does not appear to be associated with HA binding (19). In this regard, the Link module has been shown to interact with many other molecules (13). The determination of a refined structure for the free Link module, described here, will be valuable in understanding the molecular basis of these activities and in mapping the binding surfaces for its other ligands.

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The Link Module from Ovulation- and Inflammation-associated Protein TSG-6 Changes Conformation on Hyaluronan Binding

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