Overlapping sites on the Link module of human TSG-6 mediate binding to hyaluronan and chondroitin-4-sulphate

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Abstract Link modules are hyaluronan-binding domains that are involved in the formation and stability of extracellular matrix and cell migration. We have examined the glycosaminoglycan specificity of the Link module from the arthritis-associated protein, human TSG-6, by microtitre plate-based assays employing biotinylated-hyaluronan or mono-biotinylated Link module. This domain was found to interact specifically with chondroitin-4-sulphate (C4S), with similar affinity to hyaluronan, but not with chondroitin-6-sulphate or heparin. Competition experiments indicate that C4S and hyaluronan have overlapping binding surfaces on the TSG-6 Link module. Disease-associated changes in C4S expression may influence the localisation and biological role of TSG-6.

Key words: TSG-6; Link module; Proteoglycan tandem repeat; Hyaluronan; Chondroitin-4-sulphate; Inflammation

1. Introduction

The associations of glycosaminoglycans (GAGs) with proteins are of functional importance in a wide range of normal and disease processes. For example, the interactions of hyaluronan[(1)] with hyaluronan-binding matrix proteins and receptors have a crucial role in the formation and stability of the extracellular matrix (e.g. cartilage) and in regulating many aspects of cell behaviour during development, morphogenesis, tumorigenesis and inflammation [1]. These interactions with hyaluronan are often mediated by a protein domain of ≈100 amino acids in length, termed a Link module (also known as a proteoglycan tandem repeat [2]). This module has been described in extracellular matrix molecules (link protein, aggrecan, versican and brevican) and the hyaluronan receptor, CD44. The roles of these proteins in cartilage formation and cell migration have been reviewed previously [1,3]. A Link module has also been described in the arthritis-associated protein product of tumor necrosis factor (TNF)-stimulated gene-6 (TSG-6) [4].

There is no constitutive expression of TSG-6 mRNA in human fibroblasts, chondrocytes or synovial cells, but rapid onset of transcription and subsequent secretion of the gene product is seen after treatment with interleukin-1 (IL-1) or TNF [4–6]. In addition, the protein (which is ≈35 kDa and mainly comprised of contiguous Link and CUB modules [4]) has been detected in the synovial fluids from arthritis patients but not from normal individuals [5]. IL-1 and TNF are well known to have a central role in the induction of cartilage breakdown in arthritis (see [7]), therefore, TSG-6 may be involved in these processes [4,5]. However, in chondrocytes at least, TSG-6 mRNA levels are also up-regulated by transforming growth factor β1, which is thought to have a role in cartilage repair [6]. In this regard TSG-6 has been shown to potentiate plasmin inhibition by inter-α-inhibitor, with which TSG-6 forms a covalent complex [8]. TSG-6 has also been shown to have an anti-inflammatory effect in vivo by inhibiting neutrophil infiltration in a murine air pouch model [8].

Recently, we have described the determination of the 3-dimensional structure in solution, at pH 6.0, of the Link module from human TSG-6 (Link_TSG6) [3] expressed in *E. coli* [9]. In addition, we have demonstrated that the recombinant Link module interacts specifically with hyaluronan (at pH 6.0) by using a microtitre plate assay with biotinylated-hyaluronan (bHA) as a probe [3]. Elucidation of the tertiary structure of the Link module allowed the identification of a putative hyaluronan-binding surface on TSG-6 [3].

In this study, we demonstrate that the TSG-6 Link module also interacts in a specific fashion with chondroitin-4-sulphate (C4S), but not with chondroitin-6-sulphate (C6S) or heparin, at a site overlapping its hyaluronan-binding surface.

2. Materials and methods

2.1. Materials

C4S (5×10^4 Da) and C6S (5×10^4 Da) were obtained from Calbiochem, UK (Cat. Nos. 230687 and 2307, respectively). Human umbilical cord hyaluronan (mean molecular weight 4.4×10^6 Da), heparin (3×10^3 Da), and C4S/C6S (a 70 : 30 mixture of 4.6×10^4 Da C4S and C6S) were purchased from Sigma, UK (Cat. Nos. H1876, H3400 and C8529, repectively). NHS-LC-biotin and trifluoroacetic acid (TFA) were supplied by Pierce, UK. Acetonitrile was purchased from Rathburn, UK. Microtitre plates and development reagents used in the binding assay were as described previously [3]. The Link module from human TSG-6 (denoted Link_TSG6), corresponding to amino acids 36–133 in the preprotein [4], was expressed and purified as described before [9,10]. Biotinylated hyaluronan (250 kDa) was prepared as in Kohda et al. [3]. The G1 domain of aggrecan, purified from porcine laryngeal cartilage by the method of Bonnet et al. [11], was a kind gift of Ms. Sarah Howat and Dr. Mike Bayliss (Kennedy Institute of Rheumatology, London).

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Abbreviations: bA-Link_TSG6, mono-biotinylated Link_TSG6; bHA, biotinylated hyaluronan; C4S, chondroitin-4-sulphate; C6S, chondroitin-6-sulphate; DS_{equiv}, molar disaccharide equivalents; ESI-MS, electrospray ionisation mass spectrometry; GAG, glycosaminoglycan; HPLC, high-performance liquid chromatography; IL-1, interleukin-1; Link_TSG6, the Link module from TSG-6; TFA, trifluoroacetic acid; TNF, tumor necrosis factor; TSG-6, TNF-stimulated gene-6

(1) Hyaluronan, a high molecular weight GAG found in most animal tissues, consists of the repeating disaccharide [D-glucuronic acid (β1→3) *N*-acetyl-D-glucosamine (β1→4)]. Unlike other GAGs (e.g. chondroitin sulphate, heparan sulphate) hyaluronan is not found attached covalently to core proteins.

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2.2. Biotinylation of Link_TSG6

Equal volumes (400 μl) of Link_TSG6 (2.5 mg/ml in H_2O) and a fresh solution of NHS-LC-biotin (222 μg/ml) in 100 mM $NaHCO_3$ pH 8.5 were mixed for 1 h at room temperature. The reaction mixture was centrifuged (5 min, 12000×*g*) to remove any precipitate (hydrolysed biotin reagent) and 2 ml of solvent A [0.1% (v/v) trifluoroacetic acid (TFA)] was added to the supernatant. Biotinylated protein was purified by high-performance liquid chromatography (HPLC) on a 250×7.0-mm Aquapore RP-300 (300 Å, 7 μm) column (Applied Biosystems, UK), equilibrated in 80% (v/v) solvent A, 20% (v/v) solvent B [80% (v/v) acetonitrile, 0.09% (v/v) TFA] at a flow rate of 2.5 ml/min. After injection of the sample initial conditions were maintained for 10 min, followed by linear gradinets of 20–40, 40–45 and 45–95% (v/v) solvent B (in solvent A) over 15, 10 and 5 min, respectively. The absorbance at 220 nm was continuously monitored and peaks (I–IV) were collected manually. Protein was recovered from these peaks by drying in a SpeedVac (Savant) and analysed by electrospray ionisation mass spectrometry (ESI-MS) as described in Day et al. [9]. Mono-biotinylated species were resuspended in H_2O and repurified by HPLC using the conditions described above. These species were stored as HPLC eluates at 4°C and aliquots were dried in a SpeedVac as required. The mono-biotinylated species, bA-Link_TSG6, was used in the binding assay described below (Section 2.4).

2.3. Binding assay with biotinylated-hyaluronan

The effect of glycosaminoglycan competition on the binding of biotinylated-hyaluronan (bHA: 250 kDa) to immobilised Link_TSG6 or G1-aggrecan was investigated using a modification of the assay described in Kohda et al. [3]. All dilutions, incubations, and washes were performed in Standard Assay Buffer (50 mM Na-acetate, 100 mM NaCl, 0.05% (v/v) Tween 20 pH 5.8) at room temperature unless otherwise stated. Plastic Linbro microtitre plates (EIA II Plus) were coated overnight with 200 μl/well of protein solution (containing 18 or 22 pmol of G1-aggrecan or Link_TSG6, respectively) in 20 mM Na_2CO_3 pH 9.6. Control wells were incubated with buffer alone. The coating solution was removed and the plates were washed 3×. Non-specific binding sites on the plate were blocked by incubation for 90 min at 37°C with 1% (w/v) bovine serum albumin, followed by three washes. A 200 μl solution of 12.5 ng bHA (33 pmol when expressed as disaccharide equivalents)[2] alone, or containing various amounts of unlabelled GAGs (hyaluronan, heparin, C4S/C6S, C4S or C6S: in the ranges 1.60 to 4380 and 13.22 to 21900 pmol DS_{equiv}, for Link_TSG6 and G1-aggrecan coated wells, respectively), was added to each well and incubated overnight. Plates were washed 3×, and 200 μl of a 1 in 10000 dilution of Extra-Avidin alkaline phosphatase was added and incubated for 30 min, followed by three washes. A 1 mg/ml solution (200 μl/well) of disodium *p*-nitrophenylphosphate, in 100 mM Tris-HCl, 100 mM NaCl, 5 mM $MgCl_2$, pH 9.3, was added to all wells and incubated until sufficient colour had developed (18–60 min). The absorbance at 405 nm was determined on a microtitre plate reader (MKII Titertek Multiscan Plus). All absorbances were corrected against blank wells which contained substrate solution alone and were then standardised to a 10 min development time.

2.4. Assay with biotinylayed Link module of TSG-6

This assay measures the binding of mono-biotinylated Link_TSG6 (bA-Link_TSG6) to microtitre wells coated with either hyaluronan or C4S in the absence or presence of unlabelled hyaluronan or C4S in solution. The assay was performed as above (Section 2.3), but with 0.2% (v/v) Tween 20 in the Standard Assay Buffer. Wells were coated separately with 5 μg hyaluronan (13.2 nmol DS_{equiv}), 6.05 μg C4S (13.2 nmol DS_{equiv}) or coating solution alone. Plates were washed and blocked as before. A 200 μl solution of 21.9 ng bA-Link_TSG6 (2 pmol), containing either no GAG or 0.1 to 100 μg hyaluronan (0.264–264 nmol DS_{equiv}), or 0.1 to 100 μg C4S (0.219–219 nmol DS_{equiv}) was added to each well. The plates were incubated, washed, and developed as descibed in Section 2.3.

[2] Due to the wide range of GAG molecular weights used in this study, and incomplete information on the size of GAG oligomer that are recognised by the proteins, they are expressed throughout as molar disaccharide equivalents (DS_{equiv}).

3. Results

The Link module from human TSG-6 (Link_TSG6) was biotinylated with NHS-LC-biotin, and purified by reverse-phase HPLC, on an Aquapore RP-300 column, as shown in Fig. 1. Four major species (peaks I–IV) elute from the column and the peak at ≈40 min is derived from the system (i.e. it is present in all blank runs). Protein was recovered from peaks I–IV by drying in a SpeedVac and analysed by ESI-MS (data not shown). Species I has a molecular mass identical to that of wild-type Link_TSG6 (10922.5 Da) [9]. The molecular masses of species II and III are both 11262.5 Da (wild-type+340 Da). Since 340 Da is the mass of the biotin moeity, species II and III correspond to mono-biotinylated forms of the protein (denoted bA- and bB-Link_TSG-6, respectively), where the biotin is likely to be attached at different sites given their different elution volumes. Species IV has a molecular mass of wild-type+680 Da, which is consistent with it being di-biotinylated.

The effects of heparin or chondroitin-sulphate on the binding of bHA to immobilised G1-aggrecan, or Link_TSG6, were investigated (Section 2.3). Fig. 2a shows that unlabelled hyaluronan can compete the bHA binding to G1-aggrecan as shown previously [3] with an IC_{50} of ≈825 pmol DS_{equiv} (310 ng). A mixture of C4S and C6S (C4S/C6S) was also found to compete for this interaction, but only when it was present at significantly higher concentrations (IC_{50} ≈21895 pmol DS_{equiv}). Heparin was found to have no effect on the interaction between bHA and G1-aggrecan even when in a very large excess (Fig. 2a). Similarly, the interaction of bHA with Link_TSG6 was not competed by heparin (Fig. 2b). This interaction, however, could be inhibited by C4S/C6S with IC_{50} values of the same order of magnitude as with hyaluronan (≈79 and 100 pmol DS_{equiv}, respectively). Therefore, the assay was performed with either C4S or C6S, separately, to test which of these components were responsible for the inhibition. As shown in Fig. 2b, high levels of C6S were necessary to produce a small reduction in bHA binding, whereas, the interaction could be completely inhibited by C4S at similar concentrations (IC_{50} ≈40 pmol DS_{equiv}) to that seen with hyaluronan. The slight inhibition seen with C6S may be due to a small amount of C4S in this GAG preparation.

The interactions of Link_TSG6 with C4S and hyaluronan were investigated further using a different plate assay as described in Section 2.4. This assay measures the effect of C4S and hyaluronan on the binding of mono-biotinylated Link_TSG6 (bA-Link_TSG6) to C4S, or hyaluronan, coated plates. From Fig. 3 it can be seen that bA-Link_TSG6 binds to hyaluronan coated wells and this interaction can be fully competed by either hyaluronan or C4S, in solution, with IC_{50} values of 1.50 and 3.73 nmol (DS_{equiv}), respectively. The other mono-biotinylated form of the Link module (bB-Link_TSG6) was also shown to interact specifically with hyaluronan (data not shown), but was not investigated further. Fig. 3 shows that bA-Link_TSG6 can bind directly to a C4S coated plate and this interaction can be competed with similar concentrations of either C4S or hyaluronan (IC_{50} values of ≈7.75 and 6.23 nmol DS_{equiv}, repectively). In this assay there is ≈2× greater binding of bA-Link_TSG6 to wells coated with C4S than with hyaluronan in the absence of competitor. Given the similarity of the competition curves with C4S and hyaluronan, in each case, the level of bA-Link_TSG6 binding may reflect different coating efficiencies of the two GAG preparations.

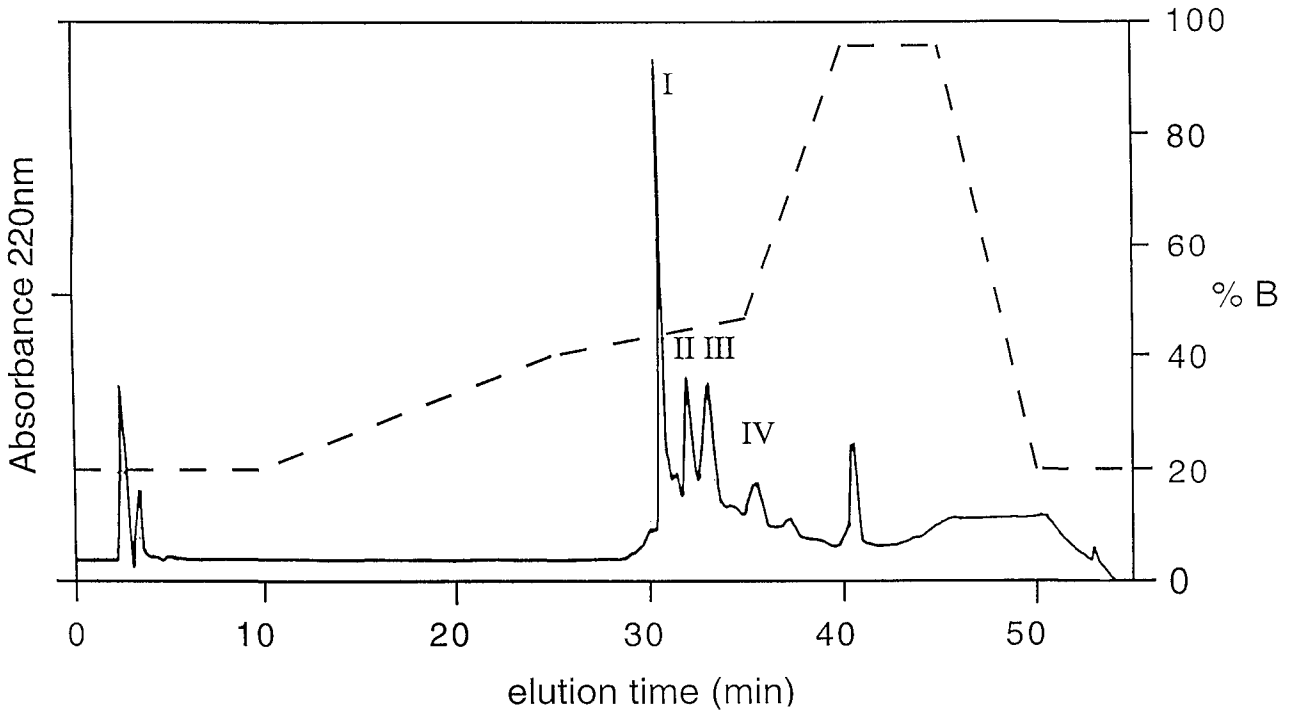

Fig. 1. Purification of biotinylated Link_TSG6 by reverse-phase HPLC on a Aquapore RP-300 column monitored at 220 nm. The elution gradient, which resolved four major species (denoted I–IV), is shown by a dotted line with the percentage of solvent B shown on the right side.

4. Discussion

We have investigated the specificity of GAG binding to the Link module of TSG-6 in comparison with that of the G1 domain of aggrecan. The binding assays were conducted at pH 5.8 as there is maximal binding of Link_TSG6, and close to maximal binding of G1-aggrecan, to hyaluronan at this pH (A.A. Parkar, J.D. Kahmann, S.L.T. Howat, M.T. Bayliss, I.D. Campbell and A.J. Day, manuscript in preparation). In a competition assay the interaction between bHA and a Link_TSG6 coated plate can be effectively inhibited by similar concentrations of hyaluronan and C4S, but not by C6S or heparin (Fig. 2b). The interaction between bHA and G1-aggrecan was also found to be unaffected by heparin, but was inhibited with C4S/C6S where the IC_{50} was >25-fold higher than with hyaluronan (Fig. 2a). In this case competition assays with the individual chondroitin sulphate isoforms were not performed. To determine whether the effect of C4S on the interaction of hyaluronan with Link_TSG6 was an artefact of the protein being immobilised on a micotitre plate, an assay was developed that employs mono-biotinylated Link_TSG6 (bA-Link_TSG6), in solution, binding to a hyaluronan coated plate (Fig. 3). In this assay both GAGs can inhibit the interaction which is consistent with the results of the previous binding assay (see Fig. 2b) and demonstrates that C4S can inhibit the binding of hyaluronan to Link_TSG6 irrespective of whether the protein is immobilised or in solution. In addition, bA-Link_TSG6 was found to bind directly to a C4S coated plate and this interaction could be inhibited by C4S and hyaluronan with similar IC_{50} values (Fig. 3). These data clearly demonstrate that C4S interacts specifically with Link_TSG6 and strongly suggests that it is a novel ligand for TSG-6. Previously, Lee et al. [4] reported that a full-length recombinant human TSG-6, expressed in a fibroblast cell line, does not bind to C4S/C6S as these species did not co-precipitate in the presence of the cationic detergent cetylpryidinium chloride, but TSG-6 did co-precipitate with hyaluronan under the same conditions. It is difficult to comment on the apparent discrepancy between the results of Lee et al. [4] and the data presented here, as their material was only partially purified (on hyaluronan-sepharose) and analysed by SDS-PAGE alone, whereas the recombinant Link module we used has been rigorously investigated [9] and its tertiary structure determined by NMR [3]. In both our plate-binding assays C4S and hyaluronan bind to Link_TSG6 competitively, indicating that their binding surfaces on the Link module are at least overlapping and possibly identical. Given the similar nature of the competition curves for the two GAGs, in both assays (Figs. 2 and 3), their affinities are also likely to be very similar. However, as the sizes of C4S and hyaluronan oligomers recognised by Link_TSG6 are not yet known, and are conceivably different, it is not possible at present to determine whether their affinities are identical.

Chondroitin sulphate and hyaluronan are highly related GAGs with the same type of glycosidic linkages. The only difference between the disaccharide unit of hyaluronan and C4S is the nature and orientation of the functional group attached at the C4 position of the amino-sugar. In hyaluronan this is a hydroxyl group in the equatorial position, whereas C4S has an axial sulphate group. The data presented here suggests that the equatorial C4 hydroxyl groups in hyaluronan may not be involved in the interaction with Link_TSG6 as they can be 'replaced' by axial sulphates in C4S without affecting the binding. Conversely, the C6 hydroxyls of hyaluronan are likely to be of functional importance in this interaction, as the presence of a sulphate group on the C6 positions (as in C6S) effectively abolishes Link_TSG6 binding. Work is currently in progress to elucidate the tertiary struc-

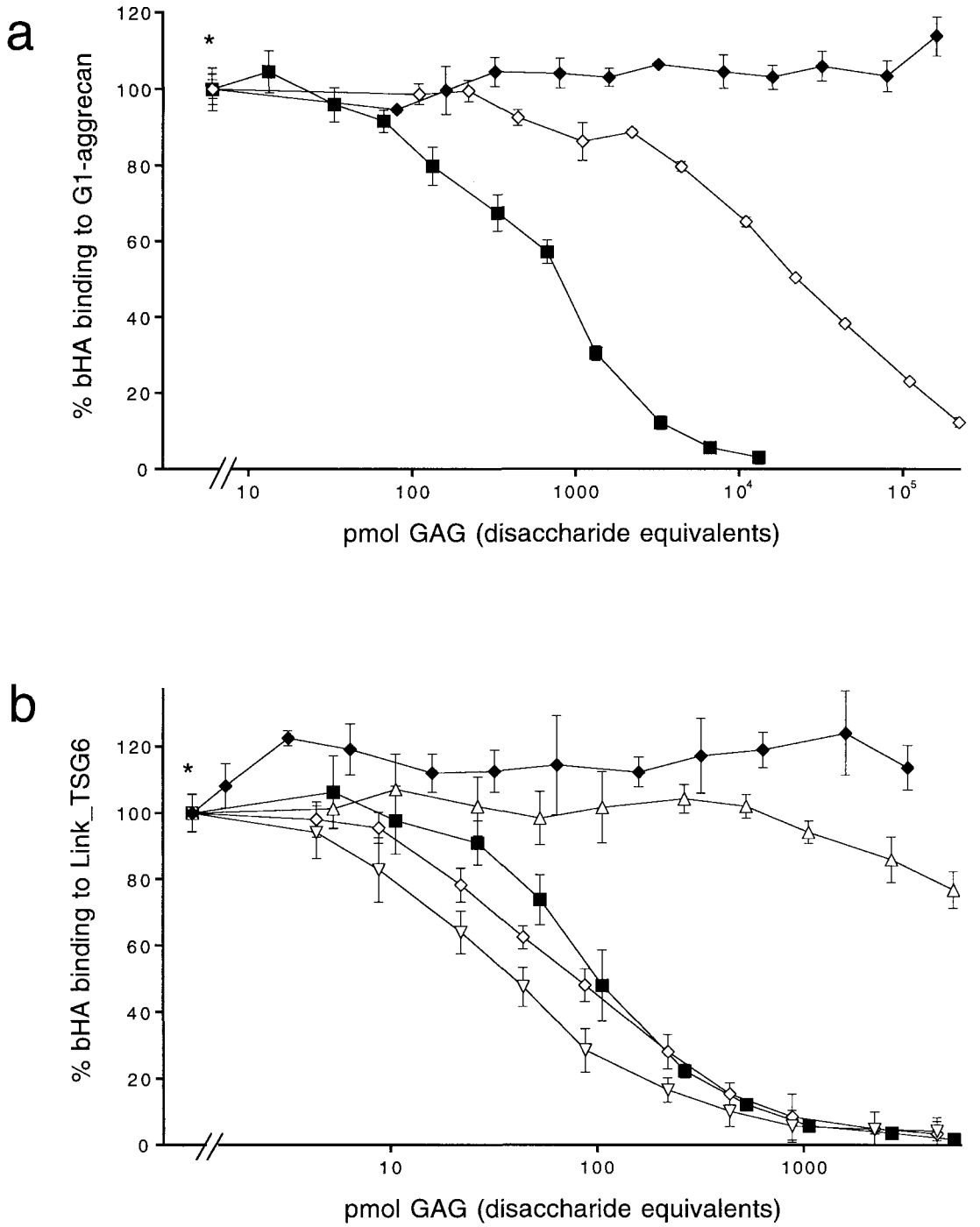

Fig. 2. Binding of bHA to G1-aggrecan (a) or Link_TSG6 (b) coated microtitre plates in the absence (*) or presence of unlabelled GAGs (■, hyaluronan; ◆, heparin; ◇, C4S/C6S; ▽, C4S; △, C6S) at pH 5.8. Binding was determined colorimetrically, by absorbace at 405 nm (after 10 min development time) and values are plotted as mean percentages ($n=3$, ±S.E.M.) of binding in the absence of competing GAG.

ture of Link_TSG6 complexed with hyaluronan. This should allow us to determine how this Link module can recognise C4S, but be unable to bind C6S.

On the basis of the competition studies presented here G1-aggrecan also binds to chondroitin sulphate, but with significantly lower affinity than to hyaluronan, and the GAG-binding sites are again likely to be overlapping. However, it has been previously shown in a similar assay, employing biotinylated-G1-aggrecan binding to a hyaluronan coated plate, that chondroitin sulphate had no effect on this interaction even when present in large excess [12]. The reason for this apparent difference is not clear, but could relate to differences in the chondroitin sulphate preparations used. Chondroitin sulphate binding to cartilage link protein and CD44 have also been investigated previously. Bovine link protein did not bind to either C4S or C6S [13]. CD44, however, has been shown to bind C4S, but in this case the interaction was $\approx 100\times$ weaker than with hyaluronan [14]. CD44 was also found to bind very

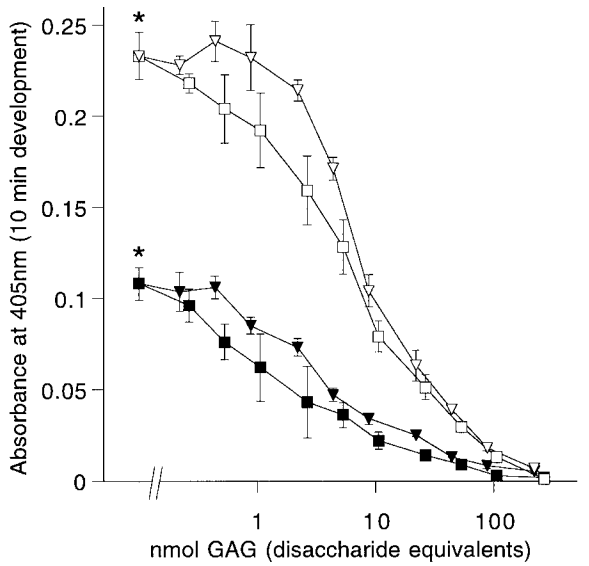

Fig. 3. Binding of mono-biotinylated Link_TSG6 to hyaluronan (closed symbols) or C4S (open symbols) coated microtitre plates in the absence (*) or presence of unlabelled hyaluronan (▩,□) or C4S (▼,▽). Values are plotted as mean absorbance ($n=3$) at 405 nm, after 10 min development time, ± S.E.M.

weakly to C6S, but not to heparin. Other studies have shown that both C4S and C6S can block the binding of soluble CD44 to cultured lymph node high endothelial cells, but ≈100-fold less effectively than hyaluronan [15]. Recently, CD44 has been shown to interact with a C4S proteoglycan, serglycin, which is secreted by hematopoetic cells and may be involved in the activation and adhesion of cytotoxic T lymphocytes [16]. The interaction between serglycin and CD44 could be inhibited by hyaluronan in a dose dependent manner indicating that the serglycin- and hyaluronan-binding sites on CD44 are likely to be overlapping. The binding of CD44 to serglycin was found to be mediated by C4S, but this interaction only had high affinity when the C4S was associated with the protein core.

As described above, TSG-6 production is induced by IL-1 and TNF in cells of articular joints [4–6]. Importantly, expression of C4S epitopes are also affected by these inflammatory mediators. IL-1 increases the level of C4S in cartilage, while reducing the production of C6S [17]. Increased levels of C4S (but not C6S) have also been found, on the GAG chains of the proteoglycan aggrecan, in articular cartilage from osteoarthritis patients compared to normals [18]. In normal human synovium, C4S is distributed evenly throughout the interstitial matrix, however, in rheumatoid synovium levels of C4S are reduced in the matrix while becoming localised to blood vessel walls [19]. In this regard, it is interesting to note that the rabbit orthologue of TSG-6 (PS4, which has 94% sequence identity with the human protein [20]) is expressed by vascular smooth muscle cells in response to IL-1 [21]. Therefore, these disease-associated changes in C4S distribution in cartilage and synovium may affect the localisation and thus the biological role of TSG-6. In addition, the expression and secretion of TSG-6 by peripheral blood monocytes is greatly up-regulated by lipopolysaccharide [4,5], which also increases the secretion of the CD44 ligand serglycin from macrophages [22]. It is possible, therefore, that TSG-6 could be involved in modulating certain aspects of CD44 function, for example the serglycin-mediated activation of cytotoxic T lymphocytes.

Here we have shown that the Link module of human TSG-6 can interact specifically with C4S at a site that is overlapping with its hyaluronan-binding surface. At present the biological relevance of the interaction between TSG-6 and C4S is not known. However, C4S-proteoglycans that are upregulated in inflammation should be considered as good candidates for TSG-6 ligands. In inflammation, changes in the expression of GAGs and GAG-binding proteins may be involved in modulating matrix breakdown and the migration of specific leukocyte populations into the tissues.

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