TSG-6 interacts with hyaluronan and aggrecan in a pH-dependent manner via a common functional element: implications for its regulation in inflamed cartilage

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Abstract Cartilage matrix is stabilised by the interactions of proteins with hyaluronan (HA). We compare the pH dependences of HA binding by aggrecan, link protein and TSG-6. Aggrecan and link protein exhibit maximal binding across a wide pH range (6.0–8.0). TSG-6, a protein that is only produced during inflammation, binds maximally at about pH 6.0 but shows a dramatic loss of function with increasing pH. TSG-6 also interacts with aggrecan, with a similar pH dependence, and this can be inhibited by HA. Thus, a common binding surface on TSG-6 may be involved in HA and aggrecan binding. We propose that TSG-6 is involved in matrix dissociation and that this is regulated by pH gradients in cartilage.

Key words: TSG-6; Aggrecan; Hyaluronan; pH-dependent ligand binding; Cartilage; Inflammation

1. Introduction

Hyaluronan (HA) is a glycosaminoglycan (GAG), of very high molecular weight (10^5–10^7 Da), comprised of the repeating disaccharide, D-glucuronic acid (β1→3) *N*-acetyl-D-glucosamine (β1→4). It is found in all organs and body fluids of adult vertebrates, particularly in connective tissues (e.g. synovial fluid, skin and cartilage), and is especially abundant during development and morphogenesis [1]. HA has a central role in the formation and stability of extracellular matrix by its interactions with HA-binding proteins and receptors [2]. These interactions are usually mediated by a common protein domain (of approximately 100 amino acids in length), termed a Link module or proteoglycan tandem repeat (see [3]). In cartilage three members of the Link module superfamily, Link protein (Lp), aggrecan and CD44, have been particularly well characterised. The roles of these molecules, and the recently discovered cartilage protein TSG-6 (the product of tumour necrosis factor (TNF)-stimulated gene-6), are reviewed in brief below.

The interaction of the proteoglycan aggrecan with HA is mediated by its N-terminal G1 domain [4,5] which is comprised of an immunoglobulin module followed by two contiguous Link modules. The same organisation of modules is found in Lp, where the Link modules are both involved in binding to HA and the immunoglobulin module mediates the interaction with G1 [6]. Electron microscopy has revealed that a dense array of alternating aggrecan and Lp molecules forms along a central hyaluronan filament [7]. These huge multimolecular structures can contain over 100 molecules of both Lp and aggrecan associated with a single HA chain, and give rise to aggregates with molecular masses of 10^8–10^9 Da (see [8]). These aggregates are hydrated, due to their high fixed negative charge resulting from the very large numbers of sulphated GAG chains on aggrecan, and constitute a space-filling gel that provides cartilage with its load bearing properties.

The major HA receptor CD44 contains a single Link module that is involved in HA binding [9,10]. In cartilage CD44 has an important role in the assembly and organisation of chondrocyte pericellular matrix by mediating the attachment of HA-proteoglycan aggregates to the cell surface [11].

TSG-6 contains a single Link module [12] that has been shown to interact specifically with HA [3,13]. There is little or no constitutive expression of TSG-6 mRNA in chondrocytes in vitro, but rapid transcription and subsequent secretion of the gene product is seen after treatment with interleukin-1 (IL-1) or TNF [14,15]. TSG-6 protein has been found at high levels in the synovial fluids of patients with various forms of arthritis, including rheumatoid (RA) and osteoarthritis (OA), but was not detected in synovial fluids from individuals without known joint disease [16]. Immunolocalisation studies have shown that there is no detectable TSG-6 in normal human articular cartilage, while in OA and RA there is significant staining of the chondrocytes and their associated pericellular matrices, especially in the deep layer of the tissue (S.L.T. Howat, A.J. Day and M.T. Bayliss, unpublished data). Therefore, it seems likely that TSG-6 is produced in cartilage during inflammation. IL-1 and TNF are well known to have a central role in the induction of cartilage breakdown in arthritis (see [17]), which suggests that TSG-6 may be involved in these processes [12,16]. In apparent contradiction to this hypothesis TSG-6 has been shown to potentiate the inhibition of plasmin (which has an important role in the activation of latent metalloproteinases involved in extracellular matrix degradation; see

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Abbreviations: bA-Link_TSG6, mono-biotinylated-Link_TSG6; b-G1, biotinylated G1; bHA, biotinylated HA; IαI, inter-α inhibitor; DS_{equiv}, molar disaccharide equivalents; G1, G1 domain of aggrecan; GAG, glycosaminoglycan; HA, hyaluronan; HEPES, *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]; IL-1, interleukin-1; Lp, link protein; Link_TSG6, the recombinant Link module from human TSG-6; NMR, nuclear magnetic resonance; OA, osteoarthritis; RA, rheumatoid arthritis; SAB, standard assay buffer; TNF, tumour necrosis factor; TSG-6, TNF-stimulated gene-6

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[18]) by inter-α inhibitor (IαI), with which TSG-6 forms a covalent complex [19].

Recently we have expressed the Link module from TSG-6 (denoted Link_TSG6) in *E. coli* [20,21] and this has been used to determine its tertiary structure by nuclear magnetic resonance (NMR) spectroscopy [3]. In addition, we have demonstrated that Link_TSG6 interacts with HA using microtitre plate assays [3,13]. Here we show that the pH dependence of HA binding by Link_TSG6 is strikingly different to that of Lp and the G1 domain of aggrecan (G1). Aggrecan is identified as a novel ligand for TSG-6 and the pH dependence of this interaction is shown to be very similar to that of HA binding to Link_TSG6. Our results have important implications for cartilage where different pH environments may modulate alternative functions of TSG-6.

2. Materials and methods

2.1. Materials

Microtitre plates, development reagents, human umbilical cord HA (mean molecular weight 4.4×10^6 Da), 250 kDa biotinylated HA (bHA), the Link module from human TSG-6 (denoted Link_TSG6) and mono-biotinylated Link_TSG6 (bA-Link_TSG6) used in the binding assays were as described previously [3,13]. Lp, G1 and biotinylated G1 (b-G1) were prepared from porcine laryngeal cartilage as described before [4,22]; Lp was stored in 4 M guanidine-HCl, 50 mM Na-acetate, 1 mM Na_2-EDTA, pH 5.8, due to its low solubility in water. Both G1 and b-G1, following biotinylation in the presence of HA, were purified by gel filtration under dissociative conditions (i.e. to remove HA) [4,22]. These preparations re-chromatographed as monomers under associative conditions and were shown to be free from contaminating HA by the assay of Fosang et al. [22] after digestion of protein with papain. Recombinant protein domains from human fibronectin (^{1}F1-^{2}F1 and ^{4}F1-^{5}F1) used as binding assay controls were kindly provided by Dr Jennifer Potts (Department of Biochemistry, University of Oxford).

2.2. Plate binding assays

Microtitre plate binding assays were carried out to investigate the effect of pH on the interactions of HA with Link_TSG6, G1 and Lp; and whether Link_TSG6 interacts directly with G1. The assays, which are based on those described previously [3,13], all determine colorimetrically the level of binding of biotinylated ligand (i.e. bHA, bA-Link_TSG6 or b-G1) to wells coated with either HA or protein.

2.2.1. Standard assay. All dilutions, incubations and washes were performed in standard assay buffer (SAB: 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, or HA, in 20 mM Na_2CO_3 pH 9.6. Control wells were treated with buffer alone. The coating solution was removed and the plates were washed three times with SAB. Non-specific binding sites were blocked by incubation with 1% (w/v) bovine serum albumin for 90 min at 37°C, followed by three washes. Biotinylated ligand (200 μl) was added to each well and incubated for 4 h or overnight. Plates were washed three times and 200 μl of a 1 in 10 000 dilution of Extra-Avidin alkaline phosphatase (in SAB) was added and incubated for 30 min, followed by three washes. A 1 mg/ml solution (200 μl/well) of disodium *p*-nitrophenyl-phosphate, 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. The absorbance at 405 nm was determined on a microtitre plate reader (MKII Titertek Multiscan Plus). All absorbances were corrected against blank wells and were then standardised to a 10 min development time.

2.2.2. pH dependence of hyaluronan interactions. The effect of pH on the interaction of bHA with plates coated with Link_TSG6 (22 pmol/well), G1 (18 pmol/well) or Lp (2.5 pmol/well) was investigated using a modification of the above assay. Plates were coated, blocked and washed as described for the standard assay. Following this, further dilutions, incubations and washes were performed in 100 mM NaCl, 0.05% (v/v) Tween 20, buffered to a specific pH: 50 mM Na-acetate was used from pH 3.5 to 6.5 and 50 mM Na-HEPES (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]) between pH 6.0 and 8.0. The pH values of all buffers (for washing) and bHA solutions were individually checked and adjusted if necessary. Wells were re-washed, incubated with 12.5 ng (33 pmol DS_{equiv})[2] bHA and washed three times in buffer of the appropriate pH. The amount of bHA binding was determined as described above for the standard assay. This assay was repeated at pH 5.8 (Na-acetate buffer) and pH 7.4 (Na-HEPES buffer) on plates coated with 25 pmol of Link_TSG6, G1, Lp, ^{1}F1-^{2}F1 or ^{4}F1-^{5}F1 per well.

The effect of pH on the binding of bA-Link_TSG6 to HA was also investigated. Plates were coated with 5 μg/well (13.3 nmol DS_{equiv}) HA, blocked and washed as described for the standard assay. Wells were then re-washed, incubated with 2 pmol bA-Link_TSG6 and washed three times in the appropriate pH buffer as above, except that in this assay 0.2% (v/v) Tween 20 was used. An increased concentration of detergent was necessary to avoid precipitation of bA-Link_TSG6 at certain pH values. Plates were developed as before.

2.2.3. Binding of G1 to Link_TSG6. The possibility that TSG-6 could interact directly with G1 was initially investigated at pH 5.8. The binding of b-G1 (1.5 pmol/well) to plates coated with 25 pmol/well of Link_TSG6, ^{1}F1-^{2}F1 or ^{4}F1-^{5}F1 was determined under standard assay conditions. In addition the binding of bA-Link_TSG6 (2 pmol/well) to plates coated with 25 pmol/well of G1, ^{1}F1-^{2}F1 or ^{4}F1-^{5}F1 was also determined under these conditions.

The binding of b-G1 to Link_TSG6 (coated at 22 pmol/well, as described above) was then determined for a range of pH values (3.5–8.0); further dilutions, incubations (with 1.5 pmol b-G1 per well) and washes were performed in 100 mM NaCl, 0.2% (v/v) Tween 20, buffered to a specific pH (with either 50 mM Na-acetate pH 3.5–6.5 or 50 mM Na-HEPES pH 6.0–8.0) as described in Section 2.2.2. Plates were then treated as for the standard assay.

The specificity of G1-aggrecan binding to Link_TSG6 coated plates (22 pmol/well) was investigated at pH 5.8 (in SAB with 0.2% (v/v) Tween 20) by incubating 1.5 pmol b-G1 (per well) in the absence or presence of unlabelled G1 (0.1–200 pmol/well) or HA (0.125–250 nmol DS_{equiv}/well).

2.3. One-dimensional NMR spectroscopy

About 10 mg of lyophilised Link_TSG6 were resuspended in 600 μl 10% (v/v) D_20, 0.02% (w/v) NaN_3 resulting in an ∼1.5 mM protein solution. The pH was carefully adjusted to the desired value (pH 3.5 to 8.5 in intervals of 0.5) by the addition of 1 M or 0.1 M NaOH. After each pH adjustment any precipitated protein was removed by centrifugation and a one-dimensional (1-D) NMR spectrum was recorded at 35°C on a 500 MHz home-built/GE Omega spectrometer; the residual solvent signal was suppressed by presaturation. The NMR data were processed using FELIX 2.3 (Biosym Inc.), applying deconvolution and a sinesquare window function for resolution enhancement. Proton chemical shifts were referenced to the H_2O signal at 4.7 ppm.

3. Results

3.1. The pH dependence of HA binding to TSG-6 compared to aggrecan and Lp

Recently we have shown by microtitre plate assays that the recombinant Link module of human TSG-6 (Link_TSG6) interacts specifically with HA [3,13]. Here we investigate the effect of pH on this interaction and compare it with that of aggrecan and Lp with HA. Fig. 1a,b shows the effects of pH on bHA binding to G1 and Lp coated plates; giving similar shaped curves, with minimal bHA binding at pH 3.5 and close to maximal binding across the range pH 6.0–8.0. These results are consistent with those for HA binding to G1 and Lp determined previously by viscosity measurements [23,24] and analytical ultracentrifugation [25], respectively. In the case

[2] Hyaluronan and bHA concentrations are expressed throughout as molar disaccharide equivalants (DS_{equiv}), i.e. the uronic acid concentration.

of Link_TSG6 (Fig. 1c) there is also minimal binding at pH 3.5 which increases to a maximum at about pH 5.5. However, unlike G1 and Lp, increasing the pH from 6.0 to 8.0 causes a reduction in the binding of bHA to Link_TSG6 to almost zero. In order to investigate whether this loss of binding activity was an artefact of the Link_TSG6 being immobilised on the microtitre plate, an assay was performed with protein (bA-Link_TSG6) in solution binding to an HA coated plate. From Fig. 1d it can be seen that the overall shape of the binding curve is very similar to that in Fig. 1c with loss of Link_TSG6 function as the pH is increased from 6.0 to 8.0. This demonstrates that HA binding is similarly effected by pH whether Link_TSG6 is immobilised or in solution.

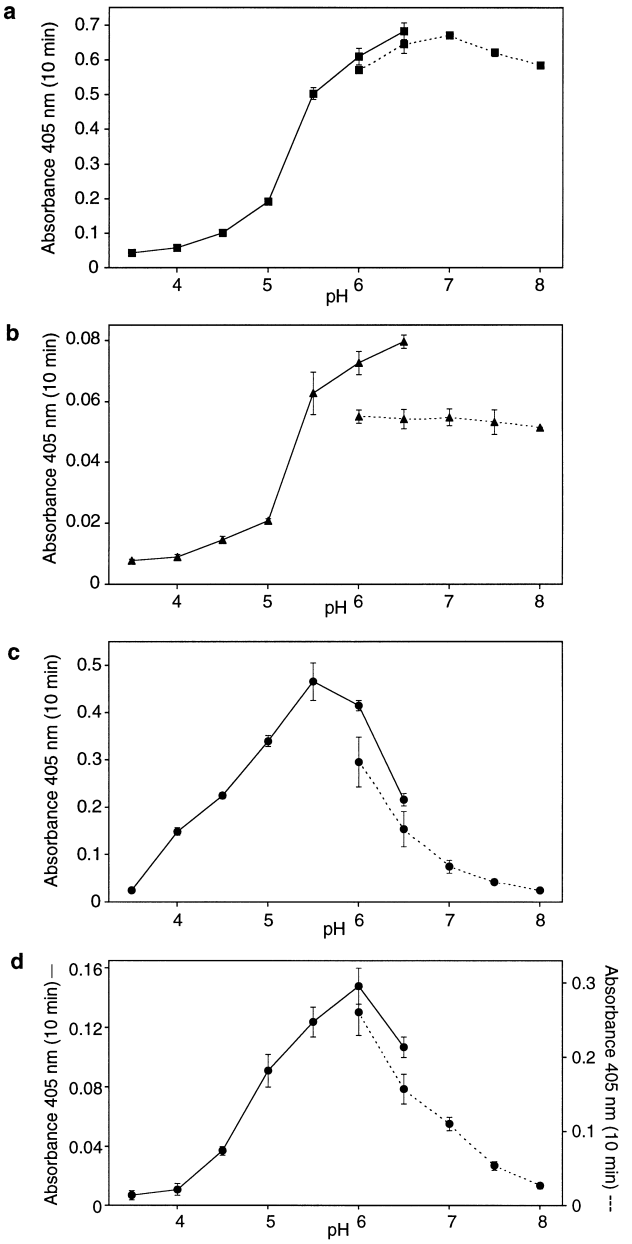

Fig. 1. The pH dependences of HA binding to G1, Lp and Link_TSG6. The binding of bHA to wells coated with G1 (a), Lp (b) or Link_TSG6 (c), and bA-Link_TSG6 binding to wells coated with HA (d), were measured colorimetrically. Values are plotted as mean absorbance ($n=3$), at 405 nm after 10 min development time ± S.E.M. The assays were performed over a range of pH values in two different buffer systems (Na-acetate, solid lines; Na-HEPES, dotted lines). In (d) absorbance values (from each of the buffers) are plotted on separate axes.

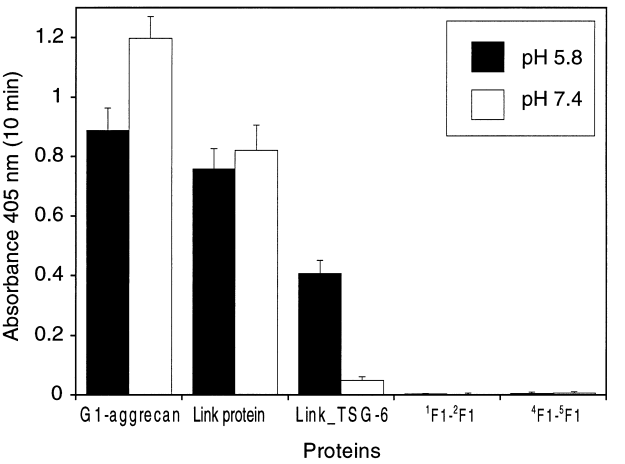

Fig. 2. The binding of biotinylated-hyaluronan (bHA), at pH 5.8 or 7.4, to plates coated with 25 pmol G1, Lp, Link_TSG6, ^{1}F1-^{2}F1 and ^{4}F1-^{5}F1. Values are plotted as mean absorbance ($n=4$) at 405 nm after 10 min development time ± S.E.M.

It should be noted that for Lp (Fig. 1b) the level of bHA binding was somewhat lower (about 30%) in the Na-HEPES buffer system than in Na-acetate and for bA-Link_TSG6 binding to HA (Fig. 1d) this was approximately two times greater in Na-HEPES than in Na-acetate. The cause of these buffer effects has not been investigated further.

To test the specificity of the bHA binding assay, microtitre plates were coated with 25 pmol per well of G1, Lp, Link_TSG6, or fragments of human fibronectin (^{1}F1-^{2}F1, ^{4}F1-^{5}F1) for which there is no evidence of HA binding. These 'module pairs' from fibronectin were chosen as controls as they were produced by recombinant expression and are both of similar size to Link_TSG6 (i.e. ~10 kDa). Fig. 2 shows that the results of this assay are consistent with those in Fig. 1: there is a similar level of binding of bHA to Lp and G1 at both pH 5.8 and 7.4, but greatly decreased binding to Link_TSG6 at pH 7.4 compared to pH 5.8. There was negligible bHA binding to the control proteins under both pH conditions. In addition, the binding of bHA to G1, Lp and Link_TSG6 at pH 5.8 could be fully competed with unlabelled HA (data not shown).

3.2. *There is no gross structural change in Link_TSG6 between pH 6 and 8*

We have recently determined the tertiary structure of Link_TSG6 by NMR spectroscopy in solution at pH 6.0 [3]. The Link module has a compact fold, comprised of two α-helices and two triple-stranded antiparallel β-sheets arranged around a large hydrophobic core which gives rise to a characteristic 1-D NMR spectrum; with methyl resonances from Val-57 shifted to high field (−0.5 and −1.1 ppm) by their close proximity to Trp-51 and Trp-88 in the core. The chemical shifts of these methyl protons are very sensitive to structural change and can be used to monitor the effect of pH on the Link_TSG6 structure. Therefore, a series of 1-D spectra were collected at a range of pH values as shown in Fig. 3. The

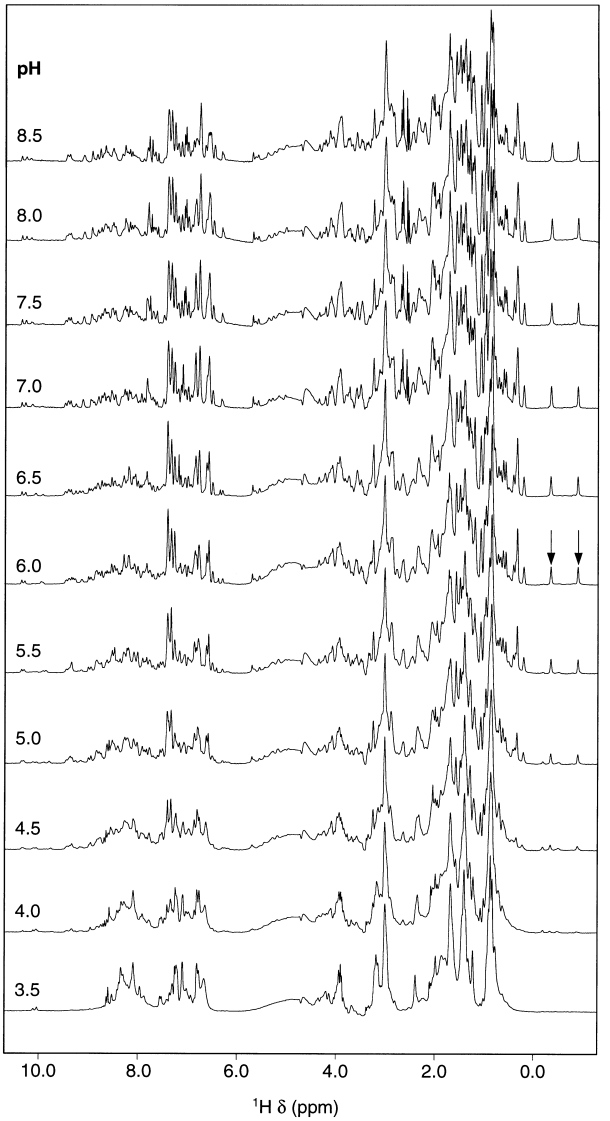

Fig. 3. One-dimensional NMR spectra of Link_TSG6 acquired at different pH values (3.5–8.5). Proton chemical shifts were referenced to H_2O at 4.7 ppm. The position of the high-field shifted methyl protons from Val-57 are indicated by arrows. The additional resonances seen between 2.5 and 3.0 ppm in the spectra at pH 7.0 and above are due to a citrate buffer impurity arising from the pH meter.

spectrum at pH 6.0 is that of the folded module with the high field shifted methyl resonances mentioned above (shown by arrows) and well-dispersed signals in the amide region (~7.5–9.5 ppm). At pH 3.5, however, the spectrum is 'collapsed' (i.e. with proton resonances at their random coil positions) with no visible signals at −0.5 or −1.1 ppm indicating that Link_TSG6 is unfolded. It is not surprising, therefore, that Link_TSG6 does not interact with HA at this pH (see Fig. 1c,d). As the pH is increased from 3.5 to 6.0, high field shifted methyls appear and increase in intensity. This demonstrates that on raising the pH there is an increasing proportion of folded protein present in the sample, that correlates with the level of Link_TSG6 binding to HA over this pH range. However, as the pH is increased from 6.0 to 8.5 there are no significant changes in the 1-D spectra (see Fig. 3) which indicates that Link_TSG6 remains fully folded under these pH conditions. Therefore, it can be concluded that the reduction in HA binding by Link_TSG6 between pH 6.0 and 8.0 is not due to a gross structural change in the Link module structure

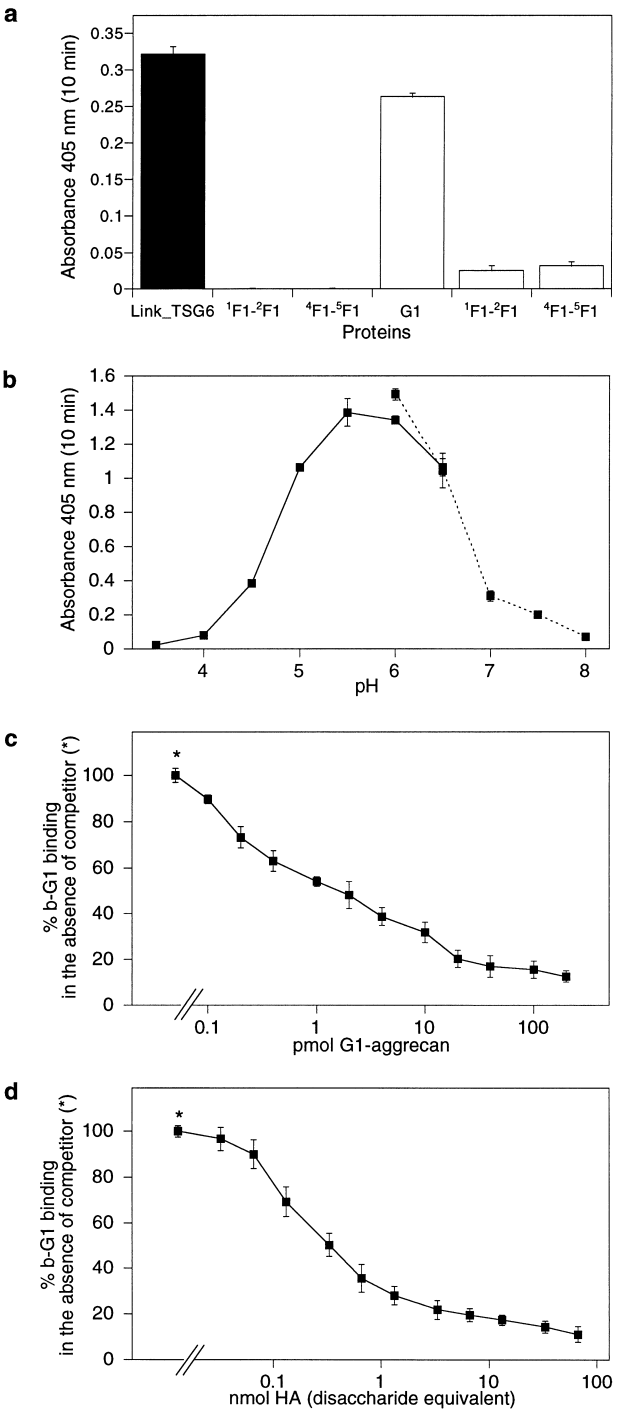

Fig. 4. The specificity and pH dependence of G1 binding to Link_TSG6. a: The level of binding of b-G1 (black bars) or bA-Link_TSG6 (white bars) to plates coated with 25 pmol/well of ^{1}F1-^{2}F1 and ^{4}F1-^{5}F1 and either Link_TSG6 or G1-aggrecan, respectively. b-d: The binding of b-G1 to plates coated with 22 pmol Link_TSG6 was determined: at a range of pHs in two buffer systems (Na-acetate, solid line; Na-HEPES, dotted line) (b); and in the absence (*) or presence of competing concentrations of unlabelled G1 (c) or HA (d). Data points are plotted as mean values ($n = 3$) ± S.E.M.

and is likely to be due to a local change in a functional element.

3.3. G1 interacts specifically with Link_TSG6

The possibility that TSG-6 could interact with aggrecan was investigated. Initially the binding of b-G1 to Link_TSG6 at pH 5.8 was compared with control proteins (^{1}F1-^{2}F1 or ^{4}F1-^{5}F1) coated on microtitre plates at 25 pmol/well. The results of this assay are shown in Fig. 4a where it can be seen that b-G1 does interact with Link_TSG6 but does not bind to the control proteins. Fig. 4a also shows that bA-Link_TSG6 binds to a G1-coated plate (25 pmol/well) with only 9.7% and 12.2% binding to wells with the same concentrations of ^{1}F1-^{2}F1 and ^{4}F1-^{5}F1, respectively. As no HA is detectable in the G1 preparations (which were purified under dissociative conditions; see Section 2.1) it is very unlikely that the interaction of Link_TSG6 with G1 results from HA contamination.

The effect of pH on the interaction of b-G1 with immobilised Link_TSG6 was investigated. As shown in Fig. 4b there is minimal binding at pH 3.5, maximum binding between pH 5.5 and 6.0 and almost complete loss of binding on increasing the pH to 8.0. The specificity of this interaction at pH 5.8 (maximal binding) was determined by binding b-G1 to plates coated with Link_TSG6 in the absence or presence of unlabelled G1. As can be seen from Fig. 4c b-G1 binding can be competed by G1, with an IC_{50} value of $\sim$1.5 pmol (i.e. the amount of b-G1 present in this assay), which indicates that this interaction is specific. In addition b-G1 binding to Link_TSG6 can be inhibited by unlabelled HA (Fig. 4d), albeit at higher concentrations than with G1 ($IC_{50} \approx 330$ pmol DS_{equiv}). This suggests that either the HA and G1 binding sites on Link_TSG6 overlap or that the HA and Link_TSG6 binding sites on G1 overlap. The former is the more likely as the pH dependences of the interactions of Link_TSG6 with G1 and HA are strikingly similar (see Figs. 1c,d and 4b). Therefore, a common pH-sensitive functional element on the TSG-6 Link module may be involved in both HA and aggrecan binding.

4. Discussion

Here we have shown that the effect of pH on HA binding by the TSG-6 Link module is different from those of aggrecan and link protein; with a dramatic reduction in HA binding seen between pH 6.0 and 8.0. In addition, we have demonstrated that Link_TSG6 interacts with the aggrecan G1 domain and that this is influenced by pH in a similar manner. This loss of ligand binding is not due to a gross structural change in Link_TSG6 and, therefore, may be of functional relevance. We discuss below how such pH-dependent ligand-binding may relate to the regulation of TSG-6 functional activity in cartilage.

Cartilage is an avascular tissue with less than 5% of its volume made up of cells (chondrocytes); the rest being composed of extracellular matrix comprised of collagen fibrils and proteoglycan aggregates [8]. In this tissue there is a relatively low O_2 tension (as low as 1%) and as a consequence of this up to 95% of chondrocyte glucose metabolism occurs by anaerobic glycolysis, leading to a significant production of lactic acid (see [26,27]). The removal of lactic acid from cartilage relies solely on diffusion through the matrix, resulting in an acidification of the tissue with the lowest pH values likely to exist around the chondrocytes. The composition of the extracellular matrix itself also has an effect on the pH of cartilage; the large numbers of fixed negative charges present on the proteoglycans lead, as a consequence of the Gibbs-Donnan equilibrium, to an average interstitial pH which is 0.5 pH units lower than that of the equilibrating synovial fluid [26]. To date there have been no direct measurements of pH in articular cartilage but when the additional effect of lactic acid production is taken into account the difference between the pH of the synovial fluid and that of the deep regions of the tissue could be as great as 1 pH unit [26]. It is perhaps not surprising, therefore, that aggrecan and Lp, which have an important role in the stability and load bearing properties of healthy cartilage, can interact maximally with HA over a wide pH range (between about 6.0 and 8.0; see Fig. 1a,b).

During inflammation (e.g. in RA) there is likely to be a significant reduction in the pH of cartilage, particularly in pericellular regions, due to increased lactic acid production by chondrocytes in response to IL-1 [27]. Simkin and Bassett [28] have shown that lactic acid is the principle determinant of local acidosis in chronically inflamed synovial joints; normal synovial fluid has a pH of about 7.3 but the pH can be as low as 6.8 during inflammation. IL-1 has a central role in arthritis promoting the autolytic breakdown of the cartilaginous matrix, while inhibiting its repair (see [17,27]). In cartilage, IL-1 increases the synthesis of some matrix metalloproteinases, cytokines and NO as well as lactate, whereas, production of collagens, aggrecan and decorin is reduced. In this regard, the overall reduction in matrix protein and proteoglycan synthesis in cartilage has been shown to be directly related to the lowering of extracellular pH [29]. IL-1 is also the major stimulator of TSG-6 production by articular chondrocytes in vitro, whilst there is no constitutive expression of this protein by these cells [14,15].

As discussed above, maximal binding of Link_TSG6 to HA has a much more restricted range, than is the case for aggrecan and Lp (between about pH 5.5 and 6.0 - see Fig. 1). In inflamed cartilage, where TSG-6 is likely to be secreted by chondrocytes, there is probably a significantly lower pH than in healthy tissue. The pH gradients that exist in cartilage could mediate exquisite regulation of TSG-6 function. It should be noted that we have not examined the pH dependency of HA (or G1) binding to full-length TSG-6 and it is possible that other regions of the protein could affect this property. However, if the pH dependency we have determined for Link_TSG6 is the same as that of the intact protein, as seems likely, TSG-6 could interact strongly with both HA and aggrecan in the vicinity of the chondrocyte but these interactions would be markedly reduced as TSG-6 diffused towards the articular surface. The concept that pH gradients may have an important regulatory role in cartilage has been suggested previously for the control of proteolytic enzymes [30,31].

The interaction between G1 and Link_TSG6 (at pH 5.8) can be inhibited by HA (Fig. 4d). This indicates that the binding surfaces on TSG-6 for these ligands are likely to be overlapping and that the Link module, at least, is unable to bind to G1 and HA at the same time. As these studies have been performed on a recombinant domain of TSG-6 some caution is necessary when extrapolating these data to functions of the full-length protein. However, these results suggest that TSG-6 could be involved in the inhibition of aggrecan

and HA complex formation, regulated by the pH of the chondrocyte pericellular environment. It is also possible that TSG-6 could play a role in the dissociation of cartilage aggregates by competition of the interaction between aggrecan with HA.

TSG-6 has been shown to form a covalent complex (120 kDa) with components of the serine protease inhibitor, IαI [32]. This IαI/TSG-6 complex has been found to have significantly increased antiplasmin activity over IαI alone [19]. This is an interesting finding as plasmin plays an important role in inflammation by activating latent metalloproteinases involved in matrix degradation (see [18]). In this regard, IαI is found in the synovial fluids of RA and OA patients, but not in controls, covalently associated with HA (see [33]). The IαI/TSG-6 complex has also been detected in the synovial fluids of patients with various forms of arthritis, but at a significantly lower level than uncomplexed TSG-6 [16]. Clearly, the IαI/TSG-6 complex present in these synovial fluids is likely to have an anti-inflammatory role. To our knowledge the presence of IαI/TSG-6 has not been examined in inflamed cartilage. The studies conducted to date on the formation, and antiplasmin activity, of the IαI/TSG-6 complex have only been carried out at pH 7.4 [19,32]. Given the dramatic effect on the interaction of Link_TSG6 with HA and aggrecan it would be interesting to know if the inhibitor function of IαI/TSG-6 is also affected by pH. This is possible as the same region of TSG-6 that binds to HA and G1 may involved in the increase in the antiplasmin activity of IαI. In this regard mutations in the Link module affect this activity [19] and these amino acids are adjacent to the putative HA binding surface [3]. This raises the intriguing possibility that TSG-6 has a number of distinct, mutually exclusive, functions that are regulated by the pH of its particular location.

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