Generation of Artificial Proteoglycans Containing Glycosaminoglycan-modified CD44

DEMONSTRATION OF THE INTERACTION BETWEEN RANTES AND CHONDROITIN SULFATE*

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All CD44 isoforms are modified with chondroitin sulfate (CS), while only those containing variably spliced exon V3 are modified with both CS and heparan sulfate (HS). The CS is added to a serine-glycine (SG) site in CD44 exon E5, while HS and CS are added to the SSGS site in exon V3. Site-directed mutagenesis and other molecular biology techniques were used to determine the minimal motifs responsible for the addition of CS and HS to CD44 (see accompanying paper (Greenfield, B., Wang, W.-C., Marquardt, H., PiepKorn, M., Wolff, E. A., Aruffo, A., and Bennett, K. L. (1999) J. Biol. Chem. 274, 2511–2517)). We have used this information to generate artificial proteoglycans containing the extracellular domain of the cell adhesion protein lymphocyte function-associated antigen-3 (LFA-3) (CD58) and CD44 motifs modified with CS or a combination of CS and HS. Analysis of the CD44-modified LFA-3 protein showed that it retains the ability to engage and trigger the function of its natural ligand CD2, resulting in T cell activation. In addition, the glycosaminoglycan-modified artificial proteoglycan is capable of binding the chemokine RANTES (regulated upon activation, normally T cell expressed and secreted) and delivering it to human T cells, resulting in enhanced T cell activation. These data demonstrate that artificial proteoglycans can be engineered with functional domains that have enhanced activity by codelivering glycosaminoglycan-binding molecules. The artificial proteoglycans were also used as a model system to explore the glycosaminoglycan binding properties of basic-fibroblast growth factor and the chemokine RANTES. While basic-fibroblast growth factor was shown to bind HS alone, this model revealed that RANTES binds not only HS, as has been demonstrated in the past, but also CS. Thus, artificial proteoglycans can be used for studying the glycosaminoglycan binding patterns of growth factors and chemokines and provide a means to manipulate the levels, types, and activity of glycosaminoglycan-binding proteins in vitro and in vivo.

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§ The abbreviations used are: GAG, glycosaminoglycan; b-FGF, basic fibroblast growth factor; CD, cluster of differentiation; CS, chondroitin sulfate; ELISA, enzyme-linked immunosorbent assay; HS, heparan sulfate; LFA-3, lymphocyte function associated antigen-3; mAb, monoclonal antibody; RANTES, regulated upon activation, normally T cell expressed and secreted; Rg, recombinant immunoglobulin; SG, serine/glycine; Th1/Th2, T helper cells; wt, wild type; aa, amino acid(s).
histocompatibility complex. However, this signal alone is not sufficient to induce a proliferative activation response. Accessory molecules expressed on the surface of T cells (such as CD2) provide a requisite second signal (costimulation signal) by binding to their respective ligands on antigen presenting cells, resulting in T cell activation and proliferation (29, 30). The T cell molecule CD2 binds to lymphocyte function-associated antigen-3 (LFA-3), which is expressed on antigen presenting cells, including B cells, memory T cells, monocytes, and dendritic cells. The combination of an antigen binding to the T cell receptor and ligation of LFA-3 to CD2 on the T cell surface results in costimulation and subsequent proliferation of T cells. The stimulation of T cells can also be augmented by the presence of growth factors or chemokines (31, 32).

We designed a recombinant artificial proteoglycan with dual costimulatory activity. The dual activity stems from LFA-3 (which binds CD2 on T cells) and from GAG-modified CD44 exon V3 (which binds growth factors and chemokines). This artificial proteoglycan provides a system to study the interaction of growth factors and chemokines with GAGs and ultimately can be used as a means of directing the levels and types of chemokines and growth factors to induce a particular physiological effect. In this manuscript, we characterize the functional domains of this artificial proteoglycan and use it as a test system to extend our knowledge regarding the GAG binding characteristics of the chemokine RANTES. In addition, we demonstrate the in vitro activity of the artificial proteoglycan in a model of T cell activation.

MATERIALS AND METHODS

Construction of LFA-3 Artificial Proteoglycan Expression Vectors—The LFA-3-Rg construct has been described previously (29). Oligonucleotide primers used for inserting the CD44 V3 eight-amino acid motif (see accompanying paper (46)) after the Ser-Gly present in the extracellular domain of LFA-3 were: LFA-3-FP-HindIII, AAGCTTGGACGAGCCATGGTTGCT; LFA-3-RP-SpeI, GGATCCCGGATAAATCTTCACTCATCATCAATACCCTGCTTGGGATACAGGT. Polymerase chain reaction product was digested with HindIII and SpeI (Boehringer Mannheim), gel-purified, and ligated into a mammalian expression vector containing the sequence for an immunoglobulin constant region (Rg) as described in the accompanying paper (46).

For constructing LFA-3 extracellular domain with complete CD44 V3 domains, primers used were LFA-3-FP-HindIII and LFA-3-RP-SpeI ACTAGTTCTGTGTCTTGAATGACCGCT. Polymerase chain reaction products were digested with HindIII and SpeI (Boehringer Mannheim), gel-purified, and ligated into HindIII and SpeI (Boehringer Mannheim) cut pCAGGS-Rg or pCAGGS-Rg expression vectors described in the accompanying paper (46).

Metabolic Labeling and Enzymatic Digestion—COS cells were purchased from American Type Culture Collection (Manassas, VA) and maintained in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) with 10% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 µg/ml), and 10 µM l-glutamine. The Rg fusion proteins were produced in COS cells, radiolabeled with [35S]NaHSO4 (NEN Life Science Products), and purified from culture supernatants using protein A-Sepharose (Repligen, Cambridge, MA) as described previously (28). The labeled protein was divided into four aliquots. One aliquot was left untreated, and the others were digested for 1 h at 37°C with 50 µl/50 µl in carbonate/bicarbonate buffer (see above) overnight at 4°C. All subsequent steps were performed at room temperature. The plates were washed three times and blocked with 2% bovine serum albumin in phosphate-buffered saline for 1 h. Recombinant human b-FGF or RANTES (R & D Systems, Minneapolis, MN) was added to the wells at the concentrations indicated under “Results” and incubated for 1 h. Goat anti-sera specific for b-FGF or RANTES (R & D Systems) at 1 µg/ml was incubated for 1 h, followed by donkey anti-goat IgG conjugated to horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA) at 1:10,000 for 1 h. Chromogenic Substrate solution (Genetic Systems Corp., Seattle, WA) was added to the wells for 15 min. The reaction was stopped by the addition of 1.0 M H2SO4, and the ratio of the absorbance at 450–630 nm was read. Inhibition of b-FGF or RANTES binding was tested by treating LFA-3-V3−Rg-coated wells with heparitinase or chondroitin ABC lyase (ICN) at concentrations of 0.33–3.3 units/ml, respectively (in phosphate-buffered saline containing 50 mM NaOAc and 1 mM CaCl2, pH 7.8), 1 h at 43°C, before proceeding with the assay as described above. Results are expressed as the mean value of triplicate wells ± S.D.

Costimulation Assays with Th1 and Th2 Clones—The tetanus toxoid-reactive human T cell (Th1) clone, H1.2, and the Dermatophagoides pteronyssinus-reactive human T cell (Th2) clone, 2DP.21, were tested for their ability to proliferate in the presence of recombinant human RANTES (PeproTech, Rocky Hill, NJ) in the presence or absence of immobilized anti-CD3 mAb and/or LFA-3-V3−Rg or LFA-3-Rg alone (31). These clones have been shown previously to be chemokine-reactive by their ability to induce migration and facilitate adhesion to endothelial cells and extracellular matrix proteins. These clones were stimulated at 1:100 dilutions in 18–24 h. The plates were then harvested and counted on a liquid scintillation counter. The results are expressed as the mean number of counts/min ± S.D. All of the costimulation experiments were performed in duplicate.

RESULTS

Generation of Artificial Proteoglycans Modified with HS and CS—Proteoglycans such as CD44 capture, concentrate, and present GAG-binding proteins, including growth factors, cytokines, and chemokines, to specialized receptors. Via this mechanism, proteoglycans play a key role in regulating the activity of these GAG-binding proteins. Therefore, the ability to generate artificial proteoglycans would result in a novel class of GAG-binding proteins. Thus, the ability to redirect the activity of GAG-binding proteins in vitro and in vivo.

In the accompanying paper (46), we demonstrated that a Rg fusion protein containing CD44 exon V3 supports CS and HS assembly and that the proteoglycan is able to bind b-FGF. We also showed that CD44 exon E5 only supports HS assembly. In addition, it was shown that the GAG assembly specificity of exon V3 and E5 could be manipulated by exchanging the eight amino acids that followed the SGSG sequence in CD44 exon V3 with the eight amino acids that followed the SG sequence in exon E5. Using the knowledge of the sequence requirement that determines the specificity of GAG assembly, we decided to

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2. D. Taub, unpublished data.
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Digestion with a combination of heparitinase and chondroitin ABC lyase is required for GAG removal (apparent molecular mass 100–220 kDa), indicating that this proteoglycan is modified with both CS and HS. Retention of a small amount of the radiolabel is observed and is due to keratan sulfate and possibly other oligosaccharide modification of the CD44 (33).

Next we explored the possibility of generating an artificial proteoglycan, which was modified with CS but not HS. This was done by constructing a chimeric gene containing a sequence encoding the CD44 V3 exon with the eight amino acids derived from CD44 exon E5, which is modified with only CS (V3E5/8aa, see accompanying paper (46)). The resulting fusion protein, LFA-3/V3E5/8aa-Rg (Fig. 2), is modified exclusively with CS and retains the ability to bind chemokines or growth factors via heparan sulfate or chondroitin sulfate to cell surface receptors.

We initiated our investigation on the preparation of artificial proteoglycans by generating an LFA-3 (CD58) recombinant fusion protein. LFA-3 was chosen for two reasons: first, it is a very well characterized single chain type I membrane protein; and second, it is a ligand for the T cell antigen CD2 and has an easily measurable costimulatory activity. The binding of a mononclonal antibody to CD3 (complexed with the T cell receptor) mimics antigen binding in the context of major histocompatibility complex to the T cell surface and induces proliferation of the T cells. This, in combination with the ligation of LFA-3 to CD2, results in costimulation and enhanced proliferation of T cells (29). The chemokine RANTES RANTES is also capable of providing a costimulatory signal in combination with anti-CD3 antibody, which results in enhanced T cell proliferation (31). The interactions between these T cell signaling molecules and their natural ligands are represented in Fig. 1A. A model of the corresponding signaling pathways, which could be activated by an artificial proteoglycan consisting of LFA-3 combined with GAG-modified CD44 (indicated by V3wt), is shown in Fig. 1B.

To generate a GAG-modified LFA-3-Rg fusion protein, the sequence coding for CD44 exon V3 (wild type) was placed between the LFA-3 extracellular domain and the Rg domain, thereby creating LFA-3/V3wt-Rg (Fig. 2). Analysis of the GAG modification on LFA-3/V3wt-Rg is shown in Fig. 3, left panel.
modification altered the CD2 binding and costimulatory properties of LFA-3. The ability of LFA-3/V3wt-Rg to drive Th1 and Th2 cell proliferation in the presence of anti-CD3 mAb showed that inclusion of the CD44-derived sequences and GAG modification does not affect the ability of the LFA-3 moiety to bind CD2 and engage its costimulatory function (Fig. 6).

The above experiments demonstrate that a recombinant artificial proteoglycan can be generated with two independent artificial proteoglycans containing CD44.

**Fig. 3.** Left panel, the chimeric LFA-3 protein containing CD44 exon V3 is modified with CS and HS. Right panel, the LFA-3 protein containing CD44 mutant V3 exon, V3E5/8aa, is modified with CS.

**Fig. 4.** GAG-modified LFA-3 interacts with growth factors. **A**, ELISA binding activity of b-FGF to recombinant proteoglycan. B-FGF binds to GAG-modified LFA-3/V3wt-Rg (□) but not LFA-3-Rg (○). Binding was detected using goat anti-serum specific for b-FGF, followed by donkey anti-goat IgG HRP. **B**, analysis of b-FGF binding to enzyme-treated recombinant proteoglycan. B-FGF binding to LFA-3/V3wt-Rg treated with heparitinase (■), chondroitin ABC lyase (○), or untreated (□) was compared by ELISA.

**Fig. 5.** A, GAG-modified LFA-3 interacts with chemokines. The binding activity of RANTES to recombinant proteoglycan was measured by ELISA. RANTES binds to GAG-modified LFA-3/V3wt-Rg (□) but not LFA-3-Rg (○). **B**, analysis of RANTES binding to enzyme-treated recombinant proteoglycan. The binding activity of RANTES to LFA-3/V3wt-Rg treated with heparitinase (■), chondroitin ABC lyase (○), both enzymes (●), or untreated (□) was compared by ELISA.
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FIG. 6. GAG-modified LFA-3 in combination with anti-CD3 mAb induces proliferation of human T cell clones. Human Th1 and Th2 clones were incubated on plates coated with LFA-3/V3wt-Rg (white columns), anti-CD3 mAb (black columns), or a combination of both LFA-3/V3wt-Rg and anti-CD3 mAb (gray columns). Cell proliferation was measured by [3H]thymidine uptake.

FIG. 7. Delivery of RANTES with the artificial proteoglycan LFA-3/V3wt-Rg enhances T cell proliferation of human Th1 and Th2 cell clones. Plots were generated using the method described in Fig. 6 with [3H]thymidine incorporation as a measure of proliferation. The LFA-3/V3wt-Rg and LFA-3/V3E5/8aa-Rg constructs reported in this study provide examples of these artificial proteoglycans. In these examples, the chimeric protein has three functional domains. The amino-terminal domain is a receptor-targeting domain, LFA-3, which binds to CD2. The second domain contains the GAG polymer (either HS and CS in the V3wt-containing protein or CS in the V3E5/8aa-containing protein). The third domain is an immunoglobulin constant region, which binds to molecules known to interact with the constant region of human IgG1. We were concerned that the inclusion of the GAG-modified domain would perturb the function of the other polypeptides in the chimera, and thus we examined in detail the functional properties of each of the domains of LFA-3/V3wt-Rg. All of the domains of this artificial proteoglycan function as expected. The CD44-V3 domain is modified with HS and CS and is capable of binding b-FGF and RANTES. The LFA-3 domain is capable of engaging CD2 and triggering a costimulatory signal, and the immunoglobulin domain binds anti-IgG1 antibodies and protein A (allowing for a simple method of purification).

The generation of artificial proteoglycans provides a system for studying the binding of growth factors and chemokines to GAGs and led us to the finding that the chemokine RANTES binds CS. The ability of RANTES to bind CS has broad implications with respect to the identity of proteoglycans responsible for chemokine presentation. Chemokines participate in the regulation of immune responses by acting as chemoattractants and activators of leukocytes. The recruitment of leukocytes to sites of inflammation requires the formation and maintenance of chemokine gradients, which are achieved by the interaction of chemokines with proteoglycans. The capture of chemokines by endothelial proteoglycans provides the mechanism to concentrate and present chemokines in biologically active form to the signaling receptors of passing leukocytes (22, 24, 25, 38). One of the proteoglycans proposed to participate in this process is CD44. The standard CD44 isoform (CD44H) contains no functional domains. Next we tested whether the two domains can act in concert to enhance a biological activity.

Delivery of RANTES with Artificial Proteoglycan LFA-3/V3wt-Rg Enhances LFA-3/CD3 T Cell Proliferation of Human Th1 and Th2 Cell Clones—Studies have demonstrated that soluble chemokines exert a costimulatory effect on T cell activation and proliferation if added in combination with the proper T cell stimulus (31). For example, the chemokine RANTES is capable of stimulating human T cell proliferation in the presence of anti-CD3 mAb. To further test the artificial proteoglycan, we examined the costimulatory effects of RANTES bound to LFA-3/V3wt-Rg on Th1 or Th2 cell clones using plates coated with anti-CD3 mAb and either LFA-3/V3wt-Rg or LFA-3-Rg. The cultures were examined 72 h later to assess effects on cell proliferation. In these studies, RANTES was incubated for 2 h on precoated plates and then washed to remove nonbound chemokine before adding T cell clones. Thus, the responses observed in these studies are only examining the presentation of RANTES by the ligands bound to the plate. Parallel samples with and without RANTES were tested, and the difference in signal given by the samples with RANTES over that of the corresponding controls without RANTES is shown in Fig. 7. These results demonstrate that the chemokine RANTES induces an increase in proliferation in response to immobilized LFA-3/V3wt-Rg and anti-CD3 mAb, as compared with LFA-3-Rg and anti-CD3 mAb. These data show that it is possible to make artificial proteoglycans with multiple functional domains and that their activity is enhanced by codelivering biologically active GAG binding molecules.

DISCUSSION

In the accompanying paper (46), we showed that a recombinant form of CD44 exon V3 alone could be expressed as a HS- and CS-modified proteoglycan. In contrast, a mutant form of this exon, in which the eight residues located downstream of the SGSG site were replaced with those located downstream of the first SG site in exon E5 (V3E5/8aa), was modified with only CS. These observations led us to examine whether we could generate artificial proteoglycans containing both CS and HS or CS only. We designed artificial proteoglycans by preparing chimeric proteins containing CD44 exon V3, which would be modified with HS and CS, or proteins containing the mutant CD44 exon V3E5/8aa, which would be modified with only CS.
chemokine RANTES but not the HS-binding growth factor b-FGF. On the other hand, CD44 isoforms containing variably spliced exon V3, which are expressed by activated monocytes, dendritic cells, and keratinocytes, are modified with HS and CS and are capable of binding both RANTES and b-FGF. These findings indicate that the alternative splicing of CD44 provides a molecular mechanism for CD44 to function as an immune regulator, by determining which growth factors and/or chemokines are localized and presented. The ability of CS-modified CD44 to bind RANTES indicates that CS-modified proteoglycans play an important role in adding diversity to the process of chemokine binding and presentation. There is evidence to suggest that CS-modified proteoglycans are more abundant than HS-modified proteoglycans (39, 40) and, as such, may play a dominant role in chemokine activity.

The artificial proteoglycan LFA-3/V3wt-Rg was tested for its ability to deliver the chemokine RANTES and enhance the biological activity of the targeting domain. Soluble chemokines have been shown to exert a costimulatory effect on T cell activation and proliferation if added in combination with the proper T cell stimulus (31). An increase in proliferation of T cell clones was observed when RANTES was added to LFA-3/V3wt-Rg and anti-CD3 mAb-coated plates as compared with immobilized LFA-3-Rg and anti-CD3 mAb. In addition, human T cells exhibited potent chemotaxis in response to C-C chemokines immobilized on LFA-3/V3wt-Rg, but not LFA-3-Rg-, coated polycarbonate filters (data not shown), strongly supporting the presentation ability of our recombinant CD44 construct. Taken together, these data demonstrate that artificial proteoglycans can be engineered such that the biological activity of the targeting domain can be enhanced by delivering GAG-binding molecules.

We attempted to generate an artificial proteoglycan by placing the eight aa located downstream of the SGG site in CD44 exon V3 downstream of a natural SG site in a polypeptide typically not modified with GAG chains. These studies were performed with LFA-3, which contains a single SG and does not normally support GAG assembly. The placement of the eight amino acids (IDDDEDPF) from CD44 exon V3 downstream of the SG site in LFA-3 did not result in CS and/or HS assembly.3 This supports the notion that the enzymes responsible for GAG assembly recognize the SG site to be modified in a three-dimensional context. This is consistent with the observations that not all SG sites support GAG assembly. Such a structural requirement for GAG assembly has been observed in the analysis of decorin (41).

The ability to generate artificial proteoglycans was attractive in light of recent evidence which indicates that proteoglycans play an important role in regulating the activity of GAG binding molecules, including growth factors and chemokines (42). This regulatory function is mediated in part by the ability of proteoglycans to bind multiple copies of GAG-binding proteins, prolonging their high local concentration, and increasing the likelihood that they will encounter, bind, and drive the oligomerization of their specific signaling receptors. There is also evidence that some GAG-binding proteins, upon interaction with GAGs, induce dimerization of their receptors, potentially changing their conformation, creating a more favorable environment for ligand driven receptor signaling (43). In addition, there appears to be a significant level of specificity in the interaction between GAG-binding proteins and GAGs. For example, certain HS-binding proteins can bind only to a subset of HS-modified proteoglycans (44). This specificity is thought to be driven by HS heterogeneity arising from distinct levels of sulfation, disaccharide composition, and chain length (45). Differences between the abilities of GAG-binding proteins to bind CS or HS also impart specificity to interactions with proteoglycans, as shown by the comparison of b-FGF and RANTES binding to CD44. Thus artificial proteoglycans containing targeting domains and/or functional domains can be used to manipulate the levels, types, and activity of GAG-binding proteins in vitro and in vivo. In addition, artificial proteoglycans provide a convenient in vitro system to study the binding relationships between GAGs and the proteins with which they interact.

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