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REVIEW ARTICLE

Molecular Organization, Structural Features, and Ligand Binding Characteristics of CD44, a Highly Variable Cell Surface Glycoprotein With Multiple Functions

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ABSTRACT **CD44 is a type I transmembrane protein and member of the cartilage link protein family. It is involved in cell-cell and cell-matrix interactions and signal transduction. Several CD44 ligands have been identified. CD44 is a major cell surface receptor for hyaluronan, a component of the extracellular matrix. It is implicated in diseases such as cancer and inflammation and therefore intensely studied. A characteristic feature of CD44 is the occurrence of many isoforms that are expressed in a cell-specific manner and differentially glycosylated. Although a number of CD44 isoforms have been characterized, the structural diversity of CD44 makes it often challenging to study (isoform-specific) CD44-ligand interactions at the molecular level of detail. The structural organization and ligand binding characteristics of CD44 are focal points of this review. On the basis of recent structural and mutagenesis studies, details of the CD44-hyaluronan interaction are beginning to be understood. Proteins 2000;39:103–111.** © 2000 Wiley-Liss, Inc.

Key words: CD44; isoforms; link proteins; proteoglycans; extracellular matrix; cellular interactions; hyaluronan binding

INTRODUCTION

CD44 is under intense investigation in many laboratories. During the period of 1998 through August 1999 a total of 654 new MEDLINE[1] entries have appeared reporting studies on CD44. Many of these investigations focus on the role of CD44 in human disease. For example, 276 of 654 reports describe CD44 in the context of cancer and metastasis. The great interest in CD44 as a disease marker and target for therapeutic intervention is based on a number of key findings. Specific isoforms of CD44 were shown to render tumor cell lines aggressively metastatic and, in addition, tumor cells often express unique patterns of CD44 isoforms.[2,3] Furthermore, CD44-ligand interactions can cause the recruitment of leukocytes to vascular endothelium at sites of inflammation,[4,5] a key event in the course of an inflammatory response. However, in vitro analysis of CD44 is often complicated by the fact that its functional profile depends, at least in part, on the expression of cell-specific isoforms and extensive posttranslational modifications[6,7] and thus on specific cellular environments.[8]

Like many cell adhesion molecules and cell surface proteins of the immune system, CD44 is a single-path transmembrane protein with extracellular domains that are flexibly linked to the transmembrane segment. Therefore, extracellular domains of these proteins can often be expressed in soluble recombinant form and studied in vitro. In consequence, significant progress has been made in recent years in understanding the three-dimensional (3D) structures of extracellular binding domains.[9] Another characteristic feature of these cell surface proteins is that they often belong to large protein families or superfamilies.[9] This is also the case for CD44 that belongs to the hyaloadherin[10] or link protein[11] superfamily (LPSF), named after cartilage link protein (CLP).[12] Members of the LPSF contain similar link (homology) modules. LPSF proteins can be extensively glycosylated and typically include additions of glycosaminoglycans (chondroitin, heparan, and keratan sulfate) and are thus termed proteoglycans.[13] The LPSF includes extracellular matrix proteins such as aggrecan or versican and proteins like CD44 or tumor necrosis factor-inducible protein (TSG-6).[14] Recently, another hyaluronan-binding protein and close homolog of CD44 (~40% sequence similarity), named LYVE-1, was identified as new member of this family.[15] Another significant event for the LPSF field has been the determination of the solution structure of the link module of TSG-6, providing a prototypic 3D structure for the LPSF.[16,17]

The following discussion focuses on what has so far been learned about the molecular structure of CD44, at both the genomic and protein level. The discussion attempts to reflect our current understanding of how structural fea-

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tures relate to ligand binding properties of CD44. Binding of the glycosaminoglycan hyaluronan to CD44 accounts for many of its biological functions, and the HA binding site in the link module of CD44 has recently been mapped.

DISCUSSION

Identification and Cloning of CD44

CD44 was initially identified as a novel cell surface protein on leukocytes by use of a monoclonal antibody (mAb).[18] It was subsequently shown that at least two other (putatively distinct) transmembrane proteins, identified using different monoclonal antibodies (mAbs), were identical to CD44.[19,20] Monoclonal antibodies in conjunction with expression cloning and, in an independent study, screening of lymphocyte complementary DNA (cDNA) libraries were used to isolate cDNA clones of a major isoform of CD44 expressed on hematopoietic cells (CD44H).[21,22] The deduced amino acid sequence of this 90 kDa form revealed a type I transmembrane protein (i.e., the N-terminus is located outside and the C-terminus inside the cell) with an extracellular domain consisting of 248 amino acids, a 21-residue transmembrane segment, and a cytoplasmic domain of 72 residues. The N-terminal ~130 residues (including the link module) showed homology to cartilage link proteins. Figure 1 shows the molecular organization of CD44. It should be noted that the 341 residues of CD44H account for a molecular mass of only ~37 kDa. The apparent molecular mass of 90 kDa is due to extensive N-linked and O-linked glycosylation of the extracellular region, emphasizing the proteoglycan nature of CD44. Other isoforms of CD44 were cloned[23,24] including an epithelial cell form of CD44 (CD44E).[24] When compared to CD44H, this 150 kDa form showed an insertion of 135 amino acids in the extracellular region.

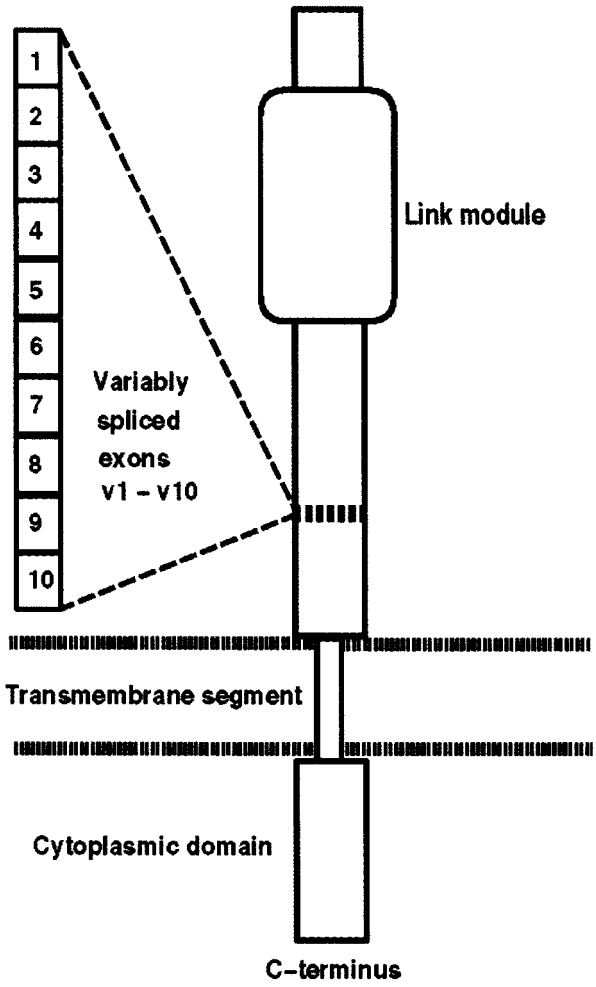

Fig. 1. Schematic representation of CD44. Variably spliced exons v1-v10 can be inserted in different combinations into the membrane-proximal extracellular region and produce a variety of CD44 isoforms. All isoforms contain the link module. The relative dimensions of the different domains do not strictly correlate with the number of residues.

CD44 Isoforms

Initial analysis of the genomic structure of CD44 revealed the presence of 19 exons (e1–e19).[25] Two additional exons, e6a (located between e5 and e6)[26] and e13a (between e13 and e14),[27] were subsequently identified. Thus, the genomic structure of CD44 consists of 21 exons, at least 11 of which can be variably spliced. These are exons e6a–e14 (corresponding to variable exons v1–v10) and e13a (v9a). As shown in Figure 1, variably spliced exons v1–v10 are located in the membrane-proximal extracellular region, approximately where N-terminal sequence homology between CD44 molecules from different species ends. Alternative splicing of these exons can give rise to a variety of CD44 isoforms, although apparently not all combinations of variably spliced exons are expressed. CD44 isoforms are widely distributed on many cells of the immune system and other tissues, for example, epithelial cells. CD44 isoforms are expressed in a cell-specific manner, and at least 30 different isoforms have been characterized. Among the most frequently occurring isoforms are CD44E and CD44H. The latter isoform does not contain variably spliced exons and is encoded by constitutively expressed exons e1–e5, e15–e17, and e19. The expression of some variably spliced exons is directly responsible for specific properties of CD44. For example, isoforms containing exon v6 are strongly implicated in tumor metastasis.[2] Tissue-specific expression of variably spliced exons gives rise to considerable structural diversity of CD44 on the cell surface.

Postranslational Modifications

The structural diversity of CD44 is amplified by extensive and often isoform-specific posttranslational modifications including N- and O-linked glycosylation and glycosaminoglycan attachment. For example, the constitutively expressed exon e5 contains two Ser-Gly motifs that support the synthesis of chondroitin but not heparan sulfate. Thus, all isoforms of CD44 are decorated with chondroitin sufate. Ser-Gly motifs occur in five exons and variably spliced exons can introduce new glycosylation sites.[28] Exon v3 contains a Ser-Gly-Ser-Gly motif that supports the synthesis of both chondroitin and heparan sulfate,[28,29] and only isoforms containing v3 are decorated with hepa-

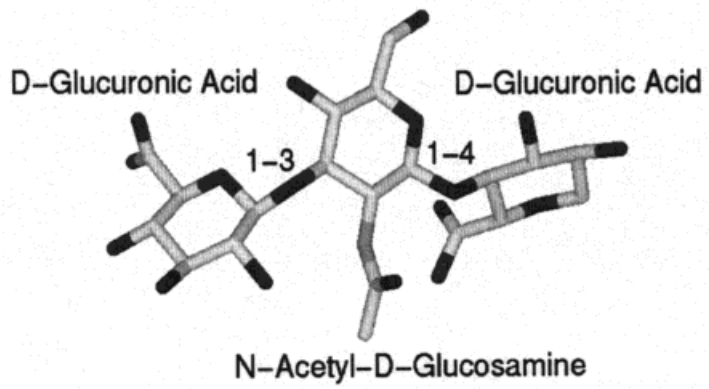

Fig. 2. Structure of a small hyaluronan fragment (Brookhaven Protein Data Bank[69] (PDB) id code 1HUA). Carbon atoms are light gray, oxygen atoms black, and nitrogen dark gray. This trisaccharide consists of two D-glucuronic acid units and N-acetyl-D-glucosamine and contains two distinct glycosidic bonds ("1–3" and "1–4").

ran sulfate (in addition to chondroitin sulfate). Furthermore, glycosylation patterns of CD44 isoforms substantially vary dependent on the cell type and cellular context. As discussed for CD44H, glycosylation and glycosaminoglycan addition are typically very extensive and can more than double the molecular weight of the proteins. It is therefore not surprising that postranslational modifications modulate binding characteristics and functional properties of CD44.[6–8]

Functional Spectrum

The structural diversity of CD44 isoforms correlates with a diverse array of overlapping yet distinct functions in cell adhesion, signal transduction, and cell-cell communication. CD44 binds to extracellular matrix components[30] and thus mediates homotypic cell-cell adhesion.[31] In addition, CD44 can trigger heterophilic adhesion events, e.g., the interaction between leukocytes and endothelial cells.[5] CD44 also acts as a signaling molecule through tyrosine kinases,[32] activation of the NF-κB pathway,[33] and induction of chemokine expression.[34] Tyrosine kinases belonging to the *src* family have been shown to associate with cytoplasmic regions of CD44. Associated kinases trigger phosphorylation-dependent signals following cross-linking of CD44 or oligomerization upon ligand binding. These signaling events can lead to activation of the NF-κB/I-κBα system and induction of proinflammatory chemokine expression in macrophages. Moreover, as a proteoglycan, CD44 can present and bind growth factors and chemokines.[35,36] For example, CD44 isoforms that contain exon v3, and are thus decorated with heparan sulfate, can recruit and present heparin-binding growth factors.[35] These findings illustrate the interplay between various signaling and cellular communication functions that involve CD44. As discussed below in more detail, the size of hyaluronan (HA) fragments recognized by CD44 is critical for signal transduction via CD44, and binding of modified HA ligands can act as a switch between adhesive and signaling functions.[37]

CD44 Ligand(s)

Several ligands for CD44 have been identified including HA and chondroitin sulfate,[38–40] collagen,[41] and the heparin-binding domain of fibronectin.[42] The role of the cytokine osteopontin as a potential CD44 ligand remains controversial.[43,44] By contrast, it has been firmly established that CD44 is the major cell surface receptor for HA.[40] Many of the functions of CD44 in cell adhesion and activation can be attributed to CD44 binding.[37] The functional role of other CD44-ligand interactions is less clear.

Hyaluronan Binding and Its Physiological Relevance

Hyaluronan is a polymeric glycosaminoglycan and major component of the extracellular matrix.[10,30] It consists of repeating D-glucuronic acid and N-acetyl-D-glucosamine disaccharide units. Figure 2 shows the structure of an HA trisaccharide.[45] For effective recognition by CD44, at least an HA hexasaccharide is required[46,47] but only a decasaccharide can replace polymeric HA bound to CD44 expressed on keratinocytes.[48] These findings may reflect differences in HA binding avidity in the systems investigated. However, evidence is accumulating that the size of HA fragments recognized by CD44 provides a physiologically important switch between its adhesive and signaling functions.[37] Binding of HA polymers to CD44 usually leads to cell adhesion[38,49] rather than activation. By contrast, recognition of low molecular weight, but not polymeric, fragments of HA, which may result from tissue damage and degradation of extracellular matrix, leads to CD44 signaling and activation of the immune system.[34,50] Thus, these differential recognition events provide, by an unknown molecular mechanism, a pathway to trigger immune responses.

Regulation of Hyaluronan Binding

The HA binding capacity of CD44 can be regulated in a number of ways. As discussed below, the HA binding

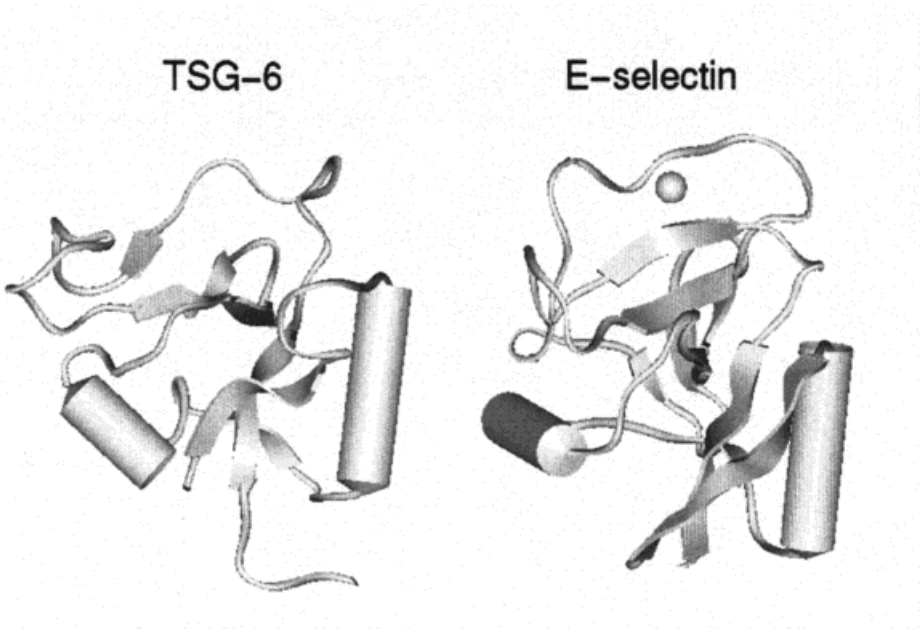

Fig. 3. Comparison of the link module of TSG-6 and the C-type lectin domain of E-selectin. TSG-6 (PDB id code 1TSG) is shown on the left and E-selectin (1ESL) on the right. Loops and regions of non-classical secondrary structure are represented as tubes, β-strands as flat ribbons, and α-helices as cylinders. The functionally important calcium position in E-selectin is shown as a sphere. The two domains were first optimally superposed[70] and then separated.

activity of CD44 and other LPSF proteoglycans mainly resides in their link modules. However, some mutations or deletions in all regions of CD44 including the extracellular, transmembrane, and cytoplasmic domains were found to affect HA binding.[8] The expression level of CD44 on the cell surface influences HA binding but, on the other hand, not all cells that express CD44 isoforms also bind HA. To further complicate matters, binding of HA to inactive cell lines can sometimes be induced by use of anti-CD44 mAbs.[51] It has become clear that many of the observed differences in binding can be attributed to isoform- and/or cell-specific differences in CD44 glycosylation.[8,51] Whether glycosylation of CD44 is directly or indirectly required for HA binding is not completely understood at present. For example, an active (i.e., HA-binding) form of CD44 was expressed in *E. coli*,[52] suggesting that glycosylation may not be critical for HA recognition. However, treatment of CD44-expressing cell lines with tunicamycin, an inhibitor of N-linked glycosylation, or genetic disruption of N-linked glycosylation sites in CD44 was found to abrogate HA binding.[53] Furthermore, several studies suggest that reduced levels of N- and O-linked glycosylation often lead to improved HA binding,[54,55] perhaps due to better accessibility of the HA binding region. By contrast, inactive CD44 isoforms are often abundantly glycosylated. Sulfation of CD44 was also found to induce or modulate the HA binding ability of CD44.[56,57] Detailed analysis of the profound influence of CD44 glycosylation on HA binding is challenging because different effects may play a role, dependent on the level of glycosylation, including induced structural perturbations, aggregation of CD44, or steric effects.

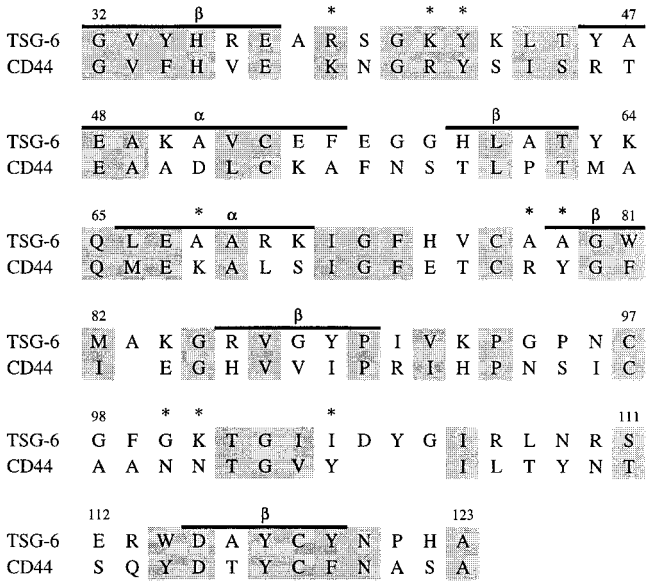

Fig. 4. Structure-oriented sequence comparison of the link modules in TSG-6 and CD44. Identical and conservatively replaced residue positions are shaded. Major secondary structure elements in TSG-6 are labeled. Residue numbers are given for CD44. Asterisks indicate residue positions in CD44 that are, on the basis of mutagenesis, important for HA binding.

Link Modules

Hyaloadherins utilize their link modules for recognition of HA.[10] The link module consists of ~100 residues and LPSF proteins may contain a varying number of link modules; CLP[11] contains two and TSG-6[14] and CD44 each

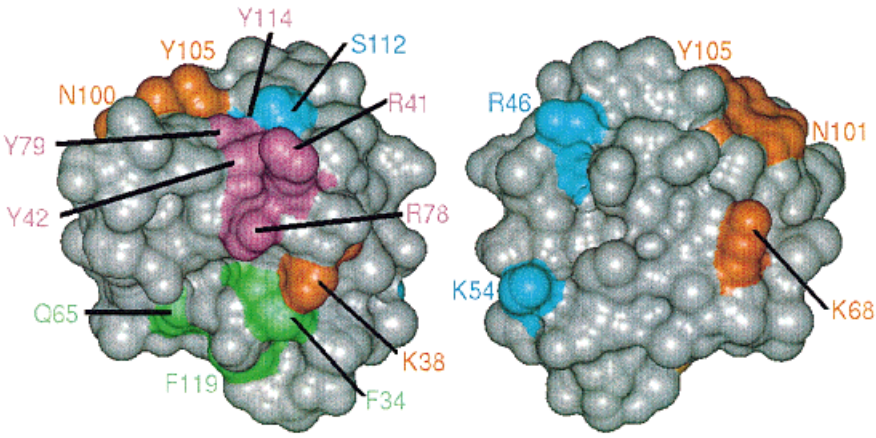

Fig. 5. Mutated residues in CD44. Residues were mapped on a molecular model of the link module, shown with solvent-accessible surface.[71] The model of the link module of CD44 was generated based on the structure of TSG-6 by comparative modeling[72] as described[59] and its sequence-structure compatibility and stereochemical quality were confirmed.[73,74] Two views of the model are shown. The view on the left is very similar to the orientation of TSG-6 in Figure 3 and the view on the right was obtained by rotation of 180° around the vertical axis. Mutated residues are color-coded according their importance following the classification in Table I (class 1 (not important): blue, 2 (important for structural integrity): green, 3A (support HA binding): red, 3B (critical for HA binding): magenta).

contain one. The link module of TSG-6, residues 36–133, was expressed in *E. coli* and shown to be sufficient for specific HA binding.[16] The link module of CD44 is critical for HA binding because site-specific mutations in this region reduce or abolish HA binding without affecting binding of CD44 mutant proteins to conformationally sensitive mAbs.[58,59] However, in contrast to TSG-6, the link module of CD44, when expressed in isolation, does not bind HA, probably because it is not correctly folded,[17] and N- and C-terminal sequence extensions are required to produce an active form (of ~160 residues).[58] Moreover, mutation of residues in basic sequence motifs[60] in CD44 outside the link module (and other modifications throughout the molecule[8]) were also found to reduce HA binding[58] suggesting that the HA binding site in CD44 may extend beyond the link module. This could be relevant because, as discussed above, regulation of adhesive versus signaling functions of CD44 appears to correlate with the size of recognized HA fragments. For example, it is possible that residues outside the link module stabilize the interaction with large HA fragments or polymers and contribute to high-avidity binding.

Prototypic Structure of the Link Module

The three-dimensional structure of the link module of TSG-6 was determined by NMR spectroscopy.[16] The structure, shown in Figure 3, forms a compact domain with a core consisting of two three-stranded anti-parallel β-sheets and two α-helices. Consistent with the modular nature of this domain, the N- and C-termini of the link module are spatially adjacent. The TSG-6 structure displayed striking similarity to the X-ray structure of the calcium-dependent (C-type) lectin domain of E-selectin[61] (Fig. 3). The selectins (E-, P-, L-) are a family of cell adhesion molecules that play an important role in the adhesion of leukocytes to activated vascular endothelium at sites of inflammation[62] (similar to one of the functions of CD44 discussed above[5]). The selectins recognize tetrasaccharide structures of the sialyl-LewisX type[62] and are members of the C-type lectin protein superfamily.[63] Carbohydrate binding to C-type lectins strictly depends on the presence of a conserved calcium site[61,64] (whereas link proteins do not require calcium for HA binding).

Hyaluronan Binding Site in CD44

Sequence comparison of LPSF proteins suggest that the structure of the TSG-6 link module provides a canonical fold for members of this family, including CD44.[16] Figure 4 shows a structure-oriented sequence comparison of the link domains of TSG-6 and CD44. Taking conservative mutations into account, the sequence similarity in the aligned region is ~50%. Conserved residues include two disulfide bonds and the majority of hydrophobic core positions. These similarities leave little doubt that the 3D structures of TSG-6 and CD44 are very similar and have made it possible to construct a comparative molecular model of the link module of CD44 on the basis of TSG-6[59] (Fig. 5). Conserved regions provided the core of the model and predicted loops and side chain conformations were added prior to energy refinement. Details of the model building and assessment procedures are provided in the original study.[59] Although approximate by definition, the model has been very useful to guide and rationalize site-specific mutagenesis experiments to identify CD44 residues important for HA binding, map the location of these residues, and outline the HA binding site in the link module.[59] Table I and Figure 5 summarize the mutagenesis and residue mapping studies. Most, if not all, of the mutagenesis results could be rationalized with the aid of the model. Four CD44 residues were identified as critical for HA binding (R41, Y42, R78, Y79) and four residues to contribute to (or support) binding (K68, N100, N101, Y105). One of these residues (R41) was originally identified by alanine scanning mutagenesis.[58] HA recognition is

TABLE I. Summary of CD44 Mutagenesis and Binding Experiments†

Residue	Mutant	MAb binding	HA binding	Class
Wild type		+	+	
F34	F34A	−	−	2
	F34Y	+	+	1
K38	K38R	+	+/−	3A
	K38S	Not	expressed	
R41	R41A	+	−	3B
Y42	Y42F	+	−	3B
	Y42S	+/−	−	
R46	R46S	+	+	
E48	E48S	Not	expressed	
K54	K54S	+	+	1
Q65	Q65S	+/−	−	2
K68	K68S	+	+/−	3A
R78	R78K	Not	expressed	
	R78S	+	−	3B
Y79	Y79F	+	−	3B
N100	N100A	+	+/−	3A
	N100R	+	+/−	
N101	N101S	+	+/−	3A
Y105	Y105F	+	+	3A
	Y105S	+	+/−	
S112	S112R	+	+	1
Y114	Y114F	+	+	1
F119	F119A	+/−	−	2
	F119Y	+	+	

†Results were originally reported in Bajorath et al.[59] Mutants were transiently expressed in COS cells as CD44-immunoglobulin fusion proteins.[59] CD44-Ig mutant proteins were tested by ELISA (enzyme-linked immunosorbent assays) for binding to conformationally sensitive mAbs ("mAb binding") against the link module of CD44 and for binding to immobilized HA ("HA binding"). Tests with conformationally sensitive anti-CD44 mAbs were carried out to identify proteins with significant structural perturbations as a consequence of the mutation. In many cases, several more or less conservative mutations were carried out in order to classify targeted residues. A score of "+" represents binding comparable to wild type CD44, "+/−" represents intermediate, and "−" non-detectable binding. Mutated residues are classified according to their importance as follows: "Class" 1, not important for HA binding or gross structural integrity (i.e., mutant proteins bind mAb and HA-like wild type CD44); class 2, important for 3D structural integrity (i.e., mutant proteins show reduced or abolished mAb and HA binding); class 3A or 3B, important or HA binding (i.e., mutant proteins show wild type-like mAb binding but reduced (A) or abolished (B) HA binding).

very sensitive to minor changes of critical residues. For example, in the case of mutant Y79F, removal of a single hydroxyl group is sufficient to completely abolish HA binding (Table I). One of the residues in the binding site region, N100, is a potential N-linked glycosylation site that, when mutated, leads to reduced but not abolished HA binding. This further illustrates the influence of glycosylation effects on the binding site and the regulation of HA binding. The results of these mutagenesis and residue mapping studies have made it possible to generate a first, albeit approximate, view of the HA binding site. As shown in Figure 6, CD44 residues that are either critical for binding or support binding cluster on the link module and these clusters form a coherent HA binding surface. The predicted binding surface is extensive and can accommodate an HA hexasaccharide or a larger structure.

Comparison of Carbohydrate Binding Sites

The location of the HA binding site in CD44 corresponds to that predicted for TSG-6[16] and largely confirmed by NMR experiments (detecting perturbation of TSG-6 residues upon HA binding).[17] Thus, at least two members of the LPSF utilize similar molecular regions for HA recognition. However, details of the interactions between CD44 and TSG-6 with HA probably differ significantly because, as shown in Figure 4, most of the residues important for HA binding to CD44 are not conserved in TSG-6[59] (and other residues may contribute). The binding site comparison can be extended to the selectins whose C-type lectin domains also recognize oligosaccharides and display structural similarity to link modules. Moreover, the selectins and CD44 fulfill similar functions in the recruitment of leukocytes to vascular endothelium at sites of inflammation. Figure 7 shows a comparison of the carbohydrate binding site in E-selectin, identified by mapping of mutants[61,65] on the X-ray structure,[61] and the modeled binding site in CD44. In E-selectin, binding of its ligand, sialyl-LewisX tetrasaccharide,[62] requires the presence of a calcium coordination sphere conserved in other members of the C-type lectin superfamily[61,64] and involves a surface patch proximal to the calcium site. This binding site is conserved in P-selectin.[66–68] CD44 does not contain a calcium-binding site. However, the region corresponding to the binding site in E-selectin also forms the center of the HA binding site in CD44. Thus, the selectins and CD44 use topologically equivalent regions for carbohydrate recognition. Although their ligands are different, aromatic and charged residues are critical for binding in both cases. The HA binding surface in CD44 is larger than in E-selectin, consistent with the size of the CD44 ligand, a hexasaccharide or larger HA structure. Taken together, the similarity of binding domains, carbohydrate binding sites, and functions suggests that the selectins and CD44 are evolutionary related, although they belong to different protein superfamilies.

CONCLUSIONS

CD44 acts as a cell adhesion and signaling molecule and is implicated in tumor metastasis and chronic inflammatory diseases. The presence of various CD44 isoforms, their proteoglycan character, cell-specific modifications, and the ability to recognize different ligands make it challenging to understand the molecular mechanisms that determine different functions of CD44. This is perhaps best illustrated by the many ways in which HA binding to CD44 can be regulated. While many investigations focus on functional or disease aspects of CD44 isoforms, we are now beginning to understand how CD44 and other hyaloadherins recognize ligands. Furthermore, studies on the structure and binding characteristics of link proteins and C-type lectins begin to unravel distinct similarities between members of these protein superfamilies. However, concerning CD44, many questions remain. For example, what are the details of CD44-HA interactions? How exactly does glycosylation influence binding? Do regions outside the link module participate in HA binding? How

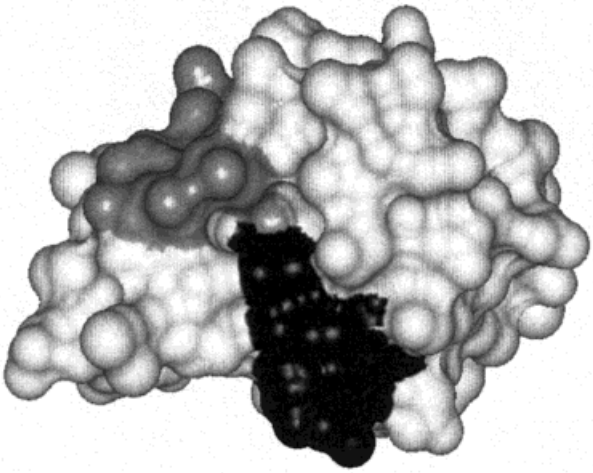

Fig. 6. Hyaluronan binding surface in CD44. A top view of the model with solvent-accessible surface is shown, obtained from the orientation in Figure 5 by ~90° rotation around the horizontal axis. The black cluster is formed by residues that are critical for the interaction with HA (R41, Y42, R78, Y79) and the gray cluster by residues that contribute to binding (K68, N100, N101, Y105). In the model, residues important for binding form a coherent binding surface.

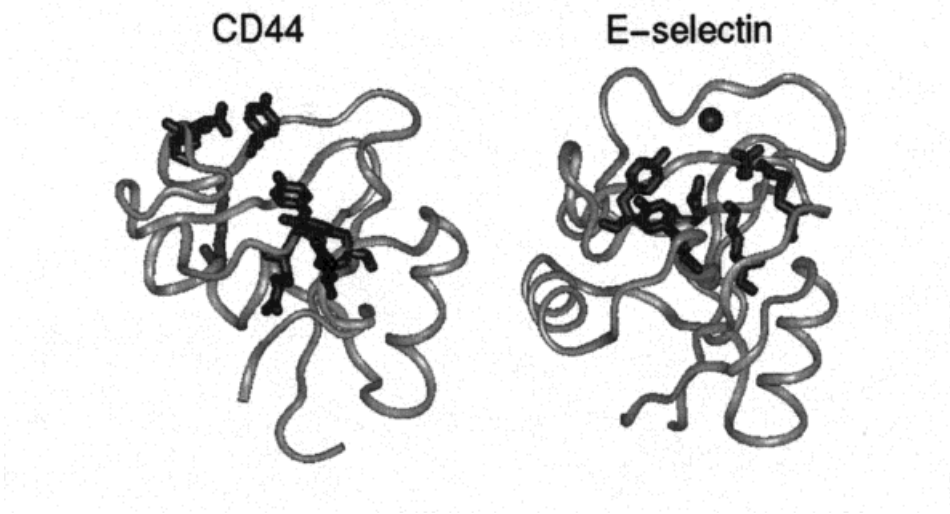

Fig. 7. Comparison of carbohydrate binding sites in CD44 and E-selectin. The CD44 model is shown on the left and the E-selectin X-ray structure on the right. The orientation of E-selectin is the same as in Figure 3. The domains were first optimally superposed and then separated. CD44 and E-selectin residues identified by mutagenesis as important for ligand binding are shown in black. Side chain conformations of residues in CD44 are only approximate.

are HA fragments of different size recognized? Why does binding of short HA fragments lead to CD44 signaling and binding of HA polymers to cell adhesion? Despite progress in this area, the molecular mechanisms that are responsible for these effects remain unknown. An important step, among others, to improve our current understanding would be the determination of atomic structures of CD44 isoforms and their complexes with HA fragments.

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