

CD44 STRUCTURE AND FUNCTION

Jayne Lesley and Robert Hyman

Cancer Biology Laboratory, The Salk Institute for Biological Studies, P.O. Box 85800, San Diego CA 92186-5800

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1. ABSTRACT

In this review we discuss the structural elements of CD44 that have been shown to be involved in specific functions. To this end, we focus primarily on experiments in which CD44 constructs are transfected into cells whose function is then assayed. The hyaluronan binding function of CD44 has been assayed in cell lines and in fusion proteins, termed CD44-Igs, consisting of the external domain of CD44 coupled to the hinge, CH2 and CH3 regions of human IgG1. These studies have shown that hyaluronan binding by CD44 is regulated by the cells in which it is expressed, and that at least part of this regulation is determined by cell specific posttranslational modifications, especially N-glycosylation, of CD44 itself. Variant isoforms of CD44 determined by alternative splicing of 11 optional exons in the middle of the gene determine additional functions of CD44, as well as contributing to the regulation of hyaluronan binding. Soluble CD44 may modulate the function of cell surface CD44. The cytoplasmic domain of CD44 contributes to ligand

binding in a way that remains obscure. It also determines membrane localization in polarized epithelial cells, and is probably involved in CD44 interactions with the cytoskeleton and in mediating post-ligand binding events.

2. INTRODUCTION

CD44 is a broadly distributed cell surface glycoprotein receptor for the glycosamino glycan hyaluronan (HA) which is a major component of extracellular spaces. It is expressed on a diverse variety of cell types including most hematopoietic cells, keratinocytes, chondrocytes, many epithelial cell types, and some endothelial and neural cells. Table 1 lists some of the many functions that have been attributed to CD44 and the types of experiments that have led to these attributions (also see reviews, refs. 1-5). In this review we discuss the structural elements shown to be involved in specific functions. Therefore, the experiments discussed are primarily studies where wild type and mutant CD44 are transfected into cells whose function is then assayed.

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Table 1. Functions of CD44 molecule

ASSAY	TRANSFECTION/ MUTATION	INHIBITION/STIMULATION BY: ANTIBODY LIGAND	REFERENCES
HA binding/adhesion	+	+ +	6-9
Regulation of HA binding	+	+	10-17
Migration on HA	+	+ +	18-20
Migration on other ECM*		+ +	21-23
ECM assembly/degradation	+	+ +	24-26
Leukocyte rolling		+ +	12,27,28
Inflammation		+	29-31
Cytokine/chemokine binding	+		32, 33
Tumor Metastasis	+	+ +	34-37
Progenitor homing/differentiation		+	38, 39
Membrane localization	+		40-42
Triton X-100 insolubility	+		43, 44
Cytoskeletal protein interactions	+		45-50
Signal transduction		+ +	45, 51
Shedding of soluble CD44	+	+	29, 31, 52-55

Columns two, three, and four indicate the types of experiments that were used to demonstrate the role of CD44 in each assay (indicated in column one). Column five lists representative publications documenting these experiments. *ECM denotes extracellular matrix.

Although most details of CD44 structure will be discussed below, a brief description of the molecule is needed. The standard or hematopoietic isoform of CD44 (CD44H) is a type 1 transmembrane molecule consisting of ~ 270 amino acids (aa) of extracellular domain (including 20 aa of leader sequence), a 21 aa transmembrane domain and a 72 aa cytoplasmic domain. The amino terminal ~180 aa are conserved among mammalian species (~85% homology). This region contains six conserved cysteines, five conserved consensus sites for N glycosylation and a region of homology with other HA binding proteins, termed the “link module” or CLP (for cartilage link protein) domain because of its resemblance to the HA binding domain of cartilage link protein (56). A membrane proximal region in the extracellular domain of ~75 aa is relatively nonconserved and includes several potential sites for carbohydrate modification. In the middle of this region, between aa 222 and 223, is a site where additional aa sequences may be inserted by alternative splicing among the eleven “variable” exons residing in the middle of the 21 exon gene. Figures 1 and 2 present the structural features that are discussed throughout this review using the aa numbering of Stamenkovic *et al* (57) for human CD44H to refer to specific residues.

Binding of the principal ligand of CD44, HA, is regulated. Though all cells that express any form of CD44 on the cell surface bear the same conserved amino terminal sequence that contains the HA recognition domain, many (most) do not bind HA constitutively. We have defined three cell activation states of CD44 that can be identified among cell lines and normal cell populations and have examined a number of potential regulatory mechanisms (see reviews 1, 2). Active cells, expressing CD44 in an active state, can bind HA constitutively. Cells expressing CD44 in an inducible state do not bind HA constitutively, but can bind HA immediately upon binding of certain CD44 specific inducing mAbs. Some of these cells can also be induced to bind HA after a period of hours by cell activation stimuli such as cytokines, phorbol

ester, and antigen or anti-CD3 (for T cells). Cells with CD44 in an inactive state cannot bind HA even in the presence of inducing mAb. The activation state of CD44 is determined by the cell in which it is expressed. The evidence is that the cDNA encoding the hematopoietic isoform of CD44 (CD44H) exhibits different functional states when it is expressed in cell lines representative of the different activation states (1, 11).

Fusion proteins consisting of the external domain of CD44 and the hinge, CH2 and CH3 regions of human IgG1 (CD44-Igs), have been used to study the ligand binding properties of CD44 free of the cellular environment (6, 9). These fusion proteins usually exhibit the same ligand binding state as the cell in which they were synthesized (11, 14, 16, 17). When fusion proteins are coated on plastic beads, HA binding is detected only above a certain threshold density. CD44-Igs derived from cells representative of different activation states show differences in the threshold density at which HA binding is detected, with the threshold for active inducible < inactive (11). Mutant fusion proteins can be assayed for function to determine structural features involved in constitutive ligand binding and in the determination of binding activation state.

Use of fusion proteins has taught us that much of the regulation of CD44 binding to HA is inherent in the structure of the molecule itself and does not require CD44 to interact directly with other cellular components. Interaction with other cellular components is still likely to be involved in regulating CD44 ligand recognition in a more dynamic way than that achieved by synthesis of a particular structure. Certainly, mediation of the consequences of ligand binding must depend on interaction of CD44 with other molecules, leading to downstream functional pathways, such as those involved in cell migration.

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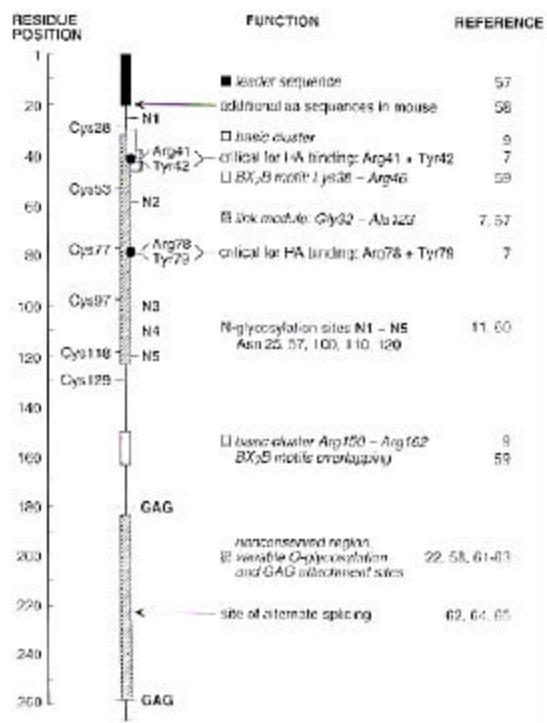


Figure 1. Features of the extracellular domain of CD44H (the standard or hematopoietic isoform, which contains none of the sequence specified by alternative splicing of variant exons). Amino acid residues are numbered according to (57). GAG denotes potential sites for the addition of glycosamino glycan side chains. BX7B denotes a motif in which B represents Arg or Lys and X₇ represents any seven non-acidic amino acids but includes one additional Arg or Lys (59).

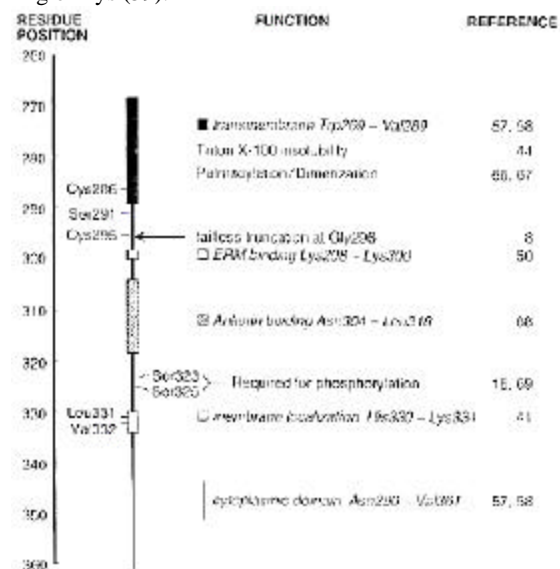


Figure 2. Features of the transmembrane and cytoplasmic domains of CD44. Amino acid residues are numbered according to (57). ERM denotes cytoskeletal associated proteins, ezrin, radixin, moesin.

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3. THE EXTRACELLULAR DOMAIN

3.1. The Conserved sequence

The amino terminal ~180 aa of CD44 is relatively conserved among mammalian species (~85% homology). As indicated in figure 1, it contains six cysteine residues which are believed to form 3 disulfide bonds and five conserved N-glycosylation consensus sequences (referred to as N1-N5, for Asn residues at positions 25, 57, 100, 110 and 120, respectively). Included in this region is ~100 aa (termed a link module) with homology (~35%) to other HA binding proteins such as aggregating proteoglycan (aggrecan), cartilage link protein and TSG-6. Two clusters of basic aa in the conserved region of CD44 were shown to mediate binding of CD44-Igs to immobilized HA by Peach *et al* (9). Fusion proteins lacking the basic cluster Arg150 to Arg162 showed reduced binding to immobilized HA. Though single mutations of these basic residues to Ala had slight effects, multiple mutations caused significant reduction in HA binding. Mutation of the more amino terminal basic cluster (Lys38 to Arg41) had more severe consequences, with the Arg41 to Ala mutation abolishing HA binding completely. Based on studies of GST-peptides from a number of HA binding proteins Western blotted with biotinylated HA, Yang *et al* (59) proposed that a motif consisting of B(X₇)B (where B is Arg or Lys, X is any non-acidic aa and X₇ includes one additional Arg or Lys) was sufficient for HA binding. This seems unlikely to be true for CD44 in view of the findings of Bajorath *et al* (7) (discussed below) and the observations of naturally occurring mutations of CD44 where one of the proposed required basic residues has been changed without loss of HA binding function (see 2, 5).

3.1.1. The Link Module

Bajorath *et al* (7) have recently published a structural model of the link module of CD44 based on the solution structure of the homologous region of TSG-6 determined by NMR (56). The structure of TSG-6 and the model of CD44 were found to be similar to the calcium-dependent (C-type) lectin fold of rat mannose-binding protein and E-selectin (7, 56). The ligand binding site of TSG-6 is not known, so mutational analysis was used to map the HA binding site of CD44 (7). Exposed residues in the vicinity of the critical Arg41 in the model of CD44 were selected for mutagenesis and mutated CD44-Igs were tested for binding to immobilized HA in an Elisa assay. Critical residues, whose mutation abolished HA binding, were two pairs, Arg41 and Tyr42 and Arg78 and Tyr79, which form a cluster at the center of the proposed binding site. Other important residues (Lys38, Lys68, Asn100, Asn101 and Tyr105) border those and form a coherent surface in the model, running along a ridge on the protein surface (7). Of

the nine residues identified as important for HA binding, only Tyr 42 is conserved in TSG-6, which also binds HA. Thus, the details of the protein carbohydrate interaction must differ substantially in these two HA binding proteins.

Since these studies with fusion proteins used full-length CD44, it is not known if the link module of CD44, like the link module of TSG-6 (56), is sufficient for HA binding, or whether other parts of CD44, such as the second basic cluster defined in the Peach study (9), are also required. Also unknown is whether a single copy of the CD44 binding domain is sufficient, since the CD44-Ig fusion protein is (at least) dimeric. It is possible that a multivalent grouping of CD44 molecules is required for efficient ligand binding. This idea is based on a number of observations: HA is a highly multivalent ligand, a repeating polymer of disaccharide units; most other HA binding proteins have two tandem link module repeats while CD44 has only one; inducing antibodies do not induce as monovalent Fab fragments but require multivalent binding, suggesting that CD44 clustering is required for antibody induced binding (70); and, most other cell surface adhesion molecules have a low intrinsic affinity for ligand and depend on multivalency to increase binding avidity (71, 72).

3.1.2. N-Glycosylation sites

Five conserved consensus sites for N-glycosylation are located in the amino terminal 120 aa of CD44. All five sites appear to be utilized in the murine and human cell lines examined so far (11, 60). Several studies have shown that cell specific N-glycosylation can modulate the ligand binding function of CD44 (11, 14-17, 60). Cell lines and normal B cells showed differences in N-glycosylation associated with different ligand binding states (13-17). In particular, CD44 from active cells had less N-glycosylation than that from inducible cells, and the CD44 of inducible cells was less N-glycosylated than that from inactive cells, based on the relative migration of their CD44 in SDS-PAGE before and after N-glycanase treatment (13-15). Inhibition of N-glycosylation with tunicamycin activated HA binding in some cell lines that were inducible or inactive (14, 15).

English *et al* (11) have shown that murine CD44H mutant constructs lacking the specific N-glycosylation sites, N1 or N5, exhibited constitutive HA binding when transfected into a cell line that has an inducible phenotype upon transfection with wild type CD44H. This suggests that cell specific modifications at those N-glycosylation sites prevent constitutive ligand recognition by wild type CD44 in this inducible cell line. These two N-glycosylation sites are outside of the link module of CD44 modeled by Bajorath *et al* (7). Perhaps specific carbohydrate modifications extending from these sites interfere directly with the ligand binding site, or indirectly modify the conformation of the binding site. It should be noted that failure to utilize sites N1 or N5 is not required for constitutive ligand binding. An active cell line that constitutively binds HA when transfected with wild type CD44H utilized both sites (11). Thus, cell specific modifications in the oligosaccharide structure at sites N1 and N5 in the inducible cell line made these N-glycans inhibitory.

Bartolozzi *et al* (60) found that mutation of any one of the five N-glycosylation sites of human CD44H

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resulted in loss of wild type CD44 mediated adhesion to HA in human cell lines in an active state. These mutations were accompanied by loss of mAb recognition of CD44 structure as well, suggesting that in this situation N-glycosylation was needed to maintain a conformation of the molecule that would allow ligand binding. In this study and that of Skelton *et al* (16), complete absence of N-glycosylation (due to tunicamycin treatment of cells or N-glycosidase F digestion of fusion proteins, respectively) inhibited HA binding, while truncation of N-glycans enhanced HA binding. The latter study concluded that the first N-linked N-acetyl-glucosamine residue was sufficient to promote HA binding, but subsequent modifications of the N-glycans, especially by alpha 2,3-linked sialic acid (see below), reduced binding affinity (16).

A specific requirement for all five N-glycosylation sites was not found in the studies of English *et al* (11). In these experiments, none of the individual mutations of Asn to Ser abolished binding, and, in fact, inactivation of sites N1 or N5 had a positive effect (binding state converted from inducible to active). One mutation in the English study (11), of Thr102 in the consensus sequence for N3, abolished constitutive HA binding in active cells, but had no effect on inducible binding. This loss of function was not due to lack of glycosylation at N3 since the mutation at Asn100, which also eliminated glycosylation at N3, did not affect constitutive binding in the same active cell line. The failure of mutation of Asn100 to affect constitutive binding in the study of English *et al* (11) is at odds with the Bajorath study (7) which found a significant reduction in HA binding by CD44-Ig when this residue was mutated. These differences may be due to differences between murine and human CD44 sequences around this region such that certain mutations affect the conformation of one CD44 differently than the other. Alternatively, they may reflect differences in oligosaccharide structure between the cell lines in the two studies. All of the results, however, are consistent with the proposal that the region around N3 contributes to the HA binding site.

A number of studies have now shown that, in many cases, removal of sialic acids (both from the cell surface and from CD44-Ig fusion proteins) enhances HA binding by inducible or inactive CD44 (11, 14, 16, 17). Coupled with the results that inhibition of N-glycosylation by tunicamycin (14, 15), truncation of N-glycans (16) and mutation of specific N-glycosylation sites (11) can all enhance HA binding, it is likely that cell specific sialylation of N-glycans is involved in restricting HA recognition by CD44. Zheng *et al* (17) showed that HA binding cells have less sialic acid on their CD44 than non-binding cells. Skelton *et al* (16) found that alpha-2,3 linked sialic acid, in particular, was associated with restriction of ligand binding, consistent with the notion that specific sialic acid modifications of N-glycans might account for their inhibitory function in certain cells.

Cell activation that results in induction of HA binding (PMA, anti-CD3, IL-5, etc.) requires several hours and probably involves new protein synthesis (73 and our unpublished results). This would allow for synthesis of CD44 with new glycosylation patterns. Hathcock *et al* (13) observed reduced N-glycosylation of CD44 upon B cell activation, while DeGrendele *et al* (12) studying T cells

did not, based on the mobility of the CD44 in SDS-PAGE. However, changes in glycosylation that result in release of inhibition of HA binding may not necessarily be detectable as a shift in SDS-PAGE. Zheng *et al* (17) showed that glucose deprivation can alter the HA binding function of some cell lines within hours, implicating synthesis of new CD44 with reduced glycosylation. These authors also found that *in vivo* growth of a tumor cell line that bound HA poorly reversibly converted it to an active HA binding state, raising the possibility that local environmental conditions may influence CD44 function, perhaps through influencing glycosylation pathways (17).

3. 1. 3. Outside the Link Module

Eleven aa amino terminal to the link module are not present in TSG-6 and were not modeled in the Bajorath study (7). This brief stretch contains the most amino terminal N-glycosylation site, N1, at Asn25 which we have shown can negatively influence HA binding as a result of cell specific glycosylation in an inducible cell environment (11). Human and murine CD44 differ here, with murine CD44 having either two or four additional aa between the leader sequence and first aa of human CD44. Also in this region is the murine allele specific determinant for the mAb recognizing CD44.1 (74).

The second basic cluster determined by Peach *et al* (9) to be involved in HA recognition is in the more carboxy terminal stretch of the conserved region as are recognition sites for a number of CD44-specific mAbs (74). Binding determinants for other CD44 specific mAbs are also found in the amino terminal conserved region of CD44 (74).

3. 2. The non-conserved region

The non-conserved region (~aa 183 to 256) shows only ~35% similarity between mammalian species. It contains potential sites for numerous carbohydrate modifications of CD44 (58, 61-63) and the site of alternative splicing which allows for the insertion of extra aa sequence from variable exons of the CD44 gene (62, 64, 65).

3. 2. 1. Carbohydrate modifications

Most of the potential sites for O-linked glycosylation are in the membrane proximal portion of the external domain of CD44. While the studies of Lesley (15) and Zheng (17) did not observe an effect on HA binding as a result of inhibition of O-glycosylation, Dasgupta *et al* (61) found that inhibition of O-glycosylation enhanced CD44H mediated HA binding in several colon carcinoma cell lines. Keratin sulfate and chondroitin sulfate have been found on CD44H from certain cell types (22, 63). Such glycosamino glycan additions have been shown to modify CD44 function. Keratin sulfate side chains on CD44H from a highly metastatic colon carcinoma cell line reduced HA binding significantly, while a less heavily keratin sulfate substituted variant line bound HA well and was less metastatic (63). Chondroitin sulfate modification of CD44 is required for the binding of fibronectin (75) and collagens (21-23, 76) and for enabling melanoma cells to migrate on collagen substrates (21, 23) and endothelial cells to migrate on fibrin substrates (22). The chondroitin sulfate modified CD44 was required for migration, but not for adhesion of melanoma cells to collagen gels, which was dependent on integrins, suggesting that interaction between the CD44-proteoglycan and integrins was involved in cell migration

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(21, 23). It is not known what CD44 isoforms are involved in these activities, but CD44H has been found to express chondroitin sulfate modifications in some cells (22).

3. 2. 2. Variable sequences

Alternative splicing of variant exons occurs only in particular cell types and activation states and, thus, must be precisely regulated. It is thought that the frequent association of abnormal isoform patterns with malignancy reflects a breakdown in this regulation (see ref. 4). The function and structure of most of the variable sequences is not known. The v6 exon conferred metastatic potential in a rat pancreatic carcinoma cell line (35), but is not necessarily associated with metastasis in many other tumors (see refs. 2-5). The presence of exons v6 and v7 has been reported to broaden the ligand specificity of CD44 to include glycosaminoglycans in addition to HA (77). V3 has a consensus motif Ser-Gly-X-Gly for glycosaminoglycan modification (78). Heparan sulfate side chains attached to CD44 at v3 have been shown to bind heparin-binding growth factors and chemokines (32, 33). Epican, a v3 containing isoform (v3-v10) expressed on keratinocytes, mediated cell-cell adhesion that was dependent on HA (79). An isoform of CD44 containing variably spliced exons v8-v10, termed CD44E or CD44R1, contains sequences rich in Ser and Thr residues which are potential sites of O-linked glycosylation (80). Extensive O-glycosylation of this isoform has been found to inhibit HA binding by CD44 (80), apparently accounting for conflicting observations concerning the HA-binding properties of this isoform (81, 82). Another study suggested that CD44R1 bound a determinant present on CD44H and CD44R1 since this isoform mediated cell aggregation that was CD44 but not HA dependent (83).

3.2.3. Soluble CD44

Soluble forms of CD44 have been reported in the serum of several species and in cell culture media from various cell lines. Circulating soluble CD44, believed to be shed from cell surfaces, increases during immune activity and tumor dissemination (53, 84). Exposure to CD44 specific antibodies induces rapid loss of CD44 from the cell surface of leukocytes exposed *in vitro* or *in vivo* (29, 31, 52). The molecular nature of the soluble CD44 and the mechanism of shedding is not known, however, metalloprotease and serine protease may be involved (52). Certain CD44 variant isoforms are reported to shed from the cell surface spontaneously more readily than CD44H (85). A recently identified variant exon, between the original v9 and v10, provides another mechanism (besides shedding) for the genesis of soluble CD44 (54). This exon contains stop codons that can result in protein truncation before the transmembrane domain. Cos cells transfected with cDNA constructs for CD44 isoforms containing the new exon secreted low molecular weight soluble CD44 into the medium. It has been proposed that soluble forms of CD44 could act as potential antagonists of their membrane-bound forms, and thus provide another mechanism of regulating CD44 function. Support for this idea is provided by a recent study of Yu and Toole (55). Murine mammary carcinoma cells transfected with cDNAs encoding soluble isoforms of CD44 displayed a marked reduction in their ability to internalize and degrade hyaluronan and a concomitant loss of tumorigenicity (55).

4. THE TRANSMEMBRANE DOMAIN

The sequence of the transmembrane domain is 100% conserved among several mammalian species. Yet this specific sequence is not absolutely required for HA binding. A cell line that binds HA constitutively upon transfection with wild type CD44H, binds HA equally well when transfected with CD44 constructs in which the CD44 transmembrane domain has been substituted with that of CD45 or of the CD3-zeta chain (44).

4. 1. Insolubility in nonionic detergents

Early studies on CD44 suggested an association of CD44 with the cytoskeleton based mainly on the fact that a proportion of CD44 was insoluble after extraction with nonionic detergents, in particular Triton X-100—a criterion which has been widely used as indicating cytoskeletal association (reviewed in 86). These studies were carried out mainly in fibroblasts, and, in fact, the CD44 of epithelial cells (40, 43) and lymphocytes (43) is entirely soluble in nonionic detergent, even after exposure of the cells to hyaluronan (43). Furthermore, tailless CD44 transfected into fibroblasts shows a Triton X-100 extraction profile that is identical to that of wild-type CD44 (40, 44). These observations indicate that insolubility in nonionic detergent cannot be taken as general evidence for an association of CD44 with the cytoskeleton since it does not occur in many cell types and occurs in fibroblasts even when nearly all of the cytoplasmic domain is absent.

In fact, the insolubility of the CD44 of fibroblasts in nonionic detergents is mediated, not by the cytoplasmic, but, rather, by the transmembrane domain. Substitution of the transmembrane domain of CD44 with that of the CD3 zeta chain or that of CD45 converts the CD44 of fibroblasts to a Triton X-100 soluble form (44). Fibroblast CD44 is soluble in detergents that solubilize glycoposphatidylinositol-linked membrane molecules that are Triton X-100 insoluble (43, 44). Triton X-100 insoluble CD44 migrates with low density lipid associated material in sucrose gradients, consistent with an association with the plasma membrane (44). Thus, the insolubility of the CD44 of fibroblasts in nonionic detergents does not reflect an association of CD44 with cytoskeletal components. Rather, it appears to reflect a difference in the lipid composition of certain fibroblasts relative to other cells. In these fibroblasts a proportion of CD44 is associated with lipid in a complex that is stable to extraction with nonionic detergent, resulting in “insolubility”.

4. 2. Palmitoylation/Dimerization

Bourguignon and colleagues (66) demonstrated that a proportion of CD44 is palmitoylated. The proportion of CD44 that undergoes palmitoylation and the extent to which CD44 palmitoylation varies under different conditions has not been determined, nor has the function of palmitoylation. Removal of palmitic acid led to a reduced ability to bind to ankyrin-coated beads in an *in vitro* assay and reacylation of CD44 restored binding ability (66). The authors inferred that palmitoylation may act to regulate attachment of CD44 to the cytoskeleton via ankyrin, which in turn may regulate HA binding (see Section 5.4.1). There are two cysteine residues that might serve as substrates for palmitoylation: Cys286 in the transmembrane domain and

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Cys295 in the cytoplasmic domain. Cell lines expressing mutant CD44 molecules in which Cys295 has been deleted (87) or mutated to Ala (88), however, still bind hyaluronan (see below, Section 5.1). Also, as noted above, constitutive HA binding can occur when the transmembrane domain of CD45, which does not contain a cysteine, is substituted for that of CD44 (44). Thus, neither potential site for palmitoylation is required for constitutive ligand binding. These results do not, however, rule out the possibility that palmitoylation contributes to some function other than ligand binding.

There is, however, evidence that Cys286 in the transmembrane domain could function to mediate HA binding in certain situations. Jurkat cells transfected with a mutant CD44 in which Cys286 has been converted to Ala do not bind HA (88). In this model, ligand binding must be induced by an inducing mAb or by overnight culture in PMA or anti-CD3 (67, 88). Liu and Sy (67) reported that dimerization of a small proportion of CD44H occurred after PMA treatment of Jurkat cells. The authors suggested that disulfide bond formation through Cys286 may serve to stabilize aggregates of CD44 in the plasma membrane, thus potentiating ligand binding (see Section 5.1).

A correlation of ability to bind soluble ligand with dimeric forms of CD44 has also been reported by Sleeman and colleagues (89). In this case, ability to bind HA correlated with the presence of variable exons. High molecular weight aggregates of CD44 that were sensitive to reduction were seen only in a minor proportion of the alternatively spliced CD44 but not in CD44H, which, in this model does not mediate binding of soluble HA. Thus, ability to form dimers correlated with ability to bind soluble ligand. In this case, it is not certain whether the transmembrane domain mediates the dimerization.

5. The cytoplasmic domain

The 72 aa CD44 cytoplasmic domain (residues 290-361, see figure 2) shows more than 85% conservation of aa sequence among species (86), implying that this domain mediates one or more essential functions. Two broad classes of functions may be envisioned (90): 1) functions leading to changes in the avidity or affinity of CD44 for its ligand (“inside-out” signaling); 2) functions consequent to ligand binding that mediate (or trigger) further downstream events (“outside-in” signaling).

5.1. Regulation of ligand binding

Initial evidence for a role for the CD44 cytoplasmic domain in mediating ligand binding came from studies by Lesley, Hyman, Kincade and colleagues (8). They demonstrated that CD44-negative mouse lymphoma cells transfected with a mutant CD44 molecule with a stop codon at Gly296 and consequently expressing only the most membrane proximal six amino acids of the CD44 cytoplasmic domain (“tailless” CD44) failed to bind soluble HA and bound immobilized HA much less well than did lymphoma cells transfected with the wild-type molecule. Cells transfected with the tailless molecule could bind soluble HA, however, if first exposed to those CD44-specific monoclonal antibodies that have the property of “inducing” HA binding. These results indicated that the CD44 cytoplasmic domain functioned in some way to mediate ligand binding and that this function could be mimicked or bypassed by treatment with inducing

antibody. The most reasonable interpretation was that the cytoplasmic domain functioned to alter either the distribution or the conformation of the CD44 molecule to allow it to bind ligand.

Similar results were obtained by Thomas and colleagues (20) who showed that melanoma cells transfected with a tailless CD44 construction bound ligand much less well than cells transfected with wild-type CD44 and were unable to migrate on an HA coated substrate.

A subsequent study showed that transfectants for a dimeric tailless CD44, made by substituting the transmembrane region of the CD3 zeta chain (which contains a cysteine capable of forming an intermolecular disulfide bond) for the transmembrane region of CD44 bound soluble HA more efficiently than intact wild type CD44 (87). Other experiments showed that for inducing antibody to overcome the tailless defect it is necessary for it to be at least bivalent; monovalent Fab fragments were unable to induce ligand binding but could do so if cross-linked by a second antibody (70).

These observations suggest that the distribution of CD44 on the cell surface is important for ligand binding, that “clustering” of CD44 molecules promotes ligand binding, and that the CD44 cytoplasmic domain acts in some way to promote the aggregation of CD44 molecules into a configuration that enhances ligand binding. This model would suggest that the affinity of an individual CD44 molecule for ligand is relatively low and that cooperative binding of polymeric ligand by several CD44 molecules is necessary for a stable interaction to occur (see Section 3.1.3). It is not certain whether mere multipoint binding is sufficient to promote the stable binding of ligand or whether something more is required, such as a specific configuration of CD44 molecules on the cell surface or a specific conformational change in the CD44 molecule itself that is promoted by inducing antibody and/or by the cytoplasmic domain.

While it is clear that truncation of the bulk of the cytoplasmic domain affects the ability of CD44 to bind ligand, it is less clear whether specific signals or interactions mediated by particular regions of the cytoplasmic domain beyond residue 295 are involved in the regulation of ligand binding. There is general agreement that truncations of the cytoplasmic domain beyond residue 318 do not affect hyaluronan binding, regardless of the cell type studied or the assay method used to determine ligand binding (68, 87, 91, 92). Lokeshwar and colleagues (68), examining the binding to hyaluronan coated plates by cos cells transiently transfected with deletion constructions, found that transfectants for mutant CD44 molecules truncated at Leu318 or thereafter bound hyaluronan while transfectants for a mutant CD44 molecule truncated at Ile303 did not. They interpreted these results to indicate that a 15 aa ankyrin binding domain (see below) located between residues 303 and 318 was essential for hyaluronan binding function. Liu and colleagues (91) found that human Jurkat lymphoma cells stably transfected with a mutant CD44 construction containing a stop codon at Gly306 did not bind soluble hyaluronan, while cells transfected with a mutant construction containing a stop codon at Thr338 were able to bind (ligand binding required that the cells be exposed to an inducing antibody and was relatively low in

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these experiments). Liao and colleagues (92) showed that Jurkat cells stably transfected with a construction containing a stop codon after His330 bound hyaluronan while cells transfected with a construction containing a stop codon after Ala309 did not bind hyaluronan (ligand binding occurred only after exposure of the cells to phorbol ester for 16 hours). In contrast, Perschl and colleagues (87), studying the constitutive binding of soluble hyaluronan to stably transfected mouse lymphoma cells found that cells transfected with a mutant construction with a stop codon at Gly306 bound ligand as well as did cells transfected with wild-type CD44. Only transfections for constructions encoding the tailless molecule containing a stop codon at Gly296 did not bind soluble hyaluronan. The residues between aa 296 and 305 did not appear critical for hyaluronan binding as transfectants for mutant CD44 molecules in which residues 292-301 or 302-323 were deleted also bound hyaluronan as well as transfectants for wild-type CD44. It should be noted that the latter deletion encompasses the ankyrin-binding region thought by Lokeshwar and colleagues (68) to be essential for hyaluronan binding.

The reasons for the discrepancy among these groups is unclear at this time. If sequence beyond residue 303 mediates a specific function, this function appears not to be required under all circumstances. Since ligand binding is seen when the most membrane proximal 16 aa of the cytoplasmic domain is expressed but not when only the most membrane proximal 6 aa are expressed and since ligand binding is seen even when portions of the sequence between the most membrane proximal 6 aa and the most membrane proximal 16 aa are deleted, provided regions further distal are included, it may be that either 1) some minimum length of cytoplasmic domain sequence of >6 and <16 aa is required to stabilize binding, with the exact nature of this sequence being irrelevant, or 2) multiple regions of the cytoplasmic domain act to stabilize binding and the stabilizing features of these sequences act in a redundant manner such that deletion of all of them is necessary to abolish ligand binding (see 87).

5. 2. Phosphorylation

CD44 is phosphorylated on serine residues; no phosphorylation on threonine or tyrosine has been observed (69, 93, 94). It is not certain whether all molecules of CD44 are necessarily phosphorylated. Camp and colleagues have demonstrated that, at least under certain conditions, a fraction of molecules exists in a non phosphorylated state (95). Mutational analysis (69, 94) indicates that Ser323 and Ser325 are the only residues of the CD44 cytoplasmic domain that are phosphorylated *in vivo*. Of these residues Ser325 is the major residue that is phosphorylated, accounting for approximately 90% of the phosphorylation on CD44 (18). However, Ser323 is required for phosphorylation of Ser325 since nonconservative mutations of Ser323 abolish phosphorylation on Ser325 (69). The kinase that carries out this serine phosphorylation is unusual in that it is relatively specific for a serine at position 325; phosphorylation is essentially abolished when Ser325 is mutated to threonine (18). While residue Ser291 has the properties of a protein kinase C consensus site and may be able to be phosphorylated *in vitro* (96), *in vivo* this site is not phosphorylated (69). There is no observable increase

in CD44 phosphorylation after phorbol ester stimulation and no additional sites are phosphorylated (69).

As pointed out above, transfectants for mutant CD44 molecules not expressing that portion of the cytoplasmic domain after aa 318 and consequently lacking Ser323 and Ser325 bind hyaluronan similarly to transfectants for wild-type CD44. This observation suggests that phosphorylation of the cytoplasmic domain has no function in regulating the ability of CD44 to bind ligand. This point has been studied in more detail by examining hyaluronan binding of transfectants for mutant CD44 molecules in which either or both phosphorylated serine residues were mutated. In a comprehensive study, either or both of the potentially phosphorylated serine residues were mutated to neutral amino acids, to create a non phosphorylated molecule, or to acidic amino acids, to create a non phosphorylated molecule in which the mutated serine residue had a similar charge and volume to phosphoserine (97). No mutation had any effect on the ability of transfectants to bind soluble hyaluronan or to adhere to hyaluronan coated plates, indicating that serine phosphorylation plays no role in regulating the ability of CD44 to bind ligand. Somewhat different results were obtained by Pure *et al* (94), using the same host cell line. In their experiments mutation of either Ser323 or Ser325 greatly reduced constitutive HA binding; however, ligand was bound in the presence of an inducing antibody. The explanation for the discrepancy is not immediately apparent. The bulk of the evidence, however, indicates that phosphorylation is not involved in regulating the ability of CD44 to bind ligand.

What, then, is the function of CD44 phosphorylation? Peck and Isacke (19) examined the ability of CD44-negative melanoma cells transfected with CD44 constructions in which Ser323 and Ser325 were mutated to neutral or acidic amino acids to bind to an HA coated substrate and to migrate on this substrate after "wounding". No effect of the mutation on adherence to the substrate was observed; however, wound closure was inhibited relative to that observed with transfectants for wild-type CD44. Similar results were observed in a series of L-cell transfectants. The mutation of Ser325 to threonine, which, as discussed above, reduces CD44 phosphorylation by approximately 90%, was sufficient to inhibit migration of melanoma cells on an HA-coated substrate (18). Melanoma cells treated with a cell permeable peptide containing a phosphorylated Ser325 residue showed only control levels of migration on HA, while cells treated with a peptide in which Ser325 was not phosphorylated migrated efficiently on HA (18). All of these cells expressed equivalent amounts of CD44 and bound ligand equally well (18).

These experiments implicate phosphorylation of the CD44 cytoplasmic domain as being involved in mediating events downstream of ligand binding that are necessary for cell migration. The inhibition of migration by peptides containing a phosphorylated Ser325 suggests that intracellular components interact specifically with the phosphorylated form of CD44 (18). While modulation of the interaction of the CD44 cytoplasmic domain with cytoskeletal components (see below) has been suggested as a possible mechanism whereby phosphorylation could affect cell motility (19), there is no direct evidence for or against this idea. In particular, it is not clear whether ligand

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binding affects the degree of phosphorylation of Ser325. Nevertheless, these experiments hint at interesting consequences of the CD44-ligand interaction which may be mediated by the CD44 cytoplasmic domain.

5. 3. Localization in the plasma membrane.

In certain cell types CD44 exhibits a nonrandom distribution in the plasma membrane. Pre-B cells transfected with CD44 and L-selectin show a complementary distribution of the two molecules, with L-selectin concentrated on microvilli and sparse on the cell body while CD44 is found infrequently on microvilli and is concentrated on the cell body (42). Domain swapping experiments in which the cytoplasmic and transmembrane domains of L-selectin were exchanged for those of CD44 and vice versa indicated that the cytoplasmic and/or transmembrane domains were responsible for maintaining this localization (42). Chimeras containing a CD44 external domain and an L-selectin cytoplasmic domain were concentrated on microvilli, while chimeras consisting of an L-selectin external domain and a CD44 transmembrane and cytoplasmic domain were excluded from microvilli and were found predominantly on the cell body.

As polarized epithelial cell cultures reach confluency, CD44 becomes concentrated at areas of cell contact along the lateral cell surfaces and is excluded from the apical surface (40). The cytoplasmic domain was shown to be responsible for this localization as polarized epithelial cells transfected with a tailless CD44 construction expressed the tailless molecule predominantly on the apical surface (40). Mutational studies (41) have identified His330-Lys334 as a localization signal that directs membrane proteins to the basolateral surface. This sorting signal appears to depend critically on the integrity of the dipeptide Leu331,Val332. Thus clearly one function of the CD44 cytoplasmic domain is to direct the molecule to specific cellular locations in particular cell types. Unresolved are what different functions CD44 may serve when localized to different areas of the plasma membrane in different cells. Also unknown are the signals that cause domains regulating plasma membrane localization to operate in some cells but not in others.

5. 4. Association with the cytoskeleton

5. 4. 1. Association with ankyrin

Kalomiris and Bourguignon (47) demonstrated that CD44 binds *in vitro* to ankyrin-coated Sepharose beads. In mapping studies using plasmid constructions in which various portions of the CD44 cytoplasmic domain had been deleted, the ankyrin binding domain was mapped to a region between Asn304 and Leu318, and it was shown that ankyrin bound to a peptide containing the 15 aa of the mouse CD44 cytoplasmic domain spanning this region (68). HA binding correlated with the ability to bind ankyrin--binding of both HA and ankyrin was absent in transfectants for a mutant CD44 constructions truncated before Asn304 but present when the region between Asn304 and Leu318 was included (68). This would seem to make a self-consistent story and implicate the binding of CD44 to ankyrin as necessary for HA binding. However, Perschl and colleagues (87) demonstrated that transfectants of a mouse lymphoma for a construction in which residues 302-323, including the putative ankyrin binding domain, was deleted bound soluble HA as well as did transfectants for wild-type CD44. The reason for this discrepancy is

unclear, but it indicates that the role of ankyrin in mediating HA binding must remain an open question.

5. 4. 2. Association with actin microfilaments

Binding of hyaluronan in the presence of inhibitors of microfilament or microtubule function has been examined in an attempt to determine whether association of CD44 with either is required for ligand binding. Conflicting results have been obtained. Liu and colleagues (91) studied phorbol ester induced induction of HA binding by Jurkat cells and observed inhibition of HA binding by cytochalasin B and by colchicine and concluded that both actin microfilaments and microtubules were involved in mediating ligand binding. Murakami and colleagues, however, found that cytochalasin B had no effect on the binding of HA by phorbol ester activated MOLT 4 cells (73). Bourguignon and colleagues observed that cytochalasin B, but not colchicine, inhibited the constitutive binding of a mouse T-cell lymphoma to HA coated plates (45).

With these conflicting results it is difficult to draw any definitive conclusions from experiments using inhibitors regarding the involvement or noninvolvement of the actin cytoskeleton or of microtubules in mediating ligand binding by CD44. It is worthwhile to point out that in no case has the amount of CD44 on the cell surface after inhibitor treatment been reported. In view of the relationship between the amount of CD44 expressed on the cell surface and the extent of hyaluronan binding, together with the fact that ligand binding is seen only above a threshold amount of CD44, this point seems crucial. A relatively small nonspecific inhibition of cell surface CD44 expression after inhibitor treatment might have a relatively large effect on measurable ligand binding if the amount of CD44 is close to the threshold.

The ERM family members (ezrin, radixin, and moesin) and the related protein merlin (neurofibromatosis 2 tumor suppressor protein) have been proposed as links between proteins of the cell surface and the actin-containing cytoskeleton (reviewed in 48). These proteins share a globular amino terminal domain that is thought to interact with membrane glycoproteins and a charged carboxyl terminal domain that interacts with F-actin. ERM proteins are concentrated at regions such as microvilli and cell adhesion junctions where actin filaments associate with the plasma membrane (49).

Immunoprecipitation experiments revealed a 140 KD protein of baby hamster kidney cells that associated with ERM proteins (49). This protein was cloned and identified as a v9, v10-containing isoform of CD44. In immunofluorescence experiments CD44 was shown to co-localize with ERM proteins (49) and with merlin (48). ERM proteins (46) and merlin (48) have been shown to bind to glutathione-S-transferase/CD44 cytoplasmic domain fusion proteins. The affinity of binding of ERM proteins to the CD44 cytoplasmic domain was quite low at physiological ionic strength but was markedly increased in the presence of phosphatidylinositol 4,5 bisphosphate, which was shown to bind to the amino terminal portion of moesin (46). This result is consistent with a model whereby phosphatidylinositides bind to the amino terminal portion of ERM proteins and alter their conformation such that affinity for CD44 is increased (46).

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A point which is still somewhat unclear is why CD44H, which is also abundant in baby hamster kidney cells, was not found associated with ERM family members in the study of Tsukita *et al* (49), since the cytoplasmic domains of variant and standard isoforms of CD44 are identical. It is possible that the v9, v10 isoform tends to form oligomers that form a more stable complex with ERM proteins than does CD44H (49). A tendency of variant CD44 isoforms to oligomerize has been noted (89), lending weight to this suggestion. Alternatively, it is possible that the binding of some unknown v9, v10 ligand could transmit a signal that alters the conformation of the CD44 cytoplasmic domain so as to increase its affinity for ERM family members.

At the present time these experiments provide the most direct evidence for an association between CD44 and the actin cytoskeleton. Whether this association occurs in all cell types and what its physiological significance may be are still open questions. A cluster of basic amino acids in the cytoplasmic domain of CD44 located near the cell membrane (Lys298-Lys300) has been shown to be essential for the binding of moesin and, presumably, the other ERM proteins (50). These amino acids can be deleted without affecting ligand binding (87), however, so it seems very unlikely that binding of ERM proteins is required for ligand binding. Rather, it is probably much more likely that the ERM proteins mediate events downstream of ligand binding such as stable cell adhesion or cell movement. The failure of CD44 to bind ERM proteins causes CD44 to be distributed evenly on the cell surface of mouse L-cells rather than to be concentrated in microvilli (50), a point which is consistent with this idea. It is perhaps noteworthy that no association between CD44, ERM proteins, and ankyrin was observed, although it is not certain whether ankyrin would remain associated with ERM proteins under the extraction conditions used (49).

5.5. Signal transduction

As discussed in Section 2, ligand binding by CD44 is a regulated process, and in the physiological situation CD44 must often be "activated" to bind ligand (e.g. 12). It is a reasonable hypothesis that intracellular signals leading to CD44 activation, "inside-out" signals, may be mediated via the CD44 cytoplasmic domain, but there is no evidence supporting this.

Similarly, there is almost no information regarding possible signals that may be transmitted by CD44 once ligand is bound. A transient increase in intracellular calcium after binding hyaluronan has been reported for a mouse T cell lymphoma, and it was suggested that this calcium mobilization was necessary for an aggregation of CD44 molecules in the cell membrane that is putatively required for cellular binding to immobilized hyaluronan (45). There is one report that p56^{lck} is associated with CD44 in T lymphocytes and that cross linking of CD44 leads to an increase in the intrinsic kinase activity of p56^{lck} and to the subsequent phosphorylation of ZAP-70 (51). If this observation can be substantiated it would provide a potential mechanism by which CD44 could initiate downstream events upon binding of ligand. However, since the cytoplasmic domain of CD44 does not possess the CXCP sequence that is considered to be a p56^{lck} binding motif, it remains unclear how p56^{lck} might associate with CD44.

6. PERSPECTIVES

CD44 is a cell surface receptor for HA that is expressed in a very diverse assortment of cell types. Each cell lineage, at each stage of differentiation or activation, must regulate the function of the receptor to suit its own requirements. Thus, a broad spectrum of functions has been attributed to CD44 (Table 1). Cell specific carbohydrate modifications of the extracellular domain have recently emerged as an important mechanism for regulating the HA binding function of CD44. Much more detailed structural analysis is required to understand how different carbohydrate structures differentially influence hyaluronan binding. Other mechanisms of regulating HA binding, especially ones that show more rapid kinetics, remain to be discovered. The ability of CD44 to serve as a cell surface proteoglycan and to increase the size of its external domain by alternative splicing might potentially expand the ligand repertoire, provide additional regulation of HA binding, and allow completely new functions, such as cytokine presentation and cooperation with other extracellular matrix receptors such as integrins. How these added properties contribute to cell function must be explored. Also, the specific intracellular signals that lead to a change in activation state and/or lead to a change in the utilization of alternative exons need to be defined. Shedding of soluble CD44 from the cell surface and/or synthesis of soluble CD44 might provide another mechanism for modulating the function of cell surface CD44. Since CD44 shedding often seems to correlate with immune activity and tumor dissemination and may have diagnostic or prognostic value, its consequences need to be examined. Very little is known about the downstream consequences of engagement of the extracellular domain of CD44. What functions are initiated by ligand binding and how are they mediated? The extreme sequence conservation of the transmembrane and cytoplasmic domains suggests essential functions which have yet to be elucidated.

7. ACKNOWLEDGMENTS

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Send correspondence to: Dr Jayne Lesley, Cancer Biology Laboratory, The Salk Institute for Biological Studies, P.O. Box 85800, San Diego CA 92186-5800, Tel:(619)-453-4100 ext.1378, Fax:(619)-457-4765, E-mail: lesley@dcbsun.salk.edu