Hyaluronan Recognition Mode of CD44 Revealed by Cross-saturation and Chemical Shift Perturbation Experiments*

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CD44 is the main cell surface receptor for hyaluronic acid (HA) and contains a functional HA-binding domain (HABD) composed of a Link module with N- and C-terminal extensions. The contact residues of human CD44 HABD for HA have been determined by cross-saturation experiments and mapped on the topology of CD44 HABD, which we elucidated by NMR. The contact residues are distributed in both the consensus fold for the Link module superfamily and the additional structural elements consisting of the flanking regions. Interestingly, the contact residues exhibit small changes in chemical shift upon HA binding. In contrast, the residues with large chemical shift changes are localized in the C-terminal extension and the first α-helix and are generally inconsistent with the contact residues. These results suggest that, upon ligand binding, the C-terminal extension and the first α-helix undergo significant conformational changes, which may account for the broad ligand specificity of CD44 HABD.

CD44 is a type I transmembrane glycoprotein with diverse functions and is expressed on the surface of many cell types (1, 2). CD44 reportedly recognizes a variety of ligands (1, 2). The most investigated aspect of CD44 function is its ability to bind hyaluronic acid (HA), a major component of the extracellular matrix (1–3). HA is a very high molecular mass glycosaminoglycan, composed of a repeating disaccharide, β-guluronic acid (β1→3) N-acetyl-d-glucosamine (β1→4) (4). The binding of CD44 to HA has been implicated in both cell adhesion to the matrix (1–3). HA is a very high molecular mass glycosaminoglycan (5). While HA exists as a high molecular mass multivalent entity consisting of the flanking regions. Interestingly, the contact residues exhibit small changes in chemical shift upon HA binding. In contrast, the residues with large chemical shift changes are localized in the C-terminal extension and the first α-helix and are generally inconsistent with the contact residues. These results suggest that, upon ligand binding, the C-terminal extension and the first α-helix undergo significant conformational changes, which may account for the broad ligand specificity of CD44 HABD.

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EXPERIMENTAL PROCEDURES

CD44 HABD and HA Sample Preparation—The human CD44 HABD gene (residues 21–178) was amplified by PCR from cDNA encoding human CD44 fused to the firefly luciferase (3, 24) and cloned into the pET11a vector (Novagen) (24). CD4 HABD was expressed in Escherichia coli BL21 (DE3) codon Plus RP (Stratagene) without affinity tags and then

geared in vivo by a variety of mechanisms, and they exhibit different biological activities (6, 7). In addition to HA, CD44 interacts with the chondroitin sulfate (ChS) proteoglycans, serglycin (8–10), versican (11), and aggrecan (12). The CD44-binding elements on these proteoglycans are ChS side chains. These ChS proteoglycans may be involved in the adhesion and activation of CD44-expressing cells.

CD44 has an N-terminal functional domain that interacts with the HA and ChS chains (11–13). This ligand recognition domain contains a homology region, termed the Link module (14, 15). Link modules are found in extracellular matrix molecules (link protein, aggrecan, versican, neurocan, and brevican) and the protein product of tumor necrosis factor-stimulated gene-6 (TSG-6). The HA-binding domains from Link module-containing proteins are divided into three subgroups as described in Ref. 16. Briefly, a single Link module, a Link module with N- and C-terminal extensions, and a pair of Link modules, which are sufficient for a high affinity interaction with HA, are classified as Types A, B, and C, respectively. In addition, a single Link module from TSG-6, belonging to Type A, interacts specifically with chondroitin 4-sulfate (Ch4S) but not with chondroitin 6-sulfate (Ch6S) (17). Bovine link protein, which contains the Type C Link module, binds to neither Ch4S nor Ch6S (18). On the other hand, CD44, which belongs to Type B, interacts with Ch4S, Ch6S, chondroitin, and other ChS chains (11, 19), suggesting that the HA-binding domain of CD44 has a broader ChS binding specificity than those of the TSG-6 and link proteins. The three-dimensional structure and ligand-binding site of Type A, i.e. the TSG-6 Link module, have been determined by NMR (20, 21). In contrast, no structural information about Type B, including CD44, is available.

In the present study, a combination of cross-saturation and chemical shift perturbation experiments has been applied to the CD44 HA-binding domain-HA complex to separate the direct intermolecular contacts from the effects due to ligand-induced conformational changes (22, 23). Significant conformational changes were observed in the C-terminal extension and the first α-helix, suggesting a correlation between the conformational changes and the broad ligand specificity of the CD44 HA-binding domain.

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† The abbreviations used are: HA, hyaluronic acid; ChS, chondroitin sulfate; TSG-6, tumor necrosis factor-stimulated gene-6; Ch4S, chondroitin 4-sulfate; Ch6S, chondroitin 6-sulfate; CS, cross-saturation; AMS, 4-acetoamido-4-maleimidylstilbene-2′-disulfonate 2Na; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; HSQC, heteronuclear single quantum coherence.
data analysis was assisted by the Sparky software (32). In the HA NOESY spectra recorded with mixing times of 60 and 100 ms, respective-
ly, the uniformly $^{13}C$-labeled protein was prepared using mix-
ing M9 medium in 99.9% $^3$H$_2$O containing $^{13}NH_4Cl$ (1 g/liter) and unlabeled or $^{13}C$-glucose (2 g/liter) supplemented with Celtone-DN powder (1 g/liter). In addition, several selective $^{15}N$-labeled proteins were prepared by growing cells in M9 medium containing $^{15}NH_4Cl$ (1 g/liter) and unlabeled or [U-$^{13}C$]glucose (2 g/liter) supplemented with Celtone-DN powder (1 g/liter). In the measurement of the diffusion coefficients of CD44 HABD, we used AMS, which binds to the sulphydryl (SEI) group (27).

HA-HABD showed an upward shift in its position on an SDS gel when it was treated with AMS in the presence of dithio-
thereitol. No such shift was observed in the absence of dithio-
thereitol (data not shown). These results indicated that CD44 HABD contains three disulfide bonds and no free SH group. Second, to evaluate the HA binding activity of CD44 HABD, surface plasmon resonance measurements were employed. The results showed that CD44 HABD has HA binding activity, and the dissociation constant ($K_d$) for CD44 HABD toward the HA 250-mer is $2.7 \times 10^{-5} \mu M$ (the $K_d$ for the monomeric CD44Rg protein, expressed in Idl-D cells and deglycosylated, toward the HA 60-mer is $5 \times 10^{-6} \mu M$ (35)). Finally, the in vitro refolding of CD44 HABD using DsbC, a disulfide isomerase with chaperone activity (36), was achieved. By SDS-PAGE, the resultant prod-
uct shown was identical to the protein prepared by the reported method, suggesting that CD44 HABD adopts a native fold. The details of the refolding method using DsbC will be reported elsewhere. Taken together, these results strongly sup-
port the idea that CD44 HABD retains the native fold and the proper functions.

**Topology of CD44 HABD**—As a first step toward elucidating the molecular basis for the ligand recognition by CD44 HABD, we investigated the topology of CD44 HABD in an unbound state. By three-dimensional NMR analyses, we obtained the main chain resonance of CD44 HABD (29) (see “Experimental Procedures”). On the basis of both the NOE connectivities observed among the main chain C$^\alpha$ and H$^N$ resonances and the chemical shift index analysis (37), we determined the secondary structure and the topology of CD44 HABD (Fig. 1, D and E). CD44 HABD was identified as a structural domain, comprising two $\alpha$-helices ($\alpha_1$, 46–56; $\alpha_2$, 63–71) and two $\beta$-sheets. Sheet I is composed of five strands: b$_1$ (34–43), b$_2$ (46–55), b$_3$ (63–68), b$_4$ (70–75), and b$_5$ (77–82). Sheet II is a two-stranded antiparallel $\beta$-sheet: $\beta_1$ (79–82) and $\beta_2$ (85–88). A comparison of the topology with that of TSG-6 indicated that CD44 LM adopts the consensus fold for the Link module superfamily (20). It should be noted that the N- and C-terminal extensions form three additional $\beta$-strands ($\beta_a$, $\beta_b$, and $\beta_g$) to sheet I of the consensus fold (Fig. 1, E, green).

**Cross-saturation Experiments**—To identify the residues of CD44 HABD that contact HA, CS experiments were performed (23) (Fig. 2A). HA oligosaccharides of defined lengths were prepared, as reported by Tawada et al. (26). HA with an aver-
age molecular mass of 6.9 kDa (termed HA$_{34}$) was selected as a saturation-donating partner from several HA oligomers for the following reasons. First, HA$_{34}$ is involved in many biological processes, such as the induction of Fas expres-
sion and the Fas-mediated apoptosis of synovial cells (38), the activation of integrins on colon cancer cells (39), and the enhancements of CD44 cleavage from tumor cells and their motility (7). Second, HA$_{34}$ is likely to fully cover the HA-binding surface of CD44 HABD. Finally, the saturation can be transferred more effec-
tively from HA$_{34}$ to the labeled CD44 HABD than from short-
chain HA oligosaccharides (e.g. the HA hexasaccharide, termed...
FIG. 1. Sequence, NMR spectra, and topology of CD44 HABD. A, schematic diagram showing the sequence of the expressed CD44 HABD. The residual N-terminal sequence derived from the multiple cloning site is designated by lowercase letters. B and C, two-dimensional $^1H$-$^15N$ HSQC spectra of uniformly $^15N$-labeled CD44 HABD in the unbound (B) and HA$_{34}$/H11011$_34$ bound (C) states. The cross-peaks are labeled with the one-letter codes for amino acids and the residue numbers. D, interstrand backbone NOE connectivities of CD44 HABD. Interstrand NOEs observed in three-dimensional $^15N$-edited and two-dimensional NOESY spectra are marked by arrows. E, topology of CD44 HABD. Strands and helices are represented by arrows and cylinders, respectively. The Link module region is depicted with a black line, and the N- and C-terminal extensions are shown with green lines.
HA residues in the complex. Pulsed-field gradient NMR experiments (33) generated diffusion coefficients of CD44 HABD in complex with HA bound and HA dissociated from complexes, and HA dissociated from complexes at 25 °C, respectively, indicating that the rotational correlation time of the HA bound complex is longer than that of the HA dissociated from complexes. Indeed, the CS phenomena were efficiently observed for the CD44 HABD-HA complex.

HA-binding Site—The main chain resonances of CD44 HABD in complex with HA were assigned by three-dimensional NMR analyses (Fig. 1C). The rf irradiation applied to the complex resulted in selective intensity losses for the CD44 HABD resonances by the CS phenomena (Fig. 2B). On the basis of the spectra for the complex with and without irradiation, the reduction ratios of the peak intensities were calculated (Fig. 3A). The residues strongly affected by the irradiation were mapped on the topology of CD44 HABD (Fig. 3D). The affected residues are distributed on both the consensus fold and the additional structural elements consisting of the flanking regions.

Conformational Changes upon HA Binding—The weighted averaged 1H and 15N chemical shift changes between the HA bound and unbound states of CD44 HABD are plotted in Fig. 3B. The residues with large chemical shift differences are also mapped on the topology of CD44 HABD. Interestingly, these residues are mostly localized on the C-terminal extension and the α1 helix, designated as the Shift region (Fig. 3D, colored in light yellow), and they are generally inconsistent with the contact residues. Significant chemical shift changes upon HA binding are observed in the 15N resonances of the residues in the Shift region (data not shown), suggesting that the Shift region may adjust to accommodate structurally different ligands. In our preliminary studies, some of the Shift region signals showed different chemical shift changes upon chondroitin binding, as compared with HA binding, in the 1H-15N HSQC spectra (data not shown), suggesting that the Shift region adopts a somewhat different conformation in the chondroitin-bound complex from that of the HA-bound complex.

Since protein-carbohydrate interactions are implicated in many biological events, from development to tumorigenesis, understanding carbohydrate recognition at an atomic resolution is of utmost importance (42). Chemical shift perturbation is a widely used NMR method to map protein interfaces. However, in this report, chemical shift perturbation would not be an appropriate method to identify the ligand-binding site of CD44 HABD because of the small chemical shift changes at the contact site and the presence of significant conformational changes in the flanking regions. Several chemical shift perturbation experiments for protein-carbohydrate complexes have been reported (21, 43–46). A common feature of these experiments is that the chemical shift perturbations of the protons on the putative ligand-binding sites are at most 0.35 ppm. This feature may reflect the fact that the common molecular scaffold of carbohydrates has no aromatic group that would cause magnetic anisotropies (Fig. 3E). On the other hand, the efficiency of CS does not depend on the presence of an aromatic group in the ligand molecules. Therefore, the CS method is especially helpful to determine interfaces of proteins with carbohydrates that have no source of ring current shifts.

In this study, we have identified the topology and the ligand recognition mode of CD44 HABD. The topology will be helpful for modeling other HA-binding domains belonging to the Type B Link module family. A combination of the CS and chemical shift perturbation methods may provide a useful means to separate the direct intermolecular contacts from the effects due to ligand-induced conformational changes.

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FIG. 3. Comparison between cross-saturation and chemical shift perturbation experiments. A, plots of the reduction ratios of the signal intensities originating from the backbone amide groups, with and without presaturation, along with the determined secondary structure. The residues with reduction ratios >0.2 are colored red, and those with reduction ratios within the 0.1–0.2 range are orange. B, plots of the weighted averaged $^1$H and $^{15}$N chemical shift changes calculated with the function $\Delta \chi = (2.25 \Delta H + 0.25 \Delta N)/2$. Residues with chemical shift changes (Δ$\chi$) > 0.6 ppm are colored cyan. C, an alignment of the CD44 HABD and TSG-6 Link module sequences, based on the secondary structure elements. The CD44 HABD residues affected by CS experiments are colored as described in the legend to A. Amino acids that could be involved directly in HA binding by the TSG-6 Link module (as described by Kahmann et al. (21)) are colored purple. Amino acid insertions are indicated with triangles. D, mapping of the residues affected in the CS and chemical shift perturbation experiments on the topology of CD44 HABD. The color usage is as described above. Ile$^{51}$, Ala$^{68}$, and Asn$^{149}$ were affected in both experiments and are doubly colored. The Shift region, which is covered with residues that are strongly affected in the chemical shift perturbation experiments, is shown in light yellow. The residues of the N-glycosylation sites are labeled with brown circles (41). The amino acids that have been investigated by site-directed mutagenesis are labeled with stars (13, 40). E, schematic representation of the chemical shift changes and CS effects due to sugar and aromatic rings.
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