CD44, a major cell surface receptor for hyaluronan (HA), contains a functional domain responsible for HA binding at its N terminus (residues 21–178). Accumulating evidence indicates that proteolytic cleavage of CD44 in its extracellular region (residues 21–268) leads to enhanced tumor cell migration and invasion. Hence, understanding the mechanisms underlying the CD44 proteolytic cleavage is important for understanding the mechanism of CD44-mediated tumor progression. Here we present the NMR structure of the HA-binding domain of CD44 in its HA-bound state. The structure is composed of the Link module (residues 32–124) and an extended lobe (residues 21–31 and 125–152). Interestingly, a comparison of its unbound and HA-bound structures revealed that rearrangement of the β-strands in the extended lobe (residues 143–148) and disorder of the structure in the following C-terminal region (residues 153–169) occurred upon HA binding, which is consistent with the results of tryptophan fluorescence studies of the CD44 HA-binding domain. The order-to-disorder transition of the C-terminal region by HA binding may be involved in the CD44-mediated cell migration.

CD44 is a type I transmembrane glycoprotein with diverse functions and is expressed on the surface of many cell types (1, 2). CD44 recognizes hyaluronic acid (HA) (3) and participates in various biological processes, such as lymphocyte rolling, tumor cell migration, and invasion (2).

HA, a major component of the extracellular matrix, is a very high molecular mass glycosaminoglycan, composed of a repeating disaccharide, D-glucuronic acid (GlcA) (β1 → 3) N-acetyl-D-glucosamine (GlcNAC) (β1 → 4) (4). Although HA exists as a high molecular mass polymer, HA fragments of various molecular sizes can be generated in vivo by a variety of mechanisms, and they exhibit different biological activities (5, 6).

The cell surface CD44 is proteolytically cleaved at the extracellular region (residues 21–268) (7). Cleavage of CD44 has been suggested to play an important role in tumor cell migration along extracellular matrix components (8, 9). Cleavage of the extracellular region is promoted by various pathways, such as those activated by extracellular Ca^{2+} influx, protein kinase C, Rho family small GTPases, and Rac and Ras oncoproteins (8, 9). Recently, it was reported that small HA oligosaccharides, generated by tumor cells constitutively expressing hyaluronidases, could induce the cleavage of the extracellular region and promote tumor migration in a CD44-dependent fashion (6, 10).

CD44 has an N-terminal functional domain that interacts with HA. This ligand recognition domain contains the link protein homology region or the Link module (residues 32–124). Link modules are found in extracellular matrix molecules, such as link protein, aggrecan, versican, neurocan, and brevican, and the protein product of tumor necrosis factor-stimulated gene 6 (11). In addition, CD44 requires N- and C-terminal extensions (residues 21–31 and 125–178, respectively) for proper folding and HA binding activity (12, 13). Recently, the topology of the secondary structures and the three-dimensional structure of CD44 HABD in the unbound state were revealed, and the N- and C-terminal extensions were found to form an additional structural lobe that intimately contacts with the Link module (14, 15). Moreover, we found from cross-saturation experiments (16) for the CD44 HABD-HA complex that the contact residues of CD44 HABD for HA are distributed in both the consensus fold for the Link module and the extended lobe (14). Interestingly, we found that the residues with large chemical shift changes induced by HA binding were mostly localized in the C-terminal extension and the first α-helix, and they generally differed from the contact residues revealed by the cross-saturation experiment. These results led us to hypothesize that the first α-helix and the C-terminal extension undergo significant conformational changes upon HA binding.
Hyaluronan-induced Structural Changes of CD44

In the present study, the solution structure of CD44 HABD in the HA-bound form has been determined by NMR. Comparisons of the structures and the backbone flexibility of CD44 HABD between its unbound and HA-bound states revealed that the C-terminal region of CD44 HABD became disordered upon HA binding. Concomitant with this structural analysis, trypsin digestion experiments of CD44 HABD in the presence and absence of HA showed that the proteolytic susceptibility in the C-terminal region increased upon HA binding. This ligand-induced order-to-disorder transition of the C terminus of CD44 HABD provides a plausible explanation for the mechanism underlying the HA-induced CD44-mediated cell migration.

MATERIALS AND METHODS

NMR Spectroscopy—CD44 HABD, corresponding to residues 21–178 of CD44, was expressed as described previously (12, 14). The expressed CD44 HABD comprises the N-terminal extension (residues 21–31), the Link module (residues 32–124), and the C-terminal extension (residues 125–178). HA hexamer (GlcA-GlcNAc-GlcA-GlcNAc-GlcA-GlcNAc) was prepared as described previously (17). The samples contained 2.0 mM 13C,15N-labeled CD44 HABD and 6.0 mM HA hexamer, in 50 mM phosphate buffer (pH 6.7) with 150 mM NaCl and 1 mM NaN3. NMR experiments were performed at 37 °C on Bruker Avance 500 and Avance 600 spectrometers. All of the NMR spectra were processed by Sparky software (18). Main chain and side chain resonance assignments were derived from an analysis of the following double and triple resonance three-dimensional experiments: HNCA, HN(CO)CA, HNCO, HNCA CB, CBCA(CO)NH, HBHA(CBCACO)NH, H(CC)ONH, C(CC)ONH, and HCCH total correlation spectra.

Structure Calculation—NOE interproton distance restraints were derived from the three-dimensional 15N- and 13C-edited NOE experiments with mixing times of 60 and 120 ms, respectively. Cross-peak intensities were used to evaluate the target distances. Dihedral ϕ and ψ angles were obtained as described (19, 20). Structures were generated with CYANA, using the CANDID method (21) for NOE spectroscopy cross-peak assignment and calibration. Two hundred structures were calculated at each iteration. The 20 lowest energy structures have been submitted to the Protein Data Bank (code 2I83). [1H]-15N NOE experiments were done using the pulse sequences adapted from standard schemes (22).

Trypsin Digestions of CD44 HABD—Trypsin digestions were performed in 50 mM Tris-HCl, 10 mM CaCl2 at pH 7.4, at 20 °C in the presence and absence of HA. The size of the added HA was 1000–2000-mer. The concentration of the CD44 HABD was 34 μM. Digestion was started by the addition of an aqueous solution of trypsin (final concentration, 850 nM). Aliquots were removed periodically, mixed with 2× denaturing loading dye, boiled for 5 min, and analyzed by Tris-Tricine SDS-PAGE. Gels were stained with Coomassie Brilliant Blue. The digests were stained with 3,5-dimethoxy-4-dihydroxycinnamic acid in 50% acetonitrile in H2O. The proteins were eluted by a gradient of 0–65% acetonitrile in 0.09% trifluoroacetic acid in H2O. The peaks were fractionated and mixed with a saturated solution of 3,5-dimethoxy-4-dihydroxycinnamic acid in 50% acetonitrile in H2O, 0.1% trifluoroacetic acid and subjected to MALDI-TOF mass spectroscopy.

RESULTS

Structure Determination of CD44 HABD in the HA-bound Form—CD44 HABD comprises the Link module (residues 32–124) and the N- and C-terminal extensions (residues 21–31 and 125–178, respectively) (Fig. 1A). Our previous studies, based on cross-saturation and chemical shift perturbation experiments, showed that the structural elements consisting of the N- and C-terminal extensions of CD44 HABD contain part of the contact surface for HA and undergo significant conformational changes upon HA binding (14). To elucidate the structural changes of CD44 HABD, the solution structure of CD44 HABD in the HA-bound state was determined by heteronuclear multidimensional NMR measurements. A summary of the structural statistics for the CD44 HABD coordinates (residues 21–152) is described in Table 1, and a superimposition of the backbone atoms for the ensemble of 10 simulated annealing structures is shown in Fig. 1B. The 10 structures were superimposed in a folded region, and residues 21–152 were defined, whereas residues 153–178 were poorly defined. The defined structure comprises a consensus fold for the Link module and an additional structural lobe formed by the N and C termini.

We performed two-dimensional 13C-filtered NOE spectroscopy experiments to observe the intermolecular NOE between CD44 HABD and HA (23). A few intermolecular NOEs were observed in the aromatic (CD44 HABD)/methyl (HA) and methyl (CD44 HABD)/ring proton (HA) regions. However, the assignments of these signals were ambiguous, because of signal overlap and broadening. We checked that the intermolecular NOEs were not included in the assigned NOE peaks derived from the three-dimensional 13C-edited or 15N-edited NOE experiments, on the basis of the chemical shifts of the observed signals in the filtered NOE spectroscopy experiment.

The secondary structural elements of the HA-bound form of CD44 HABD were defined on the basis of the backbone dihedral angles and the interstrand NOEs. A ribbon diagram of the HA-bound conformation of CD44 HABD is shown in Fig. 1C. The HA-bound conformation of CD44 HABD consists of two α-helices (α1, residues 46–56; α2, 63–72) and two β-sheets, made of six and three β-strands, respectively (β0, 23–26; β10, 29–30; β1, 33–38; β2, 58–59; β3, 80–82; β4, 85–90; β5, 103–106; β6, 116–120; β7, 127–128; β8, 144–147). These secondary structure elements are designated according to those of an unbound solution structure of CD44 HABD that was previously determined (15).

We previously identified the contact residues of CD44 HABD for HA by cross-saturation experiments (14). The residues with reduction ratios larger than 0.20 comprise Thr76, Cys77, Arg78, Tyr79, Gly80, Ile96, Cys97, Ala98, Ala99, Asn100, Asn101, Leu107, Asp115, and Gly152, and those with ratios from 0.15 to 0.20 include Leu24, Asn25, Tyr105, Ile106, Asn149, and Asp151 (Fig. 1A). Surface representations of the structure with the HA-binding sites are shown in Fig. 1D.
FIGURE 1. Structure of CD44 HABD in the HA-bound state. A, primary sequence of CD44 HABD. The α-helix and the β-strand formed in the HA-bound state are depicted as a cylinder (green) and an arrow (cyan), respectively. In the cross-saturation experiments for the CD44 HABD-HA complex, the affected residues with reduction ratios >0.20 and those with reduction ratios from 0.15 to 0.20 are highlighted in red and yellow, respectively (14).

B, stereo diagram (straight view) showing an overlay of the 10 final structures on the average coordinate positions for the backbone (N, Cα, and C') atoms of the secondary structure elements. Residues 153–178 are disordered and are not shown. The N and C termini are labeled.

C, stereo diagram (straight view) showing a ribbon representation of the minimized mean structure of CD44 HABD. The α-helices and the β-strands are shown in green and cyan, respectively. The N and C termini and the secondary structure elements are labeled. D, left panel, surface representations of CD44 HABD with the HA hexamer. Residues affected in the cross-saturation experiments (14) are mapped on the structure of CD44 HABD. Residues with reduction ratios >0.20 are colored red, and those with reduction ratios from 0.15 to 0.20 are colored yellow. The HA hexamer is depicted in a ball representation and is colored green to show a putative binding orientation. Right panel, structure rotated 90° around the vertical axis relative to the structure in the left panel. These structures were generated with the program MolMol (30).
Hyaluronan-induced Structural Changes of CD44

TABLE 1

| Structural statistics                      | Distance restraints | Dihedral angle restraints | Ramachandran statistics (%) | Root mean square deviations from the average structure* |
|--------------------------------------------|---------------------|--------------------------|-----------------------------|-----------------------------------------------------|
| Short range (|i − |j| ≤ 1) | Medium range (2 ≤ |i − |j| ≤ 4) | Long range (|i − |j| > 5) | Total | Hydrogen bonds | Φ | Ψ | protein backbone atoms (A) |
| 1541 | 114 | 351 | 2006 | 29 | 22 | 58 |

These contact residues are distributed on one side of the molecule and on both the Link module (residues 32–124) and the N- and C-terminal extensions (residues 21–31 and 125–178, respectively). The length of the contact surface for HA was about 30 Å, which corresponds to a hexameric HA, consisting of three repeating disaccharides (GlcA-GlcNAc-GlcA-GlcNAc), the minimum size of HA that efficiently binds to CD44 (24).

The backbone interstrand NOEs that correspond to those observed between β8 and β9 in the unbound state were not observed in the HA-bound state (Fig. 2, A and B). The backbone interstrand NOEs that correspond to those observed between β8 and β9 in the unbound state were not observed in the HA-bound state (Fig. 2, C and D). Because residues 158–162 go under the loop between β7 and β8 in the unbound state, we supposed that the undefined C-terminal region (resides 153–178) goes under a loop between β7 and β8. However, this supposition could not be confirmed, because of the limitation of observable NOEs.

In addition, β8 became rearranged relative to β0 upon HA binding by two residues. As shown in Fig. 2C, the backbone interstrand NOE connectivities revealed that residues 22–25 of β0 partner with residues 145–148 of β8 in parallel, in the unbound state. However, residues 22–25 of β0 partner with residues 143–146 of β8 in parallel, in the HA-bound state (Fig. 2D).

Heteronuclear [1H]-[15N] NOE experiments were performed in the unbound and HA-bound states to compare the backbone flexibility, and significant decreases of the heteronuclear NOEs at residues 162–169 were observed upon HA binding (Fig. 2, E and F). To facilitate our discussion, we refer to this region as the anchoring region. The anchoring region runs beside the α1 helix and is ordered in the unbound state. The decreases of the heteronuclear NOEs upon HA binding in the anchoring region suggest that the region became unstructured upon HA binding. Indeed, no NOEs were observed between the α1 helix and the anchoring region in the HA-bound state, and the anchoring region was unstructured in the HA-bound state (Fig. 2, A and B).

Trypsin Digestions of CD44 HABD in Its Unbound and HA-bound States—To confirm that the anchoring region of CD44 HABD became unstructured upon HA binding, a combination of proteolysis and mass spectrometry was used. We performed time course proteolytic digestions of CD44 HABD in both the absence and presence of HA (Fig. 3A). At 60 min, two major cleavage products were observed in the absence of HA on SDS-PAGE. To identify the cleavage products, the sample at 20 min was analyzed by MALDI-TOF mass spectrometry. On the basis of a comparison between the experimental and theoretical masses, these two fragments were found to be C-terminally truncated fragments (amino acids 21–154 and 21–162) (data not shown).

On the other hand, in the presence of HA, only one cleavage product was observed at 60 min. Analysis of the fragment revealed that it spanned from amino acids 21 to 162. Despite the decreased digestion at Arg154, predigested CD44 HABD (residues 21–178) in the HA-bound state disappeared rapidly, as compared with the unbound state. These results are summarized in Fig. 3B. Therefore, upon HA binding, the cleavage at the peptide bond between Arg154 and Tyr155 was suppressed and that between Arg162 and Thr163 was enhanced. The peptide bond between Arg154 and Tyr155 is in close proximity to the HA contact residues (i.e. Asn149, Asp151, and Gly152), suggesting that the decrease in the proteolytic cleavage was due to hindrance by the bound HA. In contrast, the peptide bond between Arg162 and Thr163, with increased the proteolytic susceptibility upon HA binding, is located in the anchoring region. These results confirmed the HA-induced order-to-disorder transition of the anchoring region.

DISCUSSION

Our previous cross-saturation experiments showed that the contact residues of CD44 HABD for HA were located on both the Link module and the flanking regions. In addition, the residues with significant chemical shift perturbations upon HA binding were different from the contact residues, and they were distributed in the first α-helix and the C-terminal extension. On the basis of these results, we concluded that significant conformational changes occur in the C-terminal extension upon HA binding. In this work, we elucidated the solution structure of CD44 HABD in the HA-bound state. The resultant structure comprises a consensus fold for the Link module and the extended lobe, including N- and C-terminal extensions. The residues in the C-terminal extension exhibit high root mean square deviation values, as compared with those at the N-terminal extension and the Link module, because of the intrinsic flexibility of the C-terminal extension.

Tumor necrosis factor-stimulated gene-6, which possesses the amino acid sequence of the Link module but not that of the
FIGURE 2. Comparison of CD44 HABD in its unbound and HA-bound states. A and B, ribbon representations of the unbound (A) and HA-bound (B) states of CD44 HABD. All of the α-helices are colored red. The β-strands are colored cyan and green in the Link module region (residues 32–124) and the lobular region (residues 21–31, 125–178), respectively. The C-terminal unfolded regions (residues 170–178 in A and 153–178 in B) are omitted. C and D, backbone interstrand NOEs in the unbound (C) and HA-bound (D) CD44 HABD. The labels of each amino acid are colored magenta or red, according to the side chain orientation relative to the plane of the β-sheet. To clarify the rearrangement of the β-sheet, Ile22 and Ile145 are labeled with magenta rectangles, Asp23 and Thr146 are green, Leu24 and Ile147 are red, and Asn25 and Val148 are cyan. E and F, plots of the [1H]-15N heteronuclear NOEs for the residues of CD44 HABD in the unbound (E) and HA-bound (F) states. Residues with heteronuclear NOE < 0.5 are shown in red. The backgrounds of the region corresponding to residues 162–169 are colored green. A and B were generated using MolMol (30).
extension region found in CD44, plays important roles in inflammation and ovulation through the interaction with HA. Recently, Blundell et al. have determined the three-dimensional structure of the Link module of tumor necrosis factor-stimulated gene-6 by NMR analyses, which is almost similar to that of CD44 HABD determined in the present study (26).

The comparison of the structures of CD44 HABD in its unbound and HA-bound forms showed that the overall folding of the solution structure of the HA-bound form was the same as that of the unbound form. However, significant structural differences were present in the C-terminal extension. First, the rearrangement of the parallel /H9252/8-strands occurred in the extended lobe of HABD between CD44 and LYVE-1. Ile143, Ile145, and Ile147 of CD44 HABD and the corresponding residues in LYVE-1 HABD are shown in red, to clarify the periodicity of the amino acid residues. B indicates the periodicity of the amino acid residues.

A second structural change we observed was that the anchoring region of CD44 became disordered upon HA binding. The anchoring region is colored red for HA undergoing small chemical shift changes upon HA binding and that the residues with large chemical shift changes reside in the C-terminal extension and the a1 helix. The results presented in this paper directly explain the observed large chemical shift changes.

Unfortunately, we were not able to obtain structural information about the bound HA in this study, because of significant overlaps of the 1H NMR signals from HA. As commonly seen in NMR spectra of carbohydrates. Stable isotope labeling for HA is required for further analysis of the bound conformation of HA to CD44 (27).

Although the physiological significance of the order-to-disorder transition of the anchoring region upon HA binding remains to be elucidated, the structural change might contribute to CD44-dependent cell migration. For example, the disordered state of CD44 HABD might be favored for multimerization of CD44 on cells, leading to the activation of intracellular signaling pathways related to cell migration. Furthermore, the order-to-disorder transition of CD44 HABD may be responsible for in vivo proteolytic cleavage in the anchoring region, because the transition enhances the proteolytic susceptibility of the anchoring region as shown in the trypsin proteolysis experiments (Fig. 4B). CD44 is reportedly proteolytically cleaved by metalloproteases, such as MT1-MMP (28), ADAM-10, and ADAM-17 (7). Although the anchoring region (residues 162–169) does not contain previously reported cleavage sites (Gly192-Tyr193, Gly233-Ser234, and Ser249-Gln250) in the stem region (29), the transition may affect the proteolytic susceptibility of the cleavage sites by the metalloproteases, because we confirmed that the transition also occurs in CD44 HABD with the stem region by NMR (data not shown).

We are currently investigating whether the structural change of CD44 HABD contributes to proteolytic cleavage, based on cell biology studies.

CONCLUSIONS

We determined the solution structure of CD44 HABD in the HA-bound form. A comparison of the bound state of CD44 HABD with the unbound state of the protein revealed that a rearrangement of β-strands occurred in the extended lobe of
CD44 HABD. Moreover, the C-terminal region of CD44 HABD became disordered and was released from the structural domain. This study will shed light on the HA-induced promotion mechanisms for CD44-dependent cell migration.

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