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Identification and Characterization of CD44RC, a Novel Alternatively Spliced Soluble CD44 Isoform that can Potentiate the Hyaluronan Binding Activity of Cell Surface CD44

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Abstract

Soluble CD44 proteins generated by proteolytic cleavage or aberrant intron retention have been shown to antagonize the ligand binding activity of the corresponding cell surface receptor, inducing apoptosis and inhibiting tumor growth. Interestingly, such findings appear to contradict recent studies demonstrating a correlation between the presence of high levels of soluble CD44 in the serum of cancer patients and poor prognosis. In the present study, we report the cloning of a novel, naturally occurring, differentially expressed, soluble CD44 isoform, designated CD44RC, which, in contrast to previously described soluble CD44 proteins, can dramatically enhance the hyaluronan binding activity of cell surface CD44. Sequence analysis suggests that CD44RC is generated by an alternative splicing event in which the 3′ end of CD44 exon 2 is spliced into an internal splice acceptor site present within exon 18, altering reading frame and giving rise to a soluble protein with a unique COOH terminus. Functional studies suggest that CD44RC enhances hyaluronan binding by adhering to chondroitin sulfate side-chains attached to cell surface CD44, generating a multivalent complex with increased avidity for hyaluronan.

Keywords: CD44, hyaluronan, chondroitin sulfate, alternative splicing, leukemia.

Introduction

Soluble proteins reactive with CD44 monoclonal antibodies (mAbs) can be readily detected in the serum of normal individuals and are frequently present at elevated levels in patients with various malignant diseases including lymphoma, chronic lymphocytic leukemia and cancers of the breast, colon and stomach [1–5]. Similar increases in expression are also seen in various autoimmune and inflammatory conditions including rheumatoid arthritis [6]. In the case of malignant disease, it has been proposed that the concentration of soluble CD44 in the serum may be a sensitive indicator of tumor burden [1,7,8]. In patients with lymphoma, decreases in soluble CD44 paralleled responsiveness to chemotherapy, with control levels being attained in individuals exhibiting a complete response [9]. Similarly, in animal models, the level of soluble CD44 in the circulation varied according to the rate of tumor growth and the magnitude of the anti-tumor immune response induced [10].

At present, neither the mechanism by which soluble CD44 is generated nor the precise role that the molecule plays in tumor growth and/or metastasis is clear. In normal individuals and lymphoma patients, the primary CD44 species detected in serum has a molecular mass of 70–80 kDa [3]. However, in patients with gastric or colon cancer, species of 130–190 kDa may predominate [1]. Several studies have demonstrated that CD44 can be shed from the surface of primary lymphocytes and lymphoma cells maintained in culture [11,12]. Treatment with TNF-α or IFN-γ increased release from primary lymphocytes but not lymphoma cells [13]. An endogenous protease may be involved in this process since treatment of cells with various protease inhibitors, including aprotinin and PMSF prior to culture, substantially decreased the amount of soluble material that could be detected [10,13]. Introduction of a cDNA encoding CD44H into the CD44-negative Burkitt lymphoma cell line, Namalwa, confirmed that the standard 90 kDa CD44 isoform expressed by most resting hemopoietic cells could give rise to soluble CD44 [12]. Presumably, the presence of higher molecular mass soluble CD44 proteins in the circulation of patients with various epithelial malignancies reflects the fact that such tumor cells may differentially express certain alternatively spliced CD44 isoforms that can also serve as substrates for endogenous or exogenous proteases. Although the precise site at which CD44 is cleaved by proteolyic enzymes remains to be identified, the size of the soluble protein and the fact that the molecule retains hyaluronan binding activity [12,14] suggest that it is located close to the membrane.

Proteolytic cleavage of the corresponding cell surface protein is clearly not the only mechanism by which soluble

Abbreviations: mAbs, monoclonal antibodies; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; CCII, Cool Calf II; HBSS, Hanks' balanced salt solution; PBS, phosphate-buffered saline; RT-PCR, reverse transcription polymerase chain reaction; CS'ase, chondroitinase ABC; HA'ase, hyaluronan lyase; TNF-α, tumor necrosis factor-alpha; IFN-γ, interferon-gamma; PBL, peripheral blood leukocyte.

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CD44 is generated. Specifically, there is evidence that aberrant splicing events resulting in intron retention can occur in certain tumor cell lines, giving rise to mRNA transcripts that encode soluble CD44 proteins that terminate prior to the membrane spanning domain [15–17]. When expressed in tumor cells, such proteins can bind to hyaluronan or other potential CD44 ligands blocking cellular attachment and preventing tumor growth and metastasis *in vivo* [15–17].

In the present study, we describe a novel naturally occurring alternatively spliced CD44 isoform, designated CD44RC, that encodes a soluble form of the CD44 molecule. In contrast to previously described soluble CD44 proteins, CD44RC was found to markedly enhance the hyaluronan binding function of cell surface CD44. Evidence was obtained suggesting that CD44RC mediates this effect by binding to chondroitin sulfate side-chains attached to cell surface CD44, generating a multivalent complex with increased avidity for hyaluronan.

Materials and Methods

Cell Lines

The erytholeukemic cell line K562, the histiocytic cell line U937, the promyelocytic cell line HL60, and the myelomonocytic cell line KG1 and its less mature derivative KG1a, were all obtained from the American Type Culture Collection (ATCC; Rockville, MD). K562, U937 and HL60 cells were cultured in Dulbecco's minimum essential medium (DMEM) (Stem Cell Technologies Inc., Vancouver, Canada) supplemented with 10% Cool Calf II (CCII) (Sigma, St. Louis, MO). KG1 and KG1a cells were grown in Iscove's modified Dulbecco's media supplemented with 20% fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT). All cell lines were maintained at 37°C in an atmosphere containing 5% CO_2.

Cloning of CD44RC

mRNA was isolated from the myelomonocytic cell line KG1a using a Stratagene mRNA Isolation Kit (Stratagene, La Jolla, CA). cDNA was synthesized using a Stratagene First Strand cDNA Synthesis Kit (Stratagene) and CD44 amplified by PCR (Hybaid OmniGene, Labnet, Woodbridge, NJ) (30 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 minute) using 10 μl of the first strand synthesis reaction and CD44 exon 1 (5′-GGTCTAGACCGTTCGCTCCGGACACCATGG-3′)- and exon 20 (5′-GGTCTAGATTACACCCCAATCTTCATGTCC-3′)-specific primers. A full-length CD44H cDNA template isolated from the plasmid pCDM8.CD44H [18] by digestion with *Xba*I was used as a control. The PCR products were separated on a 1% agarose gel and visualized by staining with ethidium bromide. The approximately 500-bp fragment was excised, the DNA isolated using GeneClean (BIO 101, Vista, CA), "blunted" using T4 polymerase (Gibco BRL, Gaithersburg, MD) and ligated into the EcoRV site of the vector pZERO 2.1 (Invitrogen, San Diego, CA). Restriction enzyme analysis confirmed that the majority of clones obtained had an identical insert. pZERO.CD44RC clone #2 was digested with *Xba*I and the fragment obtained cloned into the *Xba*I site of pBlueScript (KS)+. One of the clones obtained (pBS.CD44RC clone # 2.2) was completely sequenced in both directions at the University of British Columbia DNA Sequencing Facility using T3 and T7 primers.

Cellular Expression of CD44RC

The cellular expression of CD44RC was determined by reverse transcription polymerase chain reaction (RT-PCR) analysis. mRNA was isolated and cDNA generated from peripheral blood leukocytes (PBL) obtained from two healthy adult volunteers and from the cell lines KG1, KG1a, HL60 and U937 as described above. A common 5′ primer corresponding to CD44 exon 1 (5′-GGTCTAGACCGTTCGCTCCGGACACCATGG-3′) was used together with two different 3′ primers corresponding, respectively, to CD44 exon 20 (5′-GGTCTAGATTACACCCCAATCTTCATGTCC-3′) or to the junction between exon 2 and the middle of exon 18 found uniquely in CD44RC (5′-GCAATGCAAACTGCAGGTCTC-3′). The conditions employed were exactly as described above. Ten microliters of each PCR reaction was electrophoresed on a 1% agarose gel and the products visualized by ethidium bromide staining.

Generation of pCEP4.CD44RC

In order to define the functional activity of CD44RC, the cDNA was subcloned into the eukaryotic episomal expression vector pCEP4 (Invitrogen). Briefly, pBS.CD44RC clone # 2.2 was digested with *Xba*I. A fragment of approximately 500 bp containing the full-length CD44RC cDNA was isolated and ligated into the *Nhe*I site of pCEP4. The correct orientation of the cDNA was confirmed by restriction enzyme analysis and an appropriate clone (pCEP4.CD44RC #2.2.1) was picked, expanded and plasmid DNA purified using the BiggerPrep DNA Isolation Kit (5′ 3′, Boulder, CO, USA).

Expression of CD44RC

K562 cells were transfected with plasmid DNA by electroporation using the BTX ECM 600 Electroporator System (BTX, San Diego, CA). Briefly, log-phase K562 cells were harvested and resuspended in phosphate-buffered saline (PBS) at a final concentration of 1×10^7 cells/ml. Fifteen micrograms of pCEP4 or pCEP4.CD44RC plasmid DNA was added to a 400-μl aliquot of the cell suspension, transferred to a 2-mm gap cuvette, and electroporated at 250 V with a capacitance setting of 500 μF. The time constants obtained ranged from 3.0 to 3.3 msec. Immediately after electroporation, cells were diluted in 30 ml DMEM+10% CCII, transferred to a 75 cm^2 tissue culture flask (Falcon; Becton Dickinson Labware, Franklin Lakes, NJ) and incubated at 37°C in the presence of 5% CO_2. Hygromycin B (Sigma) was added 24 hours later at a final concentration of 200 μg/ml. Transfected cells were selected for a minimum of

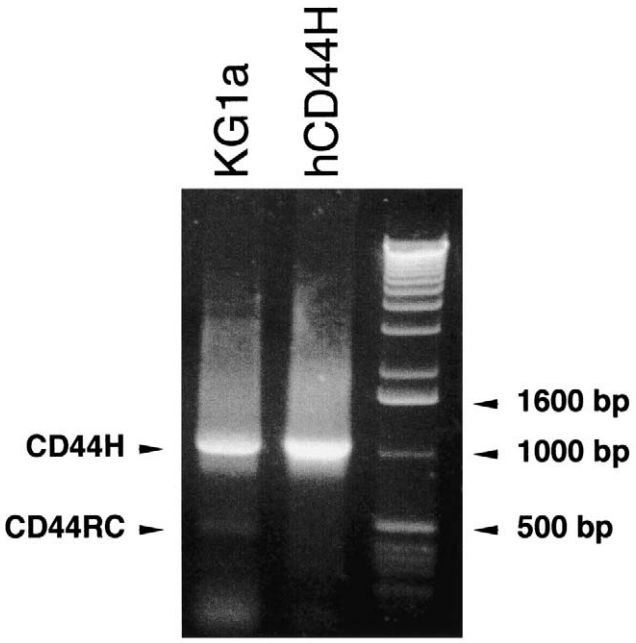

Figure 1. *Expression of CD44 isoforms in KG1a cells. CD44 cDNAs were amplified by RT-PCR from mRNA isolated from the myelomonocytic cell line KG1a using primers specific for CD44 exons 1 and 20. As a control, a CD44H cDNA isolated from the plasmid pCDM8.CD44H by digestion with XhoI was used as a template and similarly amplified using the same primer pair.*

14 days and were maintained thereafter in DMEM+10% CCII containing 200 μg/ml hygromycin. Expression of CD44RC mRNA was determined by RT-PCR analysis using the conditions and primers as described above.

In order to generate soluble CD44RC for use in further studies, 5×10^6 K562 cells transfected with pCEP4 or pCEP4.CD44RC plasmid DNA were resuspended in 10 ml DMEM without serum or hygromycin and cultured at 37°C in a 25-cm^2 flask (Falcon). Twenty-four hours later, the entire cell suspension was harvested and centrifuged at 1000 rpm for 10 minutes. The supernatant was collected, centrifuged for a further 5 minutes at 10 000 rpm to remove any cellular debris, concentrated 10-fold using an Amicon Filter (Millipore, Bedford, MD), aliquoted, and stored at −20°C until needed.

Effect of CD44RC on Cellular Adhesion to Hyaluronan

KG1 and KG1a cells from log-phase cultures were harvested and resuspended at a final concentration of 1×10^6 cells/ml and an appropriate volume of medium conditioned by K562 cells transfected with pCEP4 or pCEP4.CD44RC added to give a final concentration of 1×. In some experiments, the cells were incubated for 1 hour at 4°C with the anti-CD44 mAb 3G12 at a final concentration of 10 μg/ml and washed once with DMEM before being added to the conditioned supernatants. After incubation in the conditioned supernatants, 2×10^5 cells in a final volume of 0.5 ml were added to each of two wells in a 24-well plate (Falcon) that had been coated overnight at 4°C with human placental hyaluronan (Sigma) (5 mg/ml in PBS). After incubation for 10 minutes at room temperature, non-adherent cells were removed by gently washing each well five times with Hank's balanced salt solution (HBSS). The number of adherent cells per unit area was determined using either NIH Image (Research Services Branch, NIMH, Bethesda, MD) or by counting five random fields using an inverted phase microscope.

Mechanism CD44RC-Mediated Enhancement of Cellular Adhesion to Hyaluronan

In order to define the mechanism by which CD44RC enhances CD44-mediated adhesion to hyaluronan, KG1 cells were pretreated for 1 hour at 37°C with chondroitinase ABC (Sigma), or hyaluronan lyase (Sigma) prior to incubation in CD44RC containing supernatants. The treated cells were then washed three times with DMEM, incubated with CD44RC containing supernatant, and tested for attachment to hyaluronan as described above.

Results

Cloning and Nucleotide Sequencing of a Novel Soluble CD44

Using primers specific for CD44 exons 1 and 20, full-length CD44 cDNAs were amplified by RT-PCR from mRNA isolated from the myelomonocytic cell line KG1a. Two major products were obtained, a 1.1-kb fragment corresponding in size to CD44H and an unknown band of approximately 500 bp (Figure 1). The smaller cDNA was isolated and subcloned into pZERO2.1 and then pBlueScript (KS)+. Sequence analysis revealed a cDNA of 480 nucleotides with an ATG initiation codon at position 19 followed by an open reading frame of 396 residues (Figure 2). The first 233 nucleotides exhibit 100% sequence identity with CD44 exons 1 and 2, while the final 187 nucleotides corresponded to the last 28

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  1 ccgttcgctccggacaccATGGACAAGTTTTGGTGGCACGCAGCCTGGGGACTCTGCCTC 60
                    M  D  K  F  W  W  H  A  A  W  G  L  C  L
 61 GTGCCGCTGAGCCTGGCGCAGATCGATTTGAATATAACCTGCCGCTTTGCAGGTGTATTC 120
    V  P  L  S  L  A  Q  I  D  L  N  I  T  C  R  F  A  G  V  F
121 CACGTGGAGAAAAATGGTCGCTACAGCATCTCTCGGACGGAGGCCGCTGACCTCTGCAAG 180
    H  V  E  K  N  G  R  Y  S  I  S  R  T  E  A  A  D  L  C  K
181 GCTTTCAATAGCACCTTGCCCACAATGGCCCAGATGGAGAAAGCTCTGAGCATCGGATTT 240
    A  F  N  S  T  L  P  T  M  A  Q  M  E  K  A  L  S  I  G  F
241 GAGACCTGCAGTTTGCATTGCAGTCAACAGTCGAAGAAGGTGTGGGCAGAAGAAAAAGCT 300
    E  T  C  S  L  H  C  S  Q  Q  S  K  K  V  W  A  E  E  K  A
301 AGTGATCAACAGTGGCAATGGAGCTGTGGAGGACAGAAAGCCAAGTGGACTCAACGGAGA 360
    S  D  Q  Q  W  Q  W  S  C  G  G  Q  K  A  K  W  T  Q  R  R
361 GGCCAGCAAGTCTCAGGAAATGGTGCATTTGGTGAACAAGGAGTCGTCAGAAACTCCAGA 420
    G  Q  Q  V  S  G  N  G  A  F  G  E  Q  G  V  V  R  N  S  R
421 CCAGTTTATGACAGCTGAtgaqacaaqqaacctqcaqaatqtqqacatqaaqattqqqqt 480
    P  V  Y  D  S  *
481 gtaa 484
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Figure 2. *Nucleotide and predicted amino acid sequences of CD44RC. pBS.CD44RC clone #2.2 was fully sequenced and the predicted amino acid sequence determined. The start and stop codons are shown in bold and the putative signal peptide italicized. Exon boundaries are marked with an arrow. Cysteine residues are circled, and the putative hyaluronan binding domains boxed. The novel reading frame produced by the alternative splicing of CD44 exon 2 into exon 18 is underlined.*

residues of CD44 exon 18 and all of exon 20. Thus, this transcript appears to be generated as a result of alternative splicing occurring between the normal exon 2 splice donor and an alternative splice acceptor site (GCAG) found at position 48 within exon 18. Utilization of this site generates a frame shift producing a novel CD44 molecule with a unique COOH terminus. We have designated this CD44 isoform as CD44RC.

Upon cleavage of the 20 amino acid signal peptide, the mature CD44RC protein would consist of 119 amino acids with a predicted molecular weight of 13.1 kDa. The protein is predominantly hydrophilic, suggesting that it would be soluble in aqueous solution. The tandem hyaluronan binding domain KNGRYSISRTEAADLCK encoded by exon 2, which is present in all CD44 isoforms described to date, is also present in CD44RC (Figure 2). In addition, however, the novel COOH-terminus of CD44RC contains two further basic [B(X_6)B] amino acid motifs starting at position 92 (KKVWAEEK) and 113 (KAKWTQRR) that could potentially function as hyaluronan-binding domains (Figure 2) [19].

Cellular Expression of CD44RC

The expression of CD44RC in primary PBL and in the myeloid cell lines KG1, KG1a, HL60 and U937 was determined by RT-PCR analysis using a 5′ primer specific for CD44 exon 1 and a 3′ primer specific for exon 20. In addition to full-length CD44H, a band of approximately 500 bp corresponding in size to CD44RC can be seen in all of the cell lines and both of the primary PBL samples tested (Figure 3). The intensity of this band relative to CD44H varied considerably between the different cell types, with the highest levels being seen in KG1 and KG1 and the lowest levels being seen in primary PBL. Additional RT-PCR analysis using a 3′ primer corresponding to the unique junction created by the splicing of exon 2 into the middle of exon 18 (see Materials and Methods) confirmed the presence of CD44RC in all of the cell types tested (Figure 3).

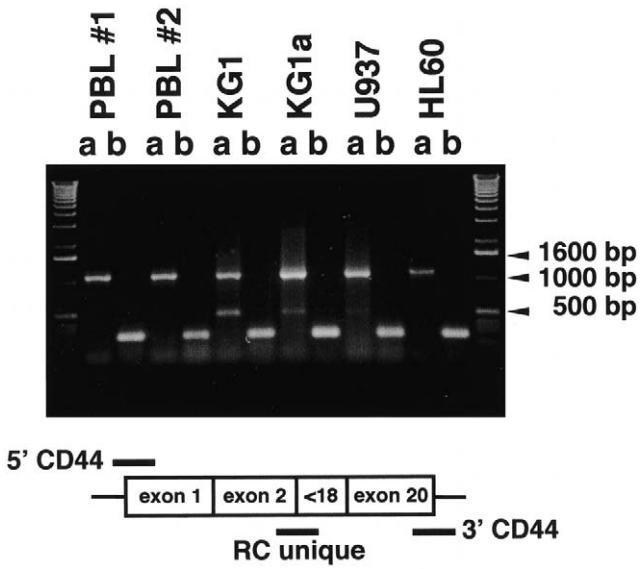

Figure 3. *Expression of CD44RC in normal PBL and various hemopoietic cell lines. RT-PCR analysis was used to determine the expression of CD44RC in normal PBL and in the hemopoietic cell lines KG1, KG1a, U937 and HL60. Two primer pairs were used: (a) full-length CD44 cDNAs were amplified using primers specific for exons 1 (5′ CD44) and 20 (3′ CD44); and (b) CD44RC transcripts were specifically amplified using primers specific for exon 1 (5′ CD44) and the unique junction generated between exons 2 and 18 (RC unique) found only in CD44RC.*

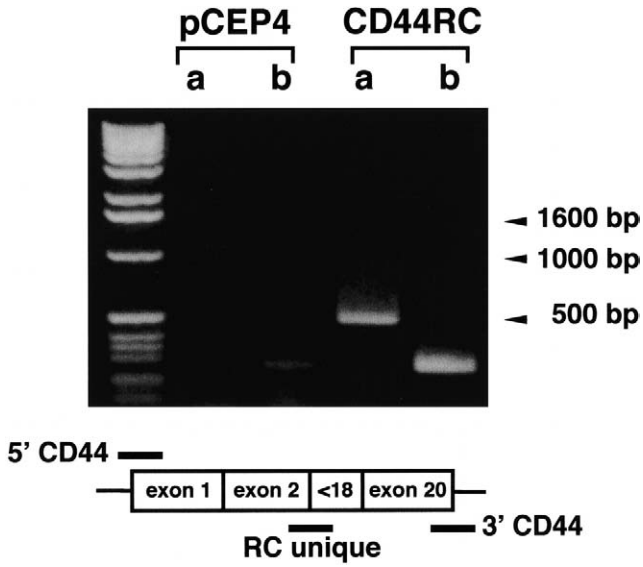

Figure 4. *Expression of CD44RC in K562.CD44RC. The expression of CD44RC in vector alone control K562 cells (pCEP4) and K562 cells transfected with pCEP4.CD44RC (CD44RC) was determined by RT-PCR. Two primer pairs were used: (a) full-length CD44 cDNAs were amplified using primers specific for exons 1 (5′ CD44) and 20 (3′ CD44); and (b) CD44RC transcripts were specifically amplified using primers specific for exon 1 (5′ CD44) and the unique junction generated between exons 2 and 18 (RC unique) found only in CD44RC.*

Functional Activity of CD44RC

The episomal expression vector pCEP4 was used to express CD44RC in the hemopoietic cell line K562. In the absence of available antibodies directed against determinants encoded by CD44 exons 1 or 2, expression of CD44RC in the transfected cells was once again determined by RT-PCR analysis using the primer sets described above. As shown in Figure 4, CD44RC mRNA is readily detected in the transfected cells. Although generally considered CD44-negative, a weak band corresponding in size to CD44H can be seen in K562 cells transfected with the control pCEP4 vector using a 5′ primer specific for CD44 exon 1 and a 3′ primer specific for exon 20. Using the 3′ primer specific for the unique junction between exons 2 and 18 present in CD44RC, high levels of CD44RC mRNA could be detected in K562 cells transfected with pCEP4.CD44RC. Very low but detectable levels of CD44RC were also observed in control K562 cells transfected with pCEP4.

To determine the effect of CD44RC on hyaluronan binding, KG1 cells were incubated with medium conditioned by K562.pCEP4 or K562.pCEP4.CD44RC and tested for their ability to bind to hyaluronan-coated plastic. As shown in

Figure 5, pretreatment of KG1 cells with CD44RC increased by approximately three-fold the proportion of these cells that bind to hyaluronan under the conditions employed. Similar treatment of KG1a cells, which exhibit a higher basal level of attachment to hyaluronan than KG1 cells, had no effect on binding. In both cases, adhesion to hyaluronan was dependent upon CD44 and could be greatly inhibited by pretreatment with mAb 3G12.

Induction of Hyaluronan Binding by CD44RC Involves the Recognition of Chondroitin Sulfate Presented by Endogenous CD44

Previously, we have demonstrated that CD44 can recognize and bind chondroitin sulfate moieties when presented in association with CD44 or other cell surface proteins [20]. In order to further characterize the molecular mechanism(s) by which CD44RC enhances the hyaluronan binding ability of KG1, cells were treated with chondroitinase ABC or hyaluronan lyase prior to incubation with CD44RC and then assayed for their ability to bind to hyaluronan-coated plastic. As shown in Figure 6, treatment with chondroitinase ABC almost completely inhibited the induction of hyaluronan binding observed following the addition of CD44RC. In contrast, treatment with hyaluronan lyase had virtually no inhibitory effect. These results suggest that CD44RC potentiates adhesion to hyaluronan by recognizing and binding to a chondroitin-sulfate-modified protein present on the surface of the KG1 cells. Since attachment of KG1 cells to hyaluronan can be almost completely blocked by mAbs directed against CD44 (Figure 5), it is likely that it is chondroitin-sulfate-modified CD44 itself that serves as a ligand for CD44RC.

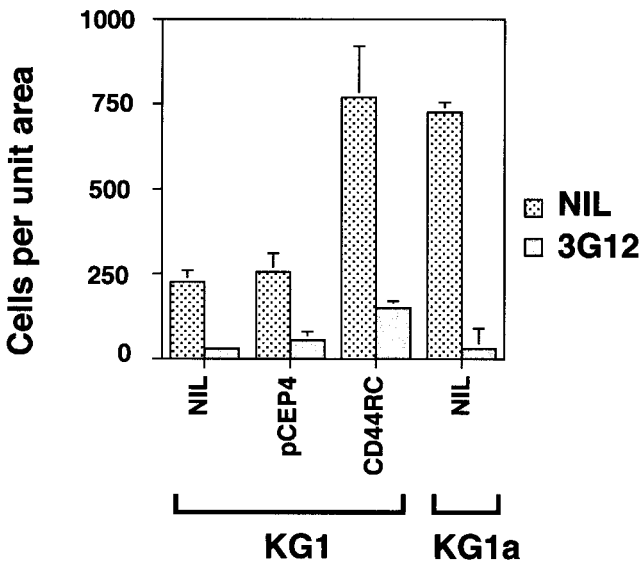

Figure 5. *Effect of CD44RC on cellular adhesion to hyaluronan. KG1 and KG1a cells were incubated with tissue culture medium or with anti-CD44 mAb 3G12 tissue culture supernatant for 1 hour at 4°C and washed prior to treatment with control tissue culture medium (NIL) or media conditioned by K562.CD44RC or K562.pCEP4 as described in Materials and Methods section. Following extensive washing, cells were assayed for their ability to adhere to hyaluronan-coated plastic. Each point represents the mean±SD of at least three independent determinations.*

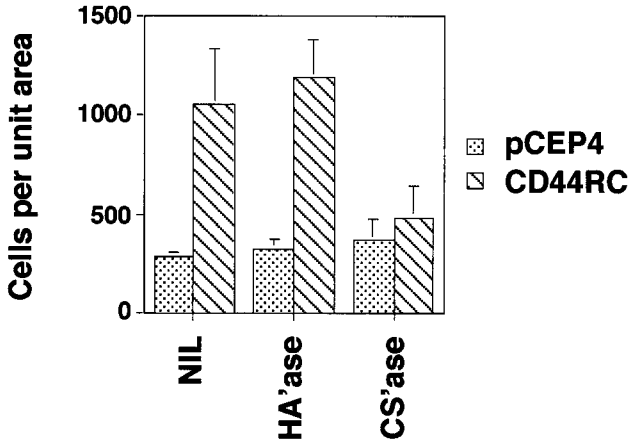

Figure 6. *Effect of chondroitinase treatment on cellular adhesion to hyaluronan induced by CD44RC. KG1 cells were pretreated for 1 hour at 37°C with control tissue culture medium (NIL), chondroitinase ABC (CS'ase), or hyaluronan lyase (HA'ase) prior to incubation with K562.pCEP4 or K562.CD44RC conditioned tissue culture supernatants as described in Materials and Methods section. Treated cells were washed extensively and then assayed for their ability to adhere to hyaluronan-coated plastic. Each point represents the mean±SD of at least three independent determinations.*

Discussion

Previous studies have suggested an important role for soluble CD44 in various cellular processes including tumor progression and metastasis [14,21]. Sy et al. [21] have shown that the enhanced growth of the CD44-negative human lymphoma line Namalwa in SCID mice induced by the transfection of CD44H can be suppressed by treatment with soluble CD44. In another recent study, Yu et al. [14] have similarly demonstrated that expression of a cDNA encoding a soluble CD44 protein within the highly metastatic murine mammary carcinoma TA3/St blocked the CD44-mediated binding and internalization of hyaluronan, inhibited tumor metastasis, and induced apoptosis *in vivo*. Such studies are in agreement with a model in which soluble CD44 antagonizes the function of the corresponding cell surface receptor by binding to and preventing recognition of the ligand hyaluronan.

CD44RC, the novel, naturally occurring soluble CD44 molecule identified in the present study had a very different effect on hyaluronan binding. Rather than blocking adhesion, pretreatment of KG1 cells with CD44RC increased the proportion of cells that bound to immobilized hyaluronan. CD44RC appears to mediate this effect by binding to and cross-linking chondroitin sulfate side-chains attached to cell surface CD44.

CD44RC includes exons 1 and 2 of the CD44 gene followed by the 3′ end of exon 18 and all of exon 20. Analysis of the sequence of exon 18 reveals a surprisingly good internal consensus splice acceptor site that appears to be utilized to generate the CD44RC cDNA. Importantly, although exon 18 encodes the transmembrane domain of

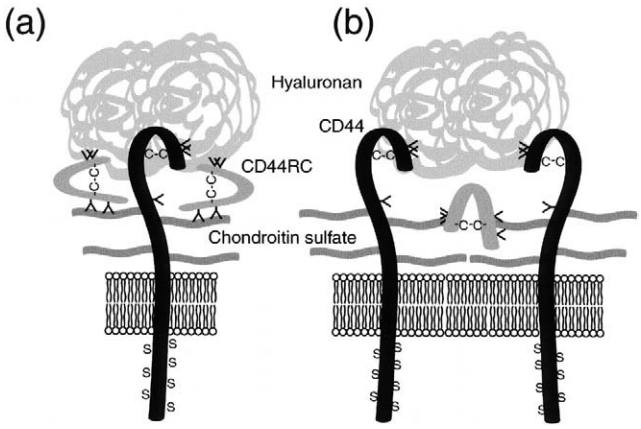

Figure 7. *Potential mechanisms by which CD44RC could enhance CD44-mediated adhesion to hyaluronan. (a) Since CD44RC contains a total of four putative hyaluronan-binding sites, the molecule may interact with chondroitin sulfate moieties on CD44, leaving one or more [B(X_7/X_6)B] motifs free. In this way, the avidity of the CD44 molecule for hyaluronan may be increased. (b) Since CD44RC is potentially multivalent, it may cross-link chondroitin sulfate moieties on CD44, inducing aggregation of the molecule in the plane of the membrane, again enhancing avidity for hyaluronan.*

cell surface CD44, utilization of the alternative exon 18 splice acceptor generates a frame shift producing a predominantly hydrophilic soluble protein. Although CD44RC has not been described previously, there are a number of other examples where alternative splicing events have been shown to result in alterations in reading frame producing biochemically and functionally distinct protein isoforms [22–26].

Previous studies have demonstrated that adhesion of CD44 and other proteins to hyaluronan is mediated by an amino acid motif in which two basic amino acid residues are separated by a stretch of six non-acidic residues [B(X_7)B] [19]. The presence of additional basic amino acids within this motif further increases its avidity for hyaluronan [19]. A tandem arrangement of the [B(X_7)B] motif is encoded by CD44 exon 2 and is present in all isoforms that have been described to date, including CD44RC. Similar motifs are also encoded by exon 5 and the alternatively spliced exon v10 [18, 27]. There is evidence that the motif present in exon v10 can contribute to the ability of CD44 isoforms containing this exon to promote cell–cell adhesion via the recognition of chondroitin sulfate side-chains presented on CD44 and other cell surface proteins [20].

Interestingly, the unique region present at the COOH terminus of CD44RC generated by the frame shift produced by the use of the alternative splice acceptor site present in exon 18 contains two [B(X_6)B] motifs. Although perhaps of lower affinity than the [B(X_7)B] motif, there is convincing evidence that peptides containing [B(X_6)B] can also bind hyaluronan [19]. Thus, it is likely that CD44RC contains two spatially distinct regions that can interact with hyaluronan and/or chondroitin sulfate. CD44RC also contains a number of cysteine residues that could potentially be linked by disulfide bonds to form covalently associated multimers.

Since both CD44H and CD44RC contain sequences encoded by exons 1 and 20, RT-PCR analysis using 5′ and 3′ primers specific for these exons constitutes a semi-quantitative means of determining the relative expression level of both isoforms within a particular cell line. Using such an approach, it appears that while primary PBL expresses fairly low levels of CD44RC, substantially higher levels are seen in various transformed hemopoietic cell lines.

The binding of hyaluronan by CD44 is of relatively of low affinity. Thus, while the precise mechanism by which CD44RC potentiates adhesion to hyaluronan remains to be determined, the data obtained in the present study suggest a model in which CD44RC enhances the *avidity* of this interaction by binding to chondroitin sulfate side-chains associated with CD44, increasing the local concentration or density of [B(X_7/X_6)B] motifs (Figure 7*a*). Increased local concentrations of [B(X_7)B] hyaluronan binding motifs could also be generated if CD44RC cross-linked chondroitin sulfate chains attached to adjacent CD44 molecules inducing aggregation or clustering of the molecule in the plane of the membrane (Figure 7*b*). In either case, the ability of CD44RC to ''activate'' the hyaluronan binding activity of cell surface CD44 would vary depending upon the concentration of the soluble protein in the local microenvironment and the proportion of cell surface CD44 molecules that are modified by the addition of chondroitin sulfate side-chains. Both of these variables could, in turn, be altered by cellular activation state and/or differentiation stage and could conceivably be affected by malignant transformation.

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