university of groningen

University Medical Center Groningen

University of Groningen

Identification of sequence motifs responsible for the adhesive interaction between exon v10-containing CD44 isoforms

Hayes, Gregory M; Chiu, Roland; Carpenito, Carmine; Dougherty, Shona T; Dougherty, Graeme J

Published in:
The Journal of Biological Chemistry

DOI:
10.1074/jbc.M206719200

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2002

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Hayes, G. M., Chiu, R., Carpenito, C., Dougherty, S. T., & Dougherty, G. J. (2002). Identification of sequence motifs responsible for the adhesive interaction between exon v10-containing CD44 isoforms. *The Journal of Biological Chemistry*, *277*(52), 50529-34. https://doi.org/10.1074/jbc.M206719200

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the “Taverne” license. More information can be found on the University of Groningen website: https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment.

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

Download date: 23-03-2025

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 52, Issue of December 27, pp. 50529–50534, 2002
Printed in U.S.A.
© 2002 by The American Society for Biochemistry and Molecular Biology, Inc.

Identification of Sequence Motifs Responsible for the Adhesive Interaction between Exon v10-containing CD44 Isoforms*

Received for publication, July 7, 2002, and in revised form, October 15, 2002
Published, JBC Papers in Press, October 28, 2002, DOI 10.1074/jbc.M206719200

Gregory M. Hayes‡§, Roland Chiu¶, Carmine Carpenito§, Shona T. Dougherty§, and Graeme J. Dougherty§‖

From the ‡Department of Pathology and Laboratory Medicine, University of British Columbia, Vancouver, British Columbia V6T 2B5, Canada, the ¶Department of Radiation Oncology, University of Maastricht, Maastricht 6200 MD, Netherlands, and the §Department of Radiation Oncology, University of California Los Angeles, Los Angeles, California 90095-1714

Previous studies have demonstrated that CD44 isoforms containing the alternatively spliced exon v10 promote cell-cell adhesion via a mechanism that involves the recognition of chondroitin sulfate side chains presented on the surface of interacting cells in association with other CD44 molecules. Sequence analysis revealed the presence within exon v10 of two motifs that may be relevant to this interaction, a B[X_7]B motif that may contribute to the recognition and binding of chondroitin sulfate and a serine-glycine motif that may serve as a site of chondroitin sulfate attachment. To determine whether either of these two motifs explain the unique adhesive activity of exon v10-containing CD44 isoforms, each was targeted by site-directed mutagenesis, and the adhesive activity of the resultant mutants was determined using a quantitative cell-cell binding assay. The data obtained demonstrate conclusively that it is the exon v10-encoded B[X_7]B motif that is solely responsible for the enhanced adhesive activity of exon v10-containing CD44 isoforms.

Numerous studies have documented a striking correlation between the presence of certain alternatively spliced isoforms of the adhesion protein CD44 on the surface of tumor cells and both metastatic propensity and poor prognosis (1). For example, in the case of colorectal carcinoma, patients with Duke's C and D tumors that express high levels of CD44R1, a CD44 isoform that contains sequences encoded by the alternatively spliced exons v8, v9, and v10, exhibit a far worse 5-year disease-free survival rate than equivalent patients with tumors that express predominantly CD44H, an isoform that lacks these differentially utilized exons (2, 3). Similar findings have been reported for a wide range of other hemopoietic and nonhemopoietic malignancies (1, 4). Recent studies from our group and others have provided insights into the molecular mechanisms that underlie this important relationship. Thus, although CD44H and the exon v10-containing CD44 isoforms CD44R1 and CD44R2 appear equivalent in their ability to bind the glycosaminoglycan hyaluronan (HA)[1] (5), only exon v10-containing CD44 isoforms possess the unique ability to directly bind to one another when expressed on the cell surface (5). The novel adhesive phenotype conferred by the inclusion of sequences encoded by exon v10 appeared not to be dependent upon the presence of HA but instead involved the recognition of chondroitin sulfate (CS) moieties presented in association with other CD44 molecules (6). This finding is in agreement with previous studies showing that CD44 can bind a variety of CS-modified protein ligands, including serglycin (7), the invariant chain of major histocompatibility complex/HLA class II (8), aggrecan (9), and versican (10).

The precise molecular nature of the CS-dependent interaction that occurs between exon v10-containing CD44 isoforms remains to be determined. Exon v10 encodes an additional B[X_7]B motif that in CD44 and other proteins has been shown to play a pivotal role in mediating the binding of HA and CS (11), although this conclusion has not been borne out in all studies (12, 13). The inclusion of an additional B[X_7]B motif in v10-containing CD44 isoforms could potentially enhance the avidity of the CD44 molecule for CS allowing homotypic adhesion between v10-containing CD44 isoforms to occur. Exon v10 also encodes a serine-glycine motif that could be differentially modified by the attachment of CS in certain cell types. Conceivably, CS moieties attached at this site may be preferentially recognized by other CD44 molecules thereby promoting cell-cell adhesion. To differentiate between these two possibilities, site-directed mutagenesis was used to abrogate each of the two functionally important motifs encoded by exon v10, and the effect on cellular adhesion was determined. The results obtained demonstrate clearly that it is the additional B[X_7]B motif encoded by exon v10 that is responsible for the enhanced cellular adhesion mediated by exon v10-containing CD44 isoforms. These data provide an explanation for the observed relationship between expression of exon v10-containing CD44 isoforms and enhanced metastatic propensity.

MATERIALS AND METHODS

Cell Lines—The adherent SV40-transformed simian fibroblastoid cell line, COS7 (14), was obtained from the American Type Culture Collection (Rockville, MD). COS7 cells were cultured in Dulbecco's minimum essential medium (DMEM) (Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (FCS, Sigma, St. Louis, MO), 1000 units/ml penicillin-streptomycin (Invitrogen), and 0.3 mg/ml L-glutamine (Invitrogen) (DMEM plus 10% FCS). Cells were maintained at 37 °C in an atmosphere containing 5% CO_2.

* This work was supported by National Institutes of Health Grant 1-RO1-CA-82296. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‖ To whom correspondence should be addressed: Dept. of Radiation Oncology, David Geffen School of Medicine, UCLA, Center for Health Sciences, Rm. B3-109, 10833 Le Conte Ave., Los Angeles, CA 90095-1714. Tel.: 310-206-8733; Fax: 310-206-1260; E-mail: graeme@radonc.ucla.edu.

[1] The abbreviations used are: HA, hyaluronan; CS, chondroitin sulfate; DAB, 3,3′-diaminobenzidine; DMEM, Dulbecco's modified Eagle's medium; FACS, fluorescence-activated cell sorter; FCS, fetal bovine serum; FITC-HA, fluorescein isothiocyanate-conjugated hyaluronan; HBSS, Hanks' balanced salt solution; mAb, monoclonal antibody; PBS, phosphate-buffered saline; PE, phycoerythrin.

This is an Open Access article under the CC BY license.

The CD4/CD8 double-positive, CD44-negative murine T cell line TIL1 was isolated and characterized as described previously (15). TIL1 cells, stably expressing human CD44H or CD44R1 were generated by retroviral-mediated gene transfer. Briefly, cDNAs encoding CD44H and CD44R1 (16) were cloned into the Moloney murine leukemia virus-based retroviral vector Jzen.1 (17) and plasmid DNA transfected into the ecotropic packaging cell line GP+E-86 (18) by calcium phosphate precipitation. Transfectants were selected in G418 (0.5 mg/ml active weight, Invitrogen), and tissue culture supernatants conditioned by the packaging line by overnight incubation in the absence of G418 were collected and used to infect TIL1 cells. Transduced TIL1 cells were selected in G418 (0.3 mg/ml active weight, Invitrogen) and checked periodically by flow cytometry to ensure the maintenance of high levels of CD44 isoform expression (see below).

Monoclonal Antibodies—The generation and characterization of the anti-CD44 mAb 4A4 has been described in detail previously (5, 15). The phycoerythrin-conjugated anti-CD44 antibody, PE-G44–26, was obtained from BD Pharmingen (San Diego, CA).

Preparation of CD44R1 Site-directed Mutants—Full-length CD44H, CD44R1, and CD44R2 cDNAs in the episomal expression vector pCDM8 (Invitrogen, Carlsbad, CA) were isolated as described in detail previously (16).

A PCR-based site-directed mutagenesis approach was used to inactivate the exon v10-encoded B[X_7]B motif in CD44R1. Briefly, PCR primers were designed to separately amplify both the 5′- and 3′-ends of CD44R1 such that a defined mutation is introduced upon ligation of the fragments to regenerate the full-length cDNA. The two primer pairs used were as follows. The 5′-end of the CD44R1 cDNA was amplified using 5′-GCAAGCTTGGTCCGCCATCCTCGTCCC-3′ (CD44 exon 1) and 5′-CCATTCGAATTGCTTGATGTC-3′ (CD44 exon v10). The 3′-end of the CD44R1 cDNA was similarly amplified using 5′-GCAATTCGAATGATGTCACAGG-3′ (CD44 exon v10) and 5′-CGCGGCCGCAATGAAACAATCAGTAGC-3′ (CD44 exon 20). A full-length CD44R1 cDNA (16) in pBluescript KS+ (Stratagene, La Jolla, CA) was used as a template. PCR reactions were carried out in a Hybaid thermocycler (Omnigene, Cambridge, MA) using a standard program (95 °C for 30 s, 56 °C for 30 s, then 72 °C for 90 s, for 35 cycles). PCR products were separated on a 1% (w/v) agarose gel, and appropriate bands were excised, purified by Geneclean (BIO 101, Inc., Vista, CA), and the ends were filled/blunted with T4 DNA polymerase (Invitrogen). The blunt fragments obtained were ligated into the *Eco*RV site of pBluescript KS+, transformed into competent *Escherichia coli* strain TOP10 (Invitrogen), and plated on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal)-containing ImMedia (Invitrogen) plates. White colonies were picked the following day, plasmid DNA was isolated, and appropriate clones were identified by restriction enzyme digestion. A fragment corresponding to the 3′-end of CD44R1 was isolated from a suitable clone by digestion with *Nsp*V and *Not*I and ligated into a pBluescript KS+ vector clone containing the 5′-end of CD44R1 that had been digested with the same two enzymes. This approach generates a full-length mutant cDNA designated CD44R1.R288S in which the first arginine residue within the exon v10 encoded B[X_7]B motif (position 288) is converted to a serine with the concomitant introduction of a *NspV* restriction site that can be used for diagnostic purposes (Fig. 1). For expression studies, the full-length CD44R1.R288S cDNA was isolated by digestion with *Hin*dIII and *Not*I and ligated into the multiple cloning site of the pCDM8 expression vector (Invitrogen).

Site-directed mutagenesis was similarly used to functionally inactivate the putative CS attachment site found at the junction of exon v10 and exon 16 in CD44R1. This was achieved using the Chameleon double-stranded site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. Briefly, a full-length CD44R1 cDNA in pBluescript KS+ was used as a template. The common 5′ primer included in the kit was used to target the *Kpn*I restriction site within the pBluescript KS+ multiple cloning site. A second unique primer, designated R1mutC, with the sequence 5′-CGTTCCTTATCAGCAGACCAAGACAC-3′, was used to convert the glycine residue in the serine-glycine motif generated at the junction of exon v10 and exon 16 to an alanine. PCR reactions were carried out using the enzyme mix and dNTPs supplied by the manufacturer, and the products were digested with *Kpn*I. Successful incorporation of the 5′ primer inactivates the *Kpn*I site in the pBluescript KS+ polylinker, thereby greatly enriching it for plasmids that carry mutated sequences after transformation into competent XLmutS, an *E. coli* strain deficient in mismatch repair. After overnight culture, miniprep plasmid DNA was recovered digested once again with *Kpn*I and retransformed into the standard bacterial host XL2-blue (Stratagene). Individual colonies were then picked, tested to ensure loss of the *Kpn*I site. This CD44R1 mutant is designated CD44R1.G355A (Fig. 1). Both CD44R1 mutants were sequenced to confirm that the desired mutations were successfully introduced.

Introduction of pCDM8 Constructs into COS7 Cells—COS7 cells were electroporated with each of the pCDM8 constructs (pCDM8 vector only, pCDM8.CD44H, pCDM8.CD44R1, pCDM8.CD44R2, pCDM8.CD44R1.R288S, and pCDM8.CD44R1.G355A) using DNA purified with the Qiagen Midi Prep DNA isolation kit (Qiagen, Valencia, CA). Log phase COS7 cells were harvested by gentle trypsinization, resuspended in DMEM plus 10% FCS, and pelleted by centrifugation. Cells were washed once in PBS and resuspended at ~2×10^7 cells/ml in PBS. 400-μl aliquots of the cell suspension were mixed with 20 μg of plasmid DNA and transferred to a 2-mm gap electroporation cuvette (BTX, San Diego, CA). Samples were incubated on ice for 10 min before being electroporated at 280 V, capacitance setting 325 microfarads, resistance R3 (48 Ω) using a BTX600 electroporator (BTX). Time constants generally ranged around 4.5 ms. The contents of each cuvette were immediately resuspended in 30 ml of prewarmed DMEM plus 10% FCS and plated in a 15-cm Integrid tissue culture dish (Falcon/Becton-Dickinson, Franklin Lakes, NJ). Cells were incubated at 37 °C for 72 h to allow expression of the introduced cDNAs.

Western Blot Analysis—Transfected COS7 cells (see above) were harvested 72 h after electroporation by brief incubation in PBS containing 5 mM EDTA, washed twice with PBS, and resuspended at 2×10^7 cells/ml in PBS containing 1% (v/v) Nonidet P-40, 1 mM EDTA, 150 mM NaCl, and 10 mM Tris (pH 7.4). After incubation on ice for 10 min with repeated vortexing, the lysates were centrifuged for 10 min to pellet nuclei and other cellular debris. Supernatant aliquots were stored at −70 °C until required. Samples were rapidly thawed, added to an equal volume of non-reducing buffer containing 125 mM Tris, 20% (v/v) glycerol, and 4.6% (w/v) SDS (pH 6.8), and incubated at 100 °C for 5 min. Total cellular proteins were separated by SDS-PAGE using a Mini Protean III apparatus (Bio-Rad, Richmond, CA) and transferred onto nitrocellulose membranes (Bio-Rad). Membranes were blocked in PBS containing 5% (w/v) skim milk protein by overnight incubation at 4 °C. After extensive washing in Hanks' balanced salt solution (HBSS), membranes were incubated in HBSS containing a 1:100 dilution of mAb 4A4 tissue culture supernatant for 4 h at room temperature. Membranes were washed in HBSS for 30 min and then incubated for a further 1 h with a 1:100 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG (Sigma). After extensive washing in HBSS, the reaction was developed in PBS containing 0.06% (w/v) 3,3′-diaminobenzidine (DAB, Sigma) and 0.012% (v/v) hydrogen peroxide (Sigma).

FACS Analysis—The expression and HA binding activity of the various wild-type and mutated CD44 constructs in transfected COS7 cells was determined by FACS analysis. Sub-confluent COS7 cell cultures were harvested 72 h after electroporation by brief incubation in PBS containing 5 mM EDTA and washed once in DMEM plus 10% FCS. Approximately 2×10^5 cells were resuspended in 0.5 ml of HBSS containing 2% FCS (HBSS plus 2% FCS), a 1:100 dilution of anti-CD44 mAb PE-G44–26 (BD Pharmingen), and a 1:1000 dilution of fluorescein isothiocyanate-conjugated hyaluronan (FITC-HA, ~50 μg/ml final), and the mixture was incubated for 1 h at 4 °C. Following repeated washing with HBSS plus 2% FCS, cells were resuspended in HBSS plus 2% FCS and analyzed on a FACSCalibur (BD Biosciences, Immunocytometry Systems, San Jose, CA).

Cell-Cell Binding Assay—Adhesive interactions between the various wild-type and mutant CD44 isoforms were quantitated using an *in vitro* cell-cell binding assay. Briefly, transfected COS7 cells were harvested 48 h after electroporation with the various pCDM8-based constructs (see above), and 2×10^4 cells in a final volume of 1 ml of DMEM plus 10% FCS were added to the wells of a 24-well plate into which had been placed tissue culture-treated glass coverslips (Nalgene Nunc International). The plates were incubated overnight at 37 °C to allow the transfected COS7 cells to attach to the coverslips, after which the culture medium was removed and the adherent monolayers were washed twice with PBS. 5×10^5 TILJNeo, TILJhCD44H, or TILJhCD44R1 cells in a final volume of 0.5 ml of PBS (no Ca^{2+}, no Mg^{2+}) were added to triplicate wells, and the plates were incubated for 15 min at 4 °C to allow the added cells to settle and interact with the adherent COS7 cells. Coverslips were then removed from the wells, washed three times in PBS, and fixed with methanol for 30 s. COS7 cells expressing CD44 were identified by indirect immunoperoxidase staining. Briefly, coverslips were incubated for 1 h with 0.5 ml of mAb 4A4 tissue culture supernatant. After extensive washing in PBS, 0.5 ml of a 1:100 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG in DMEM plus 10% FCS was added, and incubation continued for a further 1 h at room temperature. After extensive washing the coverslips were developed in PBS containing 0.06% (w/v) DAB and

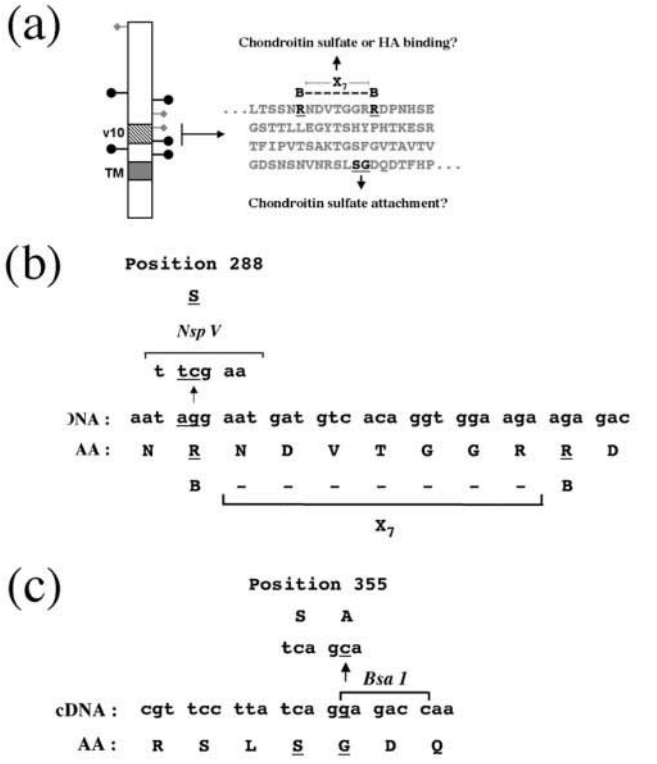

FIG. 1. **Mutation of exon v10-encoded motifs within CD44R1.** Sequence analysis identified two motifs within exon v10, a B[X_7]B motif and a serine-glycine motif, that may be responsible for the enhanced CS-dependent adhesive activity of exon v10-containing CD44 isoforms. Using a PCR-based site-directed mutagenesis approach, the arginine at position 288 within the B[X_7]B motif was converted to a serine residue generating a mutant designated CD44R1.R288S. Similarly, the glycine at position 355 within the serine-glycine motif found at the junction between exon v10 and exon 16 was converted to an alanine generating a mutant designated CD44R1.G355A.

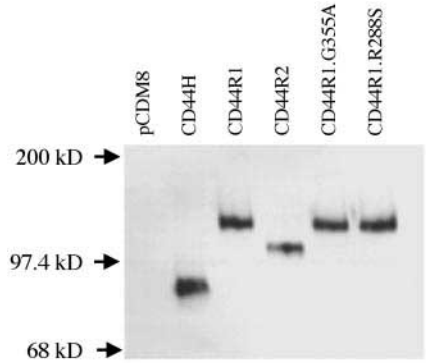

FIG. 2. **Western blot analysis of CD44 expression of COS7 cell transfectants.** Approximately 2×10^5 cell equivalents were run in each lane of a 7.5% SDS-PAGE gel, transferred to a nitrocellulose membrane, and probed with the anti-CD44 mAb 4A4 followed by a peroxidase conjugated goat anti-mouse IgG secondary antibody. The reaction was developed in PBS containing 0.06% (w/v) DAB and 0.012% (v/v) hydrogen peroxide.

0.12% (v/v) hydrogen peroxide, washed with H_2O, counterstained with hematoxylin (Sigma), and mounted onto slides using Paramount fixative (Fisher). The percentage of CD44-positive COS7 cells binding three or more TIL1 cells was determined using an inverted-phase microscope. TIL1 cells are substantially smaller and have a very different morphology than adherent COS7 cells enabling the two cell types to be readily distinguished.

To confirm the importance of CS recognition in the adhesive interaction between the various wild-type and mutant CD44 isoforms, in some experiments, transduced TIL1 cells were treated with chondroitinase ABC (Sigma, 5 units/ml final) or hyaluronidase purified from *Streptomyces hyalurolyticus* (Sigma, 20 units/ml final) for 1 h at 37 °C prior to being added to the adhesion assay, and the effect on binding to transfected COS7 cells was determined as before. To exclude the possibility that any inhibition of cellular adhesion induced by chondroitinase ABC treatment may reflect an effect of the enzyme on the ligand binding function of the CD44R1 molecule, treated TIL1 cells were also tested for their ability to bind to transfected COS7 cells that had been preincubated with HA (Sigma) at a final concentration at 1 mg/ml on ice for 1 h. As shown previously (7), assuming the CD44 molecules present on both transduced TIL1 cells and transfected COS7 cells retain ligand binding function, then HA can act as a linker or bridge promoting a high level of cell-cell adhesion that is not dependent upon the presence of CS.

RESULTS

Examination of the sequence of CD44 exon v10 revealed two motifs that could potentially contribute to the unique CS-dependent adhesive interaction that occurs between v10-containing alternatively spliced CD44 isoforms. The first is B[X_7]B, a motif that has been implicated in the HA and CS binding ability of a wide range of cell surface proteins, including CD44 itself. The second is a serine-glycine dipeptide generated at the junction of exon v10 and the "constant" exon 16 that could potentially serve as a site of CS attachment. As shown in Fig. 1, a PCR-mediated site-directed mutagenesis approach was used to inactivate each of these sites, and the ability of the mutated proteins to facilitate enhanced cell-cell adhesion was determined. Specifically, the first basic residue in the exon v10-encoded B[X_7]B motif (arginine 288) was converted to serine generating a mutant cDNA designated CD44R1.R288S. The putative serine-glycine CS attachment site was similarly inactivated by converting the glycine residue at position 355 to an alanine generating a mutant designated CD44R1.G355A.

To compare their functional activity, the wild-type and mutant CD44R1 cDNAs were cloned into the episomal expression vector pCDM8 and introduced into COS7 cells by electroporation. As shown in Fig. 2, Western blot analysis indicated that both the CD44R1.R288S and CD44R1.G355A mutants were expressed by transfected COS7 cells and that the protein products are identical in size to CD44R1. As expected, COS7 cells transfected with cDNAs encoding CD44H or the exon v10-containing isoform CD44R2 produced smaller protein products with molecular masses of ~90 and 115 kDa, respectively.

The CD44R1 mutants were next tested for their ability to bind soluble HA. COS7 cells transfected with CD44H, CD44R2, and wild-type or mutant CD44R1 cDNAs were harvested 72 h post-electroporation, and CD44 expression and HA binding activities were determined by flow cytometry. Representative dot plots for each transfectant are shown in Fig. 3. On average ~15–20% of COS7 cells transiently transfected with each of the pCDM8-based CD44 constructs expressed CD44 proteins on the cell surface as determined by reactivity with the PE-conjugated anti-CD44 mAb G44–26. Importantly, the levels of expression were similar for all the CD44 constructs tested, including the two exon v10 mutants (CD44R1.R288S and CD44R1.G355A). Double labeling with anti-CD44-PE and FITC-HA indicated that only the subset of transfected COS7 cells that express CD44 are able to bind HA and that CD44H, CD44R1, and CD44R2 appear similar in this regard. Moreover, it is evident that the mutations introduced into the CD44R1.R288S and CD44R1.G355A constructs do not adversely affect the ability of the CD44 proteins produced to bind HA.

The ability of the various wild-type and mutant CD44 proteins to mediate CS-dependent cell-cell adhesion was also determined using a quantitative cell binding assay. As shown in Fig. 4, control CD44-negative TILJneo cells lack the ability to bind to COS7 cells transfected with any of the CD44 constructs tested. In contrast, a low level of binding is evident between

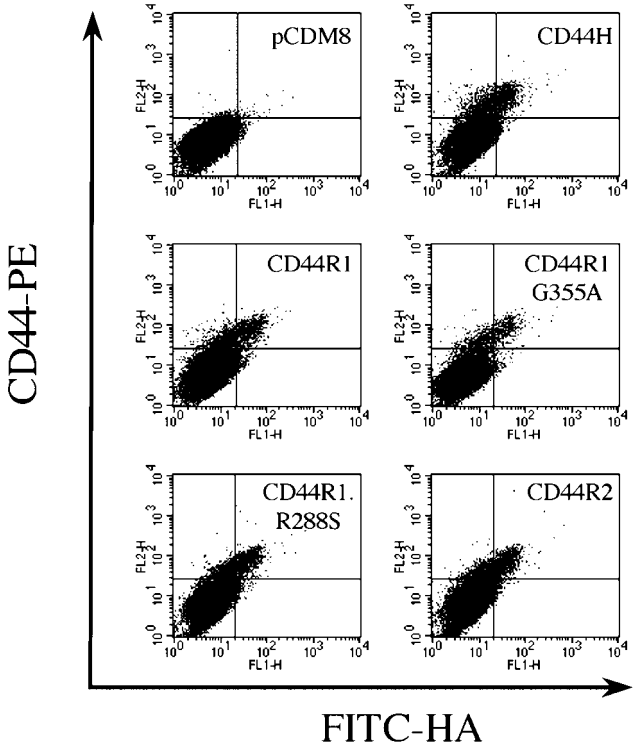

FIG. 3. **CD44 expression and HA-binding efficiency of COS7 cell transfectants.** COS7 cells were electroporated with each of the various wild-type or mutant CD44 constructs in the expression vector pCDM8. Cells were harvested 72 h after electroporation and incubated with a PE-conjugated anti-CD44 antibody and FITC-conjugated HA for ~1 h at 4 °C. Following extensive washing the labeled cells were analyzed on a FACSCalibur for CD44 expression (*FL2*) and FITC-HA binding (*FL1*).

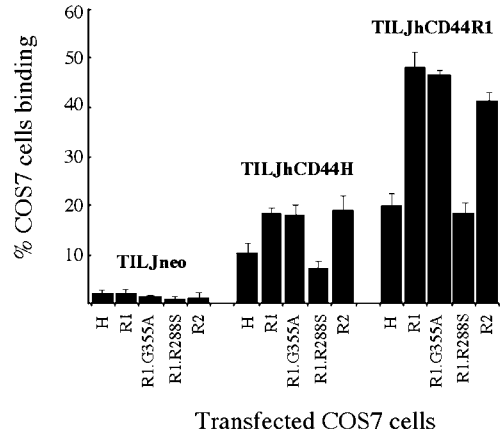

FIG. 4. **Analysis of exon v10-encoded motifs on CD44-mediated cell-cell binding.** COS7 cells electroporated with the indicated CD44 constructs in the expression vector pCDM8 were harvested after 48 h, and 2×10^4 transfected cells allowed to adhere overnight onto glass coverslips in the wells of a 24-well plate. Culture media was removed and 5×10^5 control TIL1 cells (*TILJNeo*), or TIL1 cells expressing CD44H (*TILJhCD44H*), or CD44R1 (*TILJhCD44R1*) in 0.5 ml of PBS were added and allowed to bind for 15 min at 4 °C. Unbound TIL1 were removed by gentle washing, and the coverslips were fixed and stained for CD44 expression using an indirect immunoperoxidase technique. The percentage of CD44-positive COS7 cells binding three or more TIL1 cells was determined by counting on an inverted-phase microscope.

CD44H-expressing TIL1 cells (TILJhCD44H) and COS7 cells transfected with CD44H. Significantly enhanced binding is observed between TILJhCD44H cells and COS7 cells expressing the CD44R1 isoform. A similar increased level of binding to COS7 cells expressing CD44R2 is also observed suggesting that

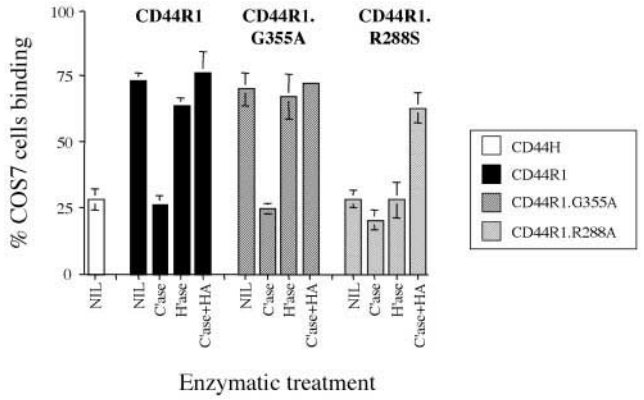

FIG. 5. **Involvement of CS in the adhesive interaction between wild-type CD44 and exon v10 site-directed CD44 mutants.** 2×10^4 COS7 cells harvested 48 h after transfection with pCDM8.CD44H, pCDM8.CD44R1, or the exon v10 site-directed CD44 mutants pCDM8.CD44R1.G355A or pCDM8.CD44R1.R288S were allowed to adhere overnight onto glass coverslips in the wells of a 24-well plate. Next day, HA was added to some wells at a final concentration of 1 mg/ml, and the plates were incubated for a further 1 h at 0 °C. Supernatants were then removed, and 5×10^5 untreated TILJhCD44R1 cells or equivalent numbers of cells that had been treated with chondroitinase ABC (5 units/ml) (*C'ase*) or hyaluronidase (20 units/ml) (*H'ase*) for 1 h at 37 °C, were added in a final volume of 0.5 ml of PBS and allowed to bind for 15 min at 4 °C. Unbound TIL1 cells were removed by gentle washing, and the coverslips were fixed and stained for CD44 expression using an indirect immunoperoxidase technique. The percentage of CD44-positive COS7 cells binding three or more TIL1 cells was determined by counting on an inverted-phase microscope.

the enhanced adhesion seen for CD44R1 can be attributed solely to the presence within this molecule of sequences encoded by exon v10, because only this additional exon is present in CD44R2. Mutation of the serine-glycine motif in CD44R1 (CD44R1.G355A) did not impact on the cell-cell binding ability of the molecule. In contrast, mutation of the B[X_7]B motif abrogated the enhanced adhesive function mediated by the presence of exon v10 reducing cell binding to the level obtained for CD44H.

High level reciprocal binding is observed for the interaction between TILJhCD44R1 and COS7 cells transfected with CD44R1 (Fig. 4). Once again, CD44R2, which contains additional sequences encoded only by exon v10, had a binding activity similar to CD44R1. As before, although mutation of the exon v10-encoded serine-glycine motif had no effect on the enhanced adhesive ability of the CD44R1 molecule, mutation of the B[X_7]B motif reduced the binding obtained to a level similar to that seen for CD44H.

As shown in Fig. 5, treatment with chondroitinase ABC also reduced the binding of TILJhCD44R1 to COS cells expressing CD44R1 or CD44R1.G355A to a level similar to that obtained for CD44H. Hyaluronidase treatment had no such effect. Importantly, the adhesive interaction between chondroitinase-treated TILJCD44R1 cells and the various wild-type and mutant CD44R1 molecules could be reconstituted by the addition of HA, which functions as a linker or bridge between the CD44 molecules present on the surface of the interacting cells. These data confirm that chondroitinase treatment affected only the presentation of CS by CD44R1 and not the ligand binding function of molecule. Finally, as expected, the low level binding of TILJhCD44R1 cells to COS cells expressing CD44R1.R288S was not further reduced by chondroitinase ABC treatment, strongly suggesting that it is the recognition of CS, mediated by the presence within exon v10 of an additional B[X_7]B motif, that is primarily responsible for the reciprocal adhesive interaction between exon v10-containing CD44 isoforms.

DISCUSSION

Although previous studies have demonstrated a correlation between the expression of certain exon v10-containing CD44 isoforms and enhanced tumor metastasis and/or poor prognosis (1, 2, 4), the molecular mechanisms that underlie this important relationship have yet to be elucidated. Some initial experiments suggested that CD44H and CD44R1 may differ in their ability to bind the extracellular matrix glycosaminoglycan HA (19, 20). However, this finding has not been borne out in all studies (5, 21, 22) and can perhaps be best explained by cell-specific differences in the post-translational modification of the two CD44 isoforms (23, 24). In this and previous studies we have shown that CD44H, CD44R1 (v8–10-containing), and CD44R2 (v10-containing) are all equivalent in their HA-binding function when expressed in either TIL1 cells or COS7 cells (5, 6). However, unlike CD44H, the exon v8–10-containing isoform CD44R1 was capable of promoting cell-cell adhesion (6, 15). Because CD44R2 has a similar activity, it was concluded that this unique function was conferred by the presence of sequences encoded by exon v10 (6). Moreover, the sensitivity of the binding process to chondroitinase indicated that CD44 exon v10-mediated cell-cell adhesion involved the recognition of CS side chains (6). Because COS7 cells transfected with CD44R1 or CD44R2 do not bind control TILJneo cells but only TIL1 cells transfected with CD44H or CD44R1, it must be assumed that the CS-modified proteoglycan recognized and bound by the exon v10-containing isoforms must be CD44 itself (6, 15).

An examination of the sequence encoded by CD44 exon v10 revealed two sites that could potentially contribute to the enhanced cellular adhesion mediated by v10-containing CD44 isoforms, namely a B[X_7]B motif that has been suggested to function as a HA/CS binding site (11) and a serine-glycine motif that could potentially serve as a site of CS attachment (25). Using site-directed mutagenesis it was shown conclusively in the present study that it is the B[X_7]B motif encoded by exon v10 that confers the unique adhesive phenotype on cells expressing exon v10-containing CD44 isoforms. When this motif is mutated, as is the case in the CD44R1.R288S construct, the CD44R1 molecule now exhibits an adhesive activity similar to CD44H, which of course completely lacks v10-encoded sequences. This result can perhaps be best explained if CD44 exon v10-containing isoforms interact with one another in reciprocal fashion. When two interacting cells both express exon v10-containing CD44 molecules, the B[X_7]B motifs in both proteins recognize and bind CS side chains covalently attached to the CD44 molecules present on the opposing cell surface promoting a high level of cellular adhesion. Conversely, if only one cell expresses exon v10-containing CD44 molecules, the interaction would be non-reciprocal in nature and a lower level of binding is expected to occur. This is exactly what is seen in the interaction between CD44R1- and CD44H-expressing cell types or when the B[X_7]B motif is mutated in one of the interacting CD44R1 proteins.

CD44H already contains two B[X_7]B motifs encoded, respectively, by exon 2 and exon 5 (26). This raises the question as to why inclusion of an additional motif in sequences encoded by exon v10 has such a profound effect on the adhesive activity of the CD44 molecule. The three B[X_7]B motifs are, of course, not identical and may differ from one another with respect to their affinity for CS. It has already been demonstrated, for example, that the inclusion of acidic amino acid residues within the motif reduces its ability to bind HA, whereas the presence of additional basic residues enhances the affinity of this interaction (11). On the other hand, it is possible that the positioning of the exon v10-encoded motif may, as a result of spatial considerations, favor interaction with CS moieties attached to CD44 proteins on the surface of interacting cells. Alternatively, the presence of an additional B[X_7]B motif may simply increase the avidity of the CD44 molecule for CS raising the level above the threshold necessary for CS binding. It is noteworthy in this regard that the binding of HA by CD44 is of low affinity and that a certain cell-specific level of CD44 expression is necessary before HA binding can occur.[2] A non-mutually exclusive possibility that incorporates both the preceding arguments could be that the exon v10-encoded B[X_7]B motif may cross-link CD44 proteins in the plane of the membrane through recognition of CS, inducing receptor aggregation thereby enhancing avidity and promoting the recognition and binding of CD44 on the surface of interacting cells. Studies to differentiate between these various alternatives are currently under way.

There are four serine-glycine sites within CD44H that could potentially be modified by attachment of CS (27, 28). Although an additional serine-glycine motif is generated at the junction of exon v10 and exon 16 in CD44R1 and CD44R2 (16), inactivation of this site by site-directed mutagenesis had no appreciable effect on the ability of v10-containing CD44 isoforms to promote enhanced cell-cell adhesion. Thus, although recognition of CS plays a critical role in the adhesive interactions mediated by the presence of CD44 exon v10 (6, 15), the actual site at which the glycosaminoglycan side chains are attached remains to be determined.

The enhanced ability of exon v10-containing CD44 isoforms to promote cell-cell adhesion may explain the observed correlation between expression of such isoforms on tumor cells and metastatic propensity and/or poor patient prognosis. The presence of exon v10-encoded sequences may promote the formation of tumor cell microemboli that survive better in the circulation and trap more readily in capillary beds. The expression of alternatively spliced CD44 isoforms may also promote the adhesion of tumor cells to vascular endothelial cells in target organs, whereas adhesive interactions between CD44 isoforms may transduce signals that protect tumor cells from various naturally occurring or therapeutically induced pro-apoptotic stimuli.

REFERENCES

1. Naot, D., Sionov, R. V., and Ish-Shalom, D. (1997) *Adv. Cancer Res.* **71,** 241–319
2. Finn, L., Dougherty, G., Finley, G., Meisler, A., Becich, M., and Cooper, D. L. (1994) *Biochem. Biophys. Res. Commun.* **200,** 1015–1022
3. Weg-Remers, S., Schuder, G., Zeitz, M., and Stallmach, A. (1998) *Ann. N. Y. Acad. Sci.* **859,** 304–306
4. Rudzki, Z., and Jothy, S. (1997) *Mol. Pathol.* **50,** 57–71
5. Dougherty, G. J., Cooper, D. L., Memory, J. F., and Chiu, R. K. (1994) *J. Biol. Chem.* **269,** 9074–9078
6. Chiu, R. K., Droll, A., Dougherty, S. T., Carpenito, C., Cooper, D. L., and Dougherty, G. J. (1999) *Exp. Cell Res.* **248,** 314–321
7. Toyama-Sorimachi, N., Sorimachi, H., Tobita, Y., Kitamura, F., Yagita, H., Suzuki, K., and Miyasaka, M. (1995) *J. Biol. Chem.* **270,** 7437–7444
8. Naujokas, M. F., Morin, M., Anderson, M. S., Peterson, M., and Miller, J. (1993) *Cell* **74,** 257–268
9. Fujimoto, T., Kawashima, H., Tanaka, T., Hirose, M., Toyama-Sorimachi, N., Matsuzawa, Y., and Miyasaka, M. (2001) *Int. Immunol.* **13,** 359–366
10. Kawashima, H., Hirose, M., Hirose, J., Nagakubo, D., Plaas, A. H., and Miyasaka, M. (2000) *J. Biol. Chem.* **275,** 35448–35456
11. Yang, B., Zhang, L., and Turley, E. A. (1993) *J. Biol. Chem.* **268,** 8617–8623
12. Bajorath, J., Greenfield, B., Munro, S. B., Day, A. J., and Aruffo, A. (1998) *J. Biol. Chem.* **273,** 338–343
13. Day, A. J., and Prestwich, G. D. (2002) *J. Biol. Chem.* **277,** 4585–4588
14. Gluzman, Y. (1981) *Cell* **23,** 175–182
15. Droll, A., Dougherty, S. T., Chiu, R. K., Dirks, J. F., McBride, W. H., Cooper, D. L., and Dougherty, G. J. (1995) *J. Biol. Chem.* **270,** 11567–11573
16. Dougherty, G. J., Landorp, P. M., Cooper, D. L., and Humphries, R. K. (1991) *J. Exp. Med.* **174,** 1–5
17. Laker, C., Stocking, C., Bergholz, U., Hess, N., De, L. J. F., and Ostertag, W. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 8458–8462
18. Markowitz, D., Goff, S., and Bank, A. (1988) *J. Virol.* **62,** 1120–1124
19. Stamenkovic, I., Aruffo, A., Amiot, M., and Seed, B. (1991) *EMBO J.* **10,** 343–348

[2] R. K. Chiu, A. Droll, S. T. Dougherty, and G. J. Dougherty, manuscript in preparation.

20. Sy, M. S., Guo, Y. J., and Stamenkovic, I. (1991) *J. Exp. Med.* **174,** 859–866
21. He, Q., Lesley, J., Hyman, R., Ishihara, K., and Kincade, P. W. (1992) *J. Cell Biol.* **119,** 1711–1719
22. Galluzzo, E., Albi, N., Fiorucci, S., Merigiola, C., Ruggeri, L., Tosti, A., Grossi, C. E., and Velardi, A. (1995) *Eur. J. Immunol.* **25,** 2932–2939
23. Bennett, K. L., Modrell, B., Greenfield, B., Bartolazzi, A., Stamenkovic, I., Peach, R., Jackson, D. G., Spring, F., and Aruffo, A. (1995) *J. Cell Biol.* **131,** 1623–1633
24. Bartolazzi, A., Nocks, A., Aruffo, A., Spring, F., and Stamenkovic, I. (1996) *J. Cell Biol.* **132,** 1199–1208
25. Mann, D. M., Yamaguchi, Y., Bourdon, M. A., and Ruoslahti, E. (1990) *J. Biol. Chem.* **265,** 5317–5323
26. Screaton, G. R., Bell, M. V., Jackson, D. G., Cornelis, F. B., Gerth, U., and Bell, J. I. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 12160–12164
27. Goldstein, L. A., Zhou, D. F., Picker, L. J., Minty, C. N., Bargatze, R. F., Ding, J. F., and Butcher, E. C. (1989) *Cell* **56,** 1063–1072
28. Stamenkovic, I., Amiot, M., Pesando, J. M., and Seed, B. (1989) *Cell* **56,** 1057–1062