Proteoglycan Forms of the Lymphocyte Homing Receptor CD44 Are Alternatively Spliced Variants Containing The v3 Exon

David G. Jackson,* John I. Bell,* Richard Dickinson,‡ Jackie Timans,‡ John Shields,‡ and Nigel Whittle‡

*Molecular Immunology Group, Instutute of Molecular Medicine, John Radcliffe Hospital, University of Oxford, Oxford OX3 9DU, United Kingdom, and ‡Cantab Pharmaceuticals, Cambridge CB4 4GN, United Kingdom

Abstract. The CD44 cell surface glycoprotein is expressed on a broad range of different tissues as multiple isoforms containing from one to ten alternatively spliced exons v1-v10 inserted within the extracellular domain. Differential glycosylation generates still further variability, yielding both N- and O-glycan–modified forms of CD44 in addition to proteoglycan-like variants containing chondroitin sulphate and heparan sulphate. These high molecular mass proteoglycan-like variants, previously identified in lymphocytes, melanomas, and keratinocytes have been implicated in cell–matrix adhesion, cell motility, and invasiveness. More recently, monocyte CD44 molecules presumed to carry glycosaminoglycan chains were shown to bind the chemokine MIP-1β (Tanaka, Y., D. H. Adams, S. Hubscher, H. Hirano, U. Siebenlist, and S. Shaw. 1993. *Nature (Lond.).* 361:79–82.) raising the intriguing possibility that proteoglycan-like CD44 variants might play a role in regulating inflammatory responses.

Here we have investigated the molecular identity of these proteoglycan-like CD44 variants by generating a panel of recombinant CD44 isoforms using a novel cassette cloning strategy. We show that both chondroitin and heparan sulphate modifications are associated specifically with isoforms ($CD44_{v3-10}$ and $CD44_{v3,8-10}$) containing the v3 alternative exon which encodes a consensus motif SGXG for GAG addition. Other isoforms ($CD44_{v10}$, $CD44_{v8-10}$, $CD44_{v7-10}$, and $CD44_{v6-10}$) are shown to lack these GAG chains but to carry extensive O-glycan modifications, most likely within the mucin-like alternative exon inserts. We also demonstrate that the majority of endogenous GAG-modified CD44 isoforms present in epithelial cells constitute v3 isoforms thus establishing that in these cells the majority of proteoglycan-like CD44 variants are generated by alternative splicing. Finally we present evidence using transfected B lymphoma cells that the GAG-modified CD44 isoforms $CD44_{v3-10}$ and $CD44_{v3,8-10}$, unlike CD44H, bind only weakly to hyaluronan. Together with the demonstration in the accompanying paper (Bennett, K., D. G. Jackson, J. C. Simon, E. Tanczos, R. Peach, B. Modrell, I. Stamenkovic, G. Plowman, and A. Aruffo. 1995. *J. Cell Biol.* 128:687–698.), that CD44 molecules containing the v3 exon bind growth factors, these results highlight a new and potentially important role for CD44 alternative splicing in the control of cell-surface proteoglycan expression.

THE CD44 cell surface glycoprotein is broadly expressed by cells of haemopoietic, epithelial, and mesothelial origin where it is thought to mediate a variety of functions including cell-extracellular matrix binding, leucocyte-endothelial transmigration, and lymphocyte activation (for review see Lesley et al., 1994). Furthermore, experimental evidence links CD44 with a possible role both in tumour metastasis (Gunthert et al., 1991) and in the control of cell migration during normal embryonic development (Wheatley et al., 1993).

Address all correspondence to Dr. David G. Jackson, University of Oxford, Molecular Immunology Group, Institute of Molecular Medicine, John Radcliffe Hospital, Headington, Oxford OX 9DU, United Kingdom. Tel.: 0865 222 332. Fax: 0865 222 502.

One of the most striking features of the CD44 glycoprotein is the extent of molecular weight heterogeneity that is apparent when CD44 antigens are immunoprecipitated from different cell types (Omary et al., 1988; Pals et al., 1989; Picker et al., 1989; Kansas et al., 1989; Brown et al., 1991; Kennel et al., 1993). Originally ascribed to posttranslational modification, this heterogeneity is now recognized to arise mainly from posttranscriptional RNA splicing (Dougherty et al., 1991; Brown et al., 1991; Hofmann et al., 1991; Jackson et al., 1992; He et al., 1992) during which up to ten alternative exons (v1–v10) may be inserted in tandem within a region of the mRNA that codes for the membrane-proximal portion of the extracellular domain (Screaton et al., 1992, 1993). The alternatively spliced CD44 isoforms which range in size

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from 110 kD to over 250 kD are largely restricted in expression to cells of epithelial origin whereas an 80–90 kD form (termed the haemopoietic form, CD44H) which lacks all ten alternative exons predominates in most cell types (for review see Lesley et al., 1994).

Although the precise functions of the alternatively spliced CD44 variants (vCD44)[1] are unknown, it is recognized that CD44H acts as the major receptor on lymphocytes and fibroblasts for hyaluronan (Culty et al., 1990; Lesley et al., 1990; Aruffo et al., 1990), an extracellular-matrix glycosaminoglycan that is highly abundant in the interstitial spaces of mesothelial tissues where it is thought to play a key role in the control of cell migration. The binding site for hyaluronan is in fact present in all CD44 isoforms where it is located within the invariant, NH_2-terminal portion of the extracellular domain that shares homology with the hyaluronan-binding domains of the tissue-matrix proteoglycans aggrecan, versican, and link protein (Stamenkovic et al., 1989; Goldstein et al., 1989). In common with these large matrix proteoglycans, all forms of CD44 also contain a highly glycosylated mucin-like domain rich in serine and threonine residues, which connects the hyaluronan-binding domain with the plasma membrane. This invariant membrane-proximal domain contains four serine-glycine motifs for potential covalent modification with glycosaminoglycans (Bourdon et al., 1985), and it has been assumed that one or more of these is the site of attachment for chondroitin sulphate and heparan sulphate chains in high molecular weight proteoglycan-like forms of CD44.

Recent reports have suggested important functions for these proteoglycan-like CD44 molecules. For example, chondroitin sulphate–modified forms of CD44 in murine melanoma cells were shown to regulate motility and invasiveness in collagen gels (Faassen et al., 1992, 1993) while another chondroitin sulphate–modified variant from human lymphocytes was shown to bind the extracellular matrix proteins fibronectin and laminin (Jalkanen and Jalkanen, 1992). Furthermore CD44 molecules isolated from human monocytes were recently shown to sequester the glycosaminoglycan (GAG)-binding monokine MIP-1β (Tanaka et al., 1993), raising the possibility that proteoglycan-like forms of CD44 might function in the binding and presentation of growth factors.

In this report we describe the results of experiments designed to characterize these proteoglycan forms of CD44 and to determine the nature of their ligands. Using a novel cassette cloning method, we have constructed a panel of recombinant CD44 alternative splice variants containing combinations of exons v3 to v10 which have been stably transfected into the $CD44^{-ve}$ B lymphoma Namalwa. First, we show that the major GAG-modified CD44 isoforms within this panel and in two other epithelial cell lines are in fact alternative splice variants containing the v3 exon. This demonstrates for the first time that alternative splicing plays a major role in the generation of proteoglycan forms of CD44. The ability of these GAG-modified forms to bind growth factors is demonstrated in the accompanying paper (Bennett et al., 1994). Second, we identify O-glycosylation as the major modification of alternative exons other than v3, and third, we describe ligand-binding studies which demonstrate that GAG-modified CD44 isoforms, when expressed in a B-lymphoma cell line, have greatly reduced affinities for hyaluronan relative to CD44H. Finally, we discuss the possible presence of a weak interaction between CD44 v3 containing isoforms and fibronectin and laminin.

1. *Abbreviations used in this paper*: vCD44, spliced CD44 variant; GAG, glycosaminoglycan.

Materials and Methods

Cell Lines and Monoclonal Antibodies

The squamous cell lung carcinoma "Hotz" was kindly provided by Dr. P. Groscurth (Zurich, Switzerland), the adenovirus 12-SV40 hybrid virus-transformed bronchial epithelial line BEAS-2B was provided by Dr. C. C. Harris, National Cancer Institute (Bethesda, MD); and the cervical carcinoma HeLa, the vulval carcinoma A-431, the colon adenocarcinoma HT29, and the mammary adenocarcinomas MCF-7 and MCF-10 were generously donated by Dr. Ken Smyth (ICRF Molecular Oncology Unit, IMM, Oxford, UK). All cell lines were grown in RPMI-1640 supplemented with 10% FCS.

The CD44 framework antibody F.10.44.2 (Dalchau et al., 1980) was a kind gift from Dr. Maria Dalchau and was purified from hybridoma culture supernatant by affinity chromatography on columns of protein A–Sepharose (Pharmacia, Sweden). The mouse monoclonal antibody 3G5 (subclass IgG_{2a}) specific for alternatively spliced isoforms of human CD44 containing the alternative v3 exon was generated as described previously (Fox et al., 1994). FITC-conjugated goat antibodies to mouse IgG and the isotype control mouse myeloma immunoglobulin UPC-10 ($IgG2_a$ Kappa) were obtained from the Sigma Chem. Co. (Poole, Dorset, UK).

Radiochemicals

$Na^{125}I$ (17 mCi/μg), $Na_2{}^{35}SO_4$ (100 mCi/mmol), and $Na_2{}^{51}CrO_4$ (250–550 mCi/mg Cr) were obtained from Amersham International Public United Company (Amersham, UK).

Preparation of "Mini-Libraries" Encoding Variably Spliced Regions from vCD44 cDNAs

DNA sequences constituting most of the extracellular domain–coding regions from alternatively spliced CD44 isoforms were generated by PCR using cDNA from the squamous cell lung carcinoma Hotz and the normal bronchial epithelial line BEAS-2B using the primers AMP1Eco (5′ CCGGAATTCCCAGTATGACACATATTGC 3′) and AMP3Eco (5′ CCGGAATT CCTTCGTGTGTGG GTAATG 3′) located in exons 3 and 15 (v10) of CD44 as described previously (Jackson et al., 1992, 1994). Products were purified on glass beads (Gene Clean, Stratech, UK), digested with EcoRI (50 U, 37°C), and cloned into EcoRI cut M13mp18 generating mini-libraries of vCD44 clones representing $CD44_{v10}$, $CD44_{v8-10}$, $CD44_{v7-10}$, $CD44_{v6-10}$, $CD44_{v3-10}$ and $CD44_{v3,8-10}$ identified by DNA sequencing using the dideoxynucleotide "chain termination" method (Sanger et al., 1977) with T7 DNA polymerase and 7-deaza-dGTP.

Preparation of CD44 Isoform Constructs and Stable Transfection of Namalwa Cells

Full-length CD44 constructs for stable expression were prepared by a novel cassette cloning strategy in which the variably spliced regions derived from the RT-PCR mini-libraries described above were inserted into a $CD44_{v10}$ clone containing unique BglII and NarI sites introduced by site-directed mutagenesis. In the following description, all exon numbering follows the convention shown in Fig. 1 *B*.

Production of Variably Spliced CD44 Fragments with Compatible Ends for Cassette Cloning. DNA clones representing the isoforms $CD44_{v8-10}$, $CD44_{v7-10}$, $CD44_{v6-10}$, $CD44_{v3,8-10}$, and $CD44_{v3-10}$ were amplified from mini-libraries of epithelial cell vCD44 DNA fragments using the primers NRW77BglII and NRW78NarI (see below for sequences) in reactions (50 μl) consisting of template DNA (100 ng), primers (0.2 μM), and deoxynucleotide triphosphates (1 mM), catalyzed by the addition of 1 U *Pyrococcus furiosus* DNA polymerase (Strategene Ltd., Cambridge, UK). The cycle parameters were 94°C, 1 min; 60°C, 3 min; and 72°C, 1 min for 30 cycles. Products were then digested with BglII and NarI (37°C) to gener-

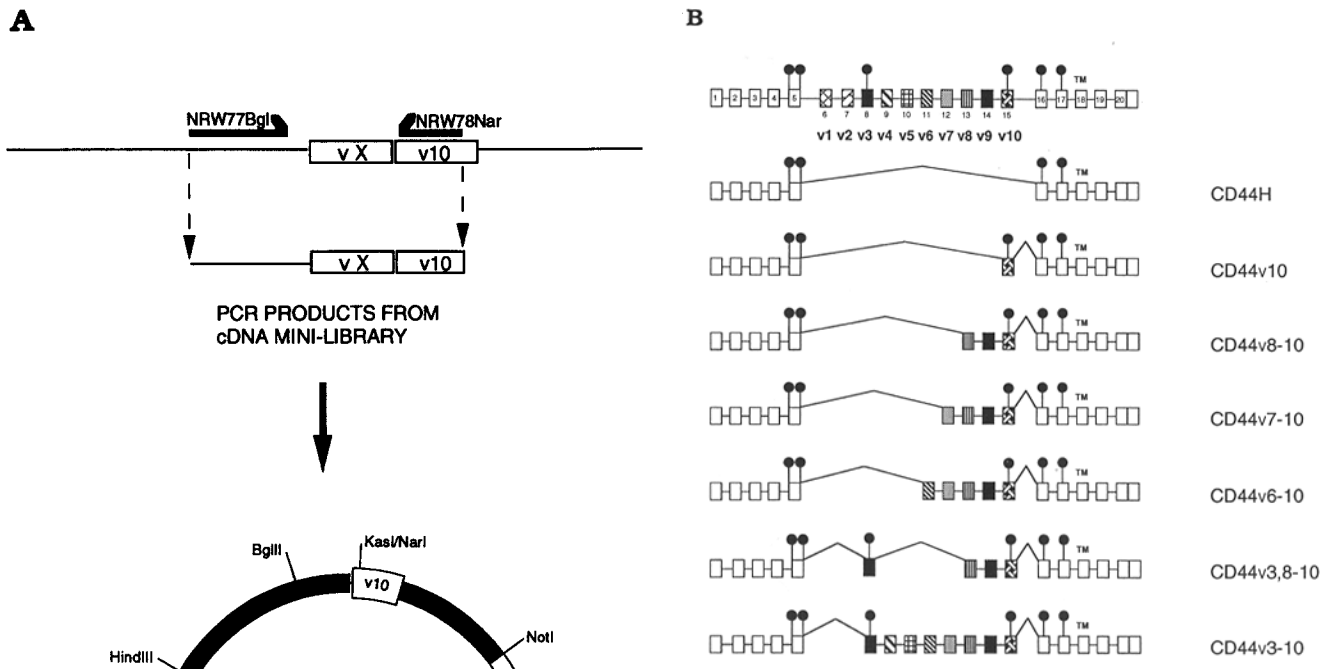

Figure 1. Construction of a panel of full-length cDNAs encoding alternatively spliced human CD44 isoforms. *A* shows the strategy employed for cloning full-length vCD44 constructs by PCR from CD44 cDNA mini-libraries and insertion into the mutagenized plasmid construct pBC^{ks}–CD44$_{v10}^{BglII/NarI*}$ (see Materials and Methods). *B* shows a diagrammatic representation of the final panel of CD44 isoform constructs including the independently derived CD44H (see Materials and Methods) used to transfect Namalwa cells. The positions of motifs for putative modification with glycosaminoglycans are indicated with the symbols (🍍).

ate the appropriate cohesive ends for ligation with the cassette vector pBC^{ks-}CD44v10$^{Bgl/Nar**}$ (Fig. 1 *A*).

Construction of the CD44$_{v10}$ Cassette Cloning Vector by Site-directed Mutagenesis. The full-length CD44$_{v10}$ cDNA described in Jackson et al. (1992) was subcloned from pCDM8 into the pBC^{ks-} phagemid (Stratagene, UK) for single stranded DNA preparation with R408 helper phage after transformation of *E. coli* XL-1 blue. Using oligonucleotide-directed mutagenesis new restriction sites for BglII and NarI/KasI were introduced, respectively, in exon 5 (nt 673-676 in Stamenkovic et al. [1989]; GAGC→ATCT) and exon 15 (AAGAA→GCGCC) using the primers NRW77-BglII (AGTGAA**agatct**AGCACTTCAGGAGG) and NRW78NarI (TGGGTCTC**ggcgcc**CACCTGTGACATCATT). The integrity of the resulting construct pBC^{ks-}CD44v10$^{Bgl/Nar**}$ (Fig. 1 *A*) was confirmed by DNA sequencing on both strands. For cassette cloning, the vector was digested overnight with BglII and NarI before ligation with the appropriate RT-PCR products containing compatible ends described above.

Expression of recombinant CD44 isoforms. Full-length constructs in the cassette vector pBC^{ks-}CD44$_{v10}^{Bgl/Nar**}$ were checked by DNA sequencing and subcloned into the HindIII/NotI cloning site of the eukaryotic expression vector pRcCMV (InVitrogen, San Diego, CA) for transfection of the CD44^{-ve} B cell lymphoma Namalwa by electroporation (960 μF, 750 V/cm). The CD44H expression construct was prepared by subcloning an existing full-length human CD44H cDNA clone (Stamenkovic et al., 1989) into the NotI–HindIII site of pRcCMV. Stable transfectants were selected first for growth in the presence of G418 (2.5 mg/ml; GIBCO BRL, UK) and second, for cell surface CD44 expression using anti-mouse Ig-coupled Dynabeads (DYNAL, Oslo, Norway) coated with the CD44mAb F.10.44.2 according to the manufacturer's instructions. The resulting cell lines were analyzed periodically for surface expression by staining with F.10.44.2 or with phycoerythrin-conjugated A3D8 mAb (Sigma, UK) followed by quantitative FACScan® analysis as described below.

Immunofluorescent Antibody Staining and Flow Cytometry

Cells (5 × 10^5) were washed once in PBS, containing 1% human serum and 0.1% NaN_3 before incubation (20 min, 4°C) with 100 μl of CD44 mAbs (10 μg/ml). After washing in PBS, cells were reincubated (30 min, 4°C) with 100 μl fluorescein-conjugated goat anti-mouse IgG (Sigma Chem. Co., UK) before recentrifugation and resuspension in PBS containing 2% (wt/vol) formaldehyde fixative. Stained cells were analyzed either by flow cytometry on a FACScan® or by microscopy using a Zeiss fluorescence microscope with automatic camera (Becton-Dickinson Immunocytometry Sys., Mountain View, CA).

Cell Surface Iodination, Biosynthetic Labeling with $^{35}(SO_4)^{2-}$ and Immunoprecipitation

Cell surface radioiodination was carried out by the lactoperoxidase/glucose oxidase catalyzed reaction as described previously (Jackson et al., 1992). In the case of adherent cells these were first detached by incubation (37°C, 30 min) in PBS, pH 7.5, 1 mM EDTA. Thereafter all cell types (0.5-1 × 10^7) were resuspended in PBS, pH 7.5, 5 mM glucose, and incubated (20 min, room temperature) with 400 mU bovine milk lactoperoxidase (E.C.1.11.1.7, Sigma, UK) and 240 mU *Aspergillus niger* glucose oxidase (EC 1.1.3.4, Sigma, UK) in the presence of 0.5 mCi $Na^{125}I$. Labeled cells were then washed three times (PBS, 1 mM NaI) to remove free ^{125}I- before solubilization in detergent (see below).

For biosynthetic incorporation of $^{35}SO_4^{2-}$ cells were incubated (37°C, 6–24 h) in sulphate free DMEM, 10% FCS containing 100 μCi/ml $Na_2{}^{35}SO_4$ (Amersham, UK). Residual unincorporated radiolabel was removed by washing labeled cells in PBS, pH 7.5.

For immunoprecipitation, radiolabeled cells were extracted into 0.5–1.5 ml Tris-HCl (20 mM) pH 7.5, containing Triton X-100 (1% wt/vol), NaCl (150 mM), EDTA (1 mM), phenylmethanesulphonylfluoride (0.5 mM), leupeptin (50 μg/ml), and iodoacetamide (5 mM) followed by centrifugation (9,000 *g*, 5 min) to remove particulate material. The resulting supernatants were precleared twice by end over end mixing with 100 μl 10% wt/vol Pansorbin (Calbiochem-Novabiochem. Corp., LaJolla, CA) followed by 100 μl 50% protein A–Sepharose CL-4B/mouse IgG (1 h, 5°C). After centrifugation, (9,000 *g*) supernatants were decanted and mixed (1 h, 5°C) with specific antibody (either F.10.44.2 or 3G5, 10 μg/ml) before the addition of 50 μl 1:1 (vol/vol) protein A–Sepharose CL-4B. Immobilized immune complexes were recovered by centrifugation and washed with TEN buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA) containing Triton X-100 (0.1%) followed by TEN buffer without Triton X-100 before enzymatic deglycosylation or SDS-PAGE.

Removal of O-linked Sugars, N-linked Sugars, and Glycosaminoglycans

CD44 isoforms immunoprecipitated from surface ^{125}I-labeled cells were treated with either N-glycosidase F (12.5 U/ml *Flavobacterium meningosepticum* enzyme, EC 3.2.2.18, Genzyme, UK) in 0.1 M Na phosphate buffer pH 8.6, or with neuraminidase (250 mU/ml *Vibrio cholerae* enzyme, EC 3.2.1.18, Calbiochem-Novabiochem Corp.) followed by O-glycosidase (5 mU/ml *Diplococcus pneumoniae* enzyme, EC 3.2.1.97, Boehringer Mannheim, UK) in 40 mM Tris-acetate buffer, pH 6.0, containing calcium acetate, for the removal of N- and O-linked carbohydrates, respectively. In addition, immunoprecipitated ^{125}I- and $^{35}SO_4^{2-}$ labeled CD44 isoforms were treated with either chondroitin ABC lyase (0.5 U/ml *Proteus vulgaris* enzyme, EC 4.2.2.4, Boehringer Mannheim, Lewes, East Sussex, UK) in 40 mM Tris acetate buffer, pH 8.0, or with heparitinase (0.1 U/ml *Flavobacterium heparinum* enzyme, EC 4.2.2.8, Seikagaku Corporation, Tokyo, Japan) in PBS, pH 7.0. Deglycosylated proteins were recovered by precipitation with 10% (wt/vol) TCA and analyzed on 7.5% acrylamide SDS-PAGE gels followed by autoradiography.

Adhesion Assays

Transfected Namalwa cells labeled with ^{51}Cr (typically 2 × 10^6 cells in 100 μl $Na_2(^{51}CrO_3)$, 1 h, 37°C) were assayed for binding to matrix components in 24-well tissue culture or 96-well microtitre plates. Plates were prepared by coating either human fibronectin (0.1 mg/ml, Calbiochem, lot No. 536892), human laminin (0.1 mg/ml, Calbiochem, lot Nos. 244791 and B09306) or hyaluronan (5 mg/ml, Sigma) overnight in PBS, pH 7.5, followed by blocking (24 h, room temperature) with either heat-denatured bovine serum albumin or with desialated human fetuin (Sigma, 1 mg/ml). For binding assays, cells were added either to 96-well plates (10^4 in 50 μl PBS, pH 7.5) or to 24-well plates (5 × 10^4 in 250 μl PBS, pH 7.5) and incubated for 60 min at 25°C or in some experiments at 5°C. In competitive-binding experiments, cells were preincubated (15 min, 5°C) with either BSA, chondroitin sulphate, heparin (Sigma, UK), soluble fibronectin (Calbiochem-Novabiochem Corp.), or with the 40-kD α chymotryptic heparin-binding fragment of fibronectin (Gibco, UK) before the adhesion assay. For the measurement of hyaluronan binding, non-adherent cells were removed during three washes of the plates (500 μl/wash) by pipetting and gentle tapping. In the case of fibronectin and laminin binding, the incubation was preceded by a brief centrifugation step (500 *g*, 5 s) and non-adherent cells were carefully removed at the end of the assay by gentle rinsing (3× 100 μl PBS) with a multichannel micropipette. In each case adherent cells were lysed by the addition of 1% Triton X-100 and samples of lysate were counted for radioactivity using an LKB 1205 Beta plate Liquid scintillation spectrometer. Results were scored for both the total number of cells bound in each well (normalized for differences in the amount of ^{51}Cr incorporated by each transfectant) and for the percentage of cells bound in each well (cpm bound/cpm added × 100).

Results

To study the functions of individual CD44 splice variants, we developed a simple and rapid strategy by which these could be generated without having to resort to conventional cDNA library screening procedures. The two basic steps in this technique which we have termed "cassette cloning" (Fig. 1 *A*) consisted of (*a*) PCR-amplification of the alternatively spliced regions from mini-libraries containing variant CD44 cDNAs, and (*b*) insertion of the products as cassettes into the homologous site of a mutagenized CD44 cDNA clone. The resulting hybrid cDNA clones (Fig. 1 *B*) were then stably transfected into the CD44^{-ve} B lymphoma Namalwa.

The two stages of the cassette cloning procedure are described in more detail in the following section.

Construction and Stable Expression of a Panel of Full-Length CD44 Isoforms

We first amplified DNA fragments encoding the alternatively spliced regions from vCD44 isoforms from mini-libraries of CD44 cDNAs prepared from the bronchial epithelial cell lines Hotz and BEAS-2B using the primers NRW77BglII and NRW78NarI located within exons 5 and 15 (v10), yielding products identified by DNA sequencing as $CD44_{v10}$, $CD44_{v7-10}$, $CD44_{v6-10}$, $CD44_{v3,8-10}$, and $CD44_{v3-10}$. These cDNA mini-libraries were initially generated by RT-PCR of total RNA using the primers AMP1Eco and AMP3Eco located within exons 3 and 15 (v10) as described in our original paper (Jackson et al., 1992) and in a more recent publication (Jackson et al., 1994).

We next mutated a full-length $CD44_{v10}$ cDNA in the phagemid pBC^{ks-} (Fig. 1 *A*), introducing unique BglII and NarI restriction sites to allow homologous insertion of the PCR-amplified vCD44 fragments described above. These new sites were created by altering the native sequences AGGAGC→AGATCT (nt 671-676 in Stamenkovic et al., 1989) and GAAGAA→GGCGCC located within CD44 exons 5 and 15 (v10), respectively, choosing substitutions that exploited codon degeneracy and thus ensured that the final constructs contained no changes in the translated amino acid sequence.

After subcloning into the eukaryotic expression vector pRcCMV, the panel of CD44 isoforms shown in Fig. 1 *B* was stably transfected into the CD44^{-ve} B cell lymphoma Namalwa yielding cell lines in which the mean level of surface expression was within 40% as determined by quantitative immunofluorescent antibody staining (Fig. 2).

Alternatively Spliced CD44 Isoforms Are Heavily O-Glycosylated

Analysis of the full panel of recombinant CD44 isoforms in Namalwa cells by surface ^{125}I-labeling and immunoprecipitation (Fig. 3) revealed a single discrete band as expected for CD44H, $CD44_{v10}$, $CD44_{v8-10}$, $CD44_{v7-10}$, and $CD44_{v6-10}$. However, both CD44v3,8-10 and CD44v3-10 migrated as indistinct high molecular weight smears, extending as far as the stacking gel in each case (see next section for full discussion). In comparison with CD44H, each CD44 isoform migrated with an apparent molecular mass that was disproportional to the size of the variable exon insert. For example the CD44v6-10 variant migrated with an apparent molecular mass of 160 kD, some 70 kD larger than CD44H (90 kD), despite the fact that the calculated mass of the v6-10 polypeptide insert is only 21.8 kD. In view of the fact that all ten human CD44 alternative exons encode polypeptide units that are rich in both serine and threonine residues

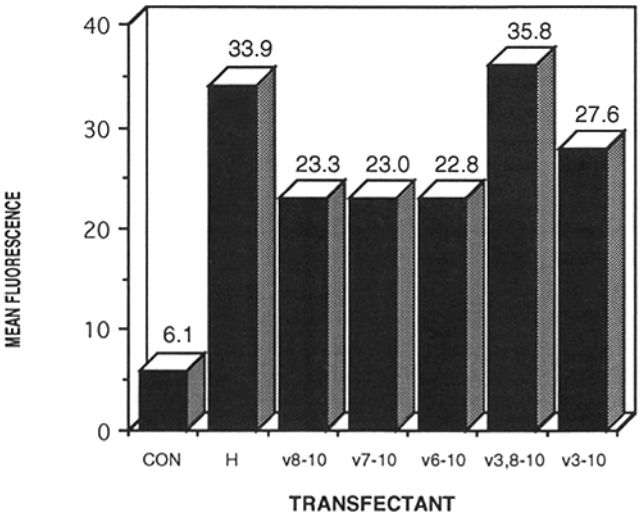

Figure 2. Quantitation of surface expression by Namalwa cells stably transfected with CD44 isoforms. Namalwa cells stably transfected with cDNAs encoding the CD44 isoforms or control untransfected Namalwa cells (*CON*) were assessed for surface CD44 expression by indirect immunofluorescent antibody staining with the pan CD44 mAb F.10.44.2 and quantitative FACS analysis as described under Materials and Methods. The values indicated are the mean fluorescent intensity (linear scale on Y-axis) for each cell population derived using the LYSYS II data analysis program (Becton-Dickinson Immunocytometry Sys.).

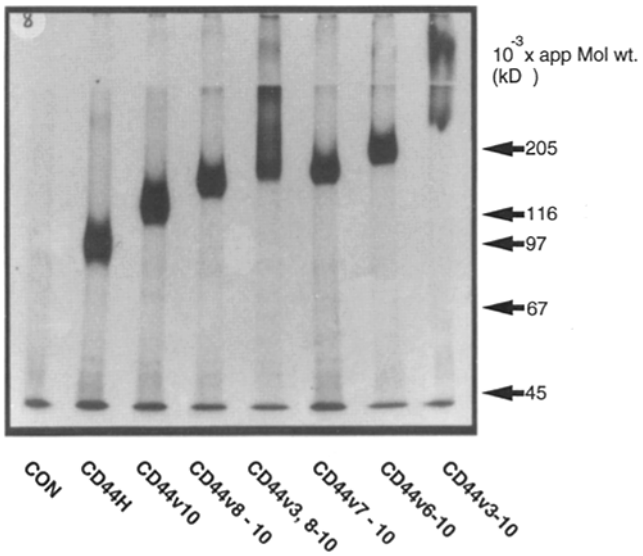

Figure 3. SDS-PAGE analysis of recombinant human CD44 isoforms expressed in Namalwa cells. Namalwa cells stably transfected with each of the human CD44 isoforms shown were surface labeled with ^{125}I before immunoprecipitation with the pan CD44 mAb F.10.44.2 and electrophoresis on a 7.5% acrylamide SDS-PAGE gel followed by autoradiography as described under Materials and Methods. The molecular weight calibration marker proteins were β-galactosidase (205 kD), myosin (160 kD), phosphorylase b (116 kD), bovine serum albumin (67 KD), and ovalbumin (45 kD).

(mean 30%; Screaton et al., 1993), we considered the possibility that the anomalous migration reflected heavy O-glycosylation. As shown in Fig. 4, treatment of ^{125}I-labeled CD44 isoforms with neuraminidase and O-glycanase resulted in a dramatic decrease in apparent molecular mass in each case (e.g., $CD44_{v10}$, 49 kD; $CD44_{v8-10}$, 32 kD; $CD44_{v7-10}$, 50 kD; $CD44_{v6-10}$, 35 kD: $CD44_{v3,8-10}$, 30 kD) compared with CD44H (16 kD). Hence the alternatively spliced isoforms must carry substantial amounts of O-linked glycans additional to the 4-5 O-glycan chains identified previously in CD44H (Lokeshwar and Bourguignon, 1991) and these presumably are attached to the variably spliced portions of the membrane-proximal domain. The alteration in mobility produced by digesting the ^{125}I-labeled CD44 isoforms with neuraminidase alone together with the fact that cleavage of O-glycans did not procede without prior neuraminidase treatment (data not shown and Lokeshwar and Bourguignon, 1991) indicates that some or all of the O-linked sugars terminate with sialic acid.

We also considered the possibility that the vCD44 isoforms might carry additional N-linked glycans. In contrast to O-glycosylation, only two CD44 alternative exons v5 and v10 contain the motif NXS/T for N-glycosylation (Screaton et al., 1992) although six such motifs are located within invariant regions of the extracellular domain common to all CD44 variants (Stamenkovic et al., 1989; Goldstein et al., 1989). Treatment with N-glycanase (Fig. 4 *B*) produced the same drop in molecular mass in each of the variant CD44 isoforms as that observed for CD44H (~20 kD) indicating that the alternatively spliced regions do not carry large amounts of additional N-glycans.

Recombinant CD44 Isoforms Containing the v3 Exon Are Heparan Sulphate and Chondroitin Sulphate Proteoglycans

In contrast to the CD44 isoforms discussed above, ^{125}I-labeled $CD44_{v3-10}$ and $CD44_{v3,8-10}$ expressed in Namalwa cells migrated as a high molecular weight smear close to the top of the resolving gel during SDS-PAGE—a common feature of proteins that are covalently modified with sulphated glycosaminoglycans. Since these two isoforms were the only ones to contain the CD44 v3 exon that encodes the consensus motif SGSG for GAG addition (Bourdon et al., 1987), it seemed likely to us that the high molecular weight smear might reflect specific modification of the v3 insert by GAG chains such as chondroitin sulphate or heparan sulphate. As an initial test of this hypothesis, ^{125}I-labeled $CD44_{v3-10}$ and v3,8-10 were digested with chondroitin ABC lyase followed by SDS-PAGE analysis to detect GAG removal as a decrease in the charge to mass ratio. The results of this analysis (Fig. 4 *b*) showed that chondroitinase treatment indeed reduced the amount of smearing associated with the v3,8-10 and v3-10 variants but had no effect on the mobility of either CD44H, $CD44_{v10}$, $CD44_{v8-10}$, $CD44_{v7-10}$, or $CD44_{v6-10}$. Treatment with heparitinase (not shown) yielded similar results. Hence the smearing associated with the v3-containing isoforms is due to the presence of both heparan and chondroitin sulphate modifications.

The presence of covalently linked GAG chains was further confirmed after biosynthetic incorporation of $^{35}SO_4^{2-}$. As shown in Fig. 5, $CD44_{v3,8-10}$ incorporated $^{35}SO_4^{2-}$ into GAG chains that were sensitive to digestion by heparitinase and to

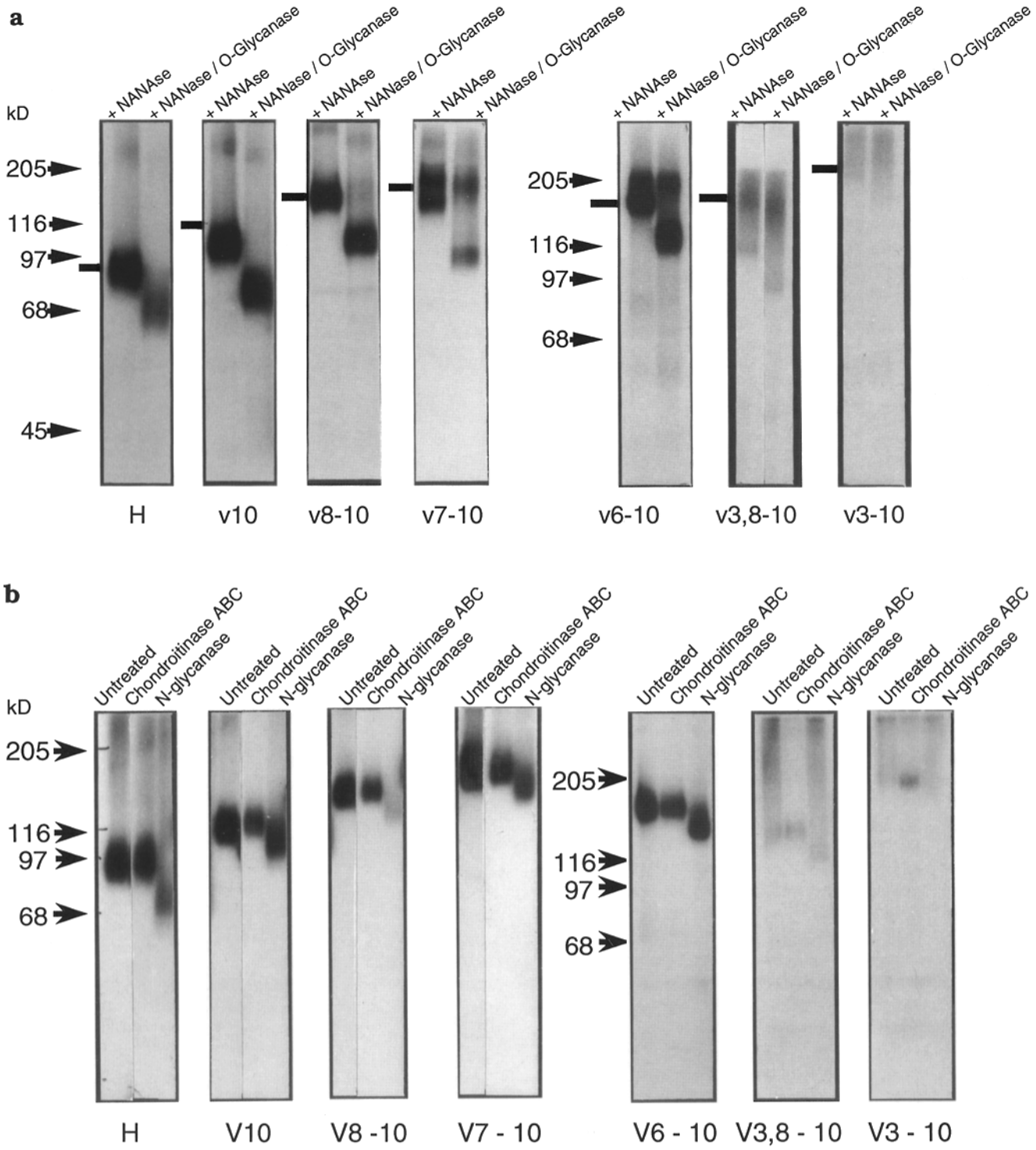

Figure 4. Enzymatic removal of O-linked sugars, N-linked sugars, and chondroitin sulphate. Recombinant human CD44 isoforms were immunoprecipitated from surface ^{125}I-labeled Namalwa cells using the pan CD44 mAb F.10.44.2 before treatment with different glycosidases as described under Materials and Methods. In the experiment shown in *a*, proteins were incubated with either neuraminidase alone (*NANAse*) or with neuraminidase followed by O-glycanase (*NANAse/O-glycanase*); the migration positions of the undigested proteins are indicated to the left of the gel in each case. In *b*, proteins were incubated with either N-glycanase or chondroitin ABC lyase as indicated. Samples were in each case electrophoresed on 7.5% SDS-PAGE gels for autoradiography. The molecular mass calibration markers were the same as those shown in Fig. 3.

a much lesser extent chondroitin ABC lyase. Similar results were obtained with $CD44_{v3-10}$ (not shown). The difficulty in detecting chondroitin sulphate by $^{35}SO_4^{2-}$ labeling despite its clear detection by ^{125}I-labeling indicates either a low turnover rate for the chondroitin sulphate chains of CD44 in Namalwa cells or a low level of sulphation. In this respect, it is interesting to note that O-sulphation is a feature of chondroitin chains whereas both O- and N-sulphation occurs during the biosynthesis of heparan chains (Kjellen and Lindahl, 1991). In contrast to the v3 variants, the two other variants analyzed, CD44H and $CD44_{v7-10}$, incorporated much smaller amounts of $^{35}SO_4^{2-}$ and this was insensitive to digestion by either chondroitin ABC lyase or heparitinase (Fig. 5).

These results demonstrate that neither CD44H nor splice variants lacking the v3 exon are modified with glycosaminoglycans when expressed in the B lymphoma Namalwa. Even the presence of the v10 exon which contains the partial GAG motif SLSG (Jackson et al., 1992) does not appear to signal GAG addition. Instead, glycosaminoglycan modification is specific to CD44 isoforms containing the v3 exon, presumably reflecting direct modification of the v3 insert itself.

Proteoglycan-like CD44 Isoforms on Epithelial Cells Are v3 Splice Variants

To determine whether the GAG-modification seen with recombinant CD44 isoforms in Namalwa cells was also a feature of native CD44 isoforms in other cell types, we studied the GAG modifications of v3-containing variants present in epithelial cells. Since these latter molecules have not yet been identified at the protein level we surveyed a panel of epithelial cell lines by surface ^{125}I-iodination and immunoprecipitation using a newly isolated v3 exon-specific mAb 3G5 (Fox et al., 1994) in addition to the pan-CD44 mAb F.10.44.2 (Dalchau et al., 1980). As shown in Fig. 6 *A*, the v3-containing variants in each of the cell lines A431, Hotz, HT29, HeLa, MCF7, and MCF10 constituted a heterogeneous group of molecules within the size range 110–250 kD representing in most cases a major proportion of total CD44. Interestingly, the variants present in Hotz and A431 cells were similar in molecular mass to the recombinant $CD44_{v3-10}$ isoform expressed in Namalwa cells and displayed the same GAG-induced smearing on SDS-PAGE (see Fig. 3).

To determine the identity of the GAG chains in more detail, CD44 isoforms biosynthetically labeled with $^{35}SO_4^{2-}$ were immunoprecipitated from A431 and Hotz cells and digested with either chondroitin ABC lyase or heparitinase. The results of these analyses (Fig. 6 *B*) showed that a discrete high molecular weight population of CD44 molecules similar if not identical in both cell lines was modified with heparan sulphate. In contrast, no significant $^{35}SO_4^{2-}$ labeling of CD44H or other lower molecular weight CD44 variants was detected in either Hotz cells or A431 cells despite the fact that these CD44 variants were clearly detectable in surface ^{125}I-labeling experiments (Fig. 6 *A*). Furthermore, the majority of the high molecular-weight heparan sulphate–modified CD44 could be immunoprecipitated with the αv3 mAb 3G5 (Fig. 6 *B*) indicating that these v3-containing splice variants are the major GAG-modified isoforms of both A431 and Hotz cells. Complete immunoprecipitation of heparan sulphate–modified CD44 from Hotz and A431 with the αv3 mAb 3G5 could not be achieved because of the low affinity of this antibody compared with F.10.44.2 as assessed in separate experiments using $CD44_{v3-10}$ using both immunoprecipitation and FACS analysis (not shown).

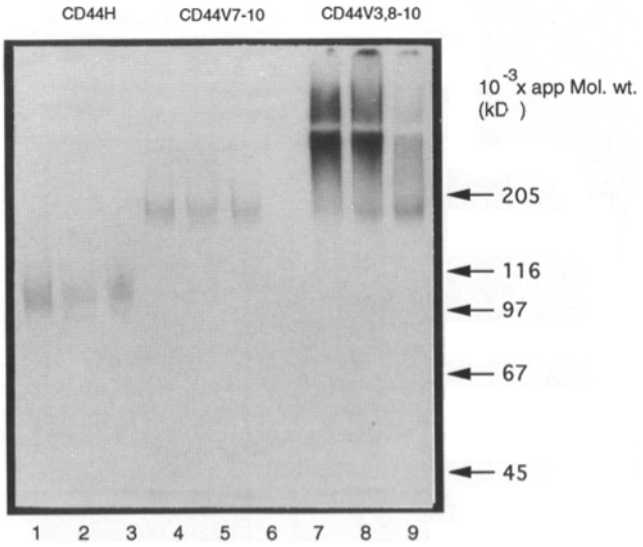

Figure 5. Recombinant human $CD44_{v3,8-10}$ expressed in Namalwa cells is modified with heparan sulphate. Namalwa cells expressing the recombinant CD44 isoforms shown were biosynthetically labeled with $Na_2{}^{35}SO_4$ in parallel followed by immunoprecipitation with the pan CD44 mAb F.10.44.2 and digestion with either chondroitin ABC lyase (lanes *2*, *5*, and *8*) or heparitinase (lanes *3*, *6*, and *9*). Lanes *1*, *4*, and *7* contain undigested controls. The same number of cells (3.3×10^5) was processed in each case. Samples were analyzed on 7.5% acrylamide SDS-PAGE gels and visualized by autoradiography. The molecular weight calibration markers were the same as those shown in Fig. 3.

Binding of CD44 Isoforms to Immobilized Hyaluronan and Extracellular Matrix Proteins

In view of the fact that CD44H previously has been proposed to function as a receptor for hyaluronan (for review see Underhill, 1992) and the extracellular matrix proteins fibronectin, collagen, and laminin, each of which contain binding sites for glycosaminoglycans (Hardingham and Fosang, 1992), we tested the binding of our panel of CD44 transfected Namalwa cells to extracellular matrix components coated on plastic tissue culture dishes.

We first measured binding of the panel of CD44 transfected Namalwa cells to hyaluronan. As shown in Fig. 7, the level of specific binding (% transfected cells bound–% control Namalwa cells bound) was severalfold higher for CD44H (24% specific binding) than for the alternatively spliced CD44 isoforms ($CD44_{v8-10}$, 9%; $CD44_{v7-10}$, 7%; $CD44_{v3-10}$, 7%; $CD44_{v6-10}$, 3%; and $CD44_{v3,8-10}$, 3% specific binding, respectively) confirming earlier findings by another group (Stamenkovic et al., 1991) who reported reduced binding by $CD44_{v8-10}$ in similar experiments. Importantly, the reduced hyaluronan binding that we observed in our experiments could not be explained by low levels of CD44 expression in the vCD44 transfected cell lines. First,

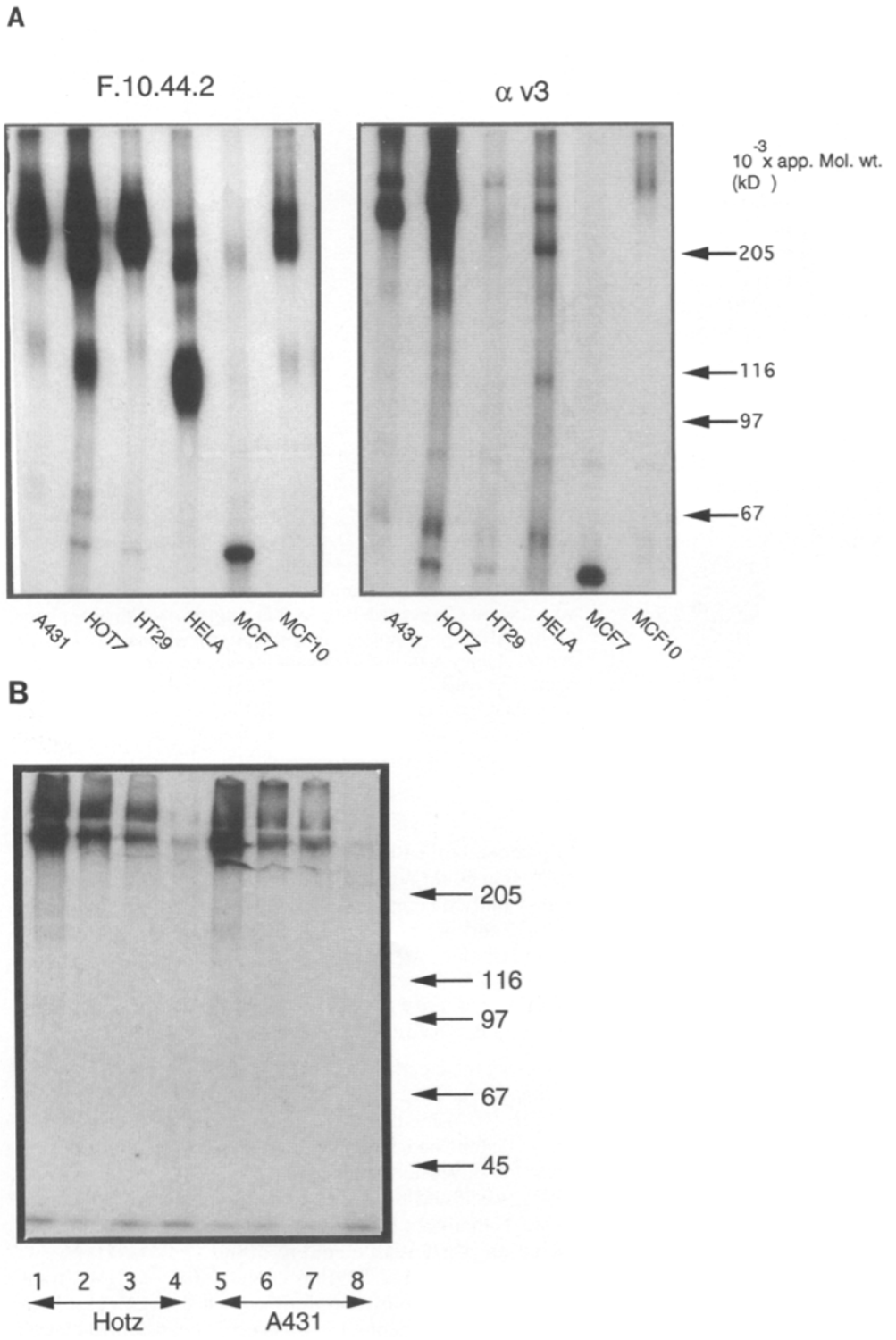

Figure 6. Epithelial cell CD44 isoforms containing the v3 exon are modified with heparan sulphate. Endogenous CD44 isoforms were immunoprecipitated from radiolabeled epithelial cell lines and analyzed on 7.5% SDS-PAGE gels either without further treatment or after treatment with either Chondroitin ABC lyase or Heparitinase as described under Materials and Methods. In *A*, each of the cell lines indicated was surface labeled with Iodine-125 and immunoprecipitated with either the pan CD44 mAb F.10.44.2 or the v3 specific mAb 3G5 as shown followed by electrophoresis on a 7.5% SDS-PAGE gel. The prominent band of 50 kD visible in the MCF7 samples cross-reacts with non-specific Ig and does not constitute an isoform of CD44 (data not shown). In *B*, the cell lines Hotz and A431 were biosynthetically labeled with $^{35}SO_4$ before immunoprecipitation with either the pan CD44 mAb F.10.44.2 (lanes *1* and *5*) or the v3 specific mAb 3G5 (all other lanes) followed by treatment with Chondroitin ABC lyase (lanes *3* and *7*) or Heparitinase (lanes *4* and *8*) and the samples electrophoresed on a 7.5% SDS-PAGE gel.

the levels of surface expression were measured for each vCD44 transfectant both 8 wk before and 11 mo after performing the assay depicted in Fig. 7 and were found to differ by no more than 30% from the CD44H expression levels (Fig. 2). Second, the binding assay has been repeated three further times yielding similar results that in each case showed a statistically significant difference between the hyaluronan-binding capacities of CD44H and vCD44 (see legend to Fig. 7).

Interestingly, none of the transfectants expressing vCD44 could be induced to bind hyaluronan by treatment with either phorbol myristate acetate or with the CD44 mAb F.10.44.2 (data not shown), conditions reported previously to induce hyaluronan binding in other CD44^{+ve} adhesion-deficient cell

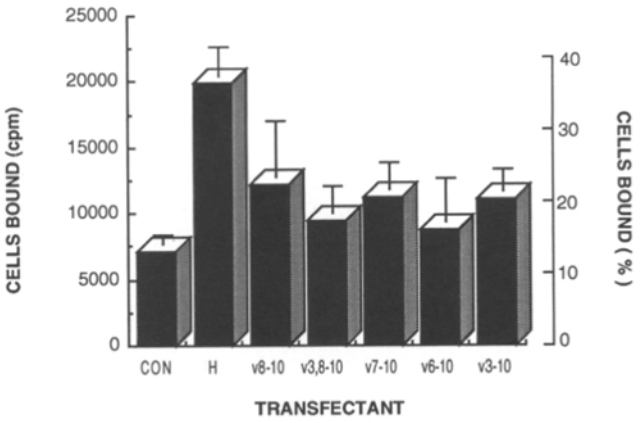

Figure 7. Binding of CD44 transfected Namalwa cells to hyaluronan. Namalwa cells stably transfected with the recombinant human CD44 isoforms shown were labeled with ^{51}Cr before incubation in plastic multiwell dishes coated with hyaluronan as described under Materials and Methods. Untransfected Namalwa cells (CON) were included as a control for CD44 independent binding. The level of non-specific background adhesion (measured using wells coated with BSA or desialated fetuin) was the same for each transfectant and was equivalent to 4,400 cpm or 8.4% binding in the experiment shown. Values shown are the mean ± SD of triplicate determinations of the number of cells bound per well, normalized for differences in ^{51}Cr incorporation by each transfectant. Values for the percentage of cells bound were calculated as described in Materials and Methods. The differences in hyaluronan binding between CD44H and vCD44 were significant to a probability $p < 0.01$ both in the experiment shown and in each of two similar experiments not shown, as calculated using a two-tailed Student's *t* test.

lines (Lesley et al., 1990; Hyman et al., 1991; Lesley et al., 1992; Liao et al., 1994). These latter results provide firm evidence that the variably spliced inserts within the membrane-proximal domain of CD44 can regulate the ligand-binding affinity of the NH_2-terminal domain.

We also compared binding of the vCD44 transfectants to fibronectin and laminin in four independent experiments using ligand immobilized in 96-well plastic microtitre dishes (data not shown). In two experiments both the GAG-modified isoforms $CD44_{v3,8-10}$ and $CD44_{v3-10}$ displayed significant binding ($p < 0.02$, Student's *t* test, $n = 5/6$) to fibronectin compared with CD44H and $CD44_{v8-10}$ (actual values $CD44_{v3,8-10}$/CD44H; 3599 ± 1294/1471 ± 371 cpm, experiment 1 and 6690 ± 2239/3454 ± 660 cpm, experiment 2). Moreover, in the case of fibronectin, binding could be partially blocked (46–73%, $n = 5$) by the addition of heparin, chondroitin sulphate, soluble $CD44_{v3,8-10}$, or a 40-kD proteolytic fragment of fibronectin containing the heparin-binding site (not shown). However in two other experiments, binding of the v3 isoforms to fibronectin was not significantly different from CD44H. Similar results were obtained when we measured the binding of CD44 to laminin (not shown). Given the extent of variability in the data, we conclude that the interaction between CD44 and both fibronectin and laminin is probably of low affinity, although when detectable this interaction appears to involve CD44-associated glycosaminoglycan chains.

Finally none of the CD44 isoforms tested bound to the GAGs chondroitin 4-sulphate, chondroitin 6-sulphate, heparan sulphate, or keratan sulphate immobilized on plastic microtitre dishes (data not shown).

Discussion

The multiple isoforms of CD44 expressed in different tissues are now known to be generated by the differential splicing of one to ten alternative exons v1-v10 that encode variable portions of the membrane-proximal extracellular domain (for review see Lesley et al., 1994). This process can in theory produce more than 1,000 different CD44 variants from a single gene (Screaton et al., 1992) representing a degree of molecular diversity exceeded only by the lymphocyte antigen receptors. In addition to primary structural differences, CD44 variants also differ in terms of glycosylation thought to arise from the addition of sugars to sites within both the invariant region of the extracellular domain encoded by constitutively spliced exons (Jalkanen et al., 1988; Camp et al., 1991; Lokeshwar and Bourguignon, 1991) and the variant region encoded by the alternatively spliced exons v1-10 (Brown et al., 1991; Screaton et al., 1993). Our studies have been directed in particular towards characterizing CD44 variants modified with glycosaminoglycans, prompted by recent suggestions that these proteoglycan-like molecules may be important in the cellular processes of motility and matrix invasion (Faassen et al., 1992, 1993). Furthermore the knowledge that other proteoglycans unrelated to CD44 may function as receptors for diverse ligands including growth factors, proteolytic enzymes, extracellular matrix proteins, and pathogenic viruses (Ruoslahti and Yamaguchi, 1991; Kjellen and Lindahl, 1991; Frevert et al., 1993; Shieh et al., 1992) led us to investigate whether similar functions might be mediated by proteoglycan-like variants of the CD44 protein.

In this present paper and the accompanying manuscript (Bennett et al., 1994), we have described the cloning, expression, and characterization of proteoglycan-like CD44 isoforms together with an investigation into their potential binding of extracellular matrix proteins and growth factors.

Proteoglycan-like Isoforms of Human CD44 Are Generated by Alternative Splicing of the v3 Exon

Previous studies involving proteoglycan molecules from various sources have identified the consensus motif SGXG (where X represents any amino acid) as the optimal site for serine modification by Xylose-Gal-Gal, the core trisaccharide unit common to both heparan sulphate and chondroitin sulphate side chains (Bourdon et al., 1987). Although the membrane-proximal region of human CD44H contains four potential motifs for GAG modification, these are of the basic SG type (Bourdon et al., 1985) only. Nevertheless it has been assumed until now that one or more of these motifs constitute the sites modified with glycosaminoglycans in high molecular weight proteoglycanlike isoforms. However, the recent identification and sequencing of the ten alternatively spliced exons of human CD44 revealed that the v3 exon contains the optimal SGSG consensus motif for GAG modification, in the context of a 17-residue sequence FSGSGIDDDEDFISSTI which is fully conserved between mouse, human, and rat. A second motif also occurs within the v10 exon, although in

contrast to the v3 site this is of the basic SG type, SLSG (Jackson et al., 1992). Hence the possibility existed that some proteoglycan-like CD44 isoforms might in fact be generated by alternative splicing. In the present study we tested this possibility by comparing the glycosylation patterns of recombinant CD44H, $CD44_{v10}$, $CD44_{v8-10}$, $CD44_{v7-10}$, $CD44_{v6-10}$, $CD44_{v3,8-10}$, and $CD44_{v3-10}$ cloned in the expression vector pRcCMV $CD44_{v10}^{Bgl/Nar^{*}}$ and stably transfected into the $CD44^{-ve}$ B lymphoma Namalwa.

We found that the two isoforms $CD44_{v3,8-10}$ and $CD44_{v3-10}$ which carry the v3 exon were indeed modified with glycosaminoglycans; both chondroitin sulphate and heparan sulphate were identified by a combination of $^{35}SO_4$ biosynthetic labeling and enzymatic deglycosylation. In contrast, we detected little if any glycosaminoglycan attached to any of the isoforms containing the v10 exon, ($CD44_{v10}$, $CD44_{v8-10}$, $CD44_{v7-10}$, and $CD44_{v6-10}$) or to CD44H. Instead these isoforms incorporated only trace levels of $^{35}SO_4$ in linkages that were stable to both chondroitinase and heparitinase digestion, indicative of either peptide sulphation or N-linked glycosylation with sulphated sugars as reported previously (Brown et al., 1991). Furthermore we found that the majority of the high molecular weight GAG-modified CD44 isoforms in two different human epithelial cell lines (the squamous cell lung carcinoma Hotz and the epidermoid carcinoma A431) also contained the v3 exon as demonstrated by biosynthetic labeling and immunoprecipitation with the CD44 exon-specific mAb 3G5. In common with the recombinant CD44 isoforms expressed in Namalwa cells, these v3-containing isoforms in epithelial cells were also modified with heparan sulphate.

These results alone suggest that the majority if not all proteoglycan-like forms of CD44 are in fact splice variants containing the v3 exon. This conclusion is further supported by the recent finding that "epican", the major heparan sulphate-modified CD44 isoform of human foreskin keratinocytes, is a v3-10 splice variant (Kugelman et al., 1992). In addition, high molecular weight heparan sulphate-modified CD44 isoforms identified in both squamous cell lung carcinoma and human foreskin keratinocytes were also identified as alternative splice variants in a recent detailed study (Brown et al., 1991).

Clearly however, not all proteoglycan-like forms of CD44 are generated by alternative splicing since it is clear that CD44H itself may be modified with glycosaminoglycans in some cell types. For example both CD44H and $CD44_{v8-10}$ may be modified with chondroitin sulphate when expressed in simian COS cells as soluble IgFc chimaeras (see accompanying manuscript [Bennett et al., 1994]). Furthermore, chondroitin sulphate-modified CD44 isoforms of 180 kD in the HT1080 fibrosarcoma cell line (Brown et al., 1991; Bennett et al., 1994) may also represent CD44H, as might some components of the high molecular mass (200–250 kD) CD44 expressed on human T and B lymphocytes (Jalkanen et al., 1988; Jalkanen and Jalkanen, 1992).

Hence covalent modification of CD44 variants with GAGs is probably regulated both by tissue-specific glycosylation and by alternative splicing. In epithelial cells and transfected Namalwa cells as shown here, CD44 molecules are modified with heparan and chondroitin sulphate and isoforms containing the v3 exon appear to be the main targets for glycosylation. In HT1080 cells and peripheral blood lymphocytes, only chondroitin sulphate is added and CD44H is probably the major target for glycosylation. One possibility is that chondroitin sulphate chains are added in tissue-specific fashion to one or more of the "invariant" glycosylation sites encoded in exons 5, 16, or 17 (Fig. 1 *B* and [Goldstein et al., 1989; Stamenkovic et al., 1989]) while heparan sulphate chains are added preferentially to the SGSG glycosylation site encoded by the v3 exon. This type of site specificity has been demonstrated for the proteoglycan syndecan in which an SGSG motif carries heparan sulphate while SG motifs carry chondroitin sulphate (Kokenyesi and Bernfield, 1994). However, we cannot rule out the alternative explanation that one or more of the invariant GAG sites in CD44H or the SLSG motif in exon v10 is cryptic and becomes exposed for modification with heparan sulphate only after insertion of the v3 exon. In either case splicing of the v3 exon clearly plays a potentially important role in regulating the expression of GAG-modified CD44 variants.

The generation of variant forms of proteoglycan molecules by the mechanism of alternative RNA splicing described here is relatively uncommon but is not unique to CD44. For example, differential RNA splicing is thought to generate variants of versican with extended GAG attachment domains expressed during chondrogenesis in the developing chick limb bud (Shinomura et al., 1993). In addition, RNA splicing generates cell surface proteoglycan forms of receptor-type protein tyrosine phosphatase in mammalian CNS (Barnea et al., 1994) and proteoglycan-like forms of type XII and XIV collagens in connective tissue (Koch et al., 1992). Insertion of the v3 exon in CD44 transcripts occurs predominantly in cells of epithelial origin and as we have shown very recently by immunohistochemical staining, many epithelial tissues express large amounts of CD44v3 protein isoforms (Fox et al., 1994). Hence the physiological function(s) of proteoglycan-like CD44 splice variants might be primarily to regulate epithelial cell–cell or cell–substratum interactions. In this respect the proteoglycan-like forms of CD44 share similar properties with the syndecans, an abundant family of cell surface proteoglycans involved in tissue organization and morphogenesis (Bernfield et al., 1992). Like the CD44 v3-containing isoforms, syndecans may be modified with either chondroitin sulphate or heparan sulphate and are also expressed predominantly on cells of epithelial origin. In addition, both molecules contain single dibasic sites for trypsin-like cleavage close to the membrane anchor (or in the case of CD44 within the membrane-proximal v10 exon, [Dougherty et al., 1991]) that may give rise to soluble forms as a result of proteolysis (Bazil and Horejsi, 1992). Finally, as demonstrated in the accompanying manuscript (Bennett et al., 1994), CD44 isoforms containing the v3 exon bind the growth factors basic fibroblast growth factor and heparin-binding epidermal growth factor via covalently linked heparan sulphate side chains, a property recently reported for a cell surface heparan sulphate proteoglycan thought to be identical to the syndecans (Kiefer et al., 1990).

Although normally absent from lymphocytes and monocytes, expression of CD44 isoforms can also be induced transiently on both cell types after antigenic stimulation in vivo (Arch et al., 1992) or after treatment with mitogenic agents or cytokines in vitro (Koopman et al., 1993; Mackay et al., 1994). It is tempting to speculate that the transient display of CD44 GAG chains on "activated" leucocytes might pro-

vide temporary sequestration of GAG-binding haemopoietic growth factors during the inflammatory response or during the early stages of an immune response.

Alternative Splicing of Exons v4-v10 Generates CD44 Molecules That Are Heavily O-Glycosylated

As distinct from the glycosaminoglycan modifications discussed above, all CD44 variants also contained extensive sialylated O-glycan modifications resulting from insertion of the variably spliced exon units. These results obtained from detailed biochemical analyses provide further evidence that the alternative exons constitute a mucin-like domain as predicted initially from their hydrophyllicity and from their high content of serine and threonine residues. Mucin-like regions in other leukocyte cell surface glycoproteins such as leukosialin (CD43) and the hinge region of the T cell molecule CD8 have been observed to adopt extended rod-like conformations in EM (Cyster et al., 1991) and hydrodynamic studies (Boursier et al., 1993). Indeed if the membrane-proximal region within the $CD44_{v3-10}$ isoform were to adopt the same conformation as the 224 residue mucin-like extracellular domain of CD43 (length 45 nm under EM; Cyster et al., 1991), then the 340–amino acid v3-10 insert would increase the length of the extracellular domain by some 60 nm. Hence, a general function of the alternative exons of CD44 may be to serve as spacer arms or act as scaffolds for the covalent attachment of sugar chains and glycosaminoglycans at critical positions for interaction with ligands in the extracellular medium.

GAG-modified CD44 Isoforms Have Reduced Affinities for Hyaluronan

Using the panel of vCD44-transfected Namalwa cells, we found that both $CD44_{v3-10}$ and $CD44_{v3,8-10}$ displayed markedly reduced binding to hyaluronan, previously identified as the major extracellular matrix ligand for CD44H. Similarly the binding affinity of $CD44_{v8-10}$, $CD44_{v7-10}$, and $CD44_{v6-10}$ for hyaluronan was considerably lower than that of CD44H despite the fact that each transfectant expressed comparable levels of CD44 on the cell surface. Hence, the insertion of variably spliced exons within the membrane proximal domain of CD44 appears to alter the binding affinity of the NH_2-terminal ligand-binding domain. Indeed even the insertion of the v10 exon alone reduces hyaluronan binding (not shown). Our results both confirm and extend the original findings of Stamenkovic et al. (1991) who demonstrated reduced binding to immobilized hyaluronan for Namalwa transfectants expressing the human epithelial variant of CD44 ($CD44_{v8-10}$) in comparison to CD44H. The inhibitory effect of the variably spliced exon units might in theory result either from conformational changes induced by the polypeptide portion of the inserts or from steric hindrance of the ligand-binding site by O-linked or N-linked sugars or by sialic acid. Alternatively, the variably spliced inserts might disrupt protein–protein interactions necessary for the formation of high affinity hyaluronan-binding sites.

Whereas the experiments with Namalwa cell transfectants described here illustrate a role for alternative splicing in the regulation of CD44 hyaluronan-binding affinity, several recent publications have demonstrated that hyaluronan binding in some cells may be regulated by changes in the so-called "activation" status of the CD44 molecule. This form of regulation has been best characterized in the mouse $CD44^{+ve}$ T cell line SAKRTLS which does not bind hyaluronan but which can be induced to do so after treatment with phorbol ester or with mAbs that induce aggregation or conformational alteration in the CD44 molecule (Hyman et al., 1991). Similar induction of hyaluronan-binding was reported in the human T cell line Jurkat transfected with CD44H and the $CD44_{v8-10}$ isoform (Liao et al., 1993). However we have been unable to demonstrate inducible binding of CD44 splice variants to hyaluronan in our panel of transfected Namalwa cells using either phorbol ester or the "activating" antibody F.10.44.2 (Liao et al., 1994). CD44 isoforms in some types of cell may therefore function as receptors for ligands other than hyaluronan.

GAG-modified CD44 Isoforms and Adhesion to Extracellular Matrix

A number of different proteoglycan molecules including decorin in bone tendon and skin, perlecan in basement membrane, and the syndecans in epithelia have been shown to bind extracellular matrix via interactions between covalently linked glycosaminoglycan side chains and proteins such as fibronectin, laminin, and collagen that possess binding sites for heparan sulphate and chondroitin sulphate (Kjellen and Lindahl, 1991). In common with such proteoglycans, some forms of the CD44 molecule have also been shown to bind components of the extracellular matrix. Most notably, Jalkanen and Jalkanen (1992) have reported that a minor 180–200-kD chondroitin sulphate–modified isoform solubilized from human lymphocytes binds to immobilized fibronectin, laminin, and type I collagen. In some experiments we also found that the chondroitin and heparan sulphate–modified isoforms $CD44_{v3,8-10}$ and $CD44_{v3-10}$ bound significantly to fibronectin and laminin and that the interaction with fibronectin was GAG dependent. However, since binding could not be reproduced in all experiments we conclude that the interaction is of low affinity.

Nevertheless it is tempting to speculate that the high molecular weight fibronectin-binding CD44 species described by Jalkanen and Jalkanen (1992) in fact constitute v3-containing isoforms. It will be necessary to determine the equilibrium binding affinities for fibronectin and laminin using soluble CD44 isoforms in order to clarify this issue.

In conclusion, we have demonstrated that proteoglycan-like forms of CD44 normally expressed in epithelial cells constitute alternatively spliced isoforms containing the v3 alternative exon and these are shown to have affinity for the extracellular matrix protein fibronectin. Together with the demonstration in the accompanying paper (Bennett et al., 1994), that proteoglycan forms of CD44 sequester GAG-binding growth factors, this identifies a new and potentially important role for the CD44 v3 exon in the regulation of cell growth and migration. However the biological function(s) of the other alternative exons v4 through v10 remain to be elucidated. Hence it is likely that other ligands for vCD44 isoforms exist and their identification represents one of the most important goals in future CD44 research.

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