

Regulated Clustering of Variant CD44 Proteins Increases Their Hyaluronate Binding Capacity

Jonathan Sleeman, Wolfgang Rudy, Martin Hofmann, Jürgen Moll, Peter Herrlich, and Helmut Ponta
Forschungszentrum Karlsruhe, Institute of Genetics, D-76021 Karlsruhe, Germany

Abstract. Cell contact with the extracellular matrix component hyaluronic acid (HA) plays an important role in many developmental, physiological, and pathological processes, although the regulation of this contact is poorly understood. CD44 proteins carry an amino acid motif that mediates affinity to HA. Artificial clustering of the smallest 85-kD isoform of CD44 (CD44s) has previously been shown to promote binding of the protein to soluble HA (Lesley, J., R. Hyman, and P.W. Kincade. 1993. *Adv. Immunol.* 54:271–335; Persche, A., J. Lesley, N. English, I. Trowbridge, and R. Hyman. 1995. *Eur. J. Immunol.* 25:495–501). Here we show that in rat pancreatic carcinoma cells, splice variants of CD44 (CD44v), but not CD44s, form molecular aggregates in the plasma membrane. We demon-

strate that reduction-sensitive dimerization of CD44v occurs, and also that larger aggregations of the protein can be stabilized by chemical cross-linking. Different CD44v proteins present on the same cell exclusively form homoaggregates. Molecular clustering does not require an intact cytoplasmic domain of the protein. The ability of cells to bind to soluble HA is upregulated more than one magnitude by the ectopic expression of CD44v4-v7, but only when the CD44v4-v7 protein forms intermolecular aggregates. Tunicamycin treatment inhibits HA binding by CD44v and at the same time destroys oligomerization. We propose that the regulation of clustering of CD44, mediated by factors including the presence of variant exons and glycosylation, allows cells in turn to regulate their HA binding properties.

ISIFORMS of the cell surface protein family CD44 have been implicated in a wide variety of physiological and pathological processes, such as T cell signaling and activation, lymphocyte recirculation, cell–cell and cell–matrix interactions, cell migration, embryogenesis, and metastasis (Herrlich et al., 1993; Lesley et al., 1993; Knudson and Knudson, 1993; Sherman et al., 1994). The different CD44 isoforms are generated by alternative splicing and/or by posttranslational modification (for review see Sherman et al., 1994). Most of the diversity is produced by the incorporation of amino acid stretches encoded by ten alternatively spliced exons into one particular part of the extracellular portion of the CD44 proteins, close to the transmembrane domain. The most common and widely expressed 85-kD isoform does not include any of these variant exons (CD44s). Expression of variant isoforms of CD44 containing sequences encoded by the variant exons (CD44v) is restricted mainly to a limited selection of epithelia in addition to certain tumor cells (Heider et al., 1993).

The CD44 isoforms CD44s and CD44v8-v10 have been shown to bind the extracellular matrix component hyal-

uronate (HA)¹ (Aruffo et al., 1990; Dougherty et al., 1994). They share this property with receptor for hyaluronic acid–mediated motility (RHAMM) and intercellular adhesion molecule-1 (ICAM-1) (Hardwick et al., 1992; McCourt et al., 1994). Evidence for the HA receptor function of CD44s (for review see Lesley et al., 1993) includes the fact that the ectopic expression of CD44s in non-HA binding cells can permit them to bind to HA, some anti-CD44 antibodies can block the binding of HA to cell surfaces, and purified CD44s protein can bind to HA in vitro. CD44 binds to a minimum of six HA sugar residues, but it has a higher affinity for larger HA molecules (Underhill et al., 1993). Molecular analysis of the binding of CD44 to HA identified an amino acid motif that is found twice in the extracellular NH₂-terminal domain shared by all CD44 proteins known to date. It occurs also in RHAMM and cartilage link protein (Yang et al., 1994).

Expression of CD44 on the surface of cells does not oblige them to bind HA (Lesley et al., 1993). Furthermore, whether the HA is soluble or immobilized affects the CD44-dependent HA binding capacity of different cell

Address all correspondence to Jonathan Sleeman, Forschungszentrum Karlsruhe, Institute of Genetics, PO Box 3640, D-76021 Karlsruhe, Germany. Tel.: (49) 7247 82 2714/4354. Fax: (49) 7247 82 3354.

1. *Abbreviations used in this paper.* DSP, dithiobis[succinimidyl]propionate; HA, hyaluronic acid; RHAMM, receptor for hyaluronic acid–mediated motility.

lines, with many cell lines being able to bind only to immobilized but not soluble HA (Lesley et al., 1993). The ability of CD44 on the cell surface to bind to HA can be experimentally and physiologically modified. Certain antibodies have the ability to stimulate HA binding by otherwise nonbinding CD44 proteins on the surface of the cell. Stimulation of cells with phorbol esters can have a similar effect (Lesley et al., 1990; Liao et al., 1993). CD44 proteins thus appear to exist in three states with respect to HA binding: non-HA binding; nonbinding unless activated, e.g., by antibodies or physiological stimuli; or constitutively binding (Lesley and Hyman, 1992). The ability of CD44 to bind to HA appears to be regulated by its cellular environment, as transfection of the same CD44s cDNA into different cell lines confers HA binding properties onto some cell lines but not others (Lesley et al., 1993).

The restricted expression of CD44 splice variants other than CD44s and their role in certain aggressive tumors indicate that these proteins possess molecular properties in addition to those exhibited by CD44s. The enormous diversity of CD44 proteins generated by alternative splicing may function either to mediate binding to new ligands, or to modulate the function of domains expressed on all CD44 proteins, such as the HA binding domain. Here we show that certain variant CD44 proteins can form multimeric complexes in the plasma membrane, and that this dramatically enhances their HA binding capacity.

Materials and Methods

Cell Culture and Antibodies

The hybridoma expressing the mAb 1.1ASML (Matzku et al., 1989), the hybridoma OX50 (Paterson et al., 1987), and derivatives of the BSp73 pancreatic tumor cell lines (BSp73ASML [ASML], and BSp73AS clones 10AS-7 [AS] and 1AS [Matzku et al., 1983]) were maintained in RPMI 1640 medium containing 10% FCS. The AS transfectants ASpSV14 (transfected with CD44v4-v7) and AS-meta2 (meta-2 now denoted CD44v6,v7; Günthert et al., 1991; Rudy et al., 1993) were maintained in RPMI 1640 medium containing 10% FCS, supplemented with 300 $\mu\text{g}/\text{ml}$ G418. Antibodies were purified from hybridoma-conditioned medium over protein G agarose (Harlow and Lane, 1988).

Construction of a Recombinant Retroviral Vector for Rat CD44v4-v7 Expression and Infection of 1AS Cells

The cDNA of rat CD44v4-v7 (Günthert et al., 1991) was subcloned into the EcoRI site of pLXSN (Miller and Rosman, 1989), and the construct was transfected into ΩE cells (Morgenstern and Land, 1990). Producer cells were selected with G418, and conditioned medium containing recombinant retrovirus was used to infect 1AS cells in the presence of 8 $\mu\text{g}/\text{ml}$ dimethylthiobromide. After selection of G418-resistant clones, the expression of CD44v4-v7 on the surface of recipient cells was confirmed by FACS[®] analysis.

Construction of CD44 Meta-Stop Plasmid and Transfection into AS Cells

The pSVmeta1 plasmid (meta-1 now denoted CD44v4-v7; Günthert et al., 1991) was cut with EcoRI and BglII to release the CD44v4-v7 insert. The insert was cut with HhaI to remove the cytoplasmic tail and the transmembrane domain. A synthetic linker with an HhaI site at the 5' end and encoding the transmembrane domain, the three arginine residues immediately COOH-terminal to the transmembrane domain, and a stop codon were synthesized (MWG, Ebersberg, Germany). A BglII site at the 3' end of this linker allowed the synthetic linker, the EcoRI-HhaI fragment encoding the NH₂ terminus of CD44v4-v7, and the original EcoRI/BglII-cut vector to be ligated together. This created an expression plasmid encoding

a CD44v4-v7 protein lacking the cytoplasmic tail, all except for the three arginine residues COOH-terminal to the transmembrane domain. This construct (CD44 meta-stop) was cotransfected with pRSVneo (Gorman, 1985) into AS cells using *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium methyl sulfate (DOTAP; Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's instructions. Transfectants were selected with 700 $\mu\text{g}/\text{ml}$ G418. Clones were picked and checked for CD44 meta-stop protein using the 1.1ASML antibody.

Immunoprecipitation

Cells were labeled with [³⁵S]methionine (500 $\mu\text{Ci}/\text{ml}$) for 4 h in methionine-free RPMI supplemented with dialyzed FCS as described (Sleeman, 1993). In Triton extractions, cells were incubated with 0.5% Triton X-100/PBS on ice for 30 min, and then the nonsoluble material was pelleted by centrifugation at top speed in an Eppendorf centrifuge. The soluble supernatant was then diluted into 10-fold more RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 7.5). The Triton-insoluble pellet was solubilized in RIPA buffer. For pulse-chase analysis, cells were incubated with [³⁵S]methionine (500 $\mu\text{Ci}/\text{ml}$) for 15 min in methionine-free RPMI supplemented with dialyzed FCS, and then washed and chased with methionine-containing medium. For cross-linking, radiolabeled cells were washed with PBS, and then treated with dithio-bis(succinimidylpropionate) (DSP) or dithio-bis(sulfosuccinimidylpropionate) (Pierce, Köln, Germany) at 1 mg/ml in PBS for 1 h at room temperature. Unreacted cross-linker was quenched by incubation with 150 mM Tris, pH 7.4, for 15 min. For immunoprecipitations, cells were lysed in RIPA buffer and aliquots were immunoprecipitated with 5 μg antibody as described (Sleeman, 1993). Immunoprecipitated proteins were separated by SDS-PAGE. Gels were treated with diphenyloxazole, as described (Sleeman, 1993) and exposed to x-ray film.

Surface Iodination

10^7 cells were resuspended in 200 μl PBS and mixed with 0.5 mCi ¹²⁵I-NaI and 10 μl lactoperoxidase (1 $\mu\text{g}/\text{ml}$). 10 μl H₂O₂ solution (diluted 1:10,000, 1:3,000, and 1:1,000 in PBS, respectively) was then added three times at 1-min intervals. The reaction was stopped by washing the cells three times in ice-cold PBS containing 5 mM NaI.

SDS-PAGE and Western Blotting

Proteins were resolved by size (Laemmli, 1970) using a resolving gel containing 7% polyacrylamide. For two-dimensional gel analysis, the first dimension was run in tube gels under nonreducing conditions. The tube gels were then incubated in reducing sample buffer for 1 h and affixed to the second dimension using 1% agarose containing 125 mM Tris, pH 6.8, and 2% SDS. For Western blotting, proteins separated by SDS-PAGE were electrically transferred to nitrocellulose (Schleicher & Schuell, Dassel, Germany) by the method of Towbin et al. (1979). Filters were washed in TBS for 10 min, and then used or stored frozen at -20°C. Blots were probed with antibody using the ECL detection system (Amersham, Braunschweig, Germany) as previously described (Sleeman, 1993).

HA Binding Assays

Coulter counter cups (Sarstedt, Heidelberg, Germany) were incubated overnight with 0.5 ml of 1 mg/ml HA in PBS, or with PBS alone for controls. After washing with PBS, the cups were incubated with 2 ml PBS/10% FCS for 30 min. Cells were harvested with PBS/5 mM EDTA, counted, and radiolabeled by incubation with [³⁵S]methionine in methionine-free medium. Where indicated, labeled cells were treated with DTT (10 mM in PBS) at room temperature for 5 min, immediately before addition to the Coulter counter cups. Tunicamycin (Sigma, Disenhofen, Germany) was added to cells as appropriate at 7.5 $\mu\text{g}/\text{ml}$ 36 h before labeling commenced, and tunicamycin was also included in the medium during the labeling incubation. The cells were washed with PBS, and then 5×10^5 cells were added to each Coulter counter cup in 0.5 ml PBS/10% FCS and incubated at room temperature for 30 min. After washing the cups three times with PBS, bound cells were lysed with PBS/0.5% Triton X-100, and the number of cells bound was calculated from quantification of radiolabel in the lysate. Each sample was performed in triplicate.

Flow Cytometry

Cells were harvested with 5 mM EDTA/PBS and resuspended in PBS/

10% FCS. Primary antibodies were applied at 5 $\mu\text{g/ml}$, and biotinylated HA was at 10 $\mu\text{g/ml}$. After incubation for 30 min, the cells were washed with PBS and incubated for an additional 30 min with fluorescently labeled secondary antibody (for antibody staining) or streptavidin (for HA staining). The cells were washed with PBS, and then analyzed by a FACS[®]tar Plus Flow Cytometer (Becton Dickinson & Co., Mountain View, CA). As negative controls, cells were stained with either isotype-matched control antibodies or with no primary antibody. No difference in staining was observed between these two controls.

Results

Hyaluronate Binding Function of CD44 Proteins

We investigated the HA binding properties of an number of cell lines derived from the BSp73 pancreatic carcinoma with respect to CD44 expression (see Table I). None of these cell lines express significant levels of RHAMM or ICAM-1 (data not shown). BSp73 clone 10AS-7 rat pancreatic carcinoma cells (hereafter called AS cells) express the CD44s isoform at high levels (Günthert et al., 1991) but bind poorly to soluble HA, while AS cells transfected with a CD44v4-v7 construct (ASpSV14; Günthert et al., 1991) bind avidly to soluble HA in a CD44v4-v7-dependent manner (Fig. 1; Sleeman et al., 1996). However, a clone of the BSp73AS cell line called 1AS (Matzku et al., 1983) did not support soluble HA binding even after transfection with CD44v4-v7 (Fig. 1; data not shown). Thus, a stable CD44v4-v7 transfectant of 1AS cells called 1ASm7, although expressing slightly more CD44v4-v7 on their surface than ASpSV14 cells, nevertheless binds very poorly to soluble HA, unlike ASpSV14 cells (Fig. 1). Clearly, the binding of CD44 to HA in these cell lines is regulated by factors other than mere expression of CD44 protein. Similar observations have been made in other cell types (e.g., Lesley et al., 1993; Moll, J., S. Khaldouanidi, J. Sleeman, I. Preuss, H. Ponta, and P. Herrlich, manuscript submitted for publication). Thus, the data in Fig. 1 raise two questions. What molecular difference between CD44s and CD44v4-v7 determines their differing ability to bind to HA? What makes CD44v4-v7 in one clone of AS cells a high-affinity soluble HA binder, while in another clone it is virtually a nonbinder?

Structural Requirements for HA Binding

Evidence for a role of CD44 protein structure in HA binding came from a comparison of binding of CD44 to either soluble or immobilized HA. Binding to immobilized HA has been considered a less stringent test of HA binding

Table I. Properties of BSp73 Pancreatic Carcinoma Cell Lines and Their Transfectants

Cell line	Parental cell line	Endogenous CD44	Transfected CD44
10AS-7 (AS)	–	CD44s	–
1AS	–	CD44s	–
ASML	–	CD44s, multiple CD44v	–
ASpSV14	10AS	CD44s	CD44sv4-v7
1ASm7	1AS	CD44s	CD44sv4-v7
AS-meta2	10AS	CD44s	CD44sv6-v7
M4.2	10AS	CD44s	CD44sv6-v7, without cytoplasmic tail

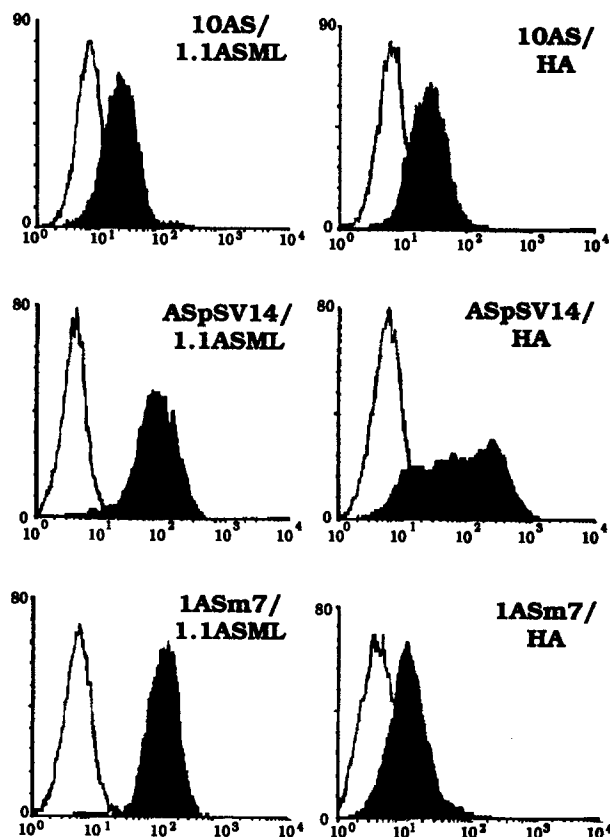


Figure 1. Binding of 10AS, ASpSV14, and 1ASm7 cells to soluble HA in relation to their expression of CD44v4-v7. FACS[®] analysis was performed as described in Materials and Methods on cells stained with 1.1ASML or with biotinylated HA. Plots of fluorescence intensity (abscissa, log scale) against cell number (ordinate, linear scale) are shown. In each case, the open trace represents background staining, while the filled-in trace represents the staining with the indicated reagent.

than binding to soluble HA, as several cell lines bind to immobilized but not to soluble HA (Lesley et al., 1993). ASpSV14 cells also bind to immobilized HA better than AS cells (Fig. 2 A). The 1ASm7 transfectant similarly shows binding ability that is higher than the parental 1AS cell line, although it still binds considerably less well to immobilized HA than ASpSV14 (Fig. 2 A). A possible explanation of these results is that the immobilization of HA enforces or facilitates CD44 proteins to adopt a conformational property, which they do not always acquire spontaneously (Lesley et al., 1993).

Although the amino acid motif permitting HA binding is linear (Yang et al., 1994), CD44 is predicted to take up a higher order structure based on disulphide bridges (Goldstein et al., 1989; Neame et al., 1986). Furthermore, CD44 is heavily glycosylated by AS cells, most of which is N-linked (Rudy et al., 1993). This glycosylation must also influence the three-dimensional structure of the protein. To determine whether the protein structure imposed by the disulphide bridges and N-linked glycosylation contributes to HA binding, we treated AS-derived cells with DTT or with tunicamycin and looked at the effect on their ability to bind HA. The presence of DTT abolished the binding to both immobilized and soluble HA (Fig. 2, B and C). In-

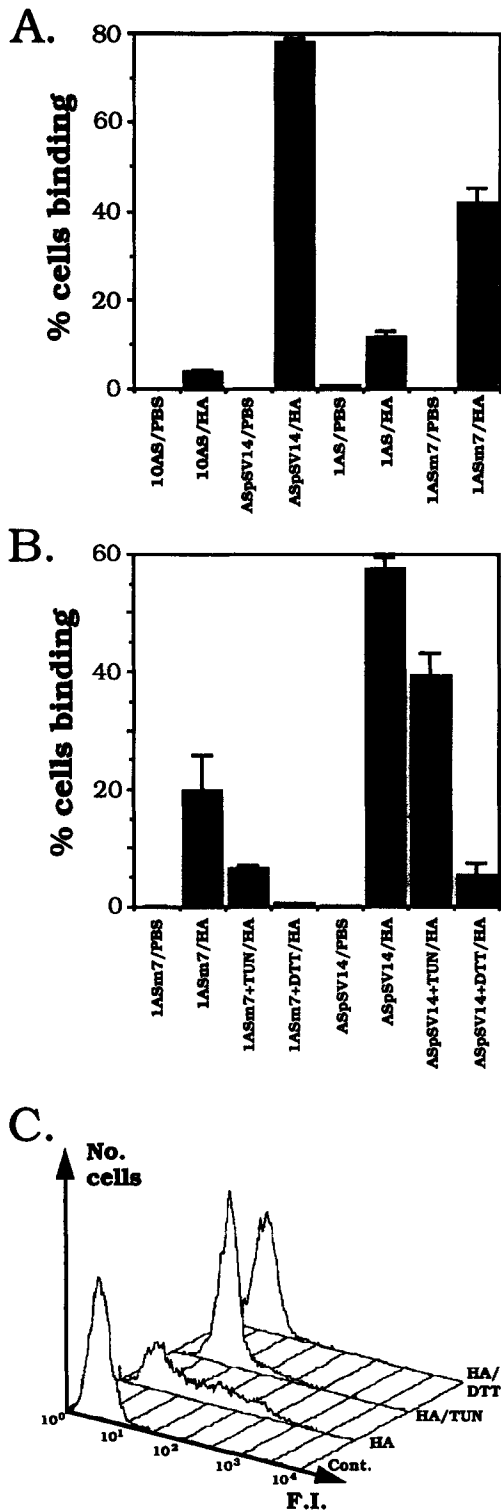


Figure 2. (A) The binding of 10AS, ASpSV14, 1AS, and 1ASm7 cells to immobilized HA. Cells were radiolabeled and applied to HA-coated (HA) or mock-coated (PBS) Coulter counter cups (5×10^5 cells per cup). Nonadherent cells were washed away. Percentage of cell adhesion was calculated with reference to the total radioactivity in 5×10^5 labeled cells by lysing the bound cells and by counting the radioactivity in the lysate. (B) The effect of tunicamycin and DTT on the ability of ASpSV14 and 1ASm7 cells to bind to HA immobilized on Coulter counter cups. The experiment was performed as described in Fig. 2 A. Cells were either mock treated, treated with 7.5 $\mu\text{g/ml}$ tunicamycin for 36 h

inhibition of N-linked glycosylation by tunicamycin also dramatically reduced the ability of ASpSV14 cells to bind to immobilized or soluble HA (Fig. 2, B and C). Furthermore, tunicamycin treatment of 1ASm7 cells reduced their ability to bind immobilized HA (Fig. 2 B). Treatment with DTT or with tunicamycin did not diminish CD44 expression on the cell surface, but in fact slightly enhanced its expression (data not shown). Cell viability was maintained throughout these experiments. We conclude that the HA binding properties of CD44 proteins depend on secondary or higher order structure that is sensitive to reduction and changes in glycosylation.

CD44v Proteins Can Homodimerize

The data in Fig. 2 suggested that CD44 protein conformation is altered by reduction, and that this destroys its ability to bind to HA. We therefore looked at the effect of reduction on the migration properties of CD44 proteins in SDS-PAGE to attempt to relate reduction-sensitive structures in CD44 proteins with HA binding capacity. In the absence of a reducing agent, a proportion of CD44v4-v7 protein from ASpSV14 cells migrated in both Western blot and immunoprecipitation/SDS-PAGE analyses at a higher apparent molecular weight ($\sim 280,000$), in addition to the 140-kD form normally detected under reducing conditions (Fig. 3). This higher molecular weight form was detected by both a CD44v6-specific antibody (1.1ASML) and by a CD44 antibody that binds to an extracellular epitope common to all CD44 proteins (OX50). A single higher molecular weight form was observed in 1.1ASML immunoprecipitations, while three bands migrating closely together were obtained with OX50 (Fig. 3 A). These were completely extracted by treatment of the cells with Triton X-100, suggesting that the higher molecular weight forms were not associated with the underlying cytoskeleton (Fig. 3 A). In similar experiments in which CD44s protein from AS cells was analysed using the OX-50 antibody, no higher molecular weight complexes were observed (Figs. 3 A and 4 B; data not shown).

Other CD44 isoforms from different cell contexts also exhibit this reduction-sensitive complex formation. Metastatic BSp73ASML cells (hereafter called ASML cells) express a number of different CD44 variant proteins including CD44v4-v7 and CD44v6-v7 as the major isoforms. CD44v6-containing proteins from ASML cells were analysed along with CD44v6-v7 protein from the AS transfectant cell line AS-meta-2 (Günther et al., 1991; Rudy et al., 1993) (Fig. 3 B). In the case of ASML, a smear of high molecular weight proteins (300,000–350,000) reactive with

before the experiment (TUN), or treated with 10 mM DTT immediately before being applied to the Coulter counter cups (DTT). (C) The effect of tunicamycin and DTT on the ability of ASpSV14 cells to bind to soluble HA. FACS[®] analysis was performed as described in Materials and Methods on cells stained with biotinylated HA. Plots of fluorescence intensity (abscissa, log scale) against cell number (ordinate, linear scale) are shown. The control cells (Cont.) demonstrate the background staining. The binding of ASpSV14 cells to HA is shown, along with cells treated with 7.5 $\mu\text{g/ml}$ tunicamycin for 36 h before the experiment (HA/TUN), and cells incubated with 10 mM DTT for 15 min immediately before the addition of HA (HA/DTT).

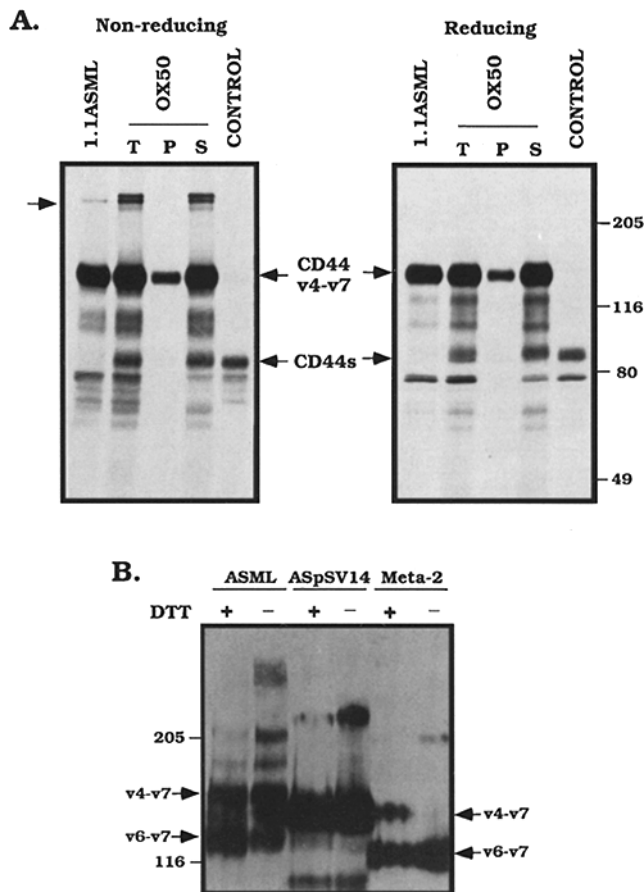


Figure 3. (A) Migration of radiolabeled immunoprecipitates of CD44v4-v7 from ASpSV14 cells on SDS-PAGE under reducing and nonreducing conditions. Both OX-50 (pan CD44) and 1.1ASML (v6-specific) antibodies precipitate higher molecular weight forms of CD44v4-v7 under nonreducing conditions (arrow). Radiolabeled cells were extracted with 0.5% Triton, and all higher molecular weight forms precipitated from total extract (T) are found only in the soluble phase (S) and not in the Triton insoluble pellet (P). The lane marked CONTROL is an OX-50 immunoprecipitate of the AS cells from which the ASpSV14 transfectant cell line is derived. Molecular weight markers are indicated (kD). (B) Detection of higher molecular weight forms of CD44v in ASML, ASpSV14, and AS-meta2 (Meta-2) cells. Cell lysates were prepared in SDS-PAGE sample buffer with and without DTT. Western blots of the lysates were probed with 1.1ASML antibody. The monomeric forms of CD44v4-v7 and CD44v6-v7 are on the left (arrows) for ASML, and on the right (arrows) for CD44v4-v7 from ASpSV14 cells and CD44v6-v7 from AS-meta2 cells, respectively. It should be noted that the monomeric forms in ASML cells migrate differently to those in transfected AS cells, due to differences in glycosylation (Rudy et al., 1993). The apparent presence of CD44v4-v7 in lysates from AS-meta2 cells treated with DTT is due to spill-over from the adjacent ASpSV14 lysate. The position of molecular weight markers is shown (kD).

1.1ASML antibody was detected in the absence of reducing reagent, in addition to the CD44 isoforms detected under reducing conditions. With AS transfectants expressing CD44v6-v7, one additional band was observed at 230 kD under nonreducing conditions, in addition to the 115-kD form also detected under reducing conditions.

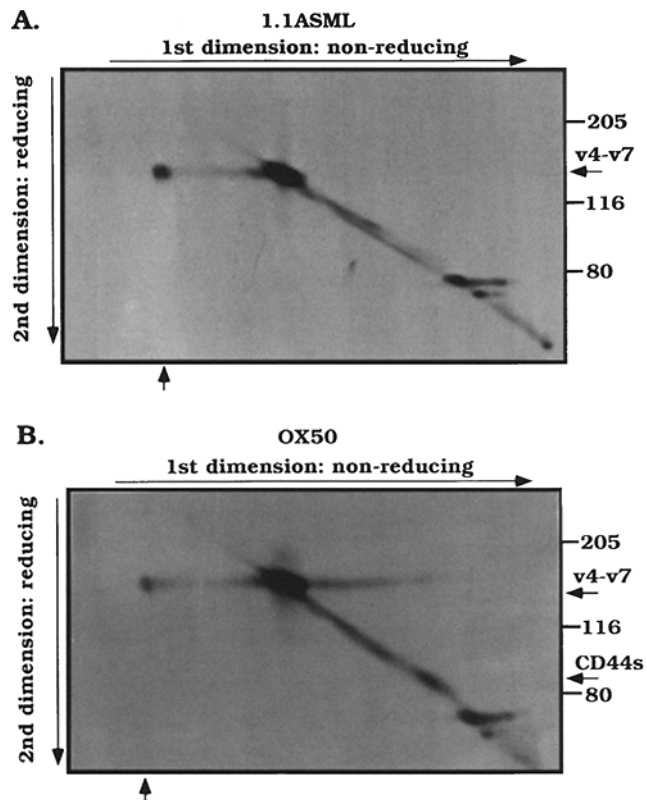


Figure 4. Nonreducing/reducing two-dimensional gel analysis of 1.1ASML and OX50 immunoprecipitates from radiolabeled lysates from ASpSV14 cells. Immunoprecipitates were resolved in a 7% nonreducing tube gel in the first dimension. After incubation of the tube gel with reducing sample buffer, the proteins within the tube gel were run into and resolved on a 7% second dimension gel. CD44s immunoprecipitated by OX50 is designated with an arrow, as is CD44v4-v7 immunoprecipitated by both 1.1ASML and OX50 (v4-v7). Only CD44v4-v7 immunoprecipitates exhibit reduction-sensitive dimerization (arrows beneath each panel). Molecular weight markers are shown (kD).

One explanation for the occurrence of high molecular weight CD44v-containing aggregates would be that CD44v proteins can interact with other molecules (potential ligands) in a reduction-sensitive manner. We investigated whether proteins other than CD44v are present in the high molecular weight moieties detected under nonreducing conditions. Radiolabeled proteins from ASpSV14 cells were immunoprecipitated with the CD44v6-specific antibody 1.1ASML or with the pan-CD44 specific OX50 antibody. The immunoprecipitates were analyzed by two-dimensional gel electrophoresis in which the first dimension was run under nonreducing conditions and the second dimension under reducing conditions. This allowed the resolution of the 280- and 140-kD forms of CD44v4-v7 in the first dimension, and the subsequent disruption by reduction and analysis of the 280-kD form in the second dimension. Fig. 4 shows that the nonreduced 280-kD form immunoprecipitated by both antibodies resolves at 140 kD after reduction, and that no other labeled proteins are contained within the complex. 1.1ASML staining of Western blots of gels from similar two dimensional analyses using unlabeled proteins showed that the resolved protein complex

contains CD44v6 epitope-carrying proteins (data not shown). No CD44s-CD44v heterodimers were observed, and no CD44s homodimers were detected by the OX50 immunoprecipitation (Fig. 4 B). These data are consistent with the hypothesis that the high molecular weight forms observed under nonreducing conditions are homodimers of CD44v proteins. The fact that the higher molecular weight forms of CD44v4-v7 and CD44v6-v7 detected under nonreducing conditions are exactly twice the molecular weight of the form detected under reducing conditions lends further support to this hypothesis.

Pulse-chase analysis showed that dimer formation occurs late after CD44v synthesis, when the protein is fully glycosylated. ASpSV14 cells were pulsed with [³⁵S]methionine for 15 min, and then a chase was performed with unlabeled methionine. At different times cells were lysed, and the CD44v4-v7 protein was immunoprecipitated with 1.1ASML. Immunoprecipitated proteins were analyzed by SDS-PAGE under nonreducing conditions. Fig. 5 A shows that immediately after the pulse, CD44v4-v7 protein migrates at a lower apparent molecular weight than the mature form because it is first labeled within the cell in an underglycosylated form (Lokeshwar and Bourguignon, 1991; Rudy et al., 1993). As the chase progressed, the protein became fully glycosylated. However, dimers of CD44v4-v7 did not appear in the chase until 1 h after the pulse, at a time when the CD44 protein is fully glycosylated and would be expected to already be on the cell surface. Fur-

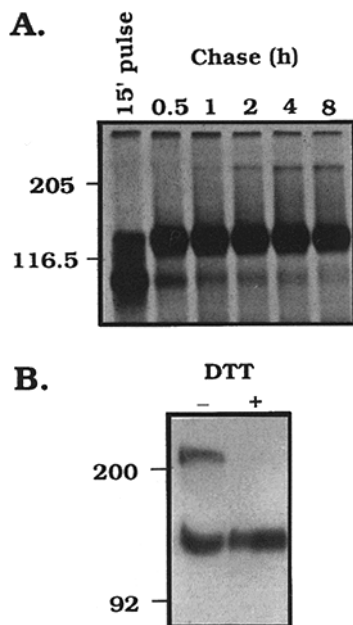


Figure 5. (A) Pulse-chase analysis of the formation of dimers by CD44v4-v7 in ASpSV14 cells. Cells were pulsed for 15 min, and then chased for 0.5, 1, 2, 4, and 8 h. After lysis in RIPA buffer, the radiolabeled proteins were immunoprecipitated with 1.1ASML and analyzed by SDS-PAGE under nonreducing conditions. Molecular weight markers are indicated (kD). (B) Surface iodination of ASpSV14 cells. After surface iodination, ASpSV14 cells were lysed in RIPA buffer and immunoprecipitated with 1.1ASML antibody. Immunoprecipitates were taken up in sample buffer with and without DTT and resolved on by 7% SDS PAGE. Molecular weight markers are indicated (kD).

ther evidence for dimer formation on the cell surface was obtained by surface iodination. Fig. 5 B shows that dimers of CD44v4-v7 are readily immunoprecipitated from surface-iodinated ASpSV14 cells, and that these dimers are reduction sensitive. These data show that the formation of dimers of CD44v is a late event after synthesis and glycosylation of the protein, and that dimers are located on the surface of the cell.

Chemical Cross-linking Reveals Higher Order Oligomers of CD44v Proteins

CD44v protein could either form dimers via a specific dimerization domain or through multivalent interactions. The latter is more likely, as evidence for clusters of CD44v protein containing multiple molecules of CD44v4-v7 was obtained from cross-linking studies. ASpSV14 cells were radiolabeled, and then treated with cross-linking reagents. The cells were subsequently lysed and immunoprecipitated with 1.1ASML. Immunoprecipitates were analyzed by SDS-PAGE. A number of cross-linking reagents were tested. After treatment with one of these, DSP, lysates of ASpSV14 cells contained complexes containing CD44v4-v7 of much higher molecular weight than CD44v4-v7 monomers and dimers. (Fig. 6 A; bands with arrows X and Y).

The cross-linker DSP contains a disulphide bridge that can be broken by reducing reagents. We could therefore investigate the constituent parts of the high molecular weight CD44v4-v7-containing complexes (X and Y) produced by DSP cross-linking. First, the two complexes and CD44v4-v7 monomeric protein were cut out of the gel. The proteins were isolated, reduced, and resolved on another SDS-PAGE gel. Fig. 6 B shows that the protein in the high molecular weight complexes resolves at exactly the same molecular weight as the monomeric CD44v4-v7 protein, and that no additional labeled proteins could be detected. Secondly, 1.1ASML immunoprecipitates of lysates made from radiolabeled, DSP-cross-linked ASpSV14 cells were analyzed on two-dimensional gels. The first dimension was run under nonreducing conditions to allow the resolution of the high molecular weight complexes containing CD44v4-v7, while the second dimension was run under reducing conditions to reverse the cross-linking, allowing visualization of the components of the complexes. Fig. 6 C shows that both complexes migrate at the same apparent molecular weight as monomeric CD44v4-v7 upon reduction, and that no additional proteins are found within the complexes. These data suggest that DSP cross-linking stabilizes homooligomers of CD44v4-v7. The chemical cross-linker dithiobis(sulfosuccinimidylpropionate), which is water soluble and cannot cross plasma membranes, showed the same CD44v cross-linking capacity as DSP (data not shown), indicating that the stabilization of oligomers occurs on the cell surface, and that oligomerization is a property of CD44v proteins in the plasma membrane.

We conclude from these data that CD44v4-v7 forms dimers and oligomeric clusters in the plasma membrane of AS cells, while CD44s does not.

CD44v Preferentially Form Homooligomers

Although CD44s is coexpressed with CD44v4-v7 on the surface of ASpSV14 cells, heterooligomerization was not

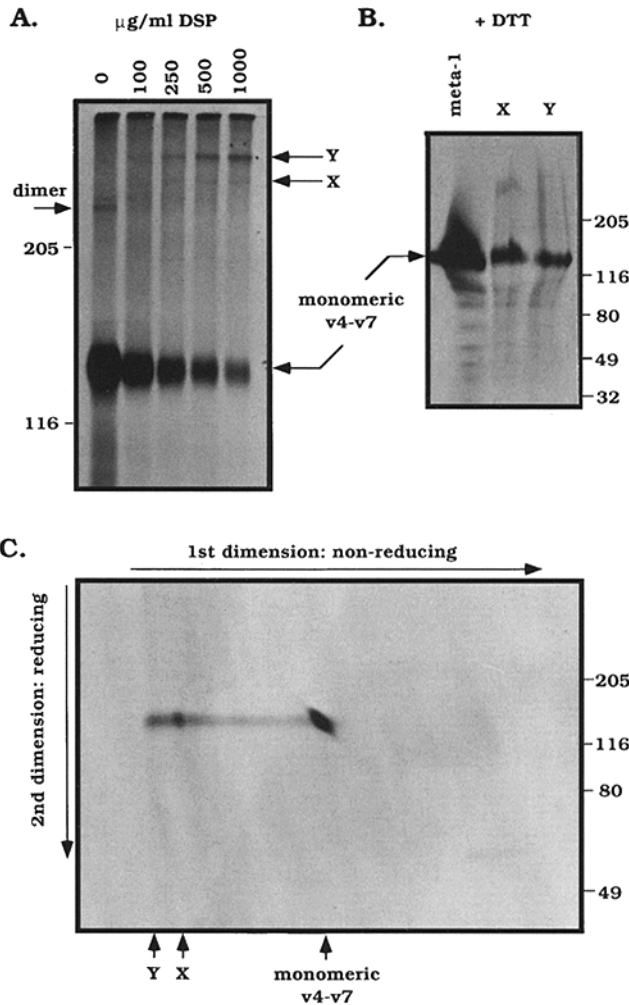


Figure 6. (A) High order oligomers of CD44v4-v7 are detected by cross-linking. Radiolabeled ASpSV14 cells were treated with 0, 100, 250, 500, and 1,000 $\mu\text{g/ml}$ DSP, lysed in RIPA buffer, and immunoprecipitated with 1.1ASML. Immunoprecipitates were analyzed by 5% SDS-PAGE. The migrations of monomeric, dimeric, and high molecular weight complexes X and Y of CD44v4-v7 are designated with arrows. Molecular weight markers are indicated (kD). (B) Monomeric CD44v4-v7 and the high molecular weight complexes X and Y in Fig. 6 A were excised from the gel, incubated with reducing sample buffer, and subjected to SDS-PAGE in a 5–20% polyacrylamide gradient. When reduced, complexes X and Y migrate at the same molecular weight as monomeric CD44v4-v7. No additional comigrating bands from complexes X and Y are observed. The migration of molecular weight markers is indicated (kD). (C) Nonreducing/reducing two-dimensional gel analysis of 1.1ASML immunoprecipitates from a lysate of radiolabeled ASpSV14 cells cross-linked with 1 mg/ml DSP. Under reducing conditions, the high molecular weight complexes X and Y resolve at the same molecular weight as monomeric CD44v4-v7 (see arrows X and Y). The migration of molecular weight markers is indicated (kD).

observed between these two proteins in the cross-linking studies described above (Fig. 6 C). This suggested, on the one hand, that CD44s may not participate in oligomer formation, and, on the other hand, that CD44v may preferentially form homooligomers. Both of these possibilities were investigated. AS cells were labeled, cross-linked with DSP, and im-

muno-precipitated with OX-50 antibody as described previously to precipitate the endogenous CD44s protein. The immunoprecipitate was subjected to two-dimensional non-reducing/reducing gel electrophoresis as described before. Fig. 7 A shows that no higher order oligomers of CD44s were observed after DSP cross-linking of AS cells. This suggests that at least in AS cells, CD44s does not participate in oligomer formation, consistent with the observation that no reduction-sensitive dimers of CD44s were detected in AS cells.

ASML cells coexpress two major forms of CD44v (120- and 150-kD) and two minor forms (180- and 195-kD), each of which contains sequences encoded by exon v6 (Günthert et al., 1991; see Fig. 3 B). Thus, by radiolabeling these cells, cross-linking them with DSP, and subsequently immunoprecipitating their proteins with 1.1ASML antibody, it should be possible to differentiate between homo- and heterooligomerization of CD44v proteins in two-dimensional nonreducing/reducing gel analysis. In the second, reducing dimension, the constituent parts of heterooligomers should resolve as spots lying along a vertical line. Homooligomers, on the other hand, should resolve as single

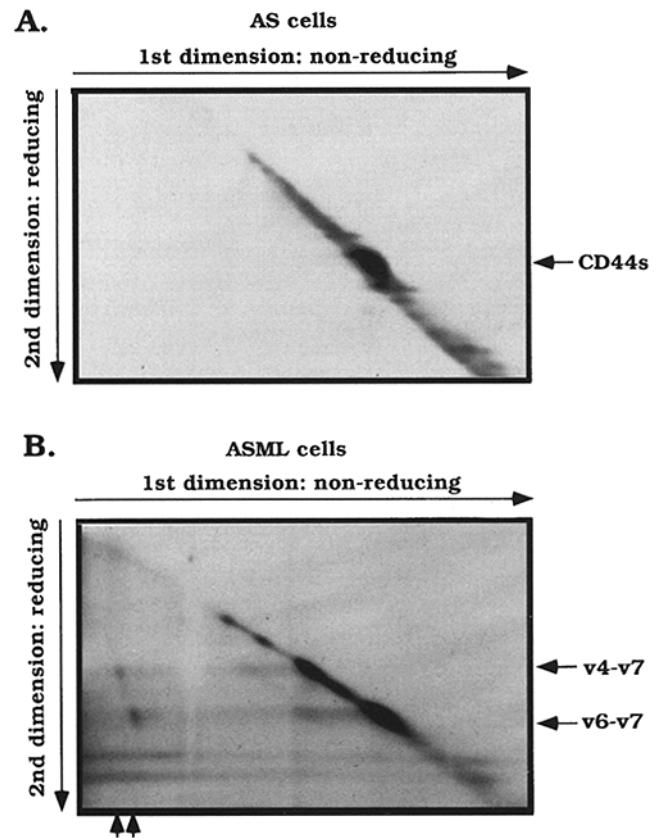


Figure 7. (A) Nonreducing/reducing two-dimensional gel analysis of radiolabeled lysates from AS cells cross-linked with 1 mg/ml DSP and immunoprecipitated by OX50. No high molecular weight complexes of CD44s can be detected. (B) Nonreducing/reducing two-dimensional gel analysis of radiolabeled lysates from ASML cells cross-linked with 1 mg/ml DSP and immunoprecipitated by 1.1ASML. The two major CD44v6-containing proteins in ASML cells (v6-v7 and v4-v7) are indicated. The resolution of cross-linked high molecular weight complexes of these proteins into monomers under reducing conditions is indicated by arrows beneath the panel.

spots. If more than one CD44v protein forms homooligomers, then the single spots corresponding to the reduced, monomeric form of CD44v protein should lie along a diagonal line. Fig. 7 B shows that the oligomeric forms of the two major CD44v6-containing proteins in ASML cells resolve as single spots after reduction and migrate at the same molecular weight as the corresponding monomeric forms. The two spots lie along a diagonal line. No other spots lie vertically above or below them, showing them to be resolved from homooligomers. No resolved spots could be detected for the minor CD44v6-containing proteins, perhaps due to insufficient sensitivity in the experiment or lack of oligomer formation by these isoforms. These results suggest that in ASML cells that coexpress multiple CD44v isoforms, homooligomers rather than heterooligomers are preferentially formed.

Our experiments, in which reducing agents destroyed CD44v-dependent soluble HA binding, linked soluble HA binding with reduction-sensitive structures in the CD44 molecule. By analyzing reduction-sensitive structural properties of CD44, we discovered CD44v homodimers and homooligomers. It therefore remained for us to determine whether this molecular association influences soluble HA binding by CD44v.

Oligomer Formation Correlates with Soluble HA Binding by CD44v4-v7

CD44v proteins molecularly associate when expressed in AS cells, while CD44s proteins do not. This clustering thus correlates directly with the affinity for soluble HA conferred by CD44v on AS cells. CD44v4-v7 expression confers negligible soluble HA binding activity on 1ASm7 cells. If the clustering of CD44v influences its ability to bind soluble HA, then CD44v4-v7 from 1ASm7 cells should not be expected to form dimers or oligomers. The analysis of clustering of CD44v4-v7 in the 1ASm7 cell line proved to strongly support a role for clustering in soluble HA binding.

1ASm7 cells were analyzed for the ability of their CD44v4-v7 protein to form dimers and oligomers using the same methods described previously. Fig. 8 A shows that CD44v4-v7 does not form dimers when expressed on the surface of 1ASm7 cells, unlike ASpSV14 cells. Similarly, CD44v4-v7 on 1ASm7 cells is not cross-linked into higher order oligomers, unlike CD44v4-v7 protein expressed on ASpSV14 cells (Fig. 8 B). These data correlate the molecular association of CD44v with its ability to bind to soluble HA and strongly suggest that soluble HA binding by CD44v is dependent on the ability of the protein to molecularly aggregate on the cell surface.

The Cytoplasmic Tail Is Not Required for HA Binding or for Dimer and Oligomer Formation by CD44v4-v7

Several studies report that the cytoplasmic tail of CD44 interacts with components of the cytoskeleton (for review see Isacke, 1994) and that the absence of a cytoplasmic tail can reduce the ability of CD44s protein to bind to HA (Thomas et al., 1992; Lesley and Hyman, 1992; Lesley et al., 1993; Liao et al., 1993). These reports prompted us to investigate whether the cytoplasmic tail influences the affinity of CD44v4-v7 for soluble HA and the ability of CD44v4-v7 to form homodimers and homooligomers.

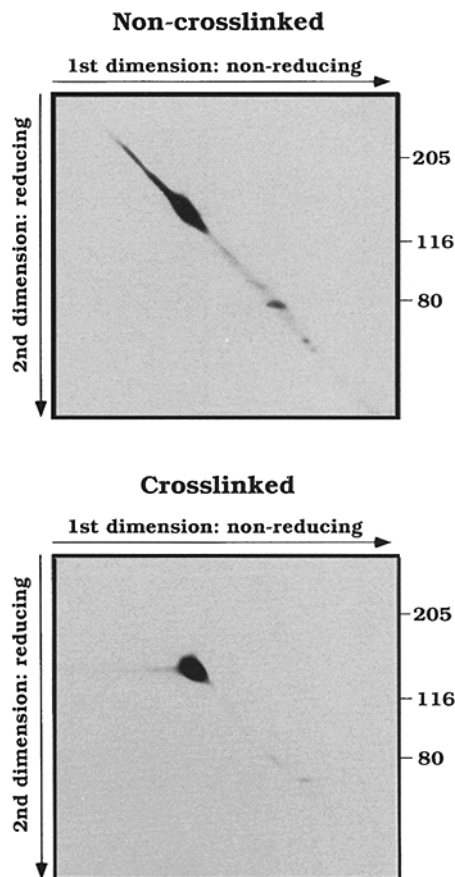


Figure 8. Nonreducing/reducing two-dimensional gel analysis of radiolabeled lysates from 1ASm7 cells immunoprecipitated by 1.1ASML, with and without cross-linking of the cells with 1 mg/ml DSP before lysis. No high molecular weight complexes can be detected. Molecular weight markers are indicated (kD).

We constructed a tail-less CD44v4-v7 expression vector (CD44 meta-stop) that lacks all but the first three amino acids of the cytoplasmic tail. This was transfected into AS cells, which do not bind to soluble HA (Fig. 1; Sleeman et al., 1996). A stable transfectant (M4.2) expressing the CD44 meta-stop protein at levels similar to the full-length CD44v4-v7 protein in ASpSV14 cells was identified using the 1.1ASML antibody. Surprisingly, the M4.2 transfectant bound to soluble HA as efficiently as the ASpSV14 cells (Fig. 9 A). However, consistent with our contention that aggregation of CD44 promotes binding to soluble HA, the tail-less CD44v4-v7 mutant protein from M4.2 cells also forms both dimers and homooligomers (Fig. 9 B). This shows that the cytoplasmic tail plays no role in the molecular homointeraction of CD44v proteins, and further suggests that dimer and oligomer formation by CD44v suffices to prevent the inhibitory effect of cytoplasmic tail truncation on soluble HA binding that has previously been reported for CD44s (Thomas et al., 1992; Lesley and Hyman, 1992; Lesley et al., 1993; Liao et al., 1993).

Glycosylation, a Prerequisite of Oligomerization and HA Binding

Inhibition of N-linked sugar addition by tunicamycin treatment reduced both the immobilized and soluble HA

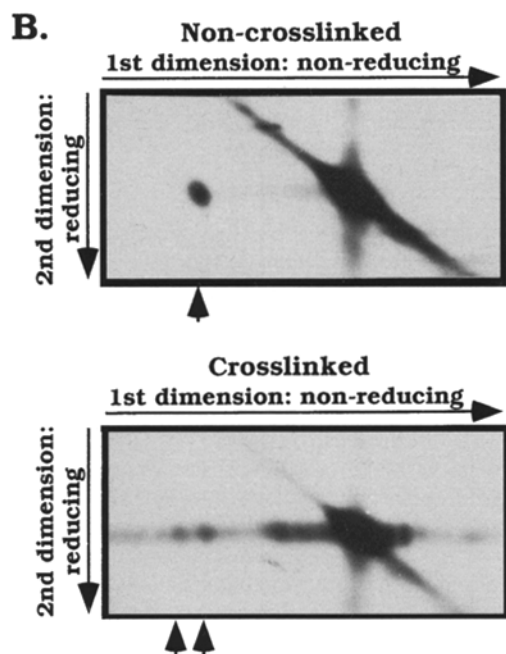
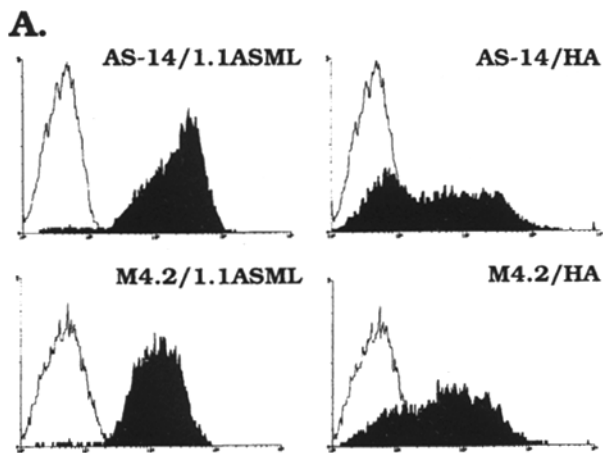


Figure 9. (A) HA binding by ASpSV14 and M4.2 cells to soluble HA in relation to their expression of CD44v4-v7. Plots of fluorescence intensity (abscissa, log scale) against cell number (ordinate, linear scale) are shown. In each case, the open trace represents background staining, while the filled-in trace represents the staining with the indicated reagent. (B) Nonreducing/reducing two-dimensional gel analysis of radiolabeled lysates from M4.2 cells, either nontreated or cross-linked with 1mg/ml DSP before lysis and immunoprecipitated by 1.1ASML. In both cases, high molecular weight complexes can readily be detected (arrows).

binding mediated by CD44v4-v7 in AS cells (Fig. 2, B and C). Furthermore, completion of the glycosylation of CD44v4-v7 preceded oligomer formation (Fig. 5 B). This prompted us to investigate whether one mechanism by which tunicamycin treatment interferes with HA binding could be the inhibition of cluster formation by CD44v4-v7. As demonstrated by Rudy et al. (1993), treatment of ASpSV14 cells with tunicamycin only completely inhibits the addition of N-linked sugars in a small proportion of the CD44v4-v7 protein (labeled ng in Fig. 10), while the

remainder of the protein remains partially N-glycosylated. Using the previously described reducing/nonreducing two-dimensional gel analysis, we found that the partially deglycosylated form and the completely non-N-linked glycosylated form of CD44v4-v7 are only weakly able to form dimers, visible only after long exposure of the gel. Moreover, neither the partially deglycosylated nor the non-N-linked glycosylated form are able to form cross-linkable oligomers (Fig. 10). This result shows that changes in glycosylation can influence the ability of CD44v4-v7 to oligomerize and provides further evidence that oligomerization enhances the HA binding capacity of CD44v4-v7.

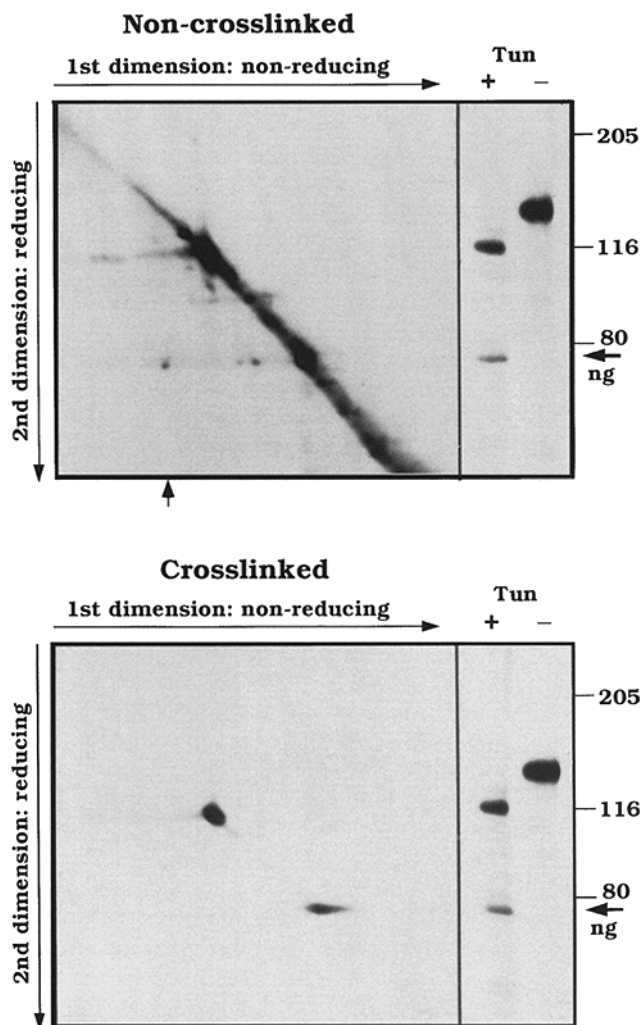


Figure 10. The effect of tunicamycin on the formation of CD44v4-v7 dimers and oligomers. ASpSV14 cells were treated with 7.5 μ g/ml tunicamycin for 36 h before the experiment, and after radiolabeling were either nontreated or cross-linked with 1mg/ml DSP before lysis. Lysates of these cells were immunoprecipitated with 1.1ASML and analyzed by nonreducing/reducing two-dimensional gel analysis. The righthand side panels show reducing SDS-PAGE analysis of 1.1ASML immunoprecipitates of radiolabeled ASpSV14 cells treated with and without tunicamycin (+ and - Tun) to indicate the efficacy of the tunicamycin treatment. The non-N-glycosylated (ng) form of CD44v4-v7 weakly forms dimers (arrow beneath the panel) but does not form oligomers. Molecular weight markers (kD) are shown.

Discussion

Our experiments provide evidence for the modulation of the HA binding ability of CD44 proteins at several levels. HA binding mediated through the NH₂-terminal amino acid motifs is determined by a reduction-sensitive protein structure, by the variant exon-promoted clustering of the protein in the plane of the plasma membrane and by glycosylation, which in turn is required for the oligomeric clustering. Given these results, we propose that the much higher, soluble HA binding capacity of CD44v compared with CD44s on AS cells that we have previously reported (Sleeman et al., 1996; Fig. 1) can be explained by the clustering of CD44v. Cellular regulation of the molecular association of CD44 proteins, e.g., by alternative splicing and specific glycosylation, likely provides plasticity in the binding response of cells to the extracellular matrix, allowing them to respond appropriately to different biological situations.

Oligomeric clustering in rat pancreatic carcinoma cells of the larger CD44 splice variants in contrast with CD44s is one of the key results of this paper. We interpret the reduction-sensitive high molecular weight forms of CD44v as evidence for homodimer formation. Several observations argue against alternative interpretations. The fact that the higher molecular weight forms of CD44v are exactly double the monomeric forms in each case speaks strongly in favor of dimer formation, as well as against an interaction of CD44v proteins with another putative molecule that, for whatever reason, failed to be radiolabeled in the two-dimensional gel analyses; interaction with such a unitary molecule would not double the size of each CD44v protein. The appearance of a triplet of higher molecular weight forms of CD44v4-7 observed with OX50 but not 1.1ASML (Fig. 3 A) probably reflects steric effects affecting 1.1ASML antibody access to the dimeric proteins in association with conformational changes in the CD44 protein after dimer formation.

Only a minor proportion of the total CD44v present forms multimers. However, this fraction of the total is extremely important for HA binding. This observation has parallels with ligand binding by integrins (Dustin et al., 1989; Diamond and Springer, 1993). The relatively low yield of cross-linked higher order oligomers of CD44v4-7 is probably due to only a small window where cross-linking of specifically interacting proteins occurs, before such complexes themselves are cross-linked into an insoluble pellet, as the cell essentially becomes fixed by the cross-linking reagent. This effect is readily seen in Fig. 6 A, where increasingly less monomeric CD44v4-7 protein is available for immunoprecipitation as the concentration of cross-linker increases. From the experiments presented here, it is not possible to ascertain whether dimers are building blocks for the formation of oligomers.

The ability of CD44v proteins to oligomerize correlates well with their affinity for soluble HA. The link is supported by three lines of evidence. First, there is a coordinated lack of soluble HA binding and CD44v oligomerization in the mutant cell line 1ASm7. Secondly, CD44v protein lacking a cytoplasmic tail forms oligomeric complexes and binds to soluble HA, in contrast with truncated CD44s protein that can do neither. Thirdly, tunicamycin

treatment inhibits both soluble HA binding and oligomer formation.

This paper focuses on the characterization of CD44v multimerization and its relationship to soluble HA binding in pancreatic carcinoma cells. The observation that CD44v forms multimers while CD44s does not is unlikely to be a general rule for all cells. A clear prediction would be that CD44s is able to spontaneously oligomerize in cell lines that support soluble HA binding in a CD44s-dependent manner.

Numerous findings by other laboratories indirectly support the notion that clustering of CD44 protein promotes its ability to bind to soluble HA. Certain antibodies can activate HA binding by CD44s, which at least in AS cells does not cluster spontaneously. These antibodies only work when binding multivalently (Lesley et al., 1993). Furthermore, chimerization of the extracellular portion of CD44s with the transmembrane region of the CD3 ζ chain caused enforced dimerization of the fusion protein via disulphide links between CD3 ζ chains, as well as enhanced HA binding (Perschl et al., 1995). Immobilized HA may in part facilitate clustering of the receptors (see also Lesley et al., 1993). Thus, CD44s can be helped to cluster and to bind soluble HA, as CD44v expressed on AS cells does spontaneously.

Several groups have shown that the presence of CD44 on the cell surface does not oblige cells to bind to HA (see Lesley et al., 1993; Moll, J., S. Khaldoiani, J. Sleeman, I. Preuss, H. Ponta, and P. Herrlich, manuscript submitted for publication). We propose which CD44 proteins which cannot form stable clusters require additional help to bind HA, be it in the form of cross-linking "activating" antibodies or by the immobilization of the HA to stabilize weak CD44-CD44 or CD44-HA interactions. This proposal would also explain the observation that surface levels of CD44 have to be above a threshold level before efficient HA binding occurs (He et al., 1992); higher surface levels of CD44 would promote intermolecular association.

Although we have clearly demonstrated here that CD44v forms aggregates on the cell surface, the nature of the protein-protein interaction remains unclear. Dimerization is reduction sensitive. While one could argue that intramolecular disulphide links may hold CD44 proteins in a conformation that allows them to dimerize via noncovalent interactions even after boiling in 2% SDS, a more attractive possibility is that dimers of CD44 are formed as a result of intermolecular disulphide bridges. None of the variant exons v4-v7 encode cysteine residues, ruling out disulphide links between the variant portions of the CD44v proteins analyzed in this study. Interestingly, DTT treatment of ASpSV14 cells, a cell line whose soluble HA binding capacity is due to transfected CD44v4-7, ablates their ability to bind to soluble HA, demonstrating that disulphide bonds are required for HA binding. Again, this could be interpreted as indicating a functional role for intramolecular loops (as proposed for the HA binding domain of cartilage link protein [Goldstein et al., 1989; Neame et al., 1986]) or for intermolecular disulphide bridges. The incorporation of variant domains into a CD44 protein may alter its conformation such that a secondary structure needed for clustering is formed or that intermolecular disulphide bonding can occur. In both cases, the exact distance of the

interacting domain from the cell surface established by variant exon sequences could enforce exclusive homooligomerization.

Truncation of the cytoplasmic portion of CD44s reduces the HA binding efficiency of the protein (Thomas et al., 1992; Lesley and Hyman, 1992; Lesley et al., 1993; Liao et al., 1993), but the truncated protein retains the ability to be activated to bind soluble HA by certain activating anti-CD44 antibodies (Lesley and Hyman, 1992). Disruption of the cytoskeleton does not interfere with HA binding by CD44 (Murakami et al., 1994), suggesting that the reduced binding capacity of cytoplasmically truncated CD44 protein may be due to reasons other than ablation of CD44-cytoskeletal interactions. Here we show that a truncated CD44v4-v7 (CD44 meta-stop) protein mediates binding to soluble HA perfectly well when transfected into AS cells, and that the CD44 meta-stop protein forms dimers and oligomers. We therefore conclude that clustering of CD44 overrides the need for a cytoplasmic tail to allow soluble HA binding, a conclusion consistent with the observation of Lesley and Hyman (1992) that a truncated CD44 protein can still be stimulated to bind HA by certain anti-CD44 antibodies, which would couple the protein into dimers.

We have shown here by tunicamycin inhibition that N-linked glycosylation is essential for the oligomerization and HA binding capacity of CD44. The fact that CD44v is hyperglycosylated with respect to CD44s in the BSp73AS system (Günthert et al., 1991; Rudy et al., 1993) may therefore be pertinent to the lack of soluble HA binding by CD44s in AS cells. Differences in glycosylation between clustered and nonclustered CD44v proteins are not necessarily extensive, however, as the CD44v4-v7 protein from ASpSV14 cells, which binds soluble HA efficiently and has the capacity to cluster, migrates similarly on SDS-PAGE as CD44v4-v7 protein from 1ASm7 cells, which does not cluster or bind soluble HA (data not shown). Highly specific tunicamycin-sensitive modifications may determine both CD44 clustering and HA binding, as the binding of both ASpSV14 and 1ASm7 to immobilized HA is also inhibited by tunicamycin treatment.

Changes in N-linked glycosylation and glycosaminoglycan addition influence HA binding by CD44 in other systems (Kato et al., 1995; Lesley et al., 1995). In these studies, tunicamycin treatment upregulated HA binding. We have also found cell lines whose HA binding capacity is upregulated by tunicamycin (Moll, J., unpublished results). However, in agreement with the results presented in this paper, another study recently showed that tunicamycin treatment of a panel of cell lines abrogated their HA binding capacity (Bartolazzi et al., 1996). Furthermore, this study showed that mutation of the N-linked sugar sites on CD44 destroyed the capacity of the CD44 protein to confer HA binding properties when expressed in non-HA-binding melanoma cells, clearly demonstrating the requirement for N-linked sugars in CD44-mediated HA binding. The diverse effects of tunicamycin underscore the complexity of the regulation of HA binding by CD44. We suggest that perhaps differences in the site of addition and constitution of the N-linked sugars may differentially influence the HA binding ability of CD44 proteins. This is supported by our observation that short times of exposure to tunicamycin slightly upregulate HA

binding in ASpSV14 cells (Sleeman, J., unpublished results), suggesting that the extent of inhibition of N-linked sugar addition by tunicamycin may be critical. For example, even long exposures to tunicamycin that downregulate HA binding do not completely inhibit addition of all of the N-linked sugars (Fig. 10). Additionally, carbohydrate groups that are not influenced by tunicamycin may become more important in determining oligomerization and HA binding levels after tunicamycin treatment. For example, CD44v4-v7 when expressed on AS cells also has O-linked carbohydrate (Rudy et al., 1993). O-linked sugars recently have been shown to inhibit HA binding (Bennett et al., 1995). Different cell lines may add a different complement of carbohydrates onto the CD44 protein, in turn affecting the ability of the protein to cluster and/or to bind to HA, permitting regulation of the HA binding activity of the CD44 protein.

In summary, this and other studies are consistent with a model in which aggregation of CD44 molecules on the cell surface enhances their HA binding properties. This molecular association is regulated by the cell to control its ability to bind HA in the extracellular matrix. Changes in glycosylation may be instrumental in this regulation. What is true for HA binding by CD44 may also be true for its ability to bind to other ligands, potentially opening a whole array of regulation to control the function of the CD44 protein family.

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