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BINDING OF CELL-SURFACE EXPRESSED CD44 TO HYALURONATE IS DEPENDENT ON SPLICING AND CELL TYPE

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SUMMARY: CD44 is a major cell-surface receptor for hyaluronate (HA). By alternative RNA-splicing a large number of CD44 variants are generated. To explore the role of CD44 splicing in the regulation of cell binding to HA, three different isoforms of CD44 were transfected in the CD44 negative B-cell lymphoma line Namalwa and in the fibroblastoid cell line COS7. We observed that whereas the standard form of CD44 (CD44s) mediated adhesion of Namalwa to HA, Namalwa transfected with CD44v3-10 or CD44v8-10 was unable to bind to either immobilized or soluble HA. After stimulation of CD44 with an activating anti-CD44 mAb or with phorbol ester, the binding of CD44s to HA was 5- to 10-fold higher than that of the other two isoforms. By contrast, COS7 cells transfected with CD44s, CD44v8-v10, or CD44v3-v10 bound equally effectively to HA. Hence, in addition to alternative splicing, cell type determines CD44 binding to HA.

CD44 is a broadly distributed family of cell surface glycoproteins that has been implicated in a number of important biological processes including lymphocyte homing, T- cell activation, hematopoiesis, and tumor metastasis (1-11). The CD44 gene consists of 20 exons. Due to alternative RNA-splicing involving at least 10 exons encoding domains of the extracellular portion of the molecule, a large number of CD44 splice variants are generated (8, 12-15).

Although unique functional properties have been attributed to certain CD44 splice variants, the precise effects of particular inserted domains on the ligand binding specificity of the CD44 molecule has remained unclear. The standard (hematopoietic) form of CD44 has been shown to function as a receptor for hyaluronate (HA) (15-18). Cell-surface HA receptors are believed to

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regulate many aspects of cell behavior such as cell differentiation, cell migration and tumor metastasis (19). Interestingly, Stamenkovic and collegues have reported that CD44E, a major CD44 splice variant on epithelium containing a 132-amino acid insert encoded by exons CD44v8-v10, is unable to bind this ligand. This suggested that CD44 splicing might regulate cell adhesion to HA (16). This finding has remained controversial, however, since studies by He et al. suggested that the murine homologue of CD44v8-v10 (CD44E) is capable of mediating binding to HA (20). Furthermore, in a recent paper Dougherty et al. (21) reported that CD44v8-v10 cloned from the human myelomonocytic leukemia line KG1a, binds avidly and specifically to HA when transfected in COS7 cells. The above controversy prompted us to further explore the role of alternative splicing of CD44 in the regulation of HA binding.

MATERIALS AND METHODS

Monoclonal antibodies (mAbs). The anti-CD44 mAbs used were NKI-P1 (3), Hermes-1 (2), and Hermes-3 (2) (kindly provided by drs C.G. Figdor, Nijmegen and E.G. Butcher, Stanford) all reactive with epitopes on the constant portion of the extracellular domain of CD44.

CD44 cDNAs. CD44s, CD44v8-v10, and CD44v3-v10 cDNAs were cloned as EcoRI-BglII-PCR fragments from HPKII cells into the EcoRI and BamHI cloning sites of the expression vector pAD-CMV2. The sequences of the inserts were identical to the CD44 sequences published by Screaton et al. (12). A schematic representation of the CD44 gene and the cDNAs is given in Fig.1.

Cell cultures and transfections. Stable Namalwa (ATCC, Rockville, MD, USA) CD44 transfectants were obtained by electroporation mediated cotransfection of CD44 cDNAs and pSVneo plasmid containing the bacterial neomycin resistance gene driven by a SV40 promotor. Cells with surface expression of CD44 were identified by indirect immunofluorescence using the CD44 mAb NKI-P1 and cloned by single cell sorting using a Becton Dickinson FACStar^{plus} flow cytometer (Mountain View, CA). At least two independent transfectants expressing each CD44 variant were used. The SV40-transformed simian fibroblastoid cell line COS7 (ATCC) was transiently transfected with the different CD44 cDNAs by electroporation and then incubated for 72h in DMEM culture medium to allow replication and expression.

Immunofluorescence. Surface expression of CD44 on Namalwa and COS transfectants was analysed by flow cytometry (FACScan Becton Dickinson) using indirect immunofluorescence (13). The fluorescein-tagged hyaluronic acid (HA-FITC) was a generous gift of Dr. Paraskevi Heldin (Dept. of Medical and Physiological Chemistry, University of Uppsala, Sweden) (22). HA-FITC binding to CD44 transfectants was performed as described by Lesley et al. (23).

Cell adhesion to immobilized hyaluronate. To study the adhesion of cells to HA, wells of a 96 wells plate (Costar, Cambridge MA, USA) were coated with 0,001-5mg/ml Rooster comb HA (Sigma Chemical Co., St. Louis) at 4°C overnight followed by 1h at 37°C. Aspecific binding was blocked by incubating the wells with 4% BSA/RPMI 1640 at 37°C for 1h. Subsequently, cells (100 ul, 1.10^6 cells/ml) were added to the wells and incubated for 1h at 37°C in 5% CO_2

in air. In some cases, cells were incubated with mAbs (for 1h on ice) or PMA (50ng/ml, for 18h at 37°C in 5% CO_2 in air), before adding the cells to the HA coated wells. After binding to HA, the wells were washed 6 times with 1% BSA/RPMI. The adherent cells were scored using a quantitative determination MTT assay (Boehringer, Mannheim, Germany) (24).

RESULTS AND DISCUSSION

Namalwa cells transfected with different CD44 variants show diffential binding to hyaluronan. cDNAs of CD44s, CD44v8-v10, and CD44v3-v10 (Fig. 1) were transfected into Namalwa cells, which in accordance with previous reports (9, 25), were negative for CD44 at both the protein (Fig. 2) and RNA level (data not shown). CD44 positive cells were cloned and several independent clones were established for each CD44 variant. Fig. 2 shows the approximately equal levels of expression of CD44 on a panel of representative CD44s, CD44v8-v10, and CD44v3-v10 transfectants.

To assess whether modification of the extracellular domain of CD44 by alternative splicing plays a role in regulating the HA receptor function of CD44, the binding of the various CD44 Namalwa transfectants to plastic immobilized and soluable HA was measured. Untransfected and mock transfected Namalwa cells, which lack CD44, were unable to bind to coated HA (Fig. 3). However, transfection of CD44s resulted in a sigificant adhesion of Namalwa to HA. This

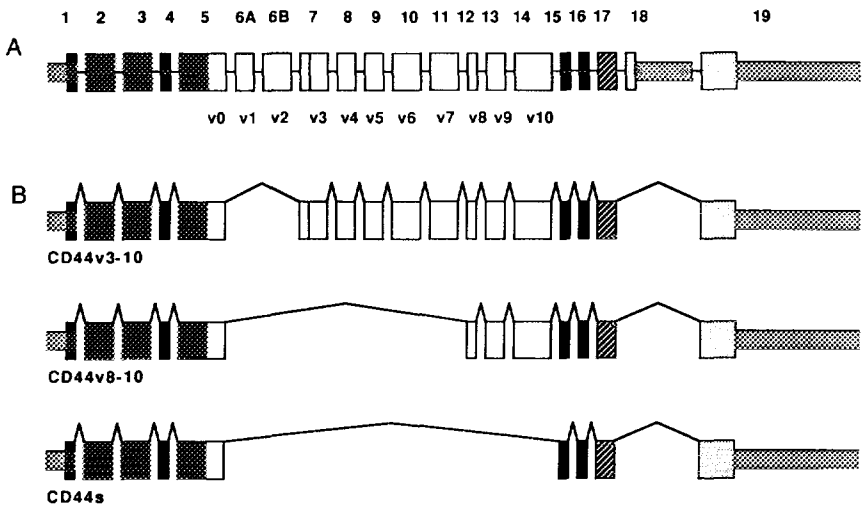

Fig. 1. Schematic representation of CD44 DNA (A) and the CD44s, CD44v8-v10, and CD44v3-v10 cDNAs (B) used for transfection.

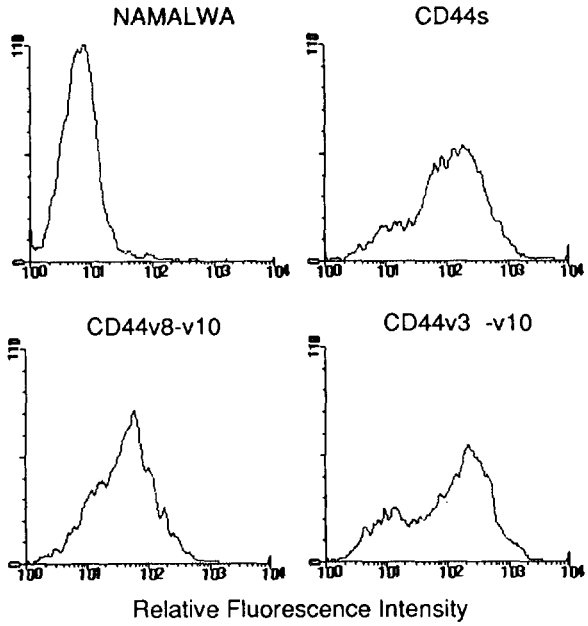

Fig. 2. Expression of CD44 on untransfected Namalwa cells and on Namalwa cells transfected with CD44s, CD44v8-v10, and CD44v3-v10. CD44 expression was analysed by FACS using mAb NKI-P1 against a standard CD44 epitope.

adhesion was completely blocked by Hermes-1, a mAb that is known to interfere with CD44-HA binding confirming the direct role of CD44 in the binding (not shown). By contrast, CD44v8-v10 or CD44v3-v10, did not support

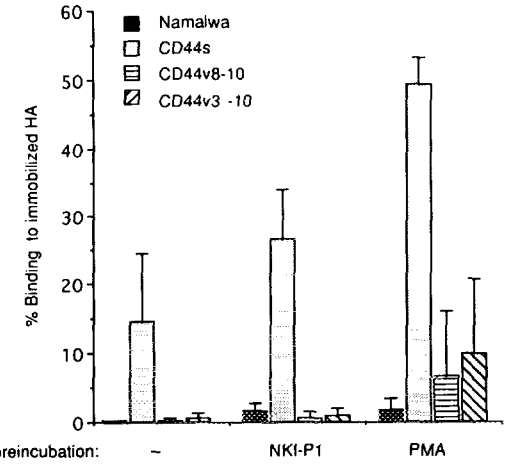

Fig. 3. Percentage of Namalwa cells transfected with different CD44 splice variants bound to immobillized HA before (-) or after treatment with the stimulating anti-CD44 mAb NKI-P1 or with PMA. Mean +/- standard deviation of 4 experiments.

adhesion of Namalwa cells to HA. Like immobilized HA, hyaluronate in solution (FITC-labeled) also bound to CD44s but hardly or not to CD44v8-v10 and CD44v3-v10 (Fig. 4).

Previous studies of CD44-HA interaction have shown that CD44 can exist on the cell surface in either an inactive, a low affinity, or a high affinity state. Activation of CD44 could be achieved PMA and by certain stimulating anti-CD44 mAbs (1, 22). To assess whether the HA binding activity of CD44v8-v10 and CD44v3-v10 is inducible, we treated the various CD44 transfectants with either the stimulating anti-CD44 mAb NKI-P1 or with the phorbol ester PMA. As shown in Fig. 3, both the antibody and the PMA treatment resulted in a strong increase in adhesion of CD44s bearing Namalwa cells. Low levels of HA binding were also induced in the CD44v8-10 and v3-v10 transfectants after PMA stimulation (Fig.3) but this binding remained 5- to 10-fold lower than that of CD44s. Thus, whereas CD44s on Namalwa cells is either constitutively active or can be readily activated to mediate binding to HA, CD44v8-v10 and CD44v3-v10 are unable to bind HA efficiently.

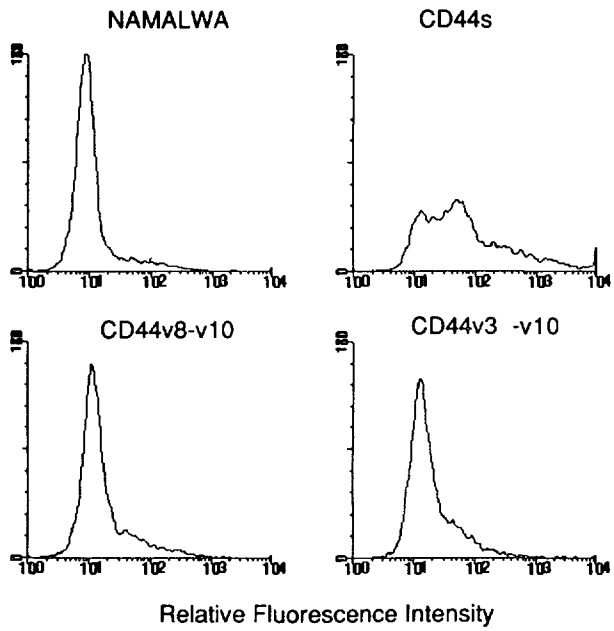

Fig.4. Binding of HA-FITC to untransfected Namalwa cells and to Namalwa cells transfected with CD44s, CD44v8-v10, and CD44v3-v10.

CD44 isoforms expressed by COS7 cells show identical hyaluronate binding. CD44s and CD44v8-v10 transfected into the Simian monkey fibroblastoid cell line COS7 have been reported to both mediate HA binding (21). We studied the interaction of CD44s, CD44v8-v10, and CD44v3-v10 transfected COS7 cells with HA-FITC (Fig. 5). FACS double staining with mAb NKI-P1 against CD44 and HA-FITC clearly showed that the transfected COS7 bound on average approximately 10-fold the amount of HA-FITC of the non-transfected cells. Interestingly, this binding was similar for cells transfected with all three CD44 cDNAs. Hence, unlike in Namalwa cells, CD44s, CD44v8-v10, and CD44v3-v10 variants transfected to COS7 cells bind HA effectively.

The molecular basis for the huge differences in HA affinity of the CD44 isoforms when expressed on Namalwa and for the cell type specific character of this difference in affinity is presently unknown. Possible mechanisms include conformational changes at the HA binding site of CD44 induced by insertion of

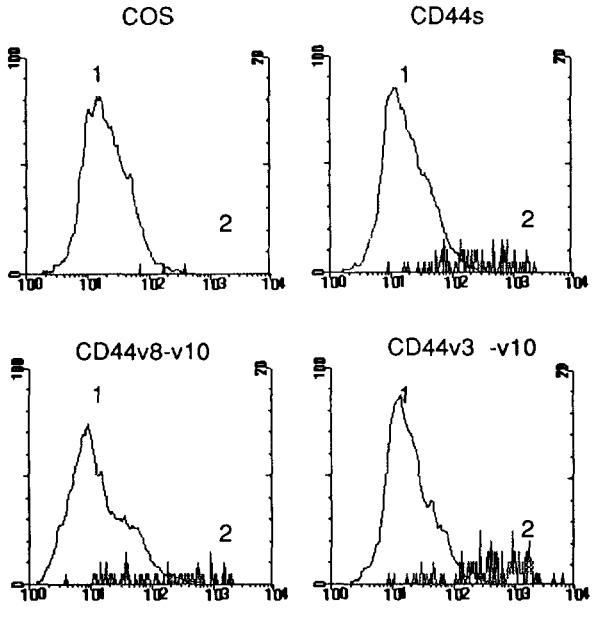

Fig. 5. Binding of HA-FITC to untransfected COS7 cells and to COS7 cells transfected with different CD44 splice variants. CD44 positive cells were identified by FACS double staining with HA-FITC and anti-CD44-PE (NKI-P1). 1, HA-FITC binding to untransfected cells; 2, HA-FITC binding to CD44 transfected cells.

the alternatively spliced domains, differences in receptor clustering on the cell cell surface, and interactions with other cell surface molecules. Also, the observed differences in affinity for HA might be caused by differential association with the cytoskeleton, which in turn might be regulated by phosphorylation of cytoplasmic serine recidues. Indeed, it has recently been shown that serine phosphorylation of CD44 regulates HA binding (26). In conclusion, our data provide further evidence that CD44 mediated cell-adhesion to HA is subject to complex regulation. This regulation involves alternative splicing as well as other unkown cell type-specific factors, and presumably allows for functional activity of CD44 at the appropriate time and location.

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