Defective Phosphorylation and Hyaluronate Binding of CD44 with Point Mutations in the Cytoplasmic Domain

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Summary

CD44 is a cell surface adhesion molecule that plays a role in leukocyte extravasation, leukopoiesis, T lymphocyte activation, and tumor metastasis. The principal known ligand for CD44 is the glycosaminoglycan hyaluronate, (HA), a major constituent of extracellular matrices. CD44 expression is required but is not sufficient to confer cellular adhesion to HA, suggesting that the adhesion function of the receptor is regulated. We recently demonstrated that CD44 in primary leukocytes is phosphorylated in a cell type- and activation state-dependent fashion. In this study we demonstrate that serines 325 and 327 within the cytoplasmic domain of CD44 are required for the constitutive phosphorylation of CD44 in T cells. Furthermore, we demonstrate that cells expressing mutated CD44 containing a serine to glycine substitution at position 325 or a serine to alanine substitution at amino acid 327 are defective in HA binding, CD44-mediated adhesion of T cells to smooth muscle cells, as well as ligand-induced receptor modulation. The effect of these mutations can be partially reversed by a monoclonal anti-CD44 antibody that enhances CD44-mediated HA binding.

CD44 is a type I transmembrane glycoprotein expressed on leukocytes, some nonleukocytes, and many tumor cells. CD44 is a receptor for hyaluronate (HA)¹, which is found ubiquitously as a constituent of extracellular matrices. Increased levels of HA are often associated with inflammation and malignancies (1-5). Although many CD44⁺ cell lines and tumors exhibit CD44-mediated HA binding, normal CD44⁺ leukocytes do not bind HA appreciably in vitro. The expression of CD44 is upregulated 10-fold on lymphocytes after antigen receptor-mediated activation (6, 7); however, this enhanced CD44 expression does not necessarily lead to the induction of HA binding in the majority of lymphoblasts. We have described cell type- and activation state-dependent variations in the N-linked glycosylation, the phosphorylation, and the cytoskeletal association of CD44 (8). Furthermore, Lesley et al. (9) demonstrated that the cytoplasmic domain of CD44 is required for optimal HA binding function. These authors also described a mAb, IRAWB14.1, that is specific for CD44. This mAb enhanced CD44-mediated HA binding by cell lines and normal CD44⁺ leukocytes that show little, if any, basal affinity for HA (9, 10). This mAb also overcame the HA binding defect of a cytoplasmic tail minus truncation mutant (9). In this study, we addressed the possibility that phosphorylation of the cytoplasmic domain of CD44 in leukocytes is at least in part responsible for the regulated adhesion function of CD44.

Materials and Methods

Antibodies. Three monoclonal anti-murine CD44 mAbs were used in these studies. The KM201 mAb directed against the HA binding domain was generously provided by Dr. Paul Kincade (Oklahoma Medical Research Foundation, Oklahoma City, OK) (11). IRAWB14.1, an anti-CD44 which enhances HA binding, was provided by Drs. Jayne Lesley and Robert Hyman (Salk Institute, La Jolla, CA) (9). IM7 was obtained from Dr. Ian Trowbridge (Salk Institute) (12). Hybridomas producing anti-CD44 mAbs were grown in serum-free medium. mAbs were partially purified from crude supernatants by precipitation in 50% saturating ammonium sulfate.

CD44 Constructs and Transfectants. A full-length, 1,388-bp cDNA clone, rlcpl, encoding the 363-amino acid hematopoietic form of CD44, was isolated by immunosdection from a murine anti-Ig activated B cell cDNA library constructed in pCDM8 (obtained from Dr. Brian Seed, Cambridge, MA) and expressed in COS-7 cells. The truncation mutant, CD44Δ1011, was generated by subcloning the HindIII-PstI fragment of rlcpl (amino acid residues 1-338) into pRC/CMV (Invitrogen, San Diego, CA). Point mutants containing a Ser → Gly substitution at residue 325 and

¹ Abbreviations used in this paper: ASM, airway smooth muscle; HA, hyaluronate.
a Ser → Ala substitution at residue 327, were generated using a
two-step PCR amplification with complementary primers con-
taining an A → G mutation at nucleotide 973 and a T → G muta-
tion at nucleotide 979, respectively. The resulting mutants were
sequenced to verify the presence of the mutations as well as the
fidelity of the PCR, and subcloned into pRc/CMV. The parental
AKR1.G10 thymoma line was transfected by electroporation with
40 μg of purified plasmid DNA. Stable subclones were isolated by
two rounds of immunoselection with IM7 using anti-rat Ig-con-
jugated magnetic beads (Dynal, Oslo, Norway) and two rounds of
immunoselection for high expression as detected by FITC-KM201
and fluorescence-activated cell sorting followed by limiting dilu-
tion cloning. The stable AKR1.G10 transfectants expressing wild
type and the cytoplasmic tail minus mutant encoding residues 1–297
were obtained from Drs. Lesley and Hyman (9). All clones were
subcloned and selected based on similar levels of expression.

Immunoprecipitation and Phosphoamino Acid Analysis of CD44.
Thymoma cells were incubated for 1 h in phosphate-free RPMI
supplemented with 5% dialyzed FCS and then loaded with 2 mCi
orthophosphate for 3.5 h (~9,000 Ci/mM; New England Nu-
clear, Boston, MA). After labeling, cells were washed and lysed
in PBS containing 1% NP-40, protease inhibitors (0.2 U/ml
aprotinin, 1 mM PMSF, and 100 μg/ml leupeptin; Sigma Chem-
ical Co., St. Louis, MO), and phosphatase inhibitors (50 mM
NaF, 50 mM KF, and 10 mM sodium pyrophosphate; Sigma
Chemical Co.). Lysates were clarified by centrifugation at 10,000 g
at 4°C for 10 min and the NP-40 soluble fraction was preclarified
with normal rat Ig and mouse anti-rat Ig conjugated to Sepharose.
Lysates were reacted with rat anti-mouse CD44 mAb, IM7, and
the immune complexes precipitated with Sepharose-mouse anti-rat
Ig. Precipitates were washed with high salt (0.6 M NaCl, 125 mM
KPO4, pH 7.4, and 0.02% NaN3), mixed detergent buffer (0.05%
NP-40, 0.1% SDS, 0.3 M NaCl, and 10 mM Tris, pH 8.6), and
PBS. Immune complexes were then eluted by boiling in 2× Laemmlli
running buffer containing 0.4% dithiothreitol and electrophoresed
on 7% polyacrylamide gels. Molecular weight standards were ob-

Figure 1. Expression of wild-
type CD44 and CD44 mutants on
stable transfectants of AKR1.G10
thymoma cells. (A) Schematic of the
primary structure of CD44. Poten-
tial sites of serine phosphorylation
within the cytoplasmic domain are
indicated. Serines are indicated by
circle (potential protein kinase C
target), triangle (potential cAMP-
dependent protein kinase target), or
squares (not a known kinase target
consensus sequence). Nucleotide
designations are based on the initi-
station start codon being 1. (B) Se-
quence of the cytoplasmic domains
of the wild-type and mutant CD44
constructs. The potential sites of
serine phosphorylation are indicated
by boldface type. (C) Flow cytom-
etry of AKR1.G10 thymoma cells
transfected with vector alone
(pRc/CMV, solid line), wild-type
CD44 construct (WT, dotted line),
and in the left panel with Δ1011
(dashed line) or Δcyt (boldface solid
line) (9), or the construct containing
the point mutations, serine 325
(boldface dashed line). In the right
panel, staining of cells transfected
with CD44 with the serine 327
point mutation is depicted by the
boldface dashed line.
tained from Bio-Rad Laboratories (Melville, NY). Gels were dried and exposed to film for 24 h. For phosphoamino acid analysis, bands corresponding to CD44 were cut out from the dried gels, reswollen in 2.5% acetic acid/10% methanol, and dehydrated in 50% methanol. The gel slice was dried by vacuum desiccation, and rehydrated in 50 mM ammonium bicarbonate, digested with 0.1 mg/ml thermolysin (Boehringer Mannheim, Indianapolis, IN) at 37°C for 12 h; the eluted peptide fragments were dried by vacuum desiccation. Lyophilized peptides were acid hydrolyzed in 6 M HCl at 100°C for 2 h in vacuo, rehydrolyzed, and resuspended in 8% acetic acid/2% formic acid, pH 1.9. The sample was then resolved on a cellulose TLC plate (Eastman Kodak, Rochester, NY) in parallel with 2 μg each of unlabeled phosphoserine, phosphothreonine, and phosphotyrosine standards. The plate was electrophoresed at 400 V for 60 min first at pH 1.9 followed by pH 3.5 for 30 min. The TLC plate was dried, stained with 1% ninhydrin in acetone to visualize the standards, and subjected to phosphorimage analysis and fluorography.

**HA Binding and Adhesion Assays.** Hyaluronic acid and hyaluronidase were purchased from Sigma Chemical Co. HA was conjugated to fluorescein by the method of deBelder and Wik (13). Cells were reacted with FITC-HA for 45 min on ice and analyzed on a flow cytometer (Coulter Corp., Hialeah, FL).

To assay for binding to insoluble HA, transfected AKR1.G10 cells were radiolabeled by overnight incubation in medium containing 2 μCi/ml [3H]thymidine (NET027X, New England Nuclear) and then washed to remove free [3H]thymidine. 10⁶ cells in 100 μl were added to 96-well microtiter plates precoated with 100 μl of 50 μg/ml HA in PBS. After 1 h, nonadherent cells were removed by washing. Adherent cells were quantitated by lysing in 0.5% Triton X-100 and counting.

Where indicated, cells were pretreated with either the blocking anti-CD44 mAb, KM201, for 45 min at 4°C or with the enhancing antibody, IRAWB14.1, for 15 min at 4°C. Sensitivity of adhesion to hyaluronidase was determined by adding hyaluronidase for 5 min at 37°C at the end of the adhesion assay before removing the nonadherent cells.

**Lymphocyte Adhesion to Airway Smooth Muscle Cells.** Airway smooth muscle cells (ASM) were isolated as previously described (14). 8 × 10⁶ transfected AKR1.G10 cells radiolabeled as described above, were added to ASM cell monolayers in 24-well culture plates and incubated for 1 h at 4°C. Where indicated, cells were pretreated with mAb as described above or with 100 μg/ml HA for 45 min at 4°C. Sensitivity to hyaluronidase was determined as described above.

**Cell Surface Modulation of CD44.** AKR.G10 transfecants were suspended at 10⁶ in cold media containing 10% FCS. 10⁶ cells were reacted with 10 μg/ml of IRAWB14.1 anti-CD44 at either 4 or 37°C. After 45 min, unbound mAb was removed by washing and the cells were stained with a FITC-conjugated, mouse anti-rat Ig at 4°C. Excess secondary antibody was removed by washing and the cells were fixed in 3.7% formaldehyde/PBS. Samples were analyzed under oil on a Leitz fluorescence microscope and photographed on ASA 400 Kodachrome film at a magnification of 1,000.

**Results**

**Serines 325 and 327 Are Required for the Constitutive Phosphorylation of CD44 in T Cells.** The cytoplasmic domain of murine CD44 contains 10 potential phosphorylation sites: 6 serines and 4 threonines (Figs. 1, A and B). Phosphoamino acid analysis of wild-type recombinant CD44 expressed in AKR1.G10 cells demonstrated that CD44 is constitutively phosphorylated on one or more serine residue(s) (Fig. 2 A). Neither phosphothreonine nor phosphotyrosine was detected. To map the site(s) of constitutive residue(s) (Fig. 2 A). Neither phosphothreonine nor phosphotyrosine was detected. To map the site(s) of constitutive phosphorylation in T cells, we established stably transfected cell lines expressing similar levels of wild-type or mutant forms of CD44 using a CD44- T cell line, AKR1.G10 (Fig. 1, B and C). These mutants encode amino acid substitutions that remove potential sites of serine phosphorylation within the CD44 intracellular domain of a cDNA clone, rkp1, of murine CD44 (Fig. 1 B). Serines 293 and 325 were replaced by glycine residues and serine 327 with an alanine residue. There were comparable levels of expression of CD44 on each of the transfected lines in comparison to staining of cells transfected with vector alone as detected by a FITC-conjugated anti-CD44 mAb and analyzed by flow cytometry (Fig. 1 C). Treatment with the enhancing anti-CD44 mAb, IRAWB14.1, did not effect the level of CD44 expressed by any of the transfected lines (data not shown).

Phosphorylation of the receptor in each transfected line was investigated by immune precipitation of CD44 from detergent lysates of ortho-32phosphate--labeled cells. Phosphorylated CD44 precipitated from cells expressing wild-type recombinant CD44 has an apparent molecular mass of ~90 kD (Fig. 2, B and C, middle lane). The Δcyt mutation, which retains only serine 293 of the cytoplasmic tail, was not phosphorylated (Fig. 2 B). Similarly, mutants that contained either a single substitution at serines 325 or 327 or three substitutions at serines 293, 325, and 327, were not phosphorylated (Fig. 2 C). These results are consistent with those recently reported for CD44 expressed in epithelial cells (15).

**T Cells Expressing Nonphosphorylated Forms of CD44 Do Not Adhere to HA.** Lesley et al. (9) demonstrated that the CD44- parental AKR1.G10 cell line does not bind appreciably to soluble or insoluble HA. Expression of wild-type recombinant CD44 confers upon the AKR1.G10 line the capacity to bind to either form of HA (Fig. 3, A and B; 9). In contrast, the line expressing a truncated form of CD44 lacking all but the first six residues of the cytoplasmic domain bound soluble HA poorly, although the binding was dramatically enhanced by treating the cells with IRAWB14.1 (9). This result is illustrated in Fig. 3. Cells transfected with vector without insert did not bind HA in the presence or absence of IRAWB14.1 (Fig. 3 A). These results indicated that the cytoplasmic domain is required for the optimal adhesion function of the extracellular domain of CD44.

We analyzed the HA binding of cells transfected with the Ser325 → Gly and Ser327 → Ala mutants of CD44 to address the possibility that phosphorylation of the cytoplasmic domain is required for the adhesion function of the extracellular domain. We compared the capacity of the aforementioned transfected cell lines expressing wild-type and mutant forms of CD44 to bind soluble FITC-HA (Fig. 3 A) and to adhere to immobilized HA (Fig. 3 B). Cells expressing the nonphosphorylated mutants of CD44 bound soluble HA minimally (Fig. 3 A, top) and did not mediate adhesion to HA-coated surfaces (Fig. 3 B). In contrast, the mutant Δ1011
which retains approximately half of the cytoplasmic domain
including serines 325 and 327, binds HA at levels similar to
wild type (Fig. 3 C, and data not shown), indicating that
the NH2-terminal half of the cytoplasmic tail is sufficient to
confer HA binding function.

We have recently developed a more physiologic assay for
CD44 adhesion function in which we quantitate CD44-
HA-mediated adhesion of T cells to ASM cell monolayers
(16). We demonstrated that activated human T cells adhere
to human ASM via integrins and CD44 (16). We also ex-
plotted the fact that murine LFA-1 does not bind to human
intercellular adhesion molecule 1 (17) whereas CD44-mediated
binding is not species specific (18) to demonstrate that CD44
is sufficient to mediate T cell adhesion to ASM (16). Thus,
the CD44+ murine thymoma cells, AKR.G10, did not ad-
here to human ASM whereas AKR.G10 cells transfected with
a wild-type construct of murine CD44 did. A comparison
of adhesion of the various transfectants in this assay dem-
strates that serines 325 (Fig. 4) and 327 (data not shown)
are required for CD44-HA-mediated T cell adhesion to
smooth muscle cell monolayers.

The defect in CD44 adhesion function of all the nonphos-
phorylated mutants, i.e., Δcyt, 325, 327, and 293/325/327,
could be in large part be reversed by treating the cells with
the anti-CD44 mAb IR-AWB14.1 (Fig. 3 A, middle, Fig. 3,
B, and C, and Fig. 4). Both constitutive and mAb-induced
HA binding in all three assays were inhibited by the blocking
anti-CD44 mAb KM201 (Fig. 3 A, bottom, and Fig. 4) or
by an excess of soluble HA (Fig. 3 B and Fig. 4). In addition,
HA binding could be reversed by subsequent digestion with
hyaluronidase (Fig. 3, B and C and Fig. 4).

Ligand-induced Modulation of CD44 Is Defective in Non-
phosphorylated Mutants. We compared the distribution of
wild-type and mutant CD44 on the cell surface and the sus-
ceptibility of wild-type and mutant receptors to ligand-induced
modulation using fluorescence microscopy. Transfectants ex-
pressing wild-type, Δcyt, or serines 327 (Fig. 5, top)
or 325 (data not shown) reacted with FITC-anti-CD44 at 4°C all
exhibited similar staining in ring patterns typical of cell sur-
face proteins that are evenly distributed on the membrane.
In contrast, the distribution of the wild-type and mutant forms
of CD44 were markedly different when the cells were in-
cubated with anti-CD44 antibodies and detected with a sec-
ondary FITC-anti-rat Ig (Fig. 5) or treated with the enhancing
anti-CD44 mAb, IR-AWB14, and FITC-HA (data not shown)
at 37°C for 1 h. Ligand binding at 37°C resulted in limited
clustering of wild-type CD44 that was evident as patches
of fluorescence over the entire surface of the cells (Fig. 5,
bottom left). In contrast, ligand binding induced a more rad-
cial modulation of CD44 in the mutations, Δcyt (Fig. 5, bottom

Figure 2. Serines 325 and 327 are required for the constitutive phosphor-
ylation of CD44 in T cells. AKR1G10 cells expressing the recombinant
forms of CD44 described in Fig. 1 were loaded with ortho-32P-phosphate
NP-40 lysates were prepared and CD44 was immunoprecipitated with
the mAb IM7 and subsequently resolved on a 7% SDS-polyacrylamide
gel (B and C). A phosphoamino acid analysis of 32P-labeled CD44 from
AKR1G10 transfected with the wild-type CD44 construct is shown (A).
center), serines 327 (Fig. 5, bottom right) and 325 (data not shown). Ligand binding to each of the mutant forms of CD44 resulted in the accumulation of the receptor into a single polar cap on the vast majority of the cells.

**Discussion**

In this study we describe two point mutations in the cytoplasmic domain of CD44 that result in the loss of phosphorylation of the receptor as well as its affinity for HA compared with wild-type CD44. These mutants provide definitive evidence that point mutations in the cytoplasmic domain of an adhesion receptor can severely limit the function of the extracellular domain of the receptor. We conclude from this study that the potential for phosphorylation of CD44 on serines 325 and/or 327 is required for optimal CD44-mediated HA binding in T cells. This is the first direct evidence that the regulation of the adhesion function of this receptor is
related to its potential to be phosphorylated. This may reflect a requirement for the phosphate group(s) per se or the negative charge that it would provide. The latter has been suggested, for example, as an explanation for the defect in the transcytosis of the poly Ig receptor with a serine to alanine mutation since substitution of the relevant serine residue with an aspartic acid residue does not result in defective transcytosis (19).

Although HA is the principal defined ligand for CD44 in vitro, adhesion in vivo involves HA in the context of complex extracellular matrices. An example of a distinction between receptor-mediated ligand recognition and adhesion to a physiologic substrate was recently published. Kansas et al. (20) demonstrated that deletion of the COOH-terminal 11-amino acids from the 17-amino acid cytoplasmic domain of L-selectin eliminated binding of lymphocytes to high endothelial venules in the in vitro frozen section assay. It also abolished leukocyte rolling in vivo in exteriorized rat mesenteric venules, but this mutation did not alter the lectin reactivity of L-selectin. In this case, pretreatment of cells with cytochalasin B also abolished adhesion without affecting carbohydrate recognition. Therefore, it was important to evaluate the effect of the serine 325 and 327 mutations on binding to HA in a more physiologically relevant assay. The movement of lymphocytes into tissues involves a cascade of adhesion receptor-mediated interactions with the endothelium that has in large part been delineated. Despite the importance of the endothelium in regulating tissue infiltration by lymphocytes, other cells such as fibroblasts or vascular or tissue smooth muscle cells which are a major source of HA may also play a role. We recently found that activated T cells can adhere to ASM cells in vitro (16). This assay was used to evaluate the effect on HA-mediated adhesion of AKR1.G10 cells ex-

Figure 4. Serine 325 is required for CD44/HA-mediated adhesion of T cell adhesion to ASM cells. ASM monolayers in 24-well culture dishes were established as previously described (14). Transfected AKR.G10 cells were radiolabeled and treated with normal rat Ig (solid bars), IRAWB14.1 (hatched bar), KM201 (shaded bar), or 100 μg/ml HA (open bars).

Figure 5. Defective ligand-induced redistribution of mutant forms of CD44 on the cell surface. Basal distribution of CD44 on the surface of AKR.G10 cells expressing wild-type (left) and the tail minus (center) and Ser327→Ala mutants of CD44 was determined by staining with IRAWB14 followed by FITC-mouse anti-rat Ig at 4°C (top). Receptor modulation was induced by incubating the cells in the presence of IRAWB14 at 37°C for 45 min and then stained with FITC-mouse anti-rat Ig (bottom).
pressing the nonphosphorylated mutants of CD44. The capacity of the various transfectants to adhere to smooth muscle cells directly correlated with their capacity to bind HA.

There are several possible mechanisms by which the mutations in the cytoplasmic domain of CD44 may affect the function of the extracellular domain. They could result in a change in the conformation of the receptor, interfere with the normal distribution or ligand-induced redistribution of the receptor or change its association with other cellular proteins. Any of these phenomena may be regulated by the state of phosphorylation of the receptor. Although we have not ruled out the possibility of an effect on the conformation of the receptor, it is worth noting that the mutant receptors are reactive with each of a panel of four mAbs to distinct epitopes of the extracellular domain (data not shown) and HA binding of the mutant receptor can be restored by mAb-induced receptor cross-linking, suggesting that the mutants can take on a functional conformation. The fact that the adhesion function of CD44 can be upregulated by treatment with an antireceptor mAb suggested that either clustering of the receptor or a conformational change in the receptor is required for optimal HA binding (21). The requirement for bivalent binding of the mAb for activation of HA binding (21) suggests that the distribution of the CD44 on the cell surface is critical to the HA adhesion function of the receptor. We demonstrate that the pattern of ligand-induced modulation of the wild-type and mutants forms of CD44 are distinct. Wild-type CD44 accumulates in clusters whereas the mutated receptors redistribute to a single polar cap. This increased susceptibility to ligand-induced modulation may reflect a difference in the ability of the wild-type and mutant forms of CD44 to associate with other proteins, including components of the cytoskeleton. Phosphorylation of the cytoplasmic domain of CD44 may mediate these changes directly or indirectly by regulating the association of CD44 with the cytoskeleton. The latter is an intriguing possibility since we previously observed an inverse relationship between the phosphorylation of CD44 and its association with the cytoskeleton (8).

Our results suggest that the function of CD44 may be subject to regulation by serine kinase and phosphatase activities. Our finding that mutation of either serine 325 or 327 expressed in T cells completely, or nearly completely, abolishes phosphorylation of CD44, although somewhat surprising, has been found to be the case in other cell types, including epithelial cells (15) and COS7 cells (Camp, R. L., and E. Puré, unpublished observation), as well. There are at least two models that would be consistent with these observations. Both serines 325 and 327 could be phosphorylated and each required for phosphorylation of either residue, or, only one of the two serines may be phosphorylated and the other serine may merely be needed for the kinase to recognize the substrate. In two-dimensional tryptic peptide maps of wild-type recombinant CD44 expressed in COS7 cells and of endogenous CD44 isolated from resident and thioglycolated elicited murine peritoneal macrophages we detected at least four distinct phosphopeptides (Puré, E., and R. L. Camp, unpublished observation). Although this could result merely from incomplete cleavage, these results are also consistent with the possibility that both serines 325 and 327 are phosphorylated since there is a predicted tryptic cleavage site located between these two serines. It is also interesting to note in this regard that neither serine 325 nor 327 is located within a previously defined kinase target consensus sequence.

The data described herein indicate that the potential for phosphorylation of CD44 in T cells is a prerequisite for the adhesion function and normal distribution of CD44 on the cell surface. However, phosphorylation may not be sufficient since unlike in T cell lymphomas, primary resting T cells do not bind HA although ~50% of the total cellular pool of CD44 in these cells is phosphorylated (8). However, resting primary T cells express lower levels of CD44. Furthermore, primary T cells can be induced to bind HA by activation with phorbol esters that induce protein kinase C. This results in a 5-10-fold increase in the expression of CD44 (7) as well as an increase in the proportion of CD44 that is phosphorylated (Camp, R. L., and E. P. Puré, unpublished observation). Thus, the adhesion function of the receptor may reflect a combination of the level of CD44 expressed and the proportion of CD44 that is phosphorylated. Other cell type-specific mechanisms may also contribute to the regulation of CD44 mediated adhesion.

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