

# A Cysteine Residue Located in the Transmembrane Domain of CD44 Is Important in Binding of CD44 to Hyaluronic Acid

By Dacai Liu and Man-Sun Sy

*From the Institute of Pathology, Department of Dermatology and Cancer Research Center, Case Western Reserve University, School of Medicine, Cleveland, Ohio 44106-4943*

## Summary

In the transmembrane domain and cytoplasmic domain of human CD44 protein there are two cysteine residues. These two cysteines are conserved in all known mammalian CD44 proteins. The functions of these cysteine residues are not known. Site-specific mutagenesis was used to create CD44 mutant proteins lacking either one or both of these cysteine residues. Wild-type CD44 and mutant CD44 genes were transfected into CD44<sup>-</sup> Jurkat cells to establish stable transfectants. These transfectants were used to study whether these two cysteine residues are important in the binding of CD44(H) to fluorescein-conjugated hyaluronic acid (F-HA). Jurkat transfectant bearing wild-type CD44 did not bind F-HA, unless they were stimulated *in vitro* with immobilized anti-CD3 monoclonal antibody. Anti-CD3 antibody also stimulated the binding of F-HA in Jurkat CD44.C295A transfectant in which the cytoplasmic cysteine residue has been replaced with alanine. In contrast, anti-CD3 antibody failed to stimulate the binding of F-HA in Jurkat transfectant (CD44.C286A), in which the transmembrane domain cysteine 286 has been replaced with an alanine, and in Jurkat transfectant CD44.2C2A, in which both of the cysteine residues have been altered. Binding can also be induced with a monoclonal anti-CD44 antibody (F-44-10-2) in Jurkat wild-type CD44 and Jurkat CD44.C295A transfectants but not in CD44.C286A transfectant. These results provide evidence that the transmembrane domain of CD44, more specifically the cysteine residue in the transmembrane domain, is important for both activation-induced and anti-CD44 antibody-induced binding of soluble HA.

CD44 is a glycoprotein expressed on lymphocytes, monocytes, and many other cell types (1–4). There are at least 20 exons in the CD44 gene (5–8). Potentially, a large number of CD44 isoforms can be generated by differential splicing of the mRNA. The mature hematopoietic form is a 80–90-kD transmembrane protein (CD44H), and represents the basic unit of the CD44 proteins. The other CD44 isoforms are created by addition of new exons into the extracellular domain of CD44H (5–8). The hematopoietic form of CD44 is the major CD44 protein present on normal human lymphocytes and monocytes.

One of the ligands for CD44 is hyaluronic acid (HA) (9–11). HA is a proteoglycan present in extracellular matrix and in the circulation (12, 13). There is a single tandem repeat loop on the extracellular domain of CD44H, which may be important in interactions between CD44 and HA. This region is conserved in all CD44 isoforms (1–4). Two regions in the human CD44 molecules have been identified to be important in hyaluronic acid binding. One of the regions is located near the amino terminus. The other site is located near

the membrane proximal region but lies outside the link protein homologous domain (14). Using blocking monoclonal antibodies, an additional region in the CD44 molecule has been proposed to be important for binding of HA (15).

Binding of soluble HA to cell surface CD44 can be demonstrated with some, but not all CD44-bearing cells (3, 16). Normal lymphocytes or monocytes do not bind HA, despite the expression of high levels of CD44 (16). Stimulation of normal murine splenic B cells with anti-IgD and dextran generated a population of B cells which expressed the highest levels of cell surface CD44. However, these B cells did not bind fluorescein-conjugated hyaluronic acid (F-HA). In contrast, splenic B cells stimulated with IL-5 expressed either equivalent or lower levels of CD44 but bound significant levels of F-HA (17). Therefore, simple upregulation of CD44 expression is insufficient to ensure binding of F-HA, and level of CD44 expression does not always correlate with binding of F-HA. Binding of low levels of HA can be induced with some anti-CD44 mAbs, indicating that conformational changes as a result of cross-linking of CD44 may be essential for binding of HA (18, 19).

Posttranslational modifications, especially N-linked glycosylation of CD44 influence binding of HA (16, 17, 20, 21).

<sup>1</sup>Abbreviations used in this paper: F-HA, fluorescein-conjugated hyaluronic acid; HA, hyaluronic acid.

Growth factors and oncogene expression also regulate interactions between CD44 and HA (22). Furthermore, the isoforms of CD44 expressed also influence binding of HA (3, 16).

The cytoplasmic domain of CD44 interacts with cytoskeletal proteins, more specifically with ankyrin (23–26). Ankyrin is important in membrane cytoskeletal interactions (27). Whether interactions between the cytoplasmic domain of CD44 and cytoskeletal proteins are important in binding of HA is controversial. Some studies suggested that the cytoplasmic domain was essential for binding (28–30). Other studies suggested that interactions between the CD44 cytoplasmic domain and cytoskeletal protein were not required for binding (14, 31). A chimeric, genetic engineered soluble human CD44 protein lacking the authentic CD44 cytoplasmic domain binds HA, suggesting that an intact CD44 cytoplasmic domain was not essential for binding (14). The reasons for these discrepancies are not known. Whether CD44 interacts with cytoplasmic skeletal proteins may depend on the nature of the cell type. The cytoplasmic domain of CD44 is subject to phosphorylation by a serine/threonine protein kinase and was reported to be essential for binding of HA (32). CD44 has GTPase activity. Binding and hydrolysis of GTP by CD44 may regulate the interactions between CD44 and cytoskeletal proteins (33). Underhill and Toole proposed that clustering of CD44 on the cell surface may be important for binding of HA (34, 35). Clusters of CD44 are more likely to make multiple interactions with a single molecule of HA than if they are dispersed over the cell surface. Large molecules of HA bind to the cell surface with a higher affinity than smaller molecules (34). In addition, when membranes are solubilized with a detergent, the binding affinity of CD44 is drastically decreased (35).

All human CD44 isoforms have identical transmembrane and cytoplasmic domains (1–4). The putative transmembrane domain of CD44H (amino acids 270 to 289) and the 18 amino acid immediately after the transmembrane domain are identical in mouse, baboon, horse, rat, mouse, and human. In the hamster, there is one conserved change from isoleucine to valine in position 272 (28). This high degree of conservation may signify the importance of this region in the functions of CD44 molecules. Within this highly conserved region of human CD44H, there are two cysteine residues. Cys 286 is located in the transmembrane domain and Cys 295 is located in the cytoplasmic domain. Whether these two cysteines are important in the functions of CD44 is not known.

Cysteine residues in the transmembrane domain and in the cytoplasmic domain of some transmembrane cell surface proteins are covalently modified with a long chain saturated fatty acid, a process known as protein palmitoylation (36–39). The functions of protein palmitoylation are not clear. Palmitoylation of proteins may facilitate protein–protein interactions and/or protein–lipid interactions. No uniform consensus function has been established for protein palmitoylation. CD44 protein in murine thymoma BW5147 (40) and in normal human peripheral blood lymphocytes (41) is modified with palmitate. There is a correlation between induction of CD44 palmitoylation and inhibition of CD3-mediated signaling in human peripheral blood lymphocytes (41).

Therefore, palmitoylation of CD44 may play a role in signal transduction.

We used site-specific mutagenesis to replace the two cysteine residues in the transmembrane domain and in the cytoplasmic domain of human CD44, either individually or together, with alanine. These constructs were then transfected into a CD44 negative human T cell leukemia cell line, Jurkat. Stable transfectants were established and used to study interactions between CD44 and HA. Our results suggest that the transmembrane domain of CD44 is important in activation induced binding of HA. More specifically, cysteine 286 within the transmembrane domain, but not cysteine 295 in the cytoplasmic domain is critical for binding of HA.

## Materials and Methods

*Cell Line and Chemicals.* Jurkat is a human leukemia cell line originally obtained from American Type Culture Collection (ATCC, Rockville, MD). All tissue culture media, fetal calf serum, antibiotics, restriction enzymes, and hygromycin were obtained from GIBCO BRL (Gaithersburg, MD). Anti-CD44 monoclonal antibody F10-44-2 was obtained from Serotec, Harlan Bioproducts (Indianapolis, IN).

*Site Specific Mutagenesis and Transfection.* There are the only two Cys residues in the entire transmembrane and cytoplasmic domain of CD44. The Unique Selection Elimination method was used to generate CD44 mutant constructs (42). Codons for Cys 286 and Cys 295 were either changed to alanine individually or in combination. All mutants have been verified by sequencing using Sequenase version 2.0 DNA sequencing Kit from United States Biochemical Co. (Cleveland, OH).

Mutated and wild-type CD44 constructs were subcloned into pCEP-4 (kindly provided by Dr. Robert Peterson of the Institute of Pathology, CWRU) which contains the CMV promoter and the hygromycin resistance gene (43). The construct was transfected into Jurkat by electroporation. Briefly, ten million cells were suspended in 0.4 ml HeBS buffer. Then 20  $\mu$ g of DNA and 200  $\mu$ g of carrier DNA were added and pulsed at setting: 260V, 960  $\mu$ F. The cells were then cultured in RPMI, 10% FCS for 2 d before selection. The transfectants were selected with 1 mg/ml hygromycin. The expression of CD44 was verified by immunofluorescent staining with anti-CD44 mAbs and analyzed by FACS.

*Biotinylation, Immunoprecipitation and Western Blotting of CD44 Proteins.* Cells were surface labeled with a biotinylation procedure as described (44). Briefly, cells ( $10^7$ /ml) were incubated with sulfosuccinimidobiotin (Pierce Co., Rockford, IL) (0.1 mg/ml) in labeling buffer (150  $\mu$ M NaCl, 0.1 M Hepes, pH 8) for 30 min at room temperature. Cells were then washed with RPMI medium supplemented with 3% FCS and lysed with 1% Triton X-100 in 10 mM Tris, pH 7.5, 150 mM NaCl, 3 mM EDTA, 50 mM iodoacetamide, 0.1%  $\text{NaN}_3$ , 1 mM PMSF, 10  $\mu$ g/ml of soybean trypsin inhibitor, 1  $\mu$ g/ml leupeptin, and 1 U/ml of aprotinin for 1 h at 4°C. The detergent solubilized materials were recovered after centrifugation and precleared with an irrelevant antibody Sepharose 4B beads, followed by immunoprecipitation with an anti-CD44 mAb A3. After extensive washing, proteins were eluted from beads by boiling in reducing SDS sample buffer and analyzed by SDS-PAGE with a 7.5% gel. Separated proteins were then transferred to a nitrocellulose membrane. The membrane was blocked with 0.5% gelatin in PBS plus 0.05% Tween 20 and 0.05% thimerosal for 1 h at room temperature. After incubation membranes were incubated with 1/1,000 or 1/500 diluted streptavidin-

horseradish peroxidase in PBS containing 1% BSA and 0.05% Tween 20. The protein bands were detected using the enhanced chemiluminescence system (Amersham Corp., Arlington Heights, IL). Prestained molecular mass markers were used as standards.

**In Vitro Induction of Binding with anti-CD3 mAb.** Jurkat cells and Jurkat cells bearing different CD44 mutant proteins ( $5 \times 10^5$ /ml) were cultured in RPMI medium (RPMI supplemented with 10% FCS and 1% antibiotics). For activation of binding with anti-CD3 mAb, cells were cultured in 24- or 96-well tissue culture plates pre-coated with different concentrations of an anti-CD3 mAb (12F6). Cultures were placed in a 5% humidified CO<sub>2</sub> incubator for 16–18 h. After culture, cells were harvested and washed extensively before staining with mAb or F-HA.

**Immunofluorescence Staining and Hyaluronate Binding Assay.** Cultured cells were harvested and washed with washing medium (PBS supplemented with 5% New born calf serum, 0.1% NaN<sub>3</sub>, pH 7.4). Single cell suspension ( $1 \times 10^6$ /ml) was incubated with affinity-purified mAbs on ice for 45 min or an isotype control antibody. Cells were washed three times with washing medium and 25  $\mu$ l FITC-conjugated goat anti-mouse IgG antibody was added for 45 min on ice. Finally, samples were washed and fixed with 1% paraformaldehyde. FITC-HA was prepared by a modified conjugation protocol described earlier (45). Briefly, hyaluronic acid sodium salt (H-4015; Sigma Chemical Co., St. Louis, MO) was dissolved in 20% DMSO and coupled to fluorescein amine by using isocyanide. 25  $\mu$ l of FITC-HA (10  $\mu$ g/ml) was added into cell samples and incubated on ice for 45 min. All samples were washed and fixed as described. Cells were analyzed in a FACScan<sup>®</sup> (Becton Dickinson, San Jose, CA). At least 5,000 cells were analyzed per sample in all experiments. All experiments were done at least three times. In all experiments two controls are always included. One control is blocking of binding of F-HA with unconjugated HA, and the other control is blocking of binding with a anti-CD44 mAb A.3.

## Results

**Generation of Stable Jurkat Transfectants Expressing Either Wild-type Human CD44H Protein or Mutant CD44H Proteins.** In the transmembrane domain of human CD44, there is one cysteine residue in position 286 (Cys286). Another cysteine residue is in the cytoplasmic domain, in position 295 (Cys295). These two cysteines are the only two cysteines in the transmembrane and cytoplasmic domain of CD44. We replaced each of the cysteines residue individually with alanine (CD44.C286A and CD44.C295A) or replaced both of the cysteine residues with alanine (CD44.2C2A) by site-specific mutagenesis (Fig. 1 A). Constructs were then transfected individually into a CD44<sup>-</sup> human leukemia cell line, Jurkat. Stable transfectants were selected and characterized by immunofluorescent staining with anti-CD44 mAbs as described in Materials and Methods.

All mutant CD44 proteins were present on the cell surface of Jurkat transfectants (Fig. 1 B). The levels of CD44 protein expression were comparable in all three mutants. Therefore, the cysteine residues are not essential for the expression of CD44 on the cell surface, and palmitoylation of the CD44 molecule is not essential for membrane localization of CD44 protein. Western blotting was used to verify the molecular mass of wild-type CD44 and mutant CD44 proteins on Jurkat transfectants. Identical numbers of Jurkat

wild-type CD44 transfectant and CD44.C286A, CD44.C295A, and CD44.2C2A transfectants were surface biotinylated and immunoprecipitated with anti-CD44 mAb. Immunoprecipitated proteins were transferred to a nitrocellulose membrane and blotted with streptavidin-conjugated horseradish peroxidase as described in Materials and Methods. Similar to the wild-type CD44 protein, the three mutant CD44 proteins also had molecular masses of 75–85 kD. (Fig. 2). Therefore, alteration of the cysteine residues did not significantly change the overall posttranslational modifications of the CD44 proteins. No high molecular mass CD44 protein was detected in any of the transfectants indicating that CD44 was not modified with glycosaminoglycans in these cells.

**Cysteine 286 in the Transmembrane Domain but not Cysteine 295 in the Cytoplasmic Domain Is Important for Binding of F-HA.** Jurkat CD44 transfectants do not bind F-HA, however, binding of high levels of F-HA can be induced by immobilized anti-CD3 mAb. We used the three mutant CD44 transfectants, CD44.C286A, CD44.C295A, and CD44.2C2A to investigate whether the cysteine residues in position 286 and 295 are essential for anti-CD3-induced binding of F-HA. First, we established that all three mutant CD44 transfectants expressed similar levels of CD3 proteins on their surface, as compared to the wild-type CD44 transfectant by immunofluorescent staining with a anti-CD3 mAb. (Results not shown.) Second, we verified that CD3 signaling pathways in these three transfectants were functional. Immobilized anti-CD3 mAbs stimulated the production of IL-2 in all three transfectants (Liu, D., and M.S. Sy, manuscript in preparation). Experiments were then carried out to determine whether immobilized anti-CD3 mAb can induce binding of F-HA in CD44.C286A, CD44.C295A or CD44.2C2A transfectants. The parental CD44 negative Jurkat was used as a negative control, and the Jurkat wild-type CD44 transfectant was used as a positive control.

Parental CD44<sup>-</sup>Jurkat did not bind F-HA in all doses of anti-CD3 mAb tested. In contrast, anti-CD3 mAb induced high levels of binding in wild-type CD44 transfectants and in CD44.C295A transfectants in a dose-dependent manner (Fig. 3, A and B). In these two cell lines, significant levels of binding can be induced with as little as 1  $\mu$ g/ml of anti-CD3 mAb. However, the levels of binding observed in CD44.C286A and CD44.2C2A transfectants were significantly reduced in all doses of anti-CD3 mAb tested. The difference was most dramatic when a lower dose of anti-CD3 mAb was used. 1  $\mu$ g/ml of anti-CD3 was unable to stimulate binding of HA in CD44.C286A and CD44.2C2A transfectants. The inability of CD44.C286A to bind F-HA after stimulation with anti-CD3 was confirmed with another independently isolated CD44.C286A transfectant. Binding of F-HA to Jurkat CD44 transfectant is specific for CD44 and hyaluronic acid because binding can be completely inhibited with unconjugated hyaluronic acid or with a monoclonal anti-CD44 antibody A.3. (16 and results not shown).

Activation of Jurkat CD44 transfectants with anti-CD3 upregulated the expression of CD44 proteins. The failure of anti-CD3 to induce binding in CD44.C286A transfec-

**A**

270      280      290      300      361

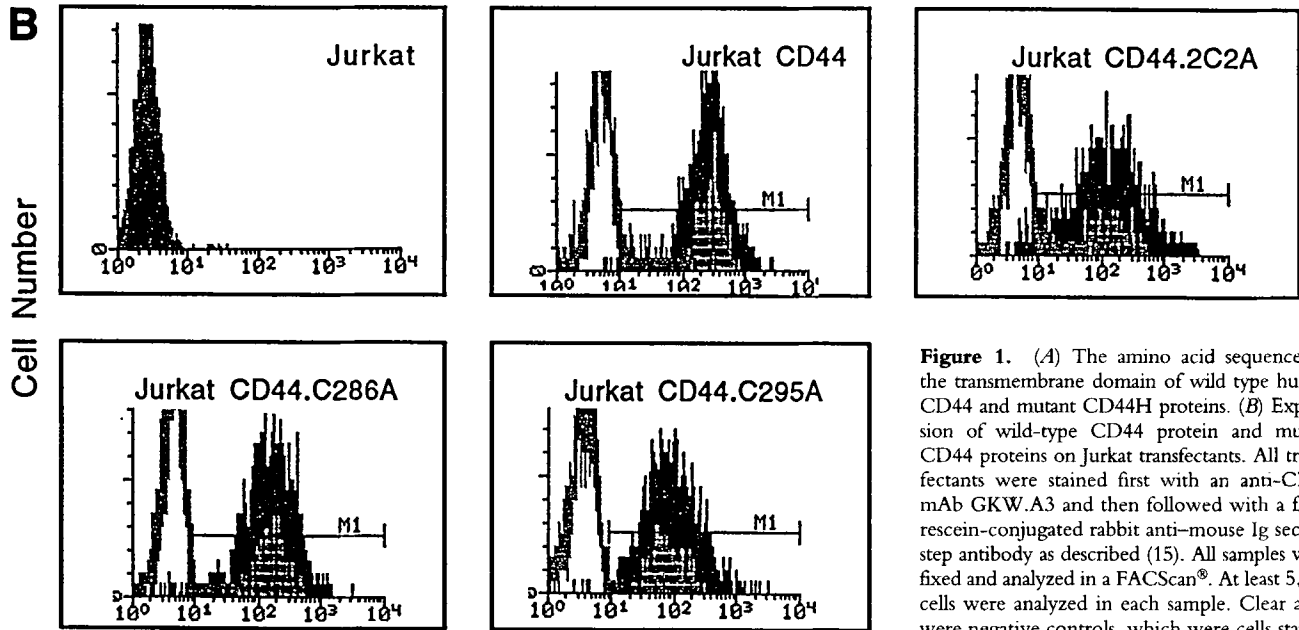
CD44    WL IILASLLALA IILAV C JAVN SRRR C GQKKK LVINGSG— DMKIG V.

          — Transmembrane domain—

CD44.C286A WL IILASLLALA IILAV A JAVN SRRR C GQKKK LVINGSG— DMKIG V.

CD44.C295A WL IILASLLALA IILAV C JAVN SRRR A GQKKK LVINGSG— DMKIG V.

CD44.2C2A WL IILASLLALA IILAV A JAVN SRRR A GQKKK LVINGSG— DMKIG V.



**Figure 1.** (A) The amino acid sequences of the transmembrane domain of wild type human CD44 and mutant CD44H proteins. (B) Expression of wild-type CD44 protein and mutant CD44 proteins on Jurkat transfectants. All transfectants were stained first with an anti-CD44 mAb GKW.A3 and then followed with a fluorescein-conjugated rabbit anti-mouse Ig second step antibody as described (15). All samples were fixed and analyzed in a FACScan<sup>®</sup>. At least 5,000 cells were analyzed in each sample. Clear areas were negative controls, which were cells stained with an irrelevant, isotype matched monoclonal antibody. F.I. = fluorescein intensity.

F.I.

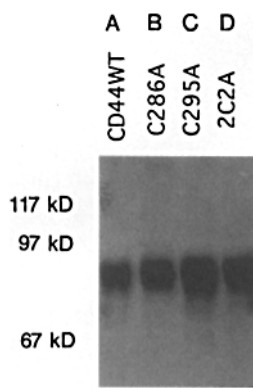
tant was not due to the lack of enhancement of CD44 expression on CD44.C286A transfectant. Culture of CD44.C286A and CD44.C295A with anti-CD3 mAb enhanced the expression of CD44 proteins to a similar degree (Fig. 4). Nevertheless, only CD44.C295A bound high levels of F-HA. Therefore, upregulation of CD44 expression by itself is insufficient to warrant binding of HA. Furthermore, anti-CD3 did not induce detectable cell surface CD44 on CD44 negative parental Jurkat T cells or Jurkat T cells transfected with a hygromycin-resistant gene without the CD44 sequence (Jurkat/pCEP) (Fig. 4). Therefore, anti-CD3 did not induce the expression of endogenous CD44 gene in Jurkat cells.

*Induction of Binding with Monoclonal Anti-CD44 Antibody.* Binding of F-HA can be induced by some anti-CD44 mAbs (17, 18). In contrast to activation-induced binding, anti-CD44 antibody-induced binding occurs rapidly and does not require in vitro culture. Induction of binding by anti-CD44 mAb may simply involve aggregation of CD44 molecules on the cell surface by the bound monoclonal antibody. We investigated whether one of these anti-CD44 antibodies, F10-44-2 induces binding of F-HA in Jurkat wild-type CD44 transfectant and in mutant CD44 transfectants. First, we established that Jurkat wild-type CD44, CD44.C286A and CD44.C295A transfectants expressed comparable levels of the epitope bound by F10-44-2. (Fig. 5 A). Incubation of Jurkat wild-type CD44 transfectant and

CD44.C295A transfectants with F10-44-2 resulted in significant levels of binding (Fig. 5 B). Surprisingly, F10-44-2 was unable to induce any detectable binding in CD44.C286A transfectant (Fig. 5 B). No binding was detected even when higher concentrations of F10-44-2 were added. (results not shown). These results provided evidence that similar to anti-CD3-induced binding, Cys286 in the transmembrane domain is also important in anti-CD44 mAb-induced binding. Therefore, induction of binding by anti-CD44 mAb is not a passive aggregation event but a cellular response, requiring the presence of the Cys 286 residue in the transmembrane domain.

## Discussion

Jurkat CD44 transfectants do not constitutively bind F-HA. However, binding can be induced by immobilized anti-CD3 mAb, PMA or a monoclonal anti-CD44 antibody (18). In this manuscript we provide evidence that a cysteine residue located in the transmembrane domain of CD44 is essential for both anti-CD3- and anti-CD44-induced binding of F-HA. The reasons why activation is required for binding of F-HA in some cells are not clear. Activation with anti-CD3 enhanced the expression of CD44 protein. However, enhancement of CD44 expression by itself is insufficient to allow binding. Treatment of Jurkat CD44.C286A



**Figure 2.** Western blotting of wild-type CD44 protein and mutant CD44 proteins on Jurkat Transfectants. Transfectants were first biotinylated and immunoprecipitated with anti-CD44 mAb. Immunoprecipitated proteins were then transferred to nitrocellulose membrane. Western blottings were carried out as described in Materials and Methods. Lane *A* was from the wild-type CD44 transfectant; lane *B* was from the CD44.C286A transfectant; lane *C* was from the CD44.C295A transfectant; lane *D* was from the CD44.2C2A transfectant. Molecular masses are marked on the left.

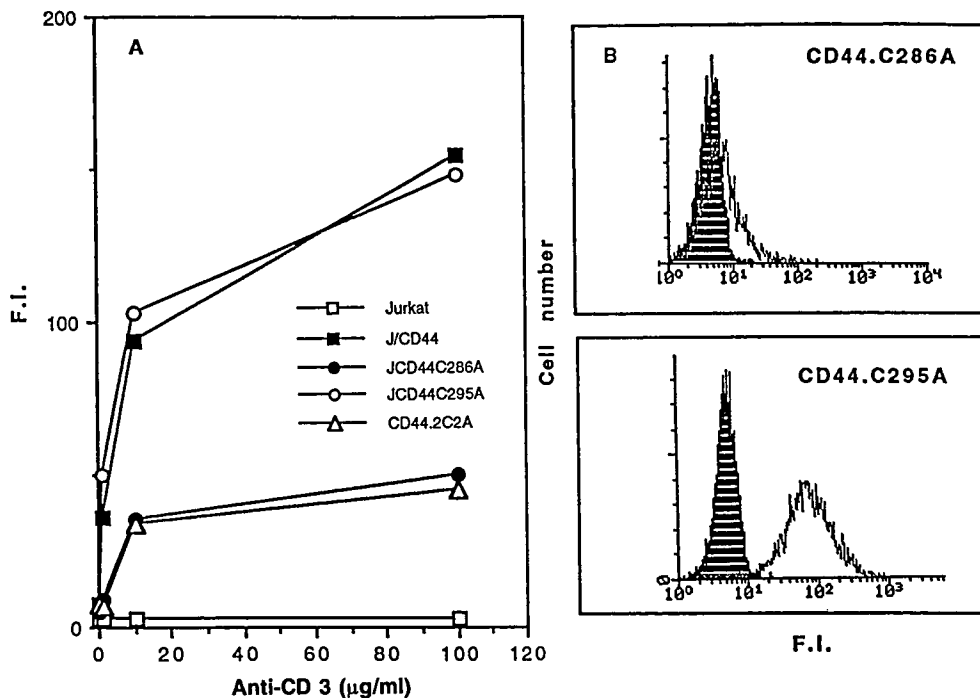
mutant transfectants with anti-CD3 mAb also increased the expression of CD44, to a level comparable to wild-type CD44-bearing cells. Nevertheless, CD44.C286A mutants bound less F-HA than wild-type CD44 Jurkat transfectants or Jurkat CD44.C295A transfectants.

Since all the CD44 constructs used in our studies are placed under the control of CMV promoter, the molecular mechanisms responsible for the upregulation of CD44 expression in these Jurkat transfectants are not known. In addition to anti-CD3 treatment, PMA treatment upregulated the expression of CD44 on Jurkat transfectants (18). Since activation with anti-CD3 mAb did not induce the expression of the endogenous CD44 gene, therefore, the binding studies described here can be attributed solely to the transfected, and expressed CD 44 gene products.

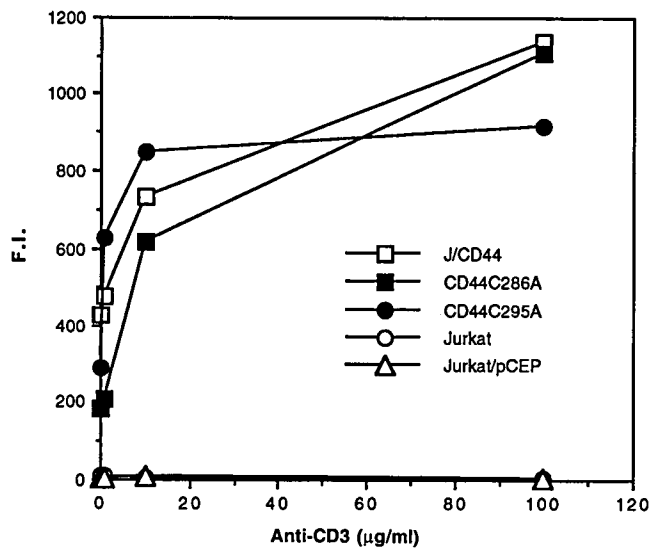
When stimulated with a sub-optimal anti-CD3 concentration (1  $\mu\text{g/ml}$ ), CD44.C286A transfectants were completely inactive. Low levels of binding of F-HA can be in-

duced in CD44.C286A and CD44.2C2A transfectants with higher doses of anti-CD3 mAb. Some other mechanisms could compensate for the lack of Cys 286 when the cells were stimulated with high doses of anti-CD3. The failure of anti-CD3 mAb to induce binding on CD44.C286A transfectants was not because these transfectants did not respond to anti-CD3 mAb. Jurkat CD44.C286A transfectant and Jurkat wild-type CD44 transfectants expressed comparable levels of CD3 complex. Anti-CD3 mAb induced the production of IL-2 by CD44.C286A transfectants, indicating that all the necessary CD3 signal transduction components are present in CD44.C286A transfectants. CD44.C286A and wild-type CD44 transfectants reacted equally to four different anti-CD44 mAb (results not shown). Therefore, simply replacing the Cys286 residue did not significantly alter the overall conformation of the protein.

Glycosylation of CD44 is important in the binding of F-HA. Treatment of cells with tunicamycin enhanced the binding of F-HA and also significantly reduced the molecular mass of CD44 protein. (16, 20, 21). Western blotting of CD44 proteins from wild-type CD44 and CD44.C286A mutant transfectants failed to reveal any obvious change in the molecular size of these two proteins. Furthermore, immunoprecipitation followed by two-dimensional gel electrophoresis also failed to detect any significant changes in the pI of CD44.C286A protein (results not shown). Therefore, posttranslational events like N-linked and O-linked glycosylation were not grossly disrupted in CD44.C286A mutants. A more extensive biochemical study is required to rule out the possibility that minor changes in the glycosylation of CD44 may occur in CD44.C286A protein. Alteration of the cysteine residue in the transmembrane domain may also influence the stability of cell surface CD44, which

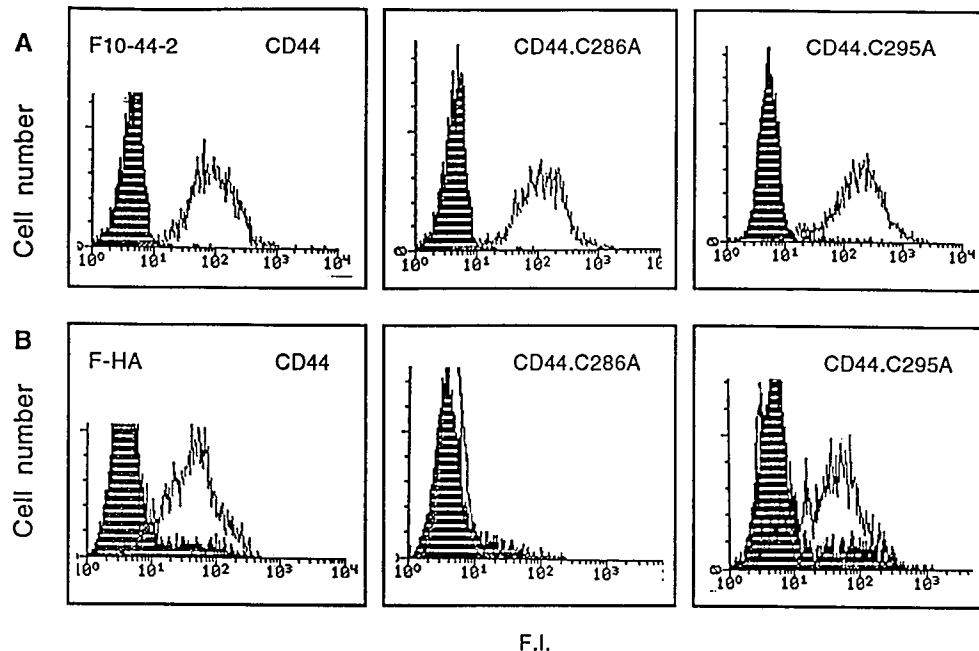


**Figure 3.** Anti-CD3 mAb does not stimulate high levels of binding in CD44.C286A transfectant. (A) Parental CD44<sup>-</sup> Jurkat, Jurkat wild-type CD44 transfectant and Jurkat CD44 mutant transfectants were cultured *in vitro* in 24-well tissue culture plates pre-coated overnight with different concentrations of purified monoclonal anti-CD3 antibody (12F6) for 18 h. After culture, cells were harvested, washed extensively, and then stained with F-HA as described (15). All samples were fixed and analyzed in a FACScan<sup>®</sup>. At least 5,000 cells were analyzed in each sample. (B) A representative histogram of binding of F-HA on Jurkat CD44.C286A and Jurkat CD44.C295A transfectants after *in vitro* stimulation with 10  $\mu\text{g/ml}$  of immobilized anti-CD3 mAb. Shaded areas were negative controls, which were cells first incubated with unconjugated HA and then stained with F-HA.



**Figure 4.** Anti-CD3 stimulation upregulates the expression of CD44 on Jurkat CD44, CD44.C286A, and CD44.C295A transfectants but not on Jurkat or Jurkat/pCEP. Jurkat CD44 transfectants or CD44 negative parental Jurkat or Jurkat T cells transfected with a hygromycin-resistant gene (CD44/pCEP) were cultured *in vitro* in 24-well tissue culture plates precoated overnight with different concentrations of purified monoclonal anti-CD3 antibody (12F6) for 18 h. After culture, cells were harvested, washed extensively, and then stained with an anti-CD44 mAb (GKW.A3) as described (15). All samples were fixed and analyzed in a FACScan®. At least 5,000 cells were analyzed in each sample.

may be critical for binding of F-HA. We ruled out this possibility by carrying metabolic labeling and pulse chase experiments. The half-life of wild-type CD44 protein on Jurkat CD44 transfectant is ~18–24 h. Replacing the Cys 286 residue did not alter the half life of CD44.C286A molecule on the cell surface (results not shown).



**Figure 5.** F10-44-2 can not induce binding in Jurkat CD44.C286A transfectant. Jurkat transfectants were harvested, washed extensively, and then counted. Equal numbers of each transfectant were divided into two microfuge tubes. 10 µl of anti-CD44 mAb (F.10-44-2; Serotec) were added to each tube and incubated on ice for 20 min. After incubation, excess antibodies were removed by washing extensively with PBS. One of the tubes was used to monitor the amount of F10-44-2 bound by adding a fluorescein-conjugated rabbit anti-mouse Ig antibody. The other tube was used to monitor the degree of binding of HA by adding F-HA. All samples were fixed and analyzed in a FACScan®. At least 5,000 cells were analyzed in each sample. Shaded areas were negative controls, which were cells first incubated with unconjugated HA and then stained with F-HA.

CD44 protein is normally present on the cell surface as a 80–95 kD monomer. The  $\zeta$  chain of murine CD3 complex exists as disulfide bonded homodimer on the cell surface. Artificial replacement of the transmembrane domain of murine CD44 with the transmembrane domain of  $\zeta$  chain of CD3 resulted in the dimerization of CD44 proteins, and binding of HA (29). Therefore, it is possible that activation by anti-CD3 mAb may induce homo-dimerization CD44 or hetero-dimerization of CD44 with another protein via Cys286. Dimerization of CD44 via Cys286 will then permit binding of HA, a situation similar to the artificial replacement of the transmembrane domain of CD44 with the transmembrane domain of the  $\zeta$  chain of CD3. Dimerization of CD44 can not occur in CD44.C286A transfectants, therefore, the cell remains unable to bind HA. We have obtained evidence that cysteine 286 in the transmembrane domain of CD44 is involved in the activation induced dimerization of CD44, and dimerization of CD44 is one of the critical events essential for binding of F-HA. (Liu, D., manuscript in preparation).

Our observation that cysteine 286 in the transmembrane domain of CD44 is essential for dimerization of CD44 raise an interesting question concerning the role of CD44 palmitoylation in the biology of CD44 molecule, and the relationship between CD44 palmitoylation and dimerization. Cysteine 286 and cysteine 295 are the only two potential site for palmitoylation in CD44. Palmitoylation of isolated murine CD44 protein from the thymoma, BW5147, has been reported to be important for binding of CD44 to ankyrin *in vitro* (26). Interactions between CD44 and ankyrin has also been reported to be essential for binding of F-HA (31). However, since Jurkat CD44.C295A transfectant can bind F-HA when stimulated with anti-CD3, therefore, palmitoylation of CD44 at cysteine 295 is not essential for binding of F-HA. It should be noted that BW5147 can consti-

tutively bind low levels F-HA, while binding in Jurkat CD44 transfectants require cellular activation. Therefore, constitutive binding of low levels of F-HA and activation-induced binding of high levels F-HA may have different cellular requirements.

In addition to the transmembrane domain, the cytoplasmic domain of CD44 is also important in the binding of F-HA (reference 19 and Liu, D., manuscript submitted for publication). Recently, we found that induction of binding in Jurkat CD44 transfectant was inhibited by cytochalasin D, an inhibitor of microtubular function (Liu, D., manuscript submitted for publication). Activation of human T cells by immobilized anti-CD3 mAb has been reported to enhance the interactions between CD44 and cytoskeletal proteins (46). Activation of Jurkat cells with anti-CD3 mAb also caused the reorganization of the cytoskeletal proteins (47). These interactions may facilitate the clustering of CD44 on the cell surface, a condition critical for binding of F-HA (34, 35).

Binding of HA can be induced by a unique group of anti-CD44 mAb (18, 19). We confirmed these findings with monoclonal anti-CD44 antibody, F10-44-2. F10-44-2 induced binding in all five Jurkat wild-type CD44 transfectants and in CD44.C295A transfectants. The levels of binding induced by anti-F44.10 were lower than the levels of binding induced with anti-CD3. Therefore, aggregation of CD44 on the cell surface may enable the cell to bind low levels of HA, but cellular activation is essential for binding of higher levels of HA. The reason why F10-44-2 could not induce binding in CD44.C286A was not because CD44.C286A bound less F10-44-2 antibody. Both wild-

type CD44 transfectant and CD44.C286A transfectants express comparable levels of F44-10-2 epitopes as demonstrated by immunofluorescent staining and FACS analysis. Therefore, antibody-induced binding is more complex than simply binding of antibody to the cell surface and cross-linking of CD44 molecules on the cell surface. This interpretation was further supported by our recent findings that antibody-induced binding was also inhibited by cytochalasin D and requires the cytoplasmic domain of CD44 (Liu, D., manuscript submitted for publication).

The results presented in this paper provide new evidence that interactions between CD44H on the cell surface and soluble HA is a complex and dynamic process. More specifically, the cysteine residue in the transmembrane domain is critical for the binding of HA. We hypothesize that in order for a cell to bind high levels of HA several cellular interactions must be achieved. These interactions may involve interactions between CD44 and CD44 or CD44 and other cytoplasmic or membrane proteins. In the absence of Cys286, one or more of these interactions may be impaired, resulting in the failure of CD44 to bind high levels HA. Binding of F-HA to cell surface provides a quick, reproducible and quantitative assay for studying interactions between CD44 and HA. However, the conformation of HA in solution may be different from HA in extracellular matrix. Therefore, our conclusions apply only to the interactions between CD44 and HA in solution. Moreover, in addition to HA, CD44 also interacts with other ligands. Whether mutations that affect interactions between CD44 and HA also affect interactions between CD44 and these other ligands is not known.

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Address correspondence to M.S. Sy, Room 933, Biomedical Research Bldg., Case Western Reserve University, School of Medicine, 10900 Euclid Ave., Cleveland, OH 44106-4943.

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