Ankyrin-binding Domain of CD44(GP85) Is Required for the Expression of Hyaluronic Acid-mediated Adhesion Function

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Abstract. GP85 is one of the most common hematopoietic isoforms of the cell adhesion molecule, CD44. CD44(GP85) is known to contain at least one ankyrin-binding site within its 70 aa cytoplasmic domain and to bind hyaluronic acid (HA) with its extracellular domain. In this study we have mapped the ankyrin-binding domain of CD44(GP85) by deleting various portions of the cytoplasmic region followed by expression of these truncated cDNAs in COS cells. The results of these experiments indicate that the ankyrin-binding domain resides between amino acids 305 and 355. Biochemical analyses, using competition binding assays and a synthetic peptide (NGGNGTVEDRKPSEL) containing 15 aa between aa 305 and aa 320, support the conclusion that this region is required for ankyrin binding. Furthermore, we have constructed a fusion protein in which this 15 aa sequence of CD44(GP85) is transplanted onto another transmembrane protein which does not bind ankyrin. Our results show that this fusion protein acquires the ability to bind ankyrin confirming that the sequence (NGGNGTVEDRKPSEL) is a critical part of the ankyrin-binding domain of CD44(GP85). In addition, we have demonstrated that deletion of this 15 aa ankyrin-binding sequence from CD44(GP85) results in a drastic reduction (>90%) of HA-binding and HA-mediated cell adhesion. These findings strongly suggest that ankyrin binding to the cytoplasmic domain of CD44(GP85) plays a pivotal role in regulating hyaluronic acid-mediated cell-cell and cell-extracellular matrix interactions.

The 85-kD mouse lymphocyte transmembrane glycoprotein, GP85 (also known as Pgp-1), is a well known T-cell differentiation antigen (66). The cDNA sequence data indicate that mouse GP85 shares 72% aa homology with human lymphocyte homing receptor CD44 (also called GP90Hc-antigen, ECMR III, and homing cellular adhesion molecule [H-CAM]) (6, 18, 19, 34, 35, 51, 69, 70). GP85 is one of the most common isoforms of CD44 found in all hematopoietic cells including T-cells, B-cells, macrophages, and granulocytes (33, 42, 66). CD44(GP85) has also been detected in many other cell types such as fibroblasts, epithelial cells, and more recently endothelial cells (14, 17, 52). To date at least 15 isoforms of CD44 have been identified. Some of these isoforms result from extensive, alternative exon splicing events (35, 57, 65).

CD44(GP85) has been shown to mediate the binding between lymphocytes and capillary high endothelial venules (HEV) during lymphocyte homing into lymphoid organs (6, 52). In addition, CD44(GP85) is involved in T- and B-cell adhesion, cell aggregation and proliferation (30, 59). Although the functional role of CD44 is not fully understood, certain isoforms have been implicated recently in tumor metastasis (1, 2, 29, 31, 60).

The extracellular matrix component, hyaluronic acid (HA), is one of the ligands specifically recognized by CD44-(GP85) (19, 32, 40, 54, 58, 67). CD44(GP85) mediates HA-dependent cell adhesion in many cell types including leukocytes, fibroblasts, and macrophages (67). HA-dependent cell adhesion plays an important role in mediating (a) interaction between stromal cells and lymphoid precursor cells in the bone marrow, (b) cell migration, and, (c) most likely, T-cell activation and B-cell maturation (19, 22, 30, 48, 63, 67). Recent studies suggest that certain factors, such as protein kinase C (40) and the cytoplasmic domain of CD44-(GP85) (41), may be important for the expression of HA-binding site(s). However, the molecular mechanisms involved in regulating the surface expression of HA-binding site(s) are not known.

Previously, we have demonstrated that the cytoplasmic domain of CD44(GP85) (~70 aa long) that is conserved >90% in most of the CD44 isoforms, is involved in ankyrin binding (16, 37). The ankyrin-binding site(s) is expressed at a very early stage in the biosynthesis of CD44(GP85) (43). Furthermore, the binding interaction between CD44(GP85) and ankyrin is highly specific and regulated by several factors in-
including protein kinase C-mediated phosphorylation (38), palmitoylation (12), and GTP binding (45). The fact that ankyrin preferentially accumulates underneath CD44(GP85) complex is related to ligand-induced lymphocyte activation (16, 37). However, the physiological significance of this close association between ankyrin and CD44(GP85) during HA-mediated cell–matrix adhesion is not fully understood.

In this manuscript, we have mapped the ankyrin-binding domain of CD44(GP85) by constructing several cytoplasmic deletion mutants and expressing them in COS cells. Our data indicate that deletion of a particular 15 aa sequence in the cytoplasmic domain of CD44(GP85) leads to a complete loss of ankyrin binding. Most interestingly, however, the deletion of ankyrin-binding domain also abolishes the HA-binding/HA-dependent cell adhesion capability of CD44(GP85) without significantly affecting its overall cell surface expression. Therefore, binding of ankyrin to cytoplasmic domain of CD44(GP85) may be critically important for the proper surface expression of HA-mediated adhesion in cells containing CD44(GP85).

Materials and Methods

Reagents

Rc/CMV plasmid containing the full-length mouse CD44(GP85/Pgp-1) cDNA clone was kindly provided by Dr. Eugene Butcher (Stanford University, CA). Mouse CD45(B 200; 64) cDNA was a gift from Dr. Ian Towbridge (Salk Institute, La Jolla, CA); pcDNA I was obtained from Invitrogen Co. An adaptor containing termination codons in all of the three possible reading frames with a Not I cohesive end was synthesized by the DNA facility (University of Miami, Miami, FL). Erythrocyte ankyrin, spectrin, pig brain fodrin, and 3H-HA were prepared as described previously (5, 28, 68). Rat anti-CD44(GP85) monoclonal antibody IM7, which recognizes an epitope in the extracellular domain of CD44(GP85), and rat anti-CD45 monoclonal antibody 13/2 (kindly provided by Dr. Robert Hyman, Salk Institute) were purified from the hybridoma culture supernatant by sequential ammonium sulfate fractionation and DEAE-cellulose chromatography. Anti-CD44(GP85) antibody, anti-CD45 antibody, ankyrin, and WGA (Sigma Chem. Co., St. Louis, MO) were coupled to CNBr-activated Sepharose beads (Pharmacia LKB Biotechnology, Piscataway, NJ) according to the manufacturer's procedure. The region I and scramble peptides (described below) were synthesized by Dr. Eric Smith (University of Miami, Miami, FL).

Cell Culture

COS-7 cells were obtained from Amer. Type Culture Collection (Rockville, MD) and grown routinely in DMEM containing 10% FBS, 1% glutamine, 1% penicillin, and 1% streptomycin. Mouse T-lymphoma BW5147 cells were cultured in DMEM containing 10% horse serum, 1% pipenillin, and 0.3% BSA at 4°C for 2 h. The discs were then incubated with various concentrations (10--400 ng/ml) of 125I-labeled ankyrin (5,000 cpm/ng protein) at 4°C for 5 h. Non-specific binding was ~30% of the total binding. As controls, the non-specific binding was determined in the presence of a 100-fold excess of respective unlabeled ligands and was subtracted from the total binding. Non-specific binding was ~20% of the total binding.

Binding of 125I-labeled Ankyrin to CD44(GP85) Proteins

Aliquots (10--20 ng protein) of both purified wild-type and mutant CD44(GP85) proteins bound to the anti-CD44(GP85) immunoaffinity chromatography were determined by restriction enzyme analyses and DNA sequencing. The DNA was further cut with Hind III without blunt ending and digested directly into Hind III–BamHI digested pcDNA I to generate the CD44(GP85)A305 mutant construct. The full-length 1.3-kb CD(GP85) construct was also recloned into pcDNA I as a Hind III–Not I fragment. All mutant constructs were characterized by restriction enzyme analyses and DNA sequencing.

COS Cell Transfection

COS-7 cells were transfected with pcDNA I plasmids containing various CD44(GP85) inserts using DEAE-dextran. Briefly, COS-7 cells were plated at a density of 2 × 106 cells per 100-mm dish and were transfected with 25 μg/dish plasmid DNA. Transfected cells were harvested after 48 h for analyses of CD44(GP85) expression.

Purification of CD44(GP85) Protein from Transfected COS Cells

Various CD44(GP85) proteins were purified from 8--10 100-mm dishes of COS cells transfected with one of the CD44(GP85) constructs. Control transfections consisted of either no DNA or pcDNA I vector DNA. The proteins were purified from either unlabeled or surface 125I-labeled COS cells using non-ionic detergent Triton X-100 extraction followed by sequential WGA-Sepharose and anti-CD44(GP85) immunoaffinity chromatographies, essentially according to the procedure described previously (43, 45). Protein concentrations were determined using the protein assay reagent (BioRad Labs., Hercules, CA). Purity of the protein preparations was determined by SDS-PAGE followed by silver staining and/or autoradiography.

Binding of 123I-labeled Ankyrin to Synthetic Region I and Scramble Peptides

Nitrocellulose discs (1-cm diam) were coated with μl of either the region I (GGNGTVEEDRKPSEL) or a scramble peptide (GRNETNPEGSGL-DVK) at 4°C for 16 h. After coating, the unoccupied sites on the discs were blocked by incubation with a solution containing 20 mM Tris.HCl pH 7.4 and 0.3% BSA at 4°C for 2 h. The discs were then incubated with various concentrations (20, 40, and 80 ng/ml) of 123I-labeled ankyrin (5,000 cpm/ng protein) at 4°C for 5 h. Non-specific binding was determined in presence of a 50-100-fold excess of unlabeled ankyrin and also by incubating the anti-CD44(GP85) immunobeads alone in the presence of the same concentration of 123I-labeled ankyrin. After binding, the immunobeads were washed extensively in binding buffer and the bead-bound radioactivity was counted. Non-specific binding was ~20% of the total binding.

Binding of 123I-labeled Ankyrin/Fodrin/Spectrin to Synthetic Region I and Scramble Peptides

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ried out in the presence or absence of various concentrations (1 nM-1/~M) of unlabeled competitive synthetic region I peptide (NGGNGTVEDRKP-SEL) or the scramble peptide (GRNETNPEGSGLDVK) at 4°C for 5 h under equilibrium conditions. Equilibrium conditions were determined by incubating with unlabeled competing synthetic region I peptide (NGGNGTVEDRKP-SEL) or the scramble peptide (GRNETNPEGSGLDVK) at 4°C for 5 h un-

fragment to ankyrin
Binding of 125I-labeled Band 3 Cytoplasmic Domain Fragment to Ankyrin

The human band 3 protein cytoplasmic domain fragment (=43 kD) that binds ankyrin, was isolated from the erythrocyte ghost as described previously (5). The band 3 protein fragment was 125I-labeled to a specific activity of 6 x 106 cpm/ng. The binding of the 125I-labeled band 3 fragment (=0.5 nM) to ankyrin in the presence or absence of region I or the scramble peptides was carried out as described above.

Construction and Expression of CD45Δ826/CD44
(Reg. I) Fusion Protein

The mouse CD45(B200) cDNA cloned in pcDNA I was partially digested with Kpn I, followed by complete digestion with Xho I. An oligonucleotide adaptor (5'CAACGGGCGGGTATGGGACAGCAAACCCAGTGAGCTTAA3') and its complementary strand were synthesized by DNA facility, University of Miami, Miami, FL. The adaptor codes for mouse CD45(B200) sequence corresponding to the 15 aa of the N-terminus of the ankyrin-binding domain (69, 70), a stop codon and Xho I cohesive ends. The adaptor was annealed to its complementary strand and cloned into Kpn I-Xho I digested CD45(B200)/pcDNA I plasmid. The resulting construct, CD45Δ826/CD44(Reg.I) contains 826 aa of CD45 and 15 aa of the region I of the ankyrin-binding domain. As a control, CD45-

CD44(GP85) Cytoplasmic Domain Deletion Mutants and Their Expression in COS Cells

To map the ankyrin-binding domain in CD44(GP85), we have constructed four mutants of CD44(GP85) (designated CD44[G(P85)]Δ355, CD44[G(P85)]Δ320, CD44[G(P85)]Δ305, and CD44[G(P85)]Δ292) in which various portions of the cytoplasmic domain of CD44(GP85) have been deleted from the carboxyl terminus (Fig. 1). Specifically, CD44[G(P85)]-363 (the wild-type containing full length cDNA with no deletion), CD44[G(P85)]Δ355 (a mutant cDNA with 8 aa deletion), CD44[G(P85)]Δ320 (a mutant cDNA with 43 aa deletion), CD44[G(P85)]Δ305 (a mutant cDNA with 58 aa deletion), and CD44[G(P85)]Δ292 (a mutant cDNA with 71 aa deletion) constructs encode for proteins consisting of 363, 355, 320, 305, and 292 aa, respectively. Subsequently, the CD44[G(P85)] wild-type and various mutant cDNAs were cloned into pcDNA I vector (Fig. 1) followed by expression in COS cells.

To detect surface expression of CD44(GP85), we have performed surface iodination of COS cells (transfected with CD44(GP85) wild-type and various mutant cDNAs) followed by Triton X-100 extraction and sequential WGA-Sepharose and anti-CD44(GP85) column chromatographies. Our results clearly indicate that the COS cells transfected with the wild-type CD44(GP85)363 cDNA express a surface protein of 85 kD, whose molecular mass is the same as that of the most common hemopoietic isoform, GP85 (Fig. 2, lane 3). Furthermore, we have found that the various truncated CD44(GP85) proteins are also expressed on the surface of the COS cells (Fig. 2, lanes 4-7). Most importantly, the apparent molecular mass of the CD44(GP85) mutant proteins expressed on the COS cells appear to correspond very well with the molecular mass predicted by deletion mutation.
Figure 1. A schematic illustration of the in vitro mutagenesis approach used in this study. The four cytoplasmic deletion mutants of CD44(GP85) were constructed according to the strategy described in the Materials and Methods. These constructs including CD44(GP85)363 (the wild-type containing full-length cDNA with no deletion), CD44(GP85)Δ355 (a mutant cDNA with 8 aa deletion), CD44(GP85)Δ320 (a mutant cDNA with 43 aa deletion), CD44(GP85)Δ305 (a mutant cDNA with 58 aa deletion), and CD44(GP85)Δ292 (a mutant cDNA with 71 aa deletion) were then subcloned into pcDNA I and transfected into COS cells to express proteins that encode 363, 355, 320, 305, and 292 aa, respectively.

As expected, a consistent size reduction of CD44(GP85) protein becomes detectable in the deletion mutants (e.g., CD44(GP85)Δ355 [Fig. 2, lane 4], CD44(GP85)Δ320 [Fig. 2, lane 5], CD44(GP85)Δ305 [Fig. 2, lane 6], and CD44(GP85)Δ292 [Fig. 2, lane 7]) which express proteins of ~84, 80, 78, and 76 kD, respectively. Surface expression of the CD44(GP85) protein on COS cells transfected with CD44(GP85) wild-type and mutant cDNAs appears to be specific since control samples (COS cells either untransfected [Fig. 2, lane 1] or transfected with pcDNA I vector alone [Fig. 2, lane 2]) reveal no surface expression of CD44(GP85).

In addition, the isolation procedure used in this study (i.e., Triton X-100 extraction plus sequential WGA-Sepharose, anti-CD44(GP85) column chromatographies) allows us to obtain pure CD44(GP85) protein from COS cells transfected with various cDNAs. For example, COS cells transfected with CD44(GP85)Δ320 cDNA reveal a single CD44(GP85) protein as shown by silver staining (Fig. 2, lane 8). The same purification procedure was used to obtain purified CD44(GP85) proteins from COS cells transfected with wild-type and the other three truncated CD44(GP85) cDNAs (data not shown). The availability of purified CD44(GP85) proteins from COS cells transfected with wild-type or mutant cDNAs has allowed us to perform in vitro ankyrin-binding assays for the purpose of mapping the ankyrin-binding domain of CD44(GP85).

Mapping the Ankyrin-binding Domain of CD44(GP85)

We have shown previously that the cytoplasmic domain of CD44(GP85) is involved in the interaction with ankyrin, both in vivo and in vitro (16, 37). Several posttranslational modifications appear to be required for effective CD44(GP85)-ankyrin binding (12, 38, 45). In this study we have used an in vitro binding assay to determine the effect of the four cytoplasmic domain deletions on the ability of CD44(GP85) to bind ankyrin in order to better define the ankyrin-binding domain of CD44(GP85). Specifically, purified CD44(GP85) proteins purified from COS cells transfected with wild-type and mutant cDNAs were used in an in vitro binding assay to determine the effect of the four cytoplasmic domain deletions on the ability of CD44(GP85) to bind ankyrin in order to better define the ankyrin-binding domain of CD44(GP85). Specifically, purified CD44(GP85) proteins were analyzed by SDS-PAGE followed by autoradiography or silver staining. Lanes 1-7 represent autoradiograms of proteins purified from surface 125I-labeled COS cells. (Lane 1) Untransfected control; (lane 2) pcDNA I vector alone; (lane 3) CD44(GP85)363; (lane 4) CD44(GP85)Δ355; (lane 5) CD44(GP85)Δ320; (lane 6) CD44(GP85)Δ305; (lane 7) CD44(GP85)Δ292. (lane 8) Silver staining of mutant CD44(GP85)Δ320 protein purified from unlabeled COS cells transfected with CD44(GP85)Δ320 cDNA.
Fig. 3 A, ankyrin binds specifically to both CD44(GP85) proteins, isolated from COS cells transfected with CD44(GP85) wild-type and various mutant cDNAs, were incubated with various concentrations of 125I-labeled ankyrin under equilibrium-binding conditions. As shown in Fig. 3 A, ankyrin binds specifically to both CD44(GP85) proteins, isolated from COS cells transfected with CD44(GP85) wild-type (Fig. 3 A, a) and CD44(GP85)Δ355 (mutant with an 8 aa deletion) (Fig. 3 A, b) proteins in a dose-dependent and saturable manner. Scatchard plot analyses of the equilibrium-binding isotherms shown in Fig. 3 A indicate that ankyrin binds to these proteins at a single site (Fig. 3 B, a and b) with high affinity (an apparent dissociation constant [Kd] of ≈1 nM) (Fig. 3 B, a and b) similar to that obtained for the mouse lymphoma GP85 protein (13). These results indicate that the COOH-terminal 8 cytoplasmic aa may be deleted without affecting ankyrin binding. An additional deletion of the sequence between aa 320 and 355 (i.e., CD44(GP85)Δ320 [mutant with a 43 aa deletion]) causes a slight reduction in binding of 125I-labeled ankyrin compared to the wild-type, CD44(GP85)Δ363, or the mutant, CD44(GP85)Δ355 proteins (Fig. 3 A, c). This mutant protein also binds ankyrin with a slightly lower affinity (Kd ≈2.6 nM) (Fig. 3 B, c). These results suggest that ankyrin-binding affinity has been reduced when 43 COOH-terminal aa are deleted. The sequence between aa 320 and 355 (designated as “region II” of the cytoplasmic domain of CD44(GP85)) shares a great deal of sequence homology with CD44 proteins from various species (Table I A; 7, 51, 55, 65, 70 [GenBank Acc. No. X66862]).

Most importantly, no ankyrin binding is detected after deleting 58 aa (CD44(GP85)Δ305 mutant protein) or all 71 aa (CD44(GP85)Δ292 mutant protein) of the COOH-terminal region (Fig. 3 A, d and e). These findings indicate that the ankyrin-binding domain of CD44(GP85) must reside between aa 305 and 355. The 15 aa (NGGNGTVEDRKPSEL) sequence is designated as the region I of the cytoplasmic domain of CD44(GP85) (Table I A). This region appears to share a great deal of homology with various CD44 proteins from different species (Table I A) (7, 51, 55, 65, 70 [GenBank Acc. No. X66862]).

Since human erythrocyte band 3 protein is a well established ankyrin-binding protein (3, 4, 20), we have also tested whether the region I peptide would compete with an ankyrin-binding fragment (=43 kD) derived from the cytoplasmic domain of band 3 (40) for ankyrin binding. As shown in Fig. 4 (panel A), the region I peptide binds ankyrin specifically and in a dose-dependent manner (Fig. 4, panel I A). The binding of this peptide to ankyrin is specific since it does not bind other cytoskeletal proteins such as spectrin (Fig. 4 [panel I B] or fodrin (Fig. 4 [panel I C]). A control peptide (GRNETPESGGLDVK), containing a scrambled sequence with the same amino acid composition as that of the synthetic region I peptide, does not bind either ankyrin or spectrin or fodrin (Fig. 4 [panel 2, A, B, and C]).

To examine whether region I of the cytoplasmic domain of CD44(GP85) is involved in ankyrin binding, we have tested the ability of a synthetic region I peptide (e.g., NGGNTYEDRKPSEL) to bind various cytoskeletal proteins. As shown in Fig. 4 (panel I), the region I peptide binds ankyrin specifically and in a dose-dependent manner (Fig. 4, panel I A). The binding of this peptide to ankyrin is specific since it does not bind other cytoskeletal proteins such as spectrin (Fig. 4 [panel I B] or fodrin (Fig. 4 [panel I C]). A control peptide (GRNETPESGGLDVK), containing a scrambled sequence with the same amino acid composition as that of the synthetic region I peptide, does not bind either ankyrin or spectrin or fodrin (Fig. 4 [panel 2, A, B, and C]). To further analyze the role of region I in ankyrin binding, we have used the synthetic peptide corresponding to region I to compete the binding of pure mouse T-lymphoma CD44(GP85) to ankyrin. As shown in Fig. 5 A, the synthetic peptide competes effectively with CD44(GP85) to bind ankyrin with an apparent inhibition constant (K0) ≈50 nM. However, the control peptide with the scrambled sequence does not compete at all with CD44(GP85) to bind ankyrin (Fig. 5 A). Since human erythrocyte band 3 protein is a well established ankyrin-binding protein (3, 4, 20), we have also tested whether the region I peptide would compete with an ankyrin-binding fragment (=43 kD) derived from the cytoplasmic domain of band 3 (40) for ankyrin binding. As shown in Fig. 5 B, the region I peptide also competes with the 43-kD ankyrin-binding domain of erythrocyte band 3 in a dose-dependent manner with an apparent inhibition constant (K0) ≈200 nM. The scrambled sequence peptide does not compete with the 43-kD fragment of band 3 for ankyrin binding (Fig. 5 B).
Table I. Sequence Comparisons between CD44 Isoforms and Band 3/Na+/K+ ATPase α Subunit Proteins

| Region | Mouse CD44 | Human CD44 | Rat CD44 | Hamster CD44 | Bovine CD44 | Horse CD44 |
|-------|------------|------------|----------|--------------|-------------|------------|
| A     | 3°N G G N G T V E D R K P S E 3°L | 3°N G S G N G A V E D R K P S G 3°L | 3°N G S G N G T M E E R K P S G 3°L | 3°N G S G N G K V E D R K P S G 3°L | 3°N G S G N G A V E D R K P S G 3°L | 3°N G S G N G T V E D R K P S E 3°L |
| B     | 3°N G G N G T V E D R K P S E 3°L | 3°N G S G N G A V E D R K P S G 3°L | 3°N G S G N G T M E E R K P S G 3°L | 3°N G S G N G K V E D R K P S G 3°L | 3°N G S G N G A V E D R K P S G 3°L | 3°N G S G N G T V E D R K P S E 3°L |
| C     | 3°N G G N G T V E D R K P S E 3°L | 3°N G S G N G A V E D R K P S G 3°L | 3°N G S G N G T M E E R K P S G 3°L | 3°N G S G N G K V E D R K P S G 3°L | 3°N G S G N G A V E D R K P S G 3°L | 3°N G S G N G T V E D R K P S E 3°L |

The sequence comparison of the ankyrin-binding domain of CD44(GP85) with similar sequences found in other ankyrin-binding proteins. The (1) indicates a perfect match. The bold letters and (c) indicate a conserved substitution. Gaps are introduced for the alignment.

These results suggest that region I is a critical part of the ankyrin-binding domain of CD44(GP85).

Furthermore, we have constructed a fusion protein (designated as CD45Δ826/CD44 [Reg.I]) (Table II) in which the 15 aa sequence of region I of CD44(GP85) is transplanted onto another transmembrane protein, the truncated CD45 molecule, CD45Δ826 (64). CD45, a leukocyte common antigen (also called GP180 or T-200) has been shown to bind fodrin (a spectrin-like protein) but not ankyrin (15, 62, 64). The cDNAs encoding various proteins (e.g., CD45Δ826, CD45Δ826/CD44[Reg.I], CD44[GP85]Δ363, and CD44[GP85]Δ305) are transcribed and translated in vitro using the TNT reticulocyte lysate system which generates unglycosylated polypeptides. We have previously shown that the unglycosylated precursor of CD44(GP85) binds ankyrin equally well as the mature CD44(GP85) (43). The polypeptides synthesized by in vitro transcription and translation are purified by anti-CD44(GP85) or anti-CIM5 immunoaffinity chromatography. The CD45Δ826 cDNA encodes a 105-kD protein corresponding to a polypeptide encoding 826 aa (Fig. 6 A). This protein does not display any ankyrin-binding property (Table II). The fusion protein CD45Δ826/CD44-[Reg.I] shows a slight increase in molecular mass (=2 kD) compared to CD45Δ826 protein (Fig. 6 B). Most importantly, it is able to bind ankyrin (Table II) in a manner identical to that of the unglycosylated wild-type 43-kD CD44-

Figure 4. Binding of 125I-labeled cytoskeletal proteins (e.g., ankyrin, spectrin, fodrin) to synthetic peptides. Various concentrations (20 ng/ml; 40 ng/ml; 80 ng/ml) of 125I-labeled cytoskeletal proteins including (A) ankyrin, (B) spectrin, and (C) fodrin were incubated with the nitrocellulose discs coated with either the region I peptide (NGGNGTVEDKPSL) or the scramble peptide (GRN- ETPNEGGLDKV) at 4°C for 4 h as described in the Materials and Methods. Non-specific binding was determined in the presence of a 100-fold excess of the respective unlabeled cytoskeletal proteins and subtracted from the total binding. The results represent an average of duplicate determinations for each of the ligand used. (panel 1) Binding of 125I-labeled cytoskeletal proteins to region I peptide. (panel 2) Binding of 125I-labeled cytoskeletal proteins to scramble peptide.
Effect of Cytoplasmic Deletions of CD44(GP85) on HA-mediated Binding and Adhesion in COS Cells Transfected with Various CD44(GP85) Constructs

In this set of experiments, we have attempted to correlate the effect of the ankyrin-binding domain with the expression of HA-interaction capability by COS cells transfected with various CD44(GP85) constructs. Using fluorescein-HA staining techniques, we have found that HA-binding sites are readily detectable as large patched structures on the surface of COS cells transfected with CD44(GP85)363 and CD44(GP85)-Δ355 cDNAs (Fig. 7, A and B). A reduction in HA binding, revealed as small clusters (Fig. 7 C) is observed in COS cells expressing the CD44(GP85)Δ320 protein. Most importantly, we have found that cells expressing CD44(GP85)Δ305 and CD44(GP85)Δ292 proteins, which lack the complete ankyrin-binding domain (Fig. 3 A), do not exhibit any detectable fluorescein-HA binding (Fig. 7, D and E). Further analysis, using fluorescence-conjugated anti-CD44(GP85) antibody staining techniques reveals that a uniform distribution pattern (not clustered or patched) of CD44 on the surface of COS cells expressing CD44(GP85)Δ305 and CD44(GP85)Δ292 proteins (data not shown). This result suggests that ankyrin is required for the collection of CD44 into clusters or patches.

The differential expression of HA-binding in COS cells transfected with different CD44(GP85) mutant constructs is further corroborated by 3H-HA-binding assays and cell adhesion to HA-coated plates. Our results indicate that the mutant CD44(GP85)Δ355 protein displays 3H-HA binding (Fig. 8 B, b) and cell adhesion (Table III) at levels comparable to those of the wild-type protein (Fig. 8, A, b and Table III). There is a slight reduction in both 3H-HA binding (Fig. 8 C, b) and cell adhesion (Table III) in COS cells expressing the CD44(GP85)Δ320 protein. Most importantly, a >90% decrease in 3H-HA binding and cell adhesion is observed in COS cells expressing either the CD44(GP85)-Δ305 (Fig. 8 D, b; Table III) or CD44(GP85)Δ292 proteins.

Table II. Binding of 125I-labeled Ankyrin to Various Transmembrane Proteins

| Ankyrin binding (cpm) |
|----------------------|
| CD45R826 (TM)        |
| 1159 ± 118           |
| CD45R826/CD44(Reg.1) |
| 1228 ± 163           |
| CD44(GP85)Δ305       |
| N.D.                 |

125I-labeled ankyrin (~30 ng protein) was incubated with various proteins (e.g., CD45R826; CD45R826/CD44 [Reg.1]; CD44(GP85)Δ363; and CD44(GP85)Δ305 (~0.3 nM) as described in the Materials and Methods. The results represent the specific binding and are an average of duplicate determinations from the same experiment. N.D., Not detectable.
Figure 6. Expression of various transmembrane proteins by in vitro transcription and translation. CD45Δ826, CD45Δ826/CD44(Reg.I), CD44(GP85)363, and CD44(GP85)Δ305 cDNAs were transcribed and translated in vitro and purified by immunoaffinity chromatography as described in the Materials and Methods. Purified proteins were analyzed by SDS-PAGE and fluorography. (lane A) CD45-Δ826; (lane B) CD45Δ826/CD44(Reg.I); (lane C) CD44(GP85)-363; (lane D) CD44(GP85)Δ305.

(Fig. 8 E, b; Table III). As controls, COS cells either transfected with pcDNA I vector alone or untransfected were also analyzed and show a background level of HA-mediated binding (data not shown) and cell adhesion to HA-coated plates (Table III). To verify that the loss of HA-binding sites in the COS cells transfected with certain mutants is not due to a failure to express CD44(GP85) on the cell surface, we have also quantitatively determined the expression of CD44(GP85) by incubating the COS cells with 125I-labeled anti-CD44-(GP85) antibody. Fig. 8 shows that COS cells transfected with various constructs express comparable amounts of CD44(GP85) (Fig. 8 A a, B a, C a, D a, and E a); only a small reduction in CD44(GP85) expression (≈15%) is observed in COS cells transfected with either CD44(GP85)-Δ320 (Fig. 8 C, a), CD44(GP85)Δ305 (Fig. 8 D, a) or CD44(GP85)Δ292 cDNAs (Fig. 8 E, a) when compared to the wild-type (Fig. 8 A, a) and CD44(GP85)Δ355 expression (Fig. 8 B, a). Since all of these mutant proteins are expressed on the COS cell surface (Figs. 2, 7, and 8), these results strongly suggest that ankyrin binding to the cytoplasmic cDNA were fixed with 2% paraformaldehyde at room temperature for 30 min. The fixed cells were incubated with fluorescein-conjugated HA at room temperature for 30 min as described in the Materials and Methods.

Figure 7. Fluorescence staining of fluorescein-HA-labeled COS cells expressing various CD44(GP85) proteins. COS cells transfected with various CD44(GP85) constructs (e.g., A, CD44-[GP85]363 cDNA; B, CD44(GP85)Δ355 cDNA; C, CD44(GP85)-Δ320 cDNA; D, CD44(GP85)Δ305 cDNA; E, CD44(GP85)Δ292 cDNA) were fixed with 2% paraformaldehyde at room temperature for 30 min. The fixed cells were incubated with fluorescein-conjugated HA at room temperature for 30 min as described in the Materials and Methods.
Materials and Methods. Non-specific binding was determined in the presence of 100 μg/ml of soluble HA and subtracted from the total binding. The results (cpm) bound for cells expressing CD44(GP85)363 (wild-type) vector alone (control) were labeled with Tras[35S]methionine. Labeled cells

| Table III. Cell Adhesion to HA-coated Plates |
|-----------------------------------------------|
| Cell                                      | Total specific binding (cpm) to HA-coated plates | % of cells adhering to HA-coated plates |
| Control (pcDNA I alone)                     | 0.38 ± 0.05 × 10^6 | 9.2% |
| CD44(GP85)363                               | 4.11 ± 0.26 × 10^6 | 100%* |
| CD44(GP85)A355                              | 4.18 ± 0.14 × 10^6 | 102% |
| CD44(GP85)A320                              | 3.85 ± 0.21 × 10^6 | 93.6% |
| CD44(GP85)A305                              | 0.49 ± 0.02 × 10^6 | 11.9% |
| CD44(GP85)A329                              | 0.44 ± 0.07 × 10^6 | 10.7% |

* COS cells transfected with various CD44(GP85) cDNAs or with pcDNA I vector alone (control) were labeled with Tras[35S]methionine. Labeled cells (1.2 × 10^6 cpm) were incubated with HA-coated plates as described in the Materials and Methods. Non-specific binding was determined in the presence of 100 μg/ml of soluble HA and subtracted from the total binding. The results represent an average of triplicate determinations. The amount of specific radioactivity (cpm) bound for cells expressing CD44(GP85)363 (wild-type) protein is designated as 100%.

Discussion

CD44(GP85) binds extracellular matrix components such as HA at its NH2-terminal domain and contains an ankyrin-binding site within its 70 aa long COOH-terminal domain (38, 69, 70). It has been suggested that ankyrin binding to CD44(GP85) is involved in lymphocyte activation (8, 9). However, at the present time, the relationship between the two functional domains, i.e., the ankyrin-binding and HA-mediated binding/adhesion, is not well understood. In this work, we have identified the ankyrin-binding domain in CD44(GP85). Furthermore, we have shown that the ankyrin-binding domain in the cytoplasmic tail of CD44(GP85) is very important for HA-binding and HA-mediated adhesion functions.

Ankyrin is known to link various transmembrane proteins to actin network through interaction with spectrin or fodrin (a spectrin-like protein) (4, 46). For example, in erythrocytes, ankyrin connects band 3 anion exchange protein to spectrin (3, 4, 20). In non-erythrocytes, ankyrin is shown to be associated with a number of physiologically important proteins including the Na+/K+ ATPase (49, 50), voltage-dependent (25) and amiloride-sensitive Na+ channels (57), inositol 1,4,5 triphosphate (IP3) receptor (10) and CIM4-(GP85) (16, 37) possibly via fodrin. Among these proteins, the interaction between ankyrin and CD44(GP85) may be considered as one of the most well understood in non-erythrocytes. Our laboratory has extensively characterized the interaction between CD44(GP85) and ankyrin (12, 13, 16, 37, 38, 43, 45). However, the aa sequence of CD44(GP85) involved in ankyrin binding has not yet been identified.

Since the cytoplasmic domain of CD44(GP85) is relatively short (≈70 aa) in comparison to other well characterized ankyrin-binding proteins (e.g., erythrocyte band 3 [39, 47], it offers us an excellent opportunity to map the ankyrin-binding site(s) of CD44(GP85). Our deletion mutation analysis indicates that the ankyrin-binding domain of CD44(GP85) resides in aa 305 and 355. However, at least two regions within this domain contribute to ankyrin binding. Region I, contains 15 aa between 305 and 320; and region II contains 35 aa between 320 and 355 (Table I A). The region II appears to be required for the high affinity ankyrin binding, since its deletion (e.g., CD44(GP85)3203 protein) results in a 2–2.5-fold decrease in the dissociation constant for ankyrin binding. This region is conserved in various CD44(GP85) proteins from different species (Table I A). Nevertheless, no sequence homology is detected between CD44's region II and other ankyrin-binding proteins (e.g., band 3 and Na+/K+ ATPase, etc.) using a Best fit program (Genetics Computer Group Inc., 24). It is possible that this region contains regulatory domains (e.g., protein kinase C-mediated phosphorylation, GTP binding, GTPase activity, etc.) responsible for the upregulation of (GP85)-ankyrin interaction (38, 45). Deletion of these regulatory domains could result in the observed reduction of high affinity binding between CD44(GP85) and ankyrin.

The deletion of both region I and II of CD44(GP85) leads to a complete loss of ankyrin binding. This suggests that the region I (38,49,50,70) is required for ankyrin binding. The fact that (a) the region I peptide (but not the scrambled peptide) binds ankyrin specifically and in a dose-dependent manner (Fig. 4); (b) the synthetic region I peptide (but not the scrambled peptide) competes effectively with mouse T-lymphoma CD44(GP85) for ankyrin binding (Fig. 5); and (c) a fusion protein expressing this sequence acquires the ability to bind ankyrin (Table II), clearly demonstrates that the region I sequence must be a required part of ankyrin-binding domain of CD44(GP85).

Since the region I peptide also competes effectively (K ≈200 nM) with the 43-kD ankyrin-binding fragment of human band 3 protein, a region I-like sequence may also be present in band 3 protein that is required for ankyrin binding. Using a Best fit (Genetics Computer Group Inc., 24) program to compare region I and the 43-kD ankyrin-binding do-

![Figure 8. Binding of 125I-labeled anti-CD44(GP85) antibody and 3H-HA to COS cells transfected with various CD44(GP85) constructs. COS cells transfected with various CD44(GP85) constructs (CD44(GP85)363 (A); CD44(GP85)A355 (B); CD44(GP85)A320 (C); CD44(GP85)A305 (D) and CD44(GP85)A292 (E)) were incubated with two different reagents such as 125I-labeled anti-CD44(GP85) antibody or 3H-HA as described in the Materials and Methods. Non-specific binding was determined in the presence of 100-fold excess of unlabeled anti-CD44(GP85) or HA and subtracted from the total binding. The binding of 125I-labeled anti-CD44(GP85) antibody or 3H-HA to COS cells transfected with the wild-type CD44(GP85)363 construct is designated as a control (100%). The results represent an average of duplicates using three different concentrations of each reagent (e.g., 125I-labeled anti-CD44(GP85) antibody or 3H-HA).]
main of band 3 protein, we have found a sequence between aa 178 and 192 has limited sequence similarity with region I (Table I B, a). It is interesting to note that this band 3 sequence partially overlaps with a region (between aa 174 and 186) of band 3 that is known to be involved in ankyrin binding (20). However, it has been suggested that the region between aa 174 and 186 of band 3 protein alone is not sufficient to account for all the high affinity interaction between the cytoplasmic domain (43-kD fragment) and ankyrin (20). After further sequence comparison between region I and the 43-kD fragment, we have found a second sequence in band 3 (aa 203-216) that also shares some sequence similarity with region I (Table I B, b). It is possible that both of these sequences are necessary for the high affinity binding observed between the cytoplasmic domain of band 3 and ankyrin (20).

In addition, sequence comparison reveals two segments, aa 109-123 and aa 627-640 of Na+/K+-ATPase α subunit (another well characterized ankyrin-binding protein) (49, 50, 56), that share some sequence similarity with region I (Table I C). The sequence between aa 627 and aa 640 appears to be included in one of two ankyrin-binding domains of Na+/K+-ATPase α subunit as shown by Devarajan et al. recently (23). However, at the present time no biochemical evidence is available to support the notion that another segment such as aa 109-123 of the Na+/K+-ATPase α subunit is required for ankyrin binding. Further studies are needed to precisely map the ankyrin-binding domain(s) in the Na+/K+-ATPase.

In this manuscript, we have presented a new mechanism which implicates the cytoskeletal protein, ankyrin, in the regulation of HA-mediated functions. There appears to be a strong correlation between the presence of an ankyrin-binding domain in the cytoplasmic tail of CD44(GP85) and the expression of HA-binding and HA-mediated adhesion functions. For example, cells expressing the mutant protein CD44(GP85)Δ320 that lacks region II of the ankyrin-binding domain displays a moderate decrease in both ankyrin-binding affinity and HA-mediated functions. More importantly, cells expressing the mutant proteins CD44(GP85)Δ305 and CD44(GP85)Δ292 that lack the entire ankyrin-binding domain (both region I and II), display no ankyrin binding and nearly a complete loss of HA-binding and HA-mediated adhesion functions. These findings strongly imply that the intracellular interaction of CD44(GP85) with ankyrin is required for its adhesion functions on the cell surface.

The fact that HA-binding and HA-mediated adhesion functions can be readily inhibited by cytoskeletal drugs, such as cytochalasin D (a microfilament inhibitor) or W-7 (a calmodulin inhibitor), suggests an involvement of actin and myosin-associated cytoskeletal proteins, such as ankyrin, to the cytoplasmic domain of CD44(GP85) is critically important in regulating various cell–cell and cell–extracellular matrix interactions.

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