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

Vinata B. Lokeshwar, Nevis Fregien, and Lilly Y. W. Bourguignon

Department of Cell Biology and Anatomy, University of Miami School of Medicine, Miami, Florida 33101

Abstract. GP85 is one of the most common hemopoietic 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 ankryin 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 (^{306}NGGNGTVEDRKPSE^{320}L) is a critical part of the ankryin-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 GP90^{Hermes} antigen, ECMR III, and homing cellular adhesion molecule [H-CAM][1]) (6, 18, 19, 34, 35, 51, 69, 70). GP85 is one of the most common isoforms of CD44 found in all hemopoietic 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 (55, 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-

Address all correspondence to Dr. Lilly Y. W. Bourguignon, Department of Cell Biology and Anatomy (R-124), University of Miami School of Medicine, 1600 NW 10th Avenue, Miami, FL 33101.

1. *Abbreviations used in this paper*: HA, hyaluronic acid; H-CAM, homing cellular adhesion molecule; HEV, high endothelial venules.

© The Rockefeller University Press, 0021-9525/94/08/1099/11 $2.00

cluding protein kinase C–mediated phosphorylation (38), palmityolation (12), and GTP binding (45). The fact that ankyrin preferentially accumulates underneath CD44(GP-85) capped structures suggests that the formation of an ankyrin-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 [70]). Mouse CD45(B 200; 64) cDNA was a gift from Dr. Ian Trowbridge (Salk Institute, La Jolla, CA). pcDNA I was obtained from In-Vitrogen. 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 ^{3}H-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 I3/2.3 (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% penicillin, and 1% streptomycin.

Protein Iodination

Ankyrin, fodrin, spectrin, anti-CD44(GP85) monoclonal antibody, purified lymphoma CD44(GP85), and surface proteins of COS-7 cells were iodinated using IodoGen as described (26).

Deletion Mutagenesis

A 1.3-kb cDNA containing the entire mouse CD44(GP85) coding sequence cloned into pRc/CMV (InVitrogen Co.) was digested with Pst I, Sst I, Bcl I, and Tha I to generate mutants CD44(GP85)Δ355, CD44(GP85)Δ320, CD44(GP85)Δ305, and CD44(GP85)ΔA292. The 3′ overhanging ends resulting from Pst I and Sst I digestions were blunt ended by T4 DNA polymerase. Tha I digestion leaves blunt ended DNA fragments. These blunt ended fragments (Pst I, Sst I, and Tha I) were ligated to a synthetic Not I adaptor containing stop codons in all of the three possible reading frames. The DNA fragments were then digested with Hind III; the deleted CD44-(GP85) inserts were gel isolated and ligated into Hind III–Not I digested pcDNA I (InVitrogen Co.) for expression into COS cells. Bcl I digested DNA was further cut with Hind III without blunt ending and ligated directly into Hind III–BamHI digested pcDNA I to generate the CD44(GP85)Δ305 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 2×10^{6} 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 ^{125}I-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 (Bio-Rad Labs., Hercules, CA). Purity of the protein preparations was determined by SDS-PAGE followed by silver staining and/or autoradiography.

Binding of ^{125}I-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 beads were incubated in 0.5 ml of binding buffer (20 mM Tris.HCl pH 7.4, 150 mM NaCl, 0.1% BSA and 0.05% Triton X-100) containing various concentrations (10–400 ng/ml) of ^{125}I-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 ^{125}I-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 ^{125}I-labeled Ankyrin/Fodrin/Spectrin to Synthetic Region I and Scramble Peptides

Nitrocellulose discs (1-cm diam) were coated with ≈1 μg of either the region I (NGGNGTVEDRKPSEL) 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 ^{125}I-labeled ankyrin/spectrin/fodrin (≈3,000 cpm/ng) at 4°C for 2 h in 1 ml binding buffer (20 mM Tris.HCl pH 7.4, 150 mM NaCl, 0.2% BSA). After binding, the discs were washed three times in the binding buffer and the disc bound radioactivity was estimated. 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 ~30% of the total binding. As controls, the ligands were also incubated with uncoated nitrocellulose discs to determine the binding observed due to the "stickyness" of various ligands. Nonspecific binding was observed in these controls.

Binding of ^{125}I-labeled Lymphoma CD44(GP85) to Ankyrin

CD44(GP85) was purified from the plasma membrane preparations of mouse T-lymphoma cells, by sequential WGA-Sepharose and anti-CD44-(GP85) immunoaffinity chromatographies as described previously (43, 45). ^{125}I-labeled CD44(GP85) (≈0.32 nM protein, 1.5×10^{4} cpm/ng) was incubated with 30 μl of ankyrin conjugated to Sepharose beads (≈0.75 μg protein) in 0.5 ml of the binding buffer (described above). The binding was car-

ried out in the presence or absence of various concentrations (1 nM–1 μM) of unlabeled competing synthetic region I peptide (NGGNGTVEDRKPSEL) or the scramble peptide (GRNETNPEGSGLDVK) at 4°C for 5 h under equilibrium conditions. Equilibrium conditions were determined by performing time course (e.g., 1–10 h) of the binding studies. After binding, the beads were washed in the binding buffer and the bead bound radioactivity was determined. Non-specific binding was determined in the presence of either a 100-fold excess of unlabeled ankyrin or using BSA-conjugated Sepharose beads. The non-specific binding was 20–30% of the total binding and was subtracted from the total binding.

Binding of ^{125}I-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 ^{125}I-labeled to a specific activity of 6×10^4 cpm/ng. The binding of the ^{125}I-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'CAACGGTGGCAATGGGACAGTGGAAGACAGGAAACCCAGTGAGCTCTAA3') and its complementary strand were synthesized by DNA facility, University of Miami, Miami, FL. The adaptor codes for mouse CD44(GP85) cDNA sequence corresponding to the 15 aa of the region I of the ankyrin-binding domain (69, 70), a stop codon and Kpn I 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-(B200)/pcDNA I was digested partially with Kpn I and completely with Xho I and ligated to an adaptor containing stop codon, and Kpn I and Xho I cohesive ends. Both CD45Δ826/CD44(Reg. I) and CD45Δ826 cDNA constructs were confirmed by sequencing. The CD45Δ826 and CD45-Δ826/CD44(Reg.I) constructs were transcribed and translated in vitro using the TNT coupled reticulocyte system (Promega Co., Madison, WI) and [^{35}S]methionine (1,000 cpm/pmol). The in vitro translated proteins were purified by rat anti-CD45 immunoaffinity chromatography as described previously (44). The expression and purity of the protein preparations were analyzed by SDS-PAGE followed by fluorography. As controls, CD44(GP85)363 and CD44(GP85)Δ305 proteins were also synthesized in vitro and purified using anti-CD44(GP85) immunoaffinity chromatography. Both CD45- and CD44(GP85)-related protein bound to immunoaffinity beads (≈0.3 nM protein) were incubated with 30 ng of ^{125}I-labeled ankyrin (3,000 cpm/ng) in 1 ml of the binding buffer (20 mM Tris.HCl pH 7.4, 150 mM NaCl, 0.1% BSA, and 0.05% Triton X-100) at 4°C for 5 h. Non-specific binding was determined in the presence of 3 μg of unlabeled ankyrin and was subtracted. Non-specific binding was ∼20% of the total binding. Other controls included binding of ^{125}I-labeled ankyrin to anti-CD44(GP85) or anti-CD45 immunobeads alone. No specific binding was observed in these controls.

Immunocytochemistry

COS cells (10^3 cells) were grown on coverslips and transfected with either various CD44(GP85) cDNAs or pcDNA I vector only as described above. Subsequently, cells were washed with PBS and fixed in PBS containing 2% paraformaldehyde. Fixed cells were then stained with fluorescein-labeled HA (21) (5 μg/ml) at room temperature for 30 min followed by washing with PBS three times. To detect nonspecific HA binding, cells were incubated with fluorescein-labeled HA in the presence of an excess amount of HA (100 μg/ml). No labeling was observed in the control samples. The fluorescein-labeled samples were excited with a Xenon 75-W bulb and examined using a Zeiss inverted microscope (63× oil immersion and epi-illumination). Cells were photographed with Kodak Tri-X film.

Binding of ^{125}I-labeled Anti-CD44(GP85) Antibody and ^{3}H-HA to COS Cells

COS cells (2×10^5 cells/35-mm dish) were transfected with either various CD44(GP85) constructs or pcDNA I vector as described above. 48 h after the transfections, culture medium was removed and the cells were washed in PBS containing 0.2% BSA (binding buffer). The cells were then incubated with various concentrations of either ^{125}I-labeled anti-CD44(GP85) antibody (50, 100, and 200 ng/ml) or ^{3}H-HA (0.3, 0.6, and 1.2 μg/ml) in the binding buffer at room temperature or 4°C for 2 h. Nonspecific binding was determined in the presence of a 100-fold excess of unlabeled ligand. After binding, the cells were washed three times in binding buffer, and then solubilized in 1% SDS solution. The solubilized cell extracts were counted either in a gamma counter or a liquid scintillation counter.

Cell Adhesion Assay

COS cells were transfected with various constructs as described above. 48 h after transfection, cells were labeled with Tran[^{35}S]methionine (30 μCi/ml) for 2 h as described previously (43). After labeling, cells were washed in PBS and incubated with PBS containing 5 mM EDTA at 37°C to obtain non-adherent, single cell suspensions. 1.2×10^6 cpm (≈10^5 cells) were incubated in each well of a 24-well Corning culture plate coated with HA. The HA-coated plates were prepared by incubating the culture wells with 2 mg/ml rooster comb HA at 22°C for 16 h. HA-coated plates were rinsed sequentially three times with PBS containing 2% BSA and PBS before incubating with labeled cells. Labeled cells were incubated with HA-coated wells at 4°C for 60 min. After incubation, wells were washed three times with PBS; the adherent cells were solubilized in PBS containing 1% SDS; and the well-bound radioactivity was determined by liquid scintillation counting. Non-specific binding was determined by including 200 μg/ml soluble HA during the incubation in the HA-coated wells. Non-specific binding was 10–15% of the total well-associated radioactivity and has been subtracted.

Results

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[GP85]Δ355, CD44[GP85]Δ320, CD44[GP85]Δ305, and CD44[GP85]Δ292) in which various portions of the cytoplasmic domain of CD44(GP85) have been deleted from the carboxyl terminus (Fig. 1). Specifically, 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) constructs encode for proteins consisting of 363, 355, 320, 305, and 292 aa, respectively. Subsequently, the CD44(GP85) 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

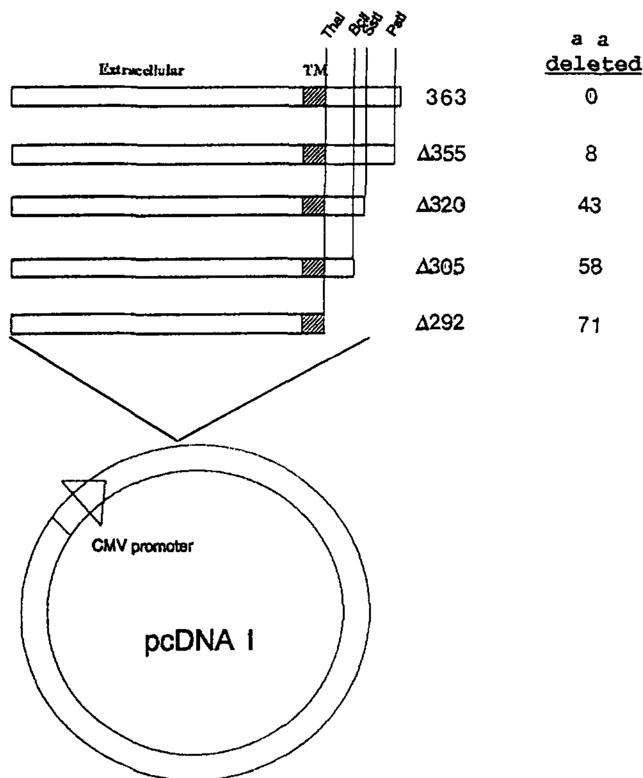

Figure 1. A schematic illustration of the in vitro mutagenesis approach used in this study. The four cytoplasmic deletion mutants of CD44(GP85) were constructed according to the strategy described in the Materials and Methods. These constructs including CD44(GP85)363 (the wild-type containing full-length 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.

analysis (Figs. 1 and 2). 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

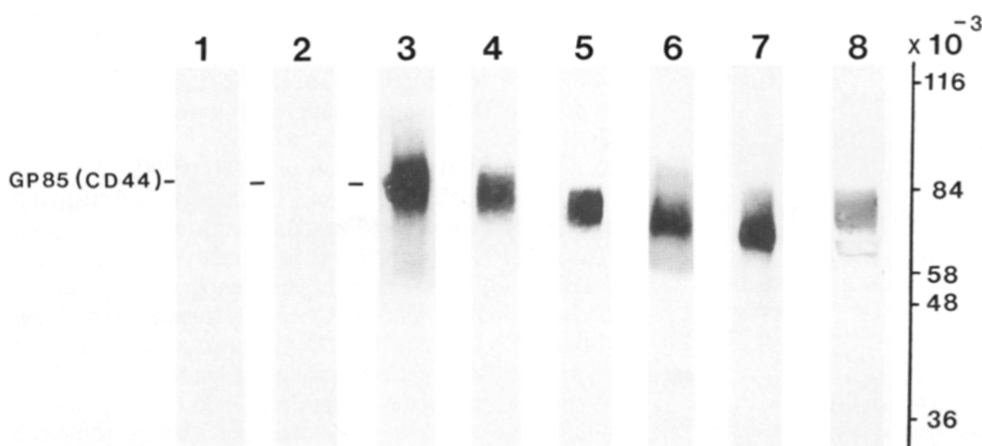

Figure 2. Expression of various CD44(GP85) proteins purified from COS cells transfected with wild type and mutant cDNAs. Various CD44-(GP85) proteins were purified from either surface ^{125}I-labeled or unlabeled COS cells transfected with various CD44(GP85) constructs by Triton X-100 solubilization, sequential WGA-Sepharose and anti-CD44(GP85) immunoaffinity chromatography as described in Materials and Methods. The purified proteins were analyzed by SDS-PAGE followed by autoradiography or silver staining. Lanes *1*–*7* represent autoradiograms of proteins purified from surface ^{125}I-labeled COS cells. (Lane *1*) Untransfected control; (lane *2*) pcDNA I vector alone; (lane *3*) CD44(GP85)363; (lane *4*) CD44(GP85)Δ358; (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.

CD44(GP85) proteins, isolated from COS cells transfected with CD44(GP85) wild-type and various mutant cDNAs, were incubated with various concentrations of ^{125}I-labeled ankyrin under equilibrium-binding conditions. As shown in Fig. 3 *A*, ankyrin binds specifically to both CD44(GP85)363 (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 [K_d] 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 ^{125}I-labeled ankyrin compared to the wild-type, CD44(GP85)363, or the mutant, CD44(GP85)Δ355 proteins (Fig. 3 *A*, *c*). This mutant protein also binds ankyrin with a slightly lower affinity (K_d ≈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 (^{306}NGGNGTVEDRKPSE^{320}L) 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 the deletion of both region I and II but not region II alone shows a complete loss of ankyrin binding, it suggests that the region I may be an important part of the ankyrin-binding domain of CD44(GP85).

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., NGGNGTVEDRKPSEL) to bind various cytoskeletal proteins. As shown in Fig. 4 (panel *1*), the region I peptide binds ankyrin specifically and in a dose-dependent manner (Fig. 4, panel *1 A*). The binding of this peptide to ankyrin is specific since it does not bind other cytoskeletal proteins such as spectrin (Fig. 4 [panel *1 B*] or fodrin (Fig. 4 [panel *1 C*]). A control peptide (GRNETNPEGSGLDVK), 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 (K_i) ≈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 (K_i) ≈200 nM. The scrambled sequence peptide does not compete with the 43-kD fragment of band 3 for ankyrin binding (Fig. 5 *B*).

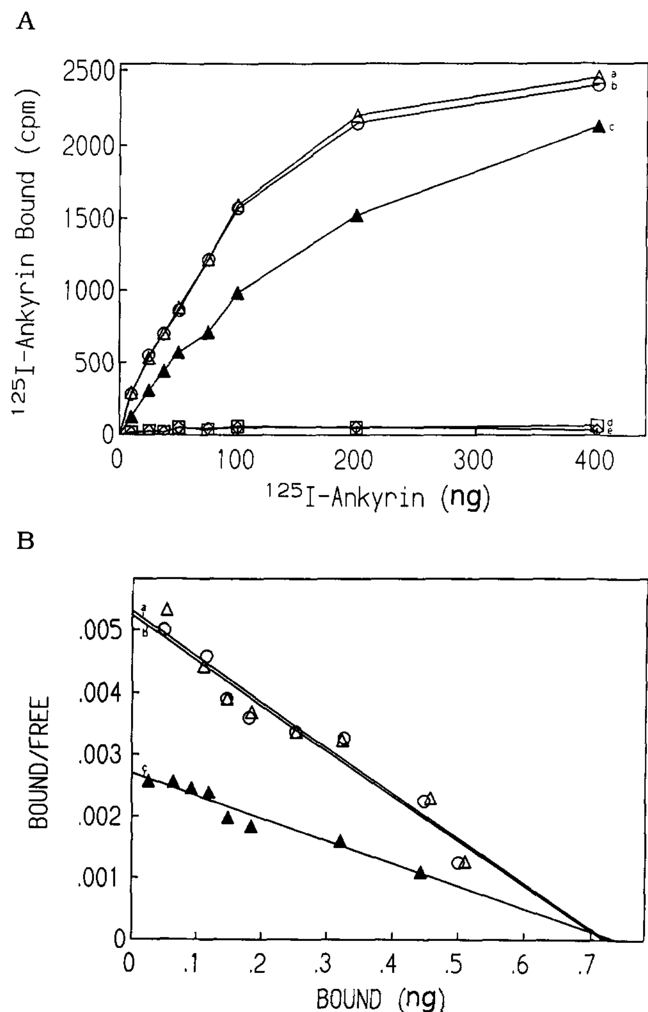

Figure 3. Binding of ^{125}I-labeled ankyrin to various CD44(GP85) proteins. Various concentrations of ^{125}I-labeled ankyrin were incubated with purified CD44(GP85)363 (the wild-type protein) or mutant proteins at 4°C for 5 h as described in the Materials and Methods. Nonspecific binding was determined in presence of 100-fold excess of unlabeled ankyrin and subtracted from the total binding. Results represent an average of triplicate determinations from the same experiment. Standard deviation is less than 5%. (*A*) Equilibrium binding between ^{125}I-labeled ankyrin and various CD44-(GP85) proteins such as CD44(GP85)363 (*a*), CD44(GP85)Δ355 (*b*), CD44(GP85)Δ320 (*c*), CD44(GP85)Δ305 (*d*), and CD44-(GP85)Δ292 proteins (*e*). (*B*) Scatchard plot analysis of the equilibrium binding data presented in Fig. 3 *A* for various CD44(GP85) proteins such as CD44(GP85)363 (*a*), CD44(GP85)Δ355 (*b*), and CD44(GP85)Δ320 proteins (*c*).

Table I. Sequence Comparisons between CD44 Isoforms and Band 3/Na+/K+ ATPase α Subunit Proteins

		Region I	Region II
A:			
Mouse CD44		^{306}N G G N G T V E D R K P S E ^{320}L	^{321}N G E A S K S Q E M V H L V N K E P S E T P D Q C . T A D E T R N L ^{355}Q
Human CD44		^{304}N **S** G N G **A** V E D R K P S **G** ^{318}L	^{319}N G E A S K S Q E M V H L V N K E **S** S E T P D Q **F I** T A D E T R N L ^{353}Q
Rat CD44		^{292}N **S** G N G T V E D R K P S E ^{306}L	^{307}N G E A S K S Q E M V H L V N K E P T E T P D Q **F I** T A D E T R N L ^{341}Q
Hamster CD44		^{305}N **S** G N G **K** V E D R K P S E ^{319}L	^{320}N G E A S K S Q E M V H L V N K E P S E T P D Q **F I** T A D E T R N L ^{354}Q
Bovine CD44		^{309}N **N** G N G T **M** E **E** R K P S **G** ^{323}L	^{324}N G E A S K S Q E M V H L V N K **G S** S E T P D Q **F M** T A D E T R N L ^{358}Q
Horse CD44		^{302}N **N** G N G **A** V **D** D R K **A** S **G** ^{316}L	^{317}N G E A S **R** S Q E M V H L V N K E **S** S E T Q D Q **F M** T A D E T R N L ^{351}Q
B:		Region I	
(a)	Mouse/human CD44	^{306}N G/S G N G T/A V E D R K P S E/G ^{320}L	
			:					
	Human band 3	^{178}A E D L G N L E G V K P A V ^{192}L	
(b)	Mouse/human CD44	^{306}N G/S G N G T/A V E D R K P S E/G ^{320}L	
		:				:			:	
	Human band 3	^{203}Q G E G G T - E G H S P S G ^{216}I	
C:			
(a)	Mouse/human CD44	^{306}N G/S G N G T/A V E D R K P S - E - ^{320}L	
			: : :	:				:		
	Human Na+/K+ATPase (α2 subunit)	-^{109}G I Q A A M E D E - P S N D N ^{123}L	
(b)	Mouse/human CD44	^{306}N G/S G N G T/A V E D R K P S E/G ^{320}L	
	Human Na+/K+ATPase (α2 subunit)	^{627}S E G N E T V E D I A A R - ^{640}L	

The sequence comparison of the ankyrin-binding domain of CD44(GP85) with similar sequences found in other ankyrin-binding proteins. The (|) indicates a perfect match. The bold letters and (:) 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]363, 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-CD45 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-

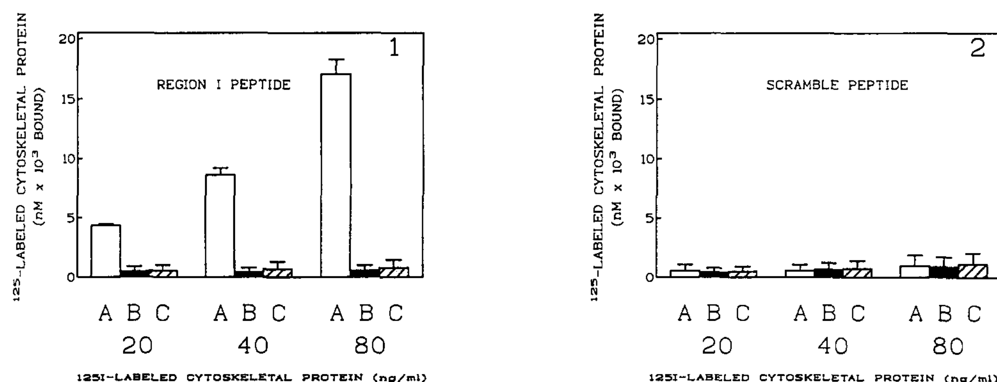

Figure 4. Binding of ^{125}I-labeled cytoskeletal proteins (e.g., ankyrin, spectrin, fodrin) to synthetic peptides. Various concentrations (20 ng/ml; 40 ng/ml; 80 ng/ml) of ^{125}I-labeled cytoskeletal proteins including (*A*) ankyrin, (*B*) spectrin, and (*C*) fodrin were incubated with the nitrocellulose discs coated with either the region I peptide (NGGNGTVEDRKPSEL) or the scramble peptide (GRNETNPEGSGLDVK) 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 ^{125}I-labeled cytoskeletal proteins to region I peptide. (panel *2*) Binding of ^{125}I-labeled cytoskeletal proteins to scramble peptide.

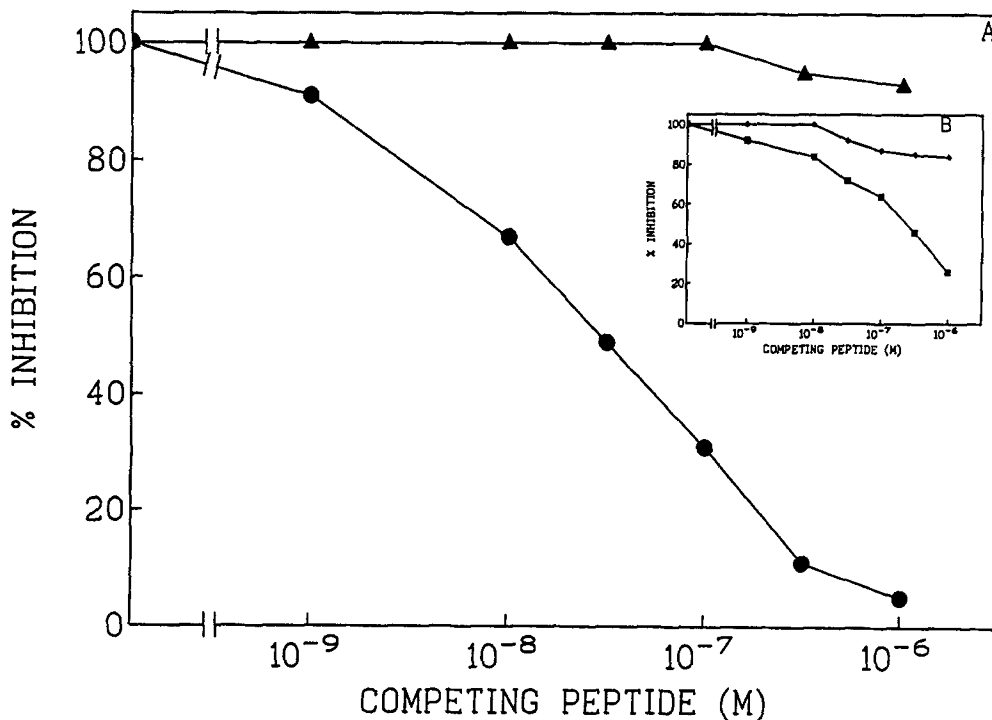

Figure 5. Binding of ^{125}I-labeled mouse T-lymphoma CD44(GP85)/band 3 cytoplasmic domain (43 kD fragment) to ankyrin. ^{125}I-labeled CD44-(GP85) or band 3 cytoplasmic domain 43-kD fragment were incubated with ankyrin in the presence of various concentrations of unlabeled synthetic region I peptide (NGGNGTVEDRKPSEL) or a scrambled peptide (GRNETNPEGSGLDVK) as described in Materials and Methods. The specific binding observed in the absence of any of the competing peptides is designated as 100%. The results represent an average of duplicate determinations for each concentration of the competing peptide used. (*A*) Binding of ^{125}I-labeled CD44(GP85) to ankyrin (●—●, region I peptide; ▲—▲, scrambled peptide); (*B*) binding of ^{125}I-labeled band 3 cytoplasmic domain (43-kD fragment) to ankyrin (■—■, region I peptide; ◆—◆, scrambled peptide).

(GP85)363 protein (Fig. 6 *C*, and Table II). In addition, as expected the 37-kD unglycosylated CD44(GP85)Δ305 protein does not bind ankyrin (Fig. 6 *D*, and Table II). Taken together, these findings support the contention that the region I sequence (NGGNGTVEDRKPSEL) is a critical part of the ankyrin-binding domain of CD44(GP85).

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 ^{3}H-HA–binding assays and cell adhesion to HA-coated plates. Our results indicate that the mutant CD44(GP85)Δ355 protein displays ^{3}H-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 ^{3}H-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 ^{3}H-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 ^{125}I-labeled Ankyrin to Various Transmembrane Proteins

		Ankyrin binding
		(*cpm*)
CD45Δ826	TM	N.D.
CD45Δ826/CD44(Reg.1)	TM Reg.1	1159 ± 118
CD44(GP85)363	TM Reg.1	1228 ± 163
CD44(GP85)Δ305	TM	N.D.

^{125}I-labeled ankyrin (~30 ng protein) was incubated with various proteins (e.g., CD45Δ826; CD45Δ826/CD44 [Reg. I]; CD44[GP85]363; 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 determination from the same experiment. N.D., Not detectable.

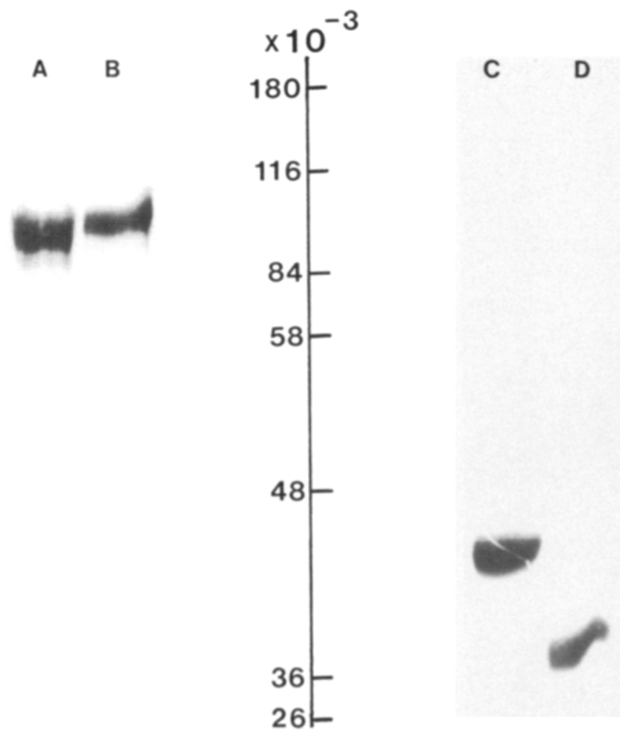

Figure 6. Expression of various transmembrane proteins by in vitro transcription and translation. CD45Δ826, CD45Δ826/CD44(Reg.I), CD44(GP85)363, and CD44(GP85)Δ305 cDNAs were transcribed and translated in vitro and purified by immunoaffinity chromatography as described in the Materials and Methods. Purified 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 ^{125}I-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

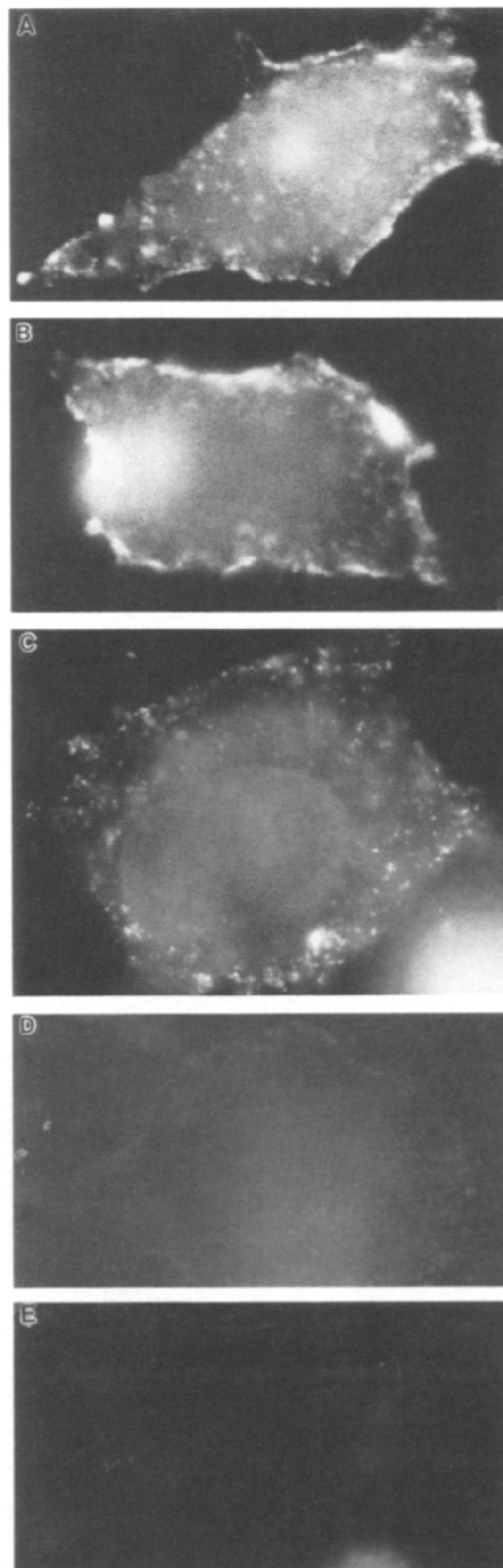

Figure 7. Fluorescence staining of fluorescein-HA-labeled COS cells expressing various CD44(GP85) proteins. COS cells transfected with various CD44(GP85) constructs (e.g., *A*, CD44-[GP85]363 cDNA; *B*, CD44[GP85]Δ355 cDNA; *C*, CD44[GP85]-Δ320 cDNA; *D*, CD44[GP85]Δ305 cDNA; *E*, CD44[GP85]Δ292 cDNA) 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.

domain of CD44(GP85) is important for both high affinity HA-binding and HA-mediated cell adhesion.

Discussion

CD44(GP85) binds extracellular matrix components such as HA at its NH_2-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 (IP_3) receptor (10) and CD44-(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

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)	$0.38 \pm 0.05 \times 10^5$	9.2%
CD44(GP85)363	$4.11 \pm 0.26 \times 10^5$	100%*
CD44(GP85)Δ355	$4.18 \pm 0.14 \times 10^5$	102%
CD44(GP85)Δ320	$3.85 \pm 0.21 \times 10^5$	93.6%
CD44(GP85)Δ305	$0.49 \pm 0.02 \times 10^5$	11.9%
CD44(GP85)Δ292	$0.44 \pm 0.07 \times 10^5$	10.7%

* COS cells transfected with various CD44(GP85) cDNAs or with pcDNA I vector alone (control) were labeled with Tran[^{35}S]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 form the total binding. The results represent an average of triplicate determinations. The amount of specific radioactivity (cpm) bound for cells expresssing CD44(GP85)363 (wild-type) protein is designated as 100%.

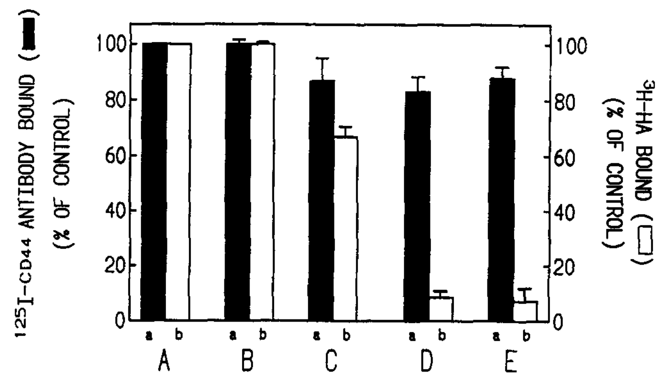

Figure 8. Binding of ^{125}I-labeled anti-CD44(GP85) antibody and ^{3}H-HA to COS cells transfected with various CD44(GP85) constructs. COS cells transfected with various CD44(GP85) constructs (CD44[GP85]363 (*A*); CD44[GP85]Δ355 (*B*); CD44[GP85]Δ320 (*C*); CD44[GP85]Δ305 (*D*) and CD44[GP85]Δ292 (*E*) were incubated with two different reagents such as ^{125}I-labeled anti-CD44(GP85) antibody or ^{3}H-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 ^{125}I-labeled anti-CD44(GP85) antibody or ^{3}H-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., ^{125}I-labeled anti-CD44[GP85] antibody or ^{3}H-HA).

to be required for the high affinity ankyrin binding, since its deletion (e.g., CD44(GP85)Δ320 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 CD44(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 (^{306}NGGNGTVEDRKPSE^{320}L) 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 the ankyrin-binding domain of CD44(GP85).

Since the region I peptide also competes effectively (K_i ≈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-

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-mediated contraction in HA-mediated functions (13). The involvement of the cytoskeleton in controlling the exposure of high affinity binding sites for hormones such as insulin and interleukin-2 has also been reported previously (11, 36). Similar observations have also been made in other adhesion-related events. For example, the cytoskeleton binding domain within the cytoplasmic tail of the integrin β_1 subunit is involved in the formation of focal adhesion plaques (53). Furthermore, cytoskeleton binding is also important for the function of a number of other adhesion molecules (e.g., ICAM-1, N-CAM, and E-cadherin) (27). Taken together, these findings strongly suggest that the binding of membrane-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.

We thank Dr. Naoko Iida and Dr. Gerard J. Bourguignon for critically reviewing this manuscript.

This work was supported by National Institutes of Health (NIH) grant GM 36353. V. B. Lokeshwar is a postdoctoral fellow of NIH (grant 1F32CA06057).

Received for publication 9 March 1994 and in revised form 12 May 1994.

References

1. Arch, R., K. Wirth, M. Hofmann, M. Ponta, S. Matzku, P. Herrlich, and M. Zoller. 1992. Participation in normal immune responses of a metastasis-inducing splice variant of CD44. *Science (Wash. DC)*. 257: 682–685.
2. Aruffo, A., I. Stamenkovic, M. Melnick, C. B. Underhill, and B. Seed. 1990. CD44 is the principle cell surface receptor for hyaluronate. *Cell*. 61:1303–1313.
3. Bennett, V. 1990. Spectrin based membrane cytoskeleton: a multipotent adaptor between plasma membrane and cytoplasm. *Physiol. Rev.* 70: 1029–1065.
4. Bennett, V. 1992. Ankyrins. *J. Biol. Chem.* 267:8703–8706.
5. Bennett, V., and P. J. Stenbuck. 1980. 3 proteins involved in membrane-cytoskeleton association in human erythrocytes: spectrin, ankyrin and band 3. *Methods Enzymol.* 96:313–325.
6. Berg, E. L., L. A. Goldstein, M. A. Jutila, M. Nakache, L. P. Picker, P. R. Streeter, N. W. Wu, D. Zhou, and E. C. Butcher. 1989. Homing receptors and vascular addressins: cell adhesion molecules that direct lymphocyte traffic. *Immunol. Rev.* 108:5–18.
7. Bosworth, B. T., T. St. John, W. M. Gallatin, and J. A. Harp. 1991. Sequence of the bovine CD44 cDNA: comparison with human and mouse sequences. *Mol. Immunol.* 28:1131–1135.
8. Bourguignon, L. Y. W. 1988. Ligand-induced surface receptor movement and transmembrane interactions. *In* Energetics of Secretion Responses. J.-W. N. Akkerman, editor. CRC Press Inc., Boca Raton, FL. Vol. I. 83–104.
9. Bourguignon, L. Y. W. 1992. Membrane skeleton linker proteins in immune cells. *In* Encyclo-pedia of immunology I. M. Rotti and P. J. Delves, editors. Academic Press, NY 1044–1046.
10. Bourguignon, L. Y. W., H. Jin, N. Iida, N. R. Brandt, and S. H. Zhang. 1993. The involvement of ankyrin in the regulation of inositol 1, 4, 5-triphosphate receptor-mediated internal Ca^{2+} release from Ca^{2+} storage vesicles in mouse T-lymphoma cells. *J. Biol. Chem.* 268:7290–7297.
11. Bourguignon, L. Y. W., W. Jy, M. H. Majercik, and G. J. Bourguignon. 1988. Lymphocyte activation and capping of hormone receptors. *J. Cell Biochem.* 37:131–150.
12. Bourguignon, L. Y. W., E. L. Kalomiris, and V. B. Lokeshwar. 1991. Acylation of the lymphoma transmembrane glycoprotein, GP85 may be required for GP85-ankyrin interaction. *J. Biol. Chem.* 266:11761–11765.
13. Bourguignon, L. Y. W., V. B. Lokeshwar, X. Chen, and G. L. Kerrick. 1993. Hyaluronic acid (HA)-induced lymphocyte signal transduction and HA receptor (GP85/CD44)-cytoskeleton interaction. *J. Immunol.* 151: 6634–6644.
14. Bourguignon, L. Y. W., V. B. Lokeshwar, J. He, X. Chen, and G. J. Bourguignon. 1992. A CD44-like endothelial cell transmembrane glycoprotein (GP116) interacts with extracellular matrix and ankyrin. *Mol. Cell. Biol.* 12:4464–4471.
15. Bourguignon, L. Y. W., S. Suchard, M. L. Nagpal, and J. Glenny. 1985. A transmembrane glycoprotein GP180 is linked to the cytoskeletal protein fodrin. *J. Cell Biol.* 101:477–487.
16. Bourguignon, L. Y. W., G. Walker, S. Suchard, and K. Balazovich. 1986. A lymphoma plasma membrane-associated protein with ankyrin like properties. *J. Cell Biol.* 102:2115–2124.
17. Brown, T., T. Bouchard, T. St. John, E. Wayner, and W. G. Caster. 1991. Human keratinocytes express a new CD44 core protein (CD44E) as a heparin sulfate intrinsic membrane proteoglycan with additional exons. *J. Cell Biol.* 113:207–221.
18. Carter, W. G., and E. A. Wayner. 1988. Characterization of the class III collagen receptor, a phosphorylated, transmembrane glycoprotein expressed in nucleated human cells. *J. Biol. Chem.* 263:4193–4201.
19. Culty, M., K. Miyake, P. W. Kincade, E. Siloraki, E. C. Butcher, and C. Underhill. 1990. The hyaluronate receptor is a member of the CD44 (H-CAM) family of cell surface glycoproteins. *J. Cell Biol.* 111:2765–2774.
20. Davis, J., S. E. Lux, and V. Bennett. 1989. Mapping the ankyrin binding site of human erythrocyte anion exchanger. *J. Biol. Chem.* 264: 9665–9672.
21. de Belder, A., and K. Wik. 1975. Preparation and properties of fluorescein labelled hyaluronate. *Carbohydr. Res.* 44:251–257.

22. Denning, S. M., P. T. Le, K. H. Singer, and B. F. Haynes. 1990. Antibodies against CD44p80 lymphocyte homing receptor molecule augment human peripheral blood T cell activation. *J. Immunol.* 144:7–15.
23. Devarajan, P., D. A. Scaramuzzino, and J. S. Morrow. 1994. Ankyrin binds two distinct cytoplasmic domains of Na^+/K^+-ATPase α subunit. *Proc. Natl. Acad. Sci. USA.* 91:2965–2969.
24. Devereux, J., P. Haeberli, and O. Smithies. 1984. A comprehensive set of sequence analysis programs for the VAS. *Nucleic Acid Res.* 122: 387–395.
25. Flucher, B. E., and M. P. Daniels. 1989. Distribution of Na^+ channels and ankyrin in neuromuscular junctions is complementary to that of acetylcholine receptors and the 43 kd protein. *Neuron.* 3:163–175.
26. Fraker, P. J., and J. C. Speck. 1978. Protein and cell membrane iodination with sparingly soluble chloramine 1,3,4,6-tetrachloro-3a,6a-diphenyl glycouril. *Biochem. Biophys. Res. Comm.* 80:849–857.
27. Geiger, B., and O. Ayalon. 1992. Cadherins. *Annu. Rev. Cell Biol.* 8:307–332.
28. Glenny, J. R., P. Glenny, and K. Weber. 1982. F-actin binding and cross-linking properties of porcine brain fodrin, a spectrin related molecule. *J. Biol. Chem.* 257:9781–9787.
29. Gunthert, V., M. Hofmann, W. Rudy, S. Reber, M. Zoller, I. Hausmann, S. Matzku, A. Wenzel, M. Ponta, and P. Herrlich. 1991. A new variant glycoprotein of CD44 confers metastatic potential to rat carcinoma cells. *Cell.* 65:13–24.
30. Haynes, B. F., M. J. Telen, L. P. Hale, and S. M. Denning. 1989. CD44-A molecule in leukocyte adherence and T-cell activation. *Immunol. Today.* 10:423–427.
31. Heider, K.-H., M. Hofmann, E. Hors, F. V. D. Berg, H. Ponta, P. Herrlich, and S. T. Pals. 1993. A human homologue of the rat metastasis associated variant of CD44 is expressed in colorectal carcinomas and adenomatous polyps. *J. Cell Biol.* 120:227–233.
32. Hyman, R., J. Lesley, and R. Schulte. 1991. Somatic cell mutants distinguish CD44 expression and hyaluronic acid binding. *Immunogenetics.* 33:392–395.
33. Isacke, C. M., C. A. Sauvage, R. Hyman, J. Lesley, R. Schulte, and I. Trowbridge. 1986. Identification and characterization of the human pgp-1 glycoprotein. *Immunogenetics.* 23:326–332.
34. Jalkanen, S. T., R. F. Bargatze, L. R. Heron, and E. C. Butcher. 1986. A lymphoid cell surface glycoprotein involved in endothelial cell recognition and lymphocyte homing in man. *Eur. J. Immunol.* 16:1195–1202.
35. Jalkanen, S., R. F. Bargatze, J. D. L. Toyos, and E. C. Butcher. 1986. Lymphocyte recognition of high endothelium: antibodies to distinct epitopes of an 85-90 kDa glycoprotein antigen differentially inhibit lymphocyte binding to lymph node, mucosal or synovial endothelial cells. *J. Cell Biol.* 105:983–990.
36. Jy, W., N. Fregien, G. J. Bourguignon, and L. Y. W. Bourguignon. 1989. Role of Ca^{2+} in the regulation of hormone receptor exposure during lymphocyte activation. *Biochim. Biophys. Acta.* 983:153–160.
37. Kalomiris, E. L., and L. Y. W. Bourguignon. 1988. Mouse T-lymphoma cells contain a transmembrane glycoprotein (GP85) that binds ankyrin. *J. Cell Biol.* 106:319–327.
38. Kalomiris, E. L., and L. Y. W. Bourguignon. 1989. Lymphoma protein kinase C is associated with the transmembrane glycoprotein, GP85 and may function in GP85-ankyrin binding. *J. Biol. Chem.* 264:8113–8119.
39. Kopito, R. R., and H. F. Lodish. 1985. Primary structure and transmembrane orientation of the murine anion exchange. *Nature (Lond.).* 316: 234–238.
40. Lesley, J., R. Schulte, and R. Hyman. 1990. Binding of hyaluronic acid to lymphoid cell lines is inhibited by monoclonal antibodies against Pgp-1. *Exp. Cell Res.* 187:224–233.
41. Lesley, J., Q. He, K. Miyake, A. Hamann, R. Hyman, and P. W. Kincade. 1992. Requirements of hyaluronic acid binding by CD44: a role for the cytoplasmic domain and activation by antibody. *J. Exp. Med.* 175: 257–266.
42. Letarte, M., S. Iturbe, and E. J. Quackenbush. 1985. A glycoprotein of molecular weight 85,000 on human cells of B-lineage: detection with a family of monoclonal antibodies. *Mol. Immunol.* 22:113–124.
43. Lokeshwar, V. B., and L. Y. W. Bourguignon. 1991. Post-translational protein modification and expression of ankyrin binding site(s) in GP85 (Pgp-1/CD44) and its biosynthetic precursors during T-lymphoma membrane biosynthesis. *J. Biol. Chem.* 266:17983–17989.
44. Lokeshwar, V. B., and L. Y. W. Bourguignon. 1992. Tyrosine phosphatase activity lymphoma CD45(GP180) is regulated by direct interaction with the cytoskeleton. *J. Biol. Chem.* 267:21551–21557.
45. Lokeshwar, V. B., and L. Y. W. Bourguignon. 1992. The lymphoma transmembrane glycoprotein CD44(GP85) is a novel guanine nucleotide-binding protein which regulates GP85(CD44)-ankyrin interaction. *J. Biol. Chem.* 267:22073–22078.
46. Luna, E. J., and A. L. Hitt. 1992. Cytoskeleton-plasma membrane interactions. Science *(Wash. DC).* 258:955–964.
47. Lux, S. E., K. M. John, R. R. Kopito, and H. F. Lodish. 1989. Cloning and characterization of band 3, the human erythrocyte anion-exchange protein (AE1). *Proc. Natl. Acad. Sci. USA.* 86:9089–9093.
48. Miyake, K., K. L. Medina, S. I. Hayashi, S. Ono, T. Hamaoka, and P. W. Kincade. 1990. Monoclonal antibodies to Pgp-1/CD44 block lymphohemopoiesis in long term bone marrow cultures. *J. Exp. Med.* 171:477–488.
49. Morrow, J. S., C. D. Cianci, A. S. Ardito, and M. Kashgarian. 1989. Ankyrin links fodrin to the alpha subunit of Na^+/K^+-ATPase in Madin-Darby Canine kidney cells and in intact renal tubule cells. *J. Cell Biol.* 108:455–465.
50. Nelson, W. J., and P. J. Veshnock. 1987. Ankyrin binding to Na^+/K^+-ATPase and implications for the organizations of membrane domains in polarized cells. *Nature (Lond.).* 328:533–536.
51. Nottenberg, C., G. Rees, and T. St. John. 1989. Isolation of mouse CD44 cDNA: structural features are distinct from the primate cDNA. *Proc. Natl. Acad. Sci. USA.* 86:8521–8527.
52. Picker, L., M. Nakache, and E. C. Butcher. 1989. Monoclonal antibodies to human lymphocyte homing receptors define a novel class of adhesion molecules on diverse cell types. *J. Cell Biol.* 109:927–937.
53. Reszka, A. A., Y. Hayashi, and A. F. Horowitz. 1992. Identification of amino acid sequences in the integrin B1 cytoplasmic domain implicated in cytoskeletal association. *J. Cell Biol.* 117:1321–1330.
54. Rudy, W., M. Hofmann, R. Schwartz-Albier, M. Zoller, K.-H. Heider, and H. Ponta. 1993. The two major/CD44 proteins expressed on a metastatic rat tumor cell line are derived from different splice variants: each one individually suffices to confer metastatic behavior. *Cancer Res.* 53:1262–1268.
55. Screaton, G. R., M. V. Bell, D. G. Jackson, F. B. Cornelis, V. Gerth, and J. I. Bell. 1992. Genomic structure of DNA coding the lymphocyte homing receptor CD44 reveals at least 12 alternatively spliced exons. *Proc. Natl. Acad. Sci. USA.* 89:12160–12164.
56. Shull, M. M., D. G. Pugh, and J. B. Lingrel. 1989. Characterization of the human Na^+/K^+-ATPase $\alpha 2$ gene and identification of the intragenic restriction fragment length polymorphism. *J. Biol. Chem.* 264:17532–17543.
57. Smith, P. R., G. Saccomani, E. H. Joe, K. J. Angelides, and D. J. Benos. 1991. Amiloride sensitive sodium channel is linked to the cytoskeleton in renal epithelial cells. *Proc. Natl. Acad. Sci. USA.* 88:6971–6975.
58. Stamenkovic, I., M. Amiot, J. M. Pesando, and B. Seed. 1991. The hemopoietic and epithelial forms of CD44 are distinct polypeptides with different adhesion potentials for hyaluronon-bearing cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:343–347.
59. St. John, T., J. Meyer, R. Idzerda, and W. M. Gallatin. 1990. Expression of CD44 confers a new adhesive phenotype on transfected cells. *Cell.* 60:45–52.
60. Tanabe, K. K., L. M. Ellis, and H. Sayer. 1993. Expression of CD44R1 adhesion molecule in colon carcinomas and metastases. *Lancet.* 341: 725–726.
61. Deleted in proof.
62. Thomas, M. L. 1989. The leukocyte common antigen family. *Annu. Rev. Immunol.* 7:339.
63. Thomas, L., H. R. Bayers, J. Vink, and I. Stamenkovic. 1992. CD44 H regulates tumor cell migration on hyaluronate-coated substrate. *J. Cell Biol.* 118:971–977.
64. Thomas, M. L., P. J. Reynolds, A. Chain, Y. Ben-Neriath, and I. S. Trowbridge. 1987. B-cell variant of mouse T200 (Ly-5): evidence for alternative mRNA splicing. *Proc. Natl. Acad. Sci. USA.* 84:5360–5363.
65. Tolg, C., M. Hofmann, P. Herrlich, and H. Ponta. 1993. Splicing choice from ten variant exons establishes CD44 variability. *Nucleic Acids Res.* 21:1225–1229.
66. Trowbridge, I. S., J. Lesley, R. Schulte, R. Hyman, and J. Trotter. 1982. Biochemical characterization and cellular distribution of a polymorphic, murine cell surface glycoprotein expressed on lymphoid tissues. *Immunogenetics.* 15:299–312.
67. Underhill, C. 1992. CD44: The hyaluronon receptor. *J. Cell Sci.* 103:293–298.
68. West, D. C., and S. Kumar. 1989. The effect of hyaluronate and its oligosaccharides on endothelial cell proliferation and monolayer integrity. *Exp. Cell Res.* 183:179–196.
69. Wolffe, E. J., W. C. Gause, C. M. Pelfrey, S. M. Holland, A. D. Steinberg, and J. T. August. 1990. The cDNA sequence of mouse Pgp-1 and homology to human CD44 cell surface antigen and proteoglycan core/link proteins. *J. Biol. Chem.* 265:341–347.
70. Zhou, P., J. Ding, L. Picker, R. Bargatze, E. Butcher, and D. Goeddel. 1989. Molecular cloning and expression of Pgp-1: the mouse homolog of the human H-CAM (Hermes) lymphocyte homing receptor. *J. Immunol.* 143:3390–3395.