A Requirement for the CD44 Cytoplasmic Domain for Hyaluronan Binding, Pericellular Matrix Assembly, and Receptor-Mediated Endocytosis in COS-7 Cells

Hong Jiang‡, Richard S. Peterson‡, Weihua Wang‡, Eckart Bartnik§, Cheryl B. Knudson‡, and Warren Knudson‡¶

‡Department of Biochemistry, Rush Medical College
Rush-Presbyterian-St. Luke's Medical Center
1653 West Congress Parkway, Chicago, IL 60612

and

§DG Thrombotic and Degenerative Joint Diseases
Aventis Pharma Deutschland GmbH
Frankfort, Germany
Running title: CD44 cytoplasmic domain required for hyaluronan interactions

Address all correspondence and reprint requests to:

Warren Knudson, Ph.D., Department of Biochemistry, Rush Medical College
Rush-Presbyterian-St. Luke's Medical Center
1653 West Congress Parkway, Chicago, IL 60612

e-mail: wknudson@rush.edu
Telephone (312) 942-7837
FAX (312) 942-3053
CD44-negative COS-7 cells were transfected with expression constructs for CD44H (the predominant CD44 isoform), CD44E (epithelial isoform), or truncation mutant derivatives lacking the carboxyl terminal 67 amino acids of the cytoplasmic domain, CD44HΔ67 and CD44EΔ67. The truncation mutant CD44HΔ67 is identical to a naturally-occurring alternatively spliced "short-tail" CD44 isoform (CD44st), that incorporates exon 19 in place of exon 20. CD44st lacks intracellular signaling motifs as well as protein domains necessary for interaction with cytoskeletal components. Transfection of COS-7 cells with each construct yielded equivalent levels of mRNA expression whereas no CD44 expression was observed in parental, non-transfected COS-7 cells. Western analysis and immunostaining of COS-7 transfectants confirmed CD44 protein expression of the truncation mutant derivatives. COS-7 cells transfected with CD44H or CD44E gained the capacity to bind fluorescein-conjugated HA (fl-HA) and assemble HA-dependent pericellular matrices in the presence of exogenously added HA and proteoglycan. In addition, the CD44H and CD44E transfected cells were able to internalize surface-bound fl-HA. COS-7 cells transfected with the vector alone, or either of the mutant CD44 isoforms, CD44HΔ67 or CD44EΔ67, did not exhibit the capacity to assemble pericellular matrices or to bind and internalize the fl-HA. Co-transfection of CD44Δ67 mutants together with CD44H reduced the size of the HA-dependent pericellular matrices. Transfection of bovine articular chondrocytes with CD44Δ67 also inhibited pericellular matrix assembly. Collectively, these results indicate an obligatory requirement for the CD44 receptor cytoplasmic domain for ligand (HA) binding, formation and retention of the pericellular matrix as well as CD44-mediated endocytosis of HA. In addition, the results suggest a potential regulatory role for the differentially expressed alternatively spliced "short-tail" CD44 isoform.
Articular cartilage is composed of specialized cells, chondrocytes, embedded in an extensive extracellular matrix. Chondrocytes function to maintain extracellular matrix composition through the synchronous coordinated synthesis and degradation of matrix components. Cell-matrix interactions play a central role in this process via the participation of matrix receptors (1). One receptor in particular, CD44, serves an important role as the primary receptor for the extracellular matrix macromolecule hyaluronan (HA) on articular chondrocytes (2-6) as well as many other cell types (7). Studies from our laboratory have demonstrated that the majority of the proteoglycan-rich pericellular matrix can be displaced by reagents that compete with CD44-HA binding such as anti-CD44 antibodies, non-sulfated chondroitin and HA hexasaccharides (HA$_6$) (2,3). Thus, CD44 serves as the critical link to the retention of HA-proteoglycan aggregates to the chondrocyte cell surface. We have also shown that CD44 mediates the binding and endocytosis of HA in articular chondrocytes (8). The internalized HA is degraded to small fragments within chloroquine-sensitive intracellular organelles (i.e., lysosomes). Additionally, the catabolic cytokine IL-1$\alpha$ stimulates the expression of chondrocyte CD44 and enhances intracellular accumulation of HA (4,9).

CD44 is a single-pass, transmembrane glycoprotein (10). The gene for CD44 consists of 20 exons, 12 of which are expressed by the most common isoform, CD44H (“hematopoetic” isoform). These 12 exons are translated into a protein with three principal domains, an extracellular domain (exons 1-5 and 16-17), a transmembrane domain (exon 18), and a 70 amino acid intracellular tail domain (3 amino acids from exon 18 and 67 amino acids encoded by exon 20) (10,11). We have shown that bovine as well as human articular chondrocytes express predominately CD44H (4,12). However, in recent studies we have also found that human articular chondrocytes as well as some human chondrosarcoma cells express varying levels (15-33%) of another alternatively spliced “short tail” isoform of CD44, CD44st (13). The CD44st isoform utilizes exon 19 as the terminating exon in place of exon 20. The presence of an early stop codon within exon 19 results in a truncated CD44 protein with the majority of the exon represented as 3'-untranslated message. Translation of CD44st mRNA results in a CD44 protein
having a cytoplasmic tail domain containing only 3 amino acids (encoded by exon 18) and, consequently, lacks intracellular signaling motifs (14,15) as well as protein domains necessary for interaction with cytoskeletal components. Currently, the biological function of the CD44st isoform is unknown leading one to query whether the expression of this unique isoform is of physiological significance. However, we have shown that IL-1α induces in a sharp, dose-dependent increase in CD44st mRNA in chondrocytes resulting in an increase in the proportion of this isoform relative to CD44H (13). This has led us to hypothesize that CD44st exerts potential modulatory effects on CD44H-mediated functions such as would be observed by a dominant-negative isoform.

In an effort to gain a better understanding of the specific functions of the CD44st isoform expressed by chondrocytes, CD44-negative COS-7 cells were transfected with expression constructs containing truncation mutant derivatives of human CD44 that, like the naturally-occurring CD44st, lack the carboxyl terminal 67 amino acids of the cytoplasmic domain. The truncation mutants were prepared by point mutation of human CD44H as well as CD44E. CD44E represent the so-called “epithelial” isoform of CD44 containing an extended extracellular protein domain due to translation of three additional alternative spliced, exons 12-14, within the extracellular domain. Articular chondrocytes were also transfected with the CD44 mutant isoform. We present results that demonstrate a mandatory requirement for the CD44 receptor cytoplasmic domain for functions of CD44 relevant to chondrocytes including the formation and retention of chondrocyte-like pericellular matrices (includes the binding of HA coupled to aggregating proteoglycan) as well as CD44-mediated endocytosis of HA.
EXPERIMENTAL PROCEDURES

Materials--Dulbecco’s modified Eagle’s medium (DMEM), Trizol, DNase I and LipofectAMINE-2000 were obtained from Life Technologies, Inc (Grand Island, NY). Fetal bovine serum (FBS) was purchased from Summit Biotechnology (Ft. Collins, CO). FuGENE was obtained from Roche (Indianapolis, IN). (GeneAmp RNA PCR kit for reverse transcription-polymerase chain reaction was purchased from Perkin-Elmer (Norwalk, CT). Specific primers for CD44 detection and mutagenesis were custom made by Integrated DNA Technologies (Coralville, IA). The expression plasmid constructs, human pCD44H and pCD44E were the same as described previously (16). pCDM8 and pTracer-SV40 vectors, as well as E.coli MC1061/P3 were obtained from Invitrogen (Carlsbad, CA). The pDsRed2-C1 vector was purchased from Clontech (Palo Alto, CA). QuikChange Site-Directed Mutagenesis kit was purchased from Stratagene (La Jolla, CA). GenePORTER transfection reagent was obtained from Gene Therapy System, Inc (San Diego, CA). Anti-human CD44 antibody BU-52 was purchased from The Binding Site (Birmingham, UK). Alkaline phosphatase-conjugated anti-mouse IgG was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Enhanced chemiluminescence substrate (diethanolamine) for Western blotting was obtained from Amersham Pharmacia Biotech (Piscataway, NJ). Agarose was from FMC BioProducts (Rockland, ME). Wizard Plus Minipreps DNA purification system, EndoFree Plasmid Maxi Kit from Qiagen (Valencia, CA) and a vacuum manifold were purchased from Promega (Madison, WI). The nuclear stain, 4′, 6-diamidino-2-phenylindole, dihydrochloride (DAPI) was obtained from Molecular Probes, Inc. (Eugene, OR). Cyanine-conjugated goat anti-mouse IgG (Cy3-AffiPure H+L IgG) was purchased from Jackson ImmunoResearch (West Grove, PA). All other enzymes and chemicals either molecular biology grade or reagent grade materials were purchased from Sigma (St. Louis, MO).

Site Specific Mutagenesis--Truncated CD44 mutants were generated by the introduction of a stop codon (Cys 295: TGT → TGA) in exon 20 of pCD44H and equivalent site within pCD44E using PCR-mediated site-directed mutagenesis (Stratagene). Briefly, the procedure was carried out
using 50 ng supercoiled double-stranded DNA (pCDM8 vector) containing inserts of human CD44H or CD44E allowed to anneal with 125 ng of two partially-overlapping synthetic oligonucleotide primers in a final reaction volume of 50 µl. The two oligonucleotide primers; Primer M ("mutagenic," 5′-GCA TTG CAG TCA ACA GTC GAA GAA-3′) and primer C ("complementary," 5′-CTT CTG CCC TCA CCT TCT TCG ACT GTT-3′) were extended using 12 cycles of amplification with Pfu Turbo DNA polymerase (30 seconds at 95 °C, 30 seconds at 60 °C and 12 minutes 72 °C). Following amplification, the products were treated with Dpn I, an endonuclease (target sequence: 5′-Gm6 ATC-3′) specific for methylated and hemi-methylated DNA. This digestion is used to cleave the parental (methylated) DNA template and select for the mutation-containing, newly-synthesized DNA. The reaction products (1µl) were then transformed into E coli MC 1061/p3. Four colonies of each transformation were picked, and DNA isolated by mini-preparation (from 3ml cultures). Automated DNA sequencing was performed at Sequetech Corp (Mountain View, CA) using a primer upstream the mutation site (5′-ACT-CAC-ATG-GGA-GTC-AAG-AAG-3′) to authenticate incorporation of the point mutation.

Transfection of COS-7 Cells—The mammalian cell line, COS-7 (simian virus 40-transformed African Green monkey kidney epithelial cells, subline M6) were transfected with cDNA encoding the hematopoietic isoform of CD44 (pCD44H), the epithelial CD44 isoform (pCD44E), mutant constructs (pCD44HΔ67 and pCD44EΔ67), or with parental vector without an inserted sequence (pCDM8). Transfectants were prepared by treating nearly confluent COS-7 cells with 0.5, 2.0 and 4.0 µg/ml of pCD44H, pCD44HΔ67, pCD44E, pCD44EΔ67 and pCDM8 using GenePORTER or LipofectAMINE-2000 transfection reagents as facilitators. In later experiments co-transfectants of pCD44H and pCD44EΔ67 were prepared using 2.0 µg/ml of each plasmid. In order to identify transfected cells, some of the cDNA constructs namely, CD44H and CD44E were subcloned into a green fluorescence protein (GFP) expressing vector (pTracer-SV40). Alternatively, the CD44 constructs in pCDM8 were co-transfected with GFP-positive empty vector (pTracer-SV40) or red fluorescence protein (RFP) empty vectors (pDsRed2-C1) at a 3:1
molar ratio of CD44-pCDM8 to empty vector. To provide for optimal transfection, the DNAs were first mixed with transport reagent at a ratio of 1:5 for GenePORTER or 1:2.2 for LipofectAMINE-2000 reagent in serum–free DMEM and incubated for 15 min to allow DNA–liposome complexes to form. The diluted DNA-transport reagent mixture (1ml) was added carefully to the cells at time of plating into 35-mm dishes as monolayer cultures at high density (90% confluence). Five hours post transfection, one volume (1ml) of medium containing 20% FCS was added to the cells, and allowed to incubate overnight in 5% CO₂ at 37 °C. Twenty-four hours post transfection, the growth medium was replaced and incubation continued for an additional 48 h.

Transfection of Bovine Articular Chondrocytes—Metacarpophalangeal joints from 18-month-old steers were obtained from a local slaughterhouse. Full thickness slices of articular cartilage were dissected under aseptic conditions and subjected to sequential pronase and collagenase digestion to liberate chondrocytes from tissues (3-5). Isolated chondrocytes were cultured for a short term as high density monolayer cultures in DMEM/Ham’s F-12 + 10% FBS. For transfection, the chondrocytes were trypsinized and resuspended in serum-free DMEM containing 5 U/ml Streptomyces hyaluronidase for 30 min following by washes in serum-free DMEM. FuGENE was incubated with the pCD44Δ67 subcloned into pTracer-SV40 or, the pTracer-SV40 empty vector for 30 min (facilitator:DNA ratio, 2.5:1) to allow complex formation (17). The lipid/DNA mixture was added dropwise to the chondrocyte suspension (15 μg DNA/10⁷ cells). FBS was added 5 hours post transfection to a final concentration of 10%. Twenty-four hours post transfection, the growth medium was replaced and incubation continued for an additional 48 h.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)—Total cytoplasmic RNA was extracted from transfected COS-7 cell monolayer cultures with Trizol reagent, purified, treated with DNase I and then subjected to RT-PCR analysis as described previously (12). Briefly, 0.50 μg of total RNA was converted to cDNA using Molony murine leukemia virus reverse transcriptase in the presence of 0.15 μM CD44-specific downstream primers (5‘-AAC CGC
GAG AAT CAA AGC CAA GGC C-3'). Aliquots of sample cDNA were amplified as templates in the presence of CD44-specific downstream primers and 0.15 μM upstream primers (5'-GAT CCA CCC CAA TTC CAT CTG TGC-3') in a PCR mixture consisting of 2 mM magnesium chloride, 200 μM of each deoxyribonucleotide, and 2.5 units of AmpliTaq DNA polymerase. The DNA was denatured by heating at 95°C for 2 min, followed by 25 cycles of 1 min at 95°C, annealing at 60°C, and extension at 72°C for 1 min (Perkin Elmer thermocycler). This reaction was followed by a final elongation step that lasted 5 min at 72°C. The amplified products were analyzed by electrophoresis on 1.5% agarose gels followed by staining with ethinium bromide. The stained products were scanned and quantified using a fluorimaging system (Fluor-S MultiImager, Bio-Rad, Hercules, CA).

Western Blot Analysis--Transfected cells were lysed with 1.0% NP-40/Tris lysis buffer (6). Samples were standardized to 10 μg protein and loaded onto 8% SDS-polyacrylamide gels for electrophoresis. Following electroblot transfer onto nitrocellulose, CD44H, CD44HΔ67, CD44E and CD44EΔ67 were detected following a 2 h incubation with anti-human CD44 monoclonal antibody (BU-52) followed by alkaline phosphatase conjugated anti-mouse IgG and enhanced chemiluminescence substrate (diethanolamine) substrate. The resultant bands were detected using a fluorimaging system (Storm FluorImager 860, Molecular Dynamics, Sunnyvale, CA).

CD44 Expression on the Cell Surface--COS-7 cells were transfected with various CD44 constructs subcloned into GFP-positive vectors or co-transfected with CD44 in pCDM8 together with a GFP-positive empty vector. Seventy-two hours post-transfection the cells were released from monolayer culture with EDTA, washed and immediately fixed with 1% paraformaldehyde in phosphate buffered saline (PBS) at room temperature for 30 min. The cells were next quenched with 0.2% glycine in PBS for 5 min and additionally blocked with 1% bovine serum
albumin (BSA) plus 10% goat serum in PBS for 1 h at room temperature. The cells were then incubated with the mouse anti-CD44 monoclonal antibody BU52, at a dilution of 1:20,000 for 1 h at room temperature. Following incubation the washed cells were next incubated with a cyanine-3-conjugated goat anti-mouse IgG (red fluorescence) for 40 min at room temperature at a 1:8000 dilution. Finally, the cells were incubated with DAPI as a nuclear counterstain. The fluorescent cells were viewed using a Nikon Ellipse E600 microscope equipped with Y-Fl Epi-fluorescence (Melville, NY). Images were captured digitally in real time using a Spot-RT camera (Diagnostic Instruments, Sterling Heights, MI) and processed using MetaView imaging software (Universal Imaging, West Chester, PA).

Visualization of Matrix Assembly --Cell-associated pericellular matrices were visualized using a particle exclusion assay (2). Endogenous matrix on bovine articular chondrocytes (3) was assessed 72 h post transfection. COS-7 cells from each transfection group were released using 0.25% trypsin and re-plated at sub-confluent densities for a 12 h incubation in DMEM containing 10% FBS. To test for matrix assembly with exogenous macromolecules, the culture medium was changed to serum-free DMEM containing 3.0 mg of aggregating proteoglycan monomer [purified from rat chondrosarcoma tumor] and 15 µg HA per ml for 12 h (16). Briefly, the culture medium was removed and replaced with a 0.75-ml suspension of formalin-fixed erythrocytes (10^8 per ml) in PBS containing 0.1% bovine serum albumin. The particles were allowed to settle for 15 min and the cells were photographed using a Zeiss inverted phase-contrast microscope with Varel and epifluorescence optics.

Binding and Uptake of Exogenous HA by Transfected COS-7 Cells--COS-7 cells were co-transfected with various CD44 constructs in pCDM8 together with a RFP-positive empty vector.
Seventy-two hours post-transfection the cells from each transfection group were EDTA-released and incubated in media containing 40 µg/ml fluorescein-HA (fl-HA, prepared using high molecular mass HA as described previously (8)). To visualize cell surface binding, the cells were incubated with fl-HA for 1 h at 4°C. In order to view internalization of the fl-HA, another group of cells were incubated with fl-HA for 3 h at 37°C (8), released using EDTA, washed three times in PBS and then incubated with 0.25% trypsin for 30 min at 37°C in order to release non-internalized fl-HA. Following fixation with 1% paraformaldehyde and the addition of DAPI nuclear counterstain, plasma membrane-bound or internalized fl-HA was then visualized using the using Nikon Ellipse E600 fluorescence microscope.
RESULTS

Construction of CD44 mock short-tail mutants pCD44Δ67 and pCD44EΔ67--Full-length CD44H and CD44E cDNAs were subcloned as Hind III-Not I fragments from pπH3H-CD44H or pπH3H-CD44E (16) into the expression vector pCDM8. The CD44 cytoplasmic domain "mock short-tail" expression mutants, pCD44HΔ67 and pCD44EΔ67, were created by the introduction of a stop codon (underlined) at cysteine 295, Cys 295 (TGT) → Stop (TGA), (Fig. 1, panels A and C) employing PCR-mediated site-directed mutagenesis. The construct pCD44HΔ67 is essentially identical to the alternatively spliced exon 19-containing CD44 isoform, CD44st (Fig. 1B). Incorporation of the point mutation within the sequence of pCD44HΔ67 and pCD44EΔ67 was verified by automated DNA sequencing (data not shown).

Expression of CD44 constructs transfected into COS-7 cells--CD44-negative COS-7 cells were transfected with expression constructs for CD44H (pCD44H), CD44E (pCD44E), or truncation mutant derivatives lacking the carboxyl terminal 67 amino acids of the cytoplasmic domain, pCD44HΔ67 and pCD44EΔ67. In order to determine optimal conditions for expression, 90% confluent COS-7 monolayers grown in 35mm wells were transiently transfected with either 0.5, 2.0, or 4.0 µg of each expression construct. Total RNA was isolated from control-untreated and transfectant cells 72-h post-transfection. CD44 isoform mRNA expression was determined by RT-PCR using specific primers for CD44 (Fig. 2). No CD44 expression was observed in COS-7 cells transfected with the pCDM8 vector alone (Fig 2, lanes 14-16). COS-7 cells transfected with either pCD44H or pCD44HΔ67 expressed a CD44H product of predicted size (600 bp, Fig 2, lanes 2-4 and 5-7, respectively). Likewise, COS-7 cells transfected with either pCD44E or pCD44EΔ67 express the larger CD44E product, containing three additional exons (exons 12-14), with a predicted size of 1004 bp (Fig 2, lanes 8-10 and 11-13, respectively). CD44 isoform mRNA increased proportionately for each construct following transfection with 0.5 or 2.0 µg of plasmid DNA. No further increases were observed with transfection of 4.0 µg of expression construct indicating optimal expression was achieved with transfections using 2.0 µg plasmid DNA.
To assess if the various CD44 constructs were being appropriately expressed as CD44 protein, total cell lysates were extracted from COS-7 cells for each transfection group. Equivalent protein aliquots (10 \( \mu \text{g} \)) were subjected to electrophoresis on an 8% SDS-polyacrylamide gel. Western blot analysis was performed using an anti-human CD44 monoclonal antibody that recognizes an epitope contained within the receptor extracellular domain, present in all of the CD44 expression constructs (Fig. 3). No CD44 epitope was detected in lanes containing lysate from control-untreated COS-7 cells (Fig. 3, lane 1) or COS-7 cells transfected with control pCDM8 vector minus an inserted sequence (Fig. 3, lane 2). This evidence confirms the absence of CD44 expression in parental COS-7 cells. Lysates from pCD44H transfected COS-7 cells revealed a band at approximately 85 kDa (Fig. 3, lane 3) which corresponds to the molecular mass of the standard form of human CD44. A faster migrating species of approximately 78 kDa was detected in lysate from pCD44H\( \Delta 67 \) transfected cells (Fig. 3, lane 4). The molecular size of the protein represented by this band agrees well with a predicted mass of a translated product of pCD44H\( \Delta 67 \), containing a truncation of the carboxyl terminal 67 amino acids (approximately 7 kDa). In a like fashion, lysate from pCD44E transfected COS-7 cells revealed a band at approximately 130 kDa, which corresponds to the predicted molecular mass of the epithelial form of CD44, CD44E (Fig. 3, lane 5). The expression of additional three exons within the extracellular domain (exons 12-14) should result in an increase of 133 amino acids and thus, an increase of approximately 16 kD. In the detergent lysates derived from the pCD44E\( \Delta 67 \) transfected cells, a faster migrating species of approximately 123 kDa was detected (Fig. 3, lane 6), again indicative of a successful truncation loss of 67 amino acids.

It was next necessary to determine whether the expressed recombinant CD44 proteins became successfully translocated to the cell surface. As shown in Figure 4 A-D, all GFP-positive COS-7 cells, transfected with pCD44H, pCD44E, pCD44H\( \Delta 67 \) and pCD44E\( \Delta 67 \), respectively, displayed brightly positive cyanine (red) immunostaining for CD44—staining that clearly outlined the
plasma membrane. Figure 4F depicts a phase contrast overlay of the same cells shown in Fig. 4D. Again it can be seen that the red staining for CD44 matches the position of the plasma membrane. No positive reaction was observed in non-transfected cells within the same field of view (GFP-negative cells) or control COS-7 cells transfected with pCDM8 vector minus an inserted DNA sequence (Fig. 4E). In addition, use of control isotype-specific control IgG or, pretreatment of these cells prior to incubation with BU-52 also resulted in a negative cell reaction (data not shown). Thus, translation of the carboxy-terminal truncated mutant CD44 proteins did not result in their enhanced degradation intracellularly or a failed translocation of these modified proteins to the plasma membrane.

Pericellular matrix assembly on live COS-7 cells transfected with CD44 expression constructs--
In an effort to determine the requirement of the cytoplasmic domain of CD44 in pericellular matrix assembly, an important chondrocyte function, transfected COS-7 cultures were supplemented with exogenous, purified HA and cartilage-derived aggrecan proteoglycan. Previous studies from our laboratory demonstrated that following successful transfection with pCD44H or pCD44E cDNA, COS-7 cells gained the capacity to capture and assemble pericellular matrices in the presence of these two matrix macromolecules (16). Consistent with our previous findings, neither untreated COS-7 cells, nor COS-7 cells transfected with control pCDM8 vector had the capacity to assemble pericellular matrices (Fig 5, panels A and B, respectively). However, COS-7 cells transfected with pCD44H or pCD44E, both exhibited the functional capacity to assemble large particle-excluding pericellular matrices in the presence of exogenous HA and proteoglycan (Fig. 5, panels C and E, respectively). When the same assay was performed on the CD44 truncation mutants, neither the pCD44HΔ67, nor the pCD44EΔ67 transfectants exhibited a capacity to assemble pericellular matrices (Fig. 5, panels D and F, respectively). Insets depict lower power views for each condition in Fig. 5 and confirm that the majority of cells within a field of view either exhibit (panels C and E) or do not exhibit (panels A, B, D and F) pericellular matrices. Thus, even though the mutant CD44 proteins are expressed on the cell surface with their extracellular proximal domains immunologically available, the
receptors are not capable of capturing, anchoring and organizing soluble HA-proteoglycan complexes.

**Binding and endocytosis of fluorescein-HA by CD44 transfected COS-7 cells**—Another important function of CD44 in chondrocytes is to internalize HA bound at the cell surface, delivering the glycosaminoglycan to lysosomes for degradation. Once the capacity of CD44-transfected COS-7 cells to participate chondrocyte-like matrix assembly was established (Fig. 5), the next step was to determine if these cells also gained the capacity for CD44-mediated endocytosis of HA. Again, COS-7 cells were transfected with pCD44H, pCD44HΔ67 or the empty vector pCDM8. However, in order to easily visualize transfected cells, a RFP-positive vector was co-transfected with each construct. Following transfection, the cells were incubated in complete media containing 40 µg/ml fl-HA. In order to distinguish internalized fl-HA, one set of cells was digested extensively with trypsin for 30 min at 37°C to remove all extracellular fl-HA bound at the cell surface. As shown in Fig. 6A, bright circumferential staining for fl-HA (green fluorescence) was visualized at the COS-7 cell surface of all pCD44H-transfected cells. COS-7 cells within the same field that were not successfully transfected (i.e., cells that were negative for red fluorescence protein) did not exhibit the capacity to bind fl-HA. Following trypsin treatment of pCD44H transfected cells, the ring of green fluorescence due to cell surface fl-HA was lost. However, staining for fl-HA remained (i.e., resistant to trypsin) in what appear to be intracellular vesicles (Fig. 6B). Similar positive binding and internalization of fl-HA was obtained with COS-7 cells transfected with pCD44E. For example, in a separate experiment, COS-7 cells were transfected with pCD44E (without the RFP vector) and incubated with a red fluorescence lysotracker reagent that concentrates in low pH intracellular vesicles. As can be seen in the trypsinized cells shown in Fig. 6C, the overlay image of green fl-HA and red lysotracker display co-localization within two of the vesicles in the image (arrows). This helps to confirm that the vesicular-localized fl-HA shown in Figs. 6B and 6C does, in fact, represent intracellular vesicles, some of which are late endosomes or lysosomes. Although the two larger lysotracker- fl-HA co-positive vesicles (Fig. 6C) stand out more clearly, smaller-sized double
positive-vesicles can also observed. No fl-HA cell surface binding (Fig. 6D) or intracellular accumulation of fl-HA (Fig. 6E) was observed in control pCDM8-transfected COS-7 cells. Thus, in order for COS-7 cells to gain a capacity to bind and internalize HA, receptors such as CD44H or CD44E must be expressed.

In the COS-7 cells transfected with pCD44HΔ67 (RFP-positive cells) little staining for cell surface-bound fl-HA was observed (Fig. 6F). However in a few cells, a thin but still discernable layer of fl-HA binding to the cell surface could be seen (Fig. 6G). This suggests that the mock short tail isoforms of CD44 still exhibit at least a limited capacity for HA binding. Nonetheless, no intracellular vesicular clusters of fl-HA were evident in pCD44HΔ67 transfectants subsequent to trypsin treatment (Fig. 6H). Like the pCD44HΔ67 transfectants, the pCD44EΔ67 also failed to display internalized fl-HA (data not shown). Thus, the presence of an intracellular tail is likely an absolute requirement for efficient HA endocytosis either because its absence limits HA binding at the cell surface and/or, its absence prevents interactions with cytosolic components necessary for the internalization events.

Co-transfection of CD44H and CD44HΔ67 isoforms and pericellular matrix assembly—Four aliquots of COS-7 cells were transfected with the pCD44H construct (2.0 µg/ml each). In addition, three of the groups were co-transfected with 0.5, 1.0 and 2.0 µg/ml of pCD44HΔ67. Western blot analysis was not able to clearly distinguish the two CD44 isoforms. However, a proportional shift of the broad band representing CD44 to lower molecular mass could be observed with increased proportion of pCD44HΔ67 to pCD44H (data not shown). At maximal time of expression (72 h) the transfected cells were assayed for their capacity to assemble chondrocyte-like pericellular matrices. As shown in Figure 7, as compared to cells transfected with pCD44H alone (panel A), transfection with increasing concentrations of pCD44HΔ67 resulted in a proportional decrease in size of the pericellular matrix (Figure 8, panels B-D). Transfection with equal concentrations of long tail and short tail constructs resulted in only a small exclusion zone surrounding the cells (panel D). A summary of data taken from three experiments that includes random field views of approximately 40 cells per condition is shown in
Table 1. Approximately 60% of the cells transfected with pCD44H alone had a morphometric ratio larger than 1.5 (matrix diameter:cell diameter). This number of matrix-positive cells is close to the transfection efficiency. Similar to the results depicted in Fig. 7, increasing the proportion of CD44HΔ67 expression resulted in a proportional decrease in the number of cells with large pericellular matrices. The proportion of cells with morphometric ratios less than 1.5 increased from 39.5% (pCD44H and no pCD44HΔ67) to 71.3% when the ratio of pCD44HΔ67 to pCD44H was 1:1. Similar to the results depicted in Fig. 5, 98.2% of the COS-7 cells transfected with pCD44HΔ67 alone had morphometric ratios less than 1.5 (Table 1). Thus, the expression of the short tail isoform not only results in the appearance of a non-ligand-binding receptor but also, its presence perturbs the function of long tail CD44 even when sufficient levels of long tail receptor are present.

Transfection of bovine articular chondrocytes with CD44HΔ67 and pericellular matrix assembly—The data shown in Figure 7 suggested that the expression of CD44HΔ67 inhibited the functions of the wild type CD44. To investigate this in a more physiological setting, primary cultures of bovine articular chondrocytes were transfected with pCD44HΔ67. Using FuGENE reagent and pre-incubation with Streptomyces hyaluronidase, transfection efficiencies in the range of 10-30% were obtained. As with the COS-7 cells, the expression of GFP allowed the identification of successfully transfected cells. Figure 8A depicts a phase contrast image of the exclusion of particles by bovine articular chondrocytes, revealing their prominent pericellular matrices. However, within the same field are three cells (arrows) devoid of a pericellular matrix. Phase contrast fluorescence microscopy of the same field (Figure 8B) reveal that the same three cells in Panel A lacking pericellular matrices were chondrocytes successfully transfected, GFP-positive cells. The insets in Figure 8A and 8B depict a separate transfection experiment. However, in this experiment the cells were plated at higher density and thus demonstrate the uniformity of pericellular matrices surrounding chondrocytes. Again, the cells present within the field that were lacking matrices (Figure 8A inset, encircled) were the same cells that were GFP-positive (Figure 8B inset, encircled) and presumably over-expressing CD44HΔ67. No effect on
matrix assembly was observed in chondrocytes transfected with pTracer plasmid alone or, CD44H subcloned into pTracer (data not shown). Further, chondrocytes transfected with any of the pTracer constructs did not elicit uptake of propidium iodide (red fluor that detects dead cells). Thus the loss of pericellular matrices observed in Figure 8A was not due to a transfection-related toxicity (e.g., GFP expression) or mere over-expression of a recombinant membrane protein.

DISCUSSION

The major findings of this study are that short tail CD44 isoforms (CD44HΔ67 and CD44EΔ67) do not support binding of HA to the plasma membrane, the assembly of pericellular matrices and consequently, do not mediate the internalization of bound HA. In addition, the over-expression of the short tail CD44 isoform together with long tail CD44 results in the inhibition CD44-mediated matrix assembly. Besides experimental applications of such results there is physiological significance as a protein identical to CD44HΔ67 is expressed in chondrocytes due to alternative splicing to generate exon 19-containing CD44.

Human articular chondrocytes naturally express both exon-19 and exon-20 containing CD44 mRNA transcripts. The proportion of exon-19 to exon-20 mRNA varies from donor to donor but typically falls within the range of 15% to 33% of the total CD44 message (13). This proportion can also be observed at the level of CD44 protein (13). We are not the first to document the expression of exon-19 containing CD44 mRNA. For example, short tail isoform mRNAs were detected in several lymphoid as well as nonlymphoid cells such as foreskin fibroblasts (18). These investigators also described the expression of CD44E long tail and CD44E tail-less transcripts in a keratinocyte cell line (18). However, the level of short tail expression in these cell types was reported as 0.5 to 1.0% of the total CD44 mRNA, much lower than what we observe in chondrocytes. Given that a naturally-occurring short tail CD44 is expressed by chondrocytes, and that the levels of expression vary considerably, we sought to determine the physiological role for the short tail CD44 isoform. Does short tail CD44 exhibit functional properties that are identical to long tail CD44, distinct from the long tail isoform or, does its
expression in some way act to modulate functions attributed to full length CD44?

One CD44 function that has received considerable attention is the regulation of HA binding. The common query is whether sites within the cytoplasmic or transmembrane domain are critical to the ability of the extracellular link domain of CD44 to bind HA. The results thus far have been controversial and inconclusive. Transfection of mouse AKR1 cells with a pCD44H∆66 construct resulted in severely reduced CD44 binding capacity for soluble HA (19), similar to what we observed for the human CD44H∆67 used in this study. However, in subsequent work, Lesley et al., demonstrated that the CD44H∆66 construct could be induced to bind fl-HA by pretreatment of transfected XJ(3)/CD44- cells with the anti-mouse CD44 monoclonal antibody IRAWB14 (20). We tested the effect of the anti-human CD44 monoclonal antibody F10-44-2 (shown to enhance HA binding in transfected Jurkat cells (21) to induce fl-HA binding to COS-7 cells transfected with pCD44H∆67. Unlike the previous report, the COS-7 transfectants continued to display little to no HA binding. Pure et al., found that constitutively phosphorylated CD44 serines residues 323 and 325 within the cytoplasmic domain were critical for efficient CD44 binding of HA when the mutated constructs were expressed in T lymphoma cells (15). However, Uff et al. performing similar mutations of cytoplasmic tail serines and expression in AKR1 cells, found no changes in the capacity of CD44 to bind HA (22). Similar conflicting results have been reported concerning the ankyrin binding motif within the cytoplasmic tail as well as clusters of basic residues that constitute the ezrin-binding motif. Some studies suggested that these sites were critical for efficient HA binding (23,24), only to be confronted by results demonstrating the lack of importance of these sites (22,25,26). Liu et al., have demonstrated that sites within the transmembrane domain of CD44 appear critical to efficient HA binding, Cysteine 286 in particular (27,28). The interaction of Cysteine 286 residues may participate in the dimerization of CD44 molecules. However, in recent studies, Lesley et al., using a domain swapping approach, have shown that a CD44 chimera containing the extracellular domain of CD44 linked to the transmembrane and cytoplasmic domain of β5 integrin (and expressed in AKR1 cells), binds fl-HA with the same capacity as wild-type CD44 (20). Their conclusion is
that the exact sequence or motifs within the transmembrane and/or cytoplasmic domain are not critical for constitutive CD44-mediated HA binding. What is critical may be merely a compatible transmembrane domain together with a minimum length of cytoplasmic domain (i.e., >6 amino acids). An exon-19 containing CD44 isoform expressed by chondrocytes or the comparable tail-less recombinant construct expressed in COS-7 cells, would not meet this minimum requirement. One conclusion that can be drawn from these observations is that the role of the short tail CD44 isoform cannot be as a “decoy” receptor because it has little native affinity to bind its ligand and unlikely competes with wild-type CD44 for HA.

Another suggested function of CD44 is to mediate the endocytosis of HA. One of the highlights of this study was that COS-7 cells transfected with either pCD44H or pCD44E gained the capacity to internalize bound HA. Although the involvement of CD44 has been implicated in several studies (29-33), including our own (8, 4, 34), to our knowledge the participation of CD44 in HA endocytosis has never been heretofore directly demonstrated by a “gain-of-function” approach. This implies that the monkey kidney epithelial cells (COS-7 cells) possess all of the remaining necessary cellular machinery to facilitate the internalization of HA. As shown in Fig. 6C, some of the internalized fl-HA co-localized with low pH intracellular vesicles. Given that Tammi et al., could not detect internalized HA co-localized within true (anti-cathepsin-D positive) lysosomes of keratinocytes (33), the vesicles observed in Fig. 6C may represent low pH, late endosomes on the path toward fusion with lysosomes. These results do not imply that CD44 is the only receptor that mediates HA internalization. Other receptors such as HARE (HA receptor for endocytosis (35)) and LYVE-1 (lymphatic vessel endothelial HA receptor (36)) have been also recently identified. HARE and LYVE-1 are likely responsible for the clearance of systemic, circulating HA. Although the expression of HARE or LYVE-1 were not directly assayed in this study, neither the control, non-transfected cells nor cells transfected with the empty pCDM8 vector, exhibited a capacity for HA binding or uptake. Thus it is likely that the parental COS-7 cells do not express significant levels of any form of HA receptors.
Neither of the short tail constructs, pCD44HΔ67 nor pCD44EΔ67 exhibited a capacity for HA binding, pericellular matrix assembly or, HA internalization. Validation for the latter point is less clear as HA binding is a pre-requisite for internalization. Further work, such as the domain swapping studies of Lesley et al., (20) will be required to determine whether distinct molecular motifs or sequence within the CD44 cytoplasmic tail actively participate in the internalization of HA. It would be expected that some level of association with the underlying actin cytoskeleton must participate in this event. However, our work with chondrocytes as well as recent studies by Tammi et al. suggest that CD44-mediated internalization of HA by keratinocytes is not associated with clatherin-coated vesicles, not associated with caveolae and not associated with pinocytosis (3, 33). In addition, the cytoplasmic tail domain of CD44 does not contain an AP-2 consensus binding sequence common to many receptors internalized via clatherin-coated vesicles (37). Thus, although a non-classical mode of CD44-mediated HA uptake is suggested, the possibility remains that this event is not under direct cellular control (i.e., via the cytoskeletal elements). The signal for HA internalization may be a reduction in steric resistance of the extracellular HA, either by reducing the size of the extracellularly-bound HA or, removal of proteoglycan bound to HA.

In this study, both pCD44H and pCD44E transfected COS-7 cells gained the constitutive capacity to bind fluorescein HA and assemble HA-dependent pericellular matrices, in the presence of exogenous HA and proteoglycan. Null transfected cells or cells transfected with the empty pcDM8 vector, did not exhibit this capacity. With the chondrocytes depicted in Fig. 8, the capacity to assemble pericellular matrices is inherent as these cells express all three components, CD44H (long tail), HA and aggrecan. These results confirm our earlier work (2,3, 16) and set the stage for examining the effects of the short tail mutant isoform of CD44. COS-7 cells co-transfected with pCD44HΔ67 and pCD44H (Fig. 7D) lost their capacity to assemble a
pericellular matrix as compared to cells transfected with pCD44H alone. This inhibition in matrix size was roughly dependent on the proportion of pCD44HΔ67 to pCD44H (Table 1). As the percentage of CD44HΔ67 increased there was a proportional shift in the cell population to cells with smaller pericellular matrices and eventually, a near complete absence of matrix. However, conclusions from these experiments alone are limited because it is impossible to know that each cell obtained an equivalent dose of both constructs at the designated proportion. However, transfection of chondrocytes with pCD44HΔ67 does not have this limitation. The chondrocytes naturally express sufficient CD44H to direct CD44-mediated functions such as matrix assembly. As with the co-transfected COS-7 cells, pCD44HΔ67 transfected chondrocytes (marked by the expression of GFP) lost their capacity to retain a pericellular matrix to the cell surface. The exact mechanism for this effect is not known. The over-expression of mutant CD44HΔ67 receptors could displace functional, endogenous wild type CD44 from plasma membrane domains necessary for HA binding. Alternatively, mutant pCD44HΔ67 could form aberrant non-functional dimers or oligomers with endogenous CD44 or, otherwise interfere with the requisite organization of CD44 necessary for HA binding. Regardless of the mechanism, the end result of over-expression of pCD44HΔ67 is an interference with cellular functions mediated via CD44, in essence, actions consistent with those of a dominant negative receptor. Thus, the use of pCD44HΔ67 will help to elucidate the role of CD44 in cells containing multiple HA receptors in addition to CD44. Future studies will also determine whether naturally occurring isoforms such as the exon 19-containing CD44 mRNA translation product serve to regulate endogenous long tail CD44H in the same fashion.
Acknowledgement: Supported in part by NIH grants P50-AR39239 (SCOR), RO1-AR43384 (WK), RO1-AR39507 (CBK) and grants from the National Arthritis Foundation.
FOOTNOTES

¶To whom correspondence should be addressed: Department of Biochemistry, Rush Medical College, Rush-Presbyterian-St. Luke's Medical Center, 1653 West Congress Parkway, Chicago, IL 60612
Chicago, Illinois 60612
Telephone # (312) 942-7837, Fax # (312) 942-3053, e-mail:wknudson@rush.edu

¹The abbreviations used are: Hyaluronan, HA; fluorescein-conjugated hyaluronan, fl-HA, Hyaluronan synthase, HAS; Dulbecco’s modified Eagle’s medium, DMEM; fetal bovine serum, FBS; hyaluronan, HA; proteoglycan, PG; glyceraldehyde-3-phosphate dehydrogenase, GAPDH; phosphate buffered saline; PBS hyaluronan binding protein, HABP; reverse transcription polymerase chain reaction, RT-PCR; GFP, green fluorescence protein; RFP, red fluorescence protein; monoclonal antibody, mAb; 4′, 6-diamidino-2-phenylindole, dihydrochloride, DAPI.
Fig. 1. **Strategy employed to generate the mock "short-tail" CD44 truncation mutant derivatives, pCD44HΔ67 and pCD44EΔ67.** Exon 18 encodes the single-pass transmembrane domain. The major CD44 isoform, CD44H, expresses the "long-tail" highly conserved 70 amino acid cytoplasmic domain encoded primarily by exon 20, (line A). Exon 19 contains an early stop codon and the resultant mRNA is represented primarily as 3’ untranslated message (line B). Translation of CD44 mRNA containing exon 19 results in a CD44 having a cytoplasmic domain containing only 3 amino acids (encoded in exon 18). The mock "short-tail" mutants pCD44HΔ67 and pCD44EΔ67 were generated by the introduction of a stop codon (Cys 295 - TGT → TGA- Stop) into exon 20-containing CD44 by PCR-mediated site-directed mutagenesis (line C).

Fig. 2. **CD44 mRNA expression in COS-7 cells transfected with CD44 expression constructs.** Aliquots of total RNA (0.5 µg) derived from COS-7 cells transiently transfected with CD44 expression constructs were reverse transcribed and PCR-amplified with CD44-specific primers for 25 cycles. The products were separated on a 1.5% agarose gel and visualized by ethidium bromide staining. Lane 1 represents φX174/Hae III DNA markers bands. Lanes 2-4 represent COS-7 cells transfected with 0.5 µg, 2.0 µg, or 4.0 µg/ml of pCD44H respectively; lanes 5-7, COS-7 cells transfected with 0.5 µg, 2.0 µg, or 4.0 µg/ml of pCD44HΔ67 expression construct; lanes 8-10, COS-7 cells transfected with 0.5 µg, 2.0 µg, or 4.0 µg/ml of pCD44E expression construct; lanes 11-13, COS-7 cells transfected with 0.5 µg, 2.0 µg, or 4.0 µg/ml of pCD44EΔ67 expression construct; lanes 14-16, COS-7 cells transfected with 0.5 µg, 2.0 µg, or 4.0 µg/ml of pCDM8 expression vector containing no insert. The RT-PCR product for the CD44E sequences are larger due to the inclusion of three additional alternatively spliced exons (exons 13-15) within the amplified region.
Fig. 3. Western blot analysis CD44H, CD44E, CD44HΔ67 and CD44EΔ67 protein expression in COS-7 cells. Following electrophoresis and electroblot transfer to nitrocellulose, the various CD44 isoforms expressed by transfected COS-7 cells were visualized following incubation with the anti-human CD44 monoclonal antibody BU-52 and development with alkaline phosphatase reagents. An 87.2 kDa standard marker protein band is depicted by an arrow (pre-stained protein that cannot be visualized in this reproduction). Lane 1 represents lysate from control, untreated COS-7 cells; lane 2, control pCDM8 transfectant lysate; lane 3, lysate from pCD44H transfected cells; lane 4, lysate from pCD44HΔ67 transfected cells; lane 5, lysate from pCD44E transfected cells and; lane 6, lysate from pCD44EΔ67 transfected cells. Horizontal lines depict the median migration position of the wild type CD44H and CD44E bands.

Fig. 4. Immunofluorescent staining of cell surface CD44. Seventy-two hours post-transfection, COS-7 cell transfectants (GFP positive cells) were fixed, washed, blocked and incubated with mouse anti-human CD44 mAb (BU52). Bound antibody was detected using cyanine-3-conjugated goat anti-mouse IgG followed by addition of DAPI nuclear counterstain. Cells transfected with either pCD44H (panel A), pCD44E (panel B), pCD44HΔ67 (panels C) or pCD44EΔ67 (panel D) all exhibited positive (red) staining for CD44. All nontransfected cells within the same field of view in panels A-D were negative for CD44. In addition, COS-7 cells transfected with pCDM8 vector alone did not exhibit immunoreactivity (panels E). Panel F is a phase contrast overlay of the same cells shown in panel D. Bars, 20μm.
Fig. 5. Matrix assembly on live COS-7 cells transfected with CD44 expression constructs. Seventy-two hours post-transfection COS-7 cell transfectants were trypsinized and allowed to re-attach overnight. Transfectants were then incubated in fresh medium containing exogenous HA and aggregating proteoglycan for 3 h. Matrices were visualized employing a particle exclusion assay. Untreated COS-7 cells or COS-7 cells transfected with control pCDM8 vector, did not have the capacity to assemble pericellular matrices (panels A and B, respectively). Cells transfected with either pCD44H (panel C) or pCD44E (panels E) both exhibited large, prominent cell associated matrices. However, cells transfected with pCD44HΔ67 (panel D), or pCD44EΔ67 (panel F) did not assemble pericellular matrices. Insets depict lower power fields of view demonstrating that the majority of cells present follow the same pattern of the higher power representative image.

Fig. 6. Binding and uptake of exogenous fl-HA by transfected COS-7 cells. Seventy-two hours post-transfection, COS-7 cell transfectants (RFP positive cells) were released from monolayers with EDTA and incubated in complete media containing 40 µg/ml fl-HA. To view cell surface binding, after 1 h of incubation at 4 °C, unbound fl-HA was removed, the cells fixed, incubated with DAPI nuclear counterstain and visualized by fluorescence microscopy. Panel A depicts two examples of RFP-positive COS-7 cells transfected with pCD44H; pCDM8-transfected cells in panel D and pCD44HΔ67 transfected cells in panels F and G. A corresponding aliquot of cells from each group was incubated for 3 h at 37 °C followed by extensive trypsinization to remove all cell surface bound fl-HA and reveal internalized (enzyme-protected) fl-HA. Two examples of trypsinized pCD44H-expressing cells are depicted in panel B, trypsinized pCDM8 in panel E and trypsinized pCD44Δ67 expressing cells in panel H. In a separate experiment COS-7 cells transfected with pCD44E (no RFP) was incubated with fl-HA for 3 h at 37 °C. During the final 30 min, red fluorescent lysotracker reagent was added, and the cells subsequently trypsinized, fixed, washed and viewed. Yellow fluorescence detected in panel C (arrows) is due to co-localization of green fl-HA and red lysotracker. Bars, 20µm.
Fig. 7. Co-transfection of CD44H and CD44HΔ67 isoforms and pericellular matrix assembly.

Seventy-two hours post-transfection COS-7 cell transfectants were trypsinized and allowed to attach overnight. Transfectants were incubated in medium containing exogenous HA and aggregating proteoglycan for 3 h. Matrices were visualized by the particle exclusion assay. Images depict a representative view of three separate experiments. Cells in all panels represent experiment groups co-transfected with same amount of pCD44H (2µg/ml) together with differing concentrations of pCD44HΔ67: 0 µg/ml (panel A), 0.5 µg/ml (panel B), 1.0 µg/ml (panel C) and 2.0 µg/ml (panel D).

Fig. 8. Transfection of bovine articular chondrocytes with CD44HΔ67 and pericellular matrix assembly.

Seventy-two hours post-transfection, pericellular matrices and cellular GFP fluorescence, associated with cells within a particular field, were visualized using a Nikon Diaphot inverted phase contrast microscope equipped with epifluorescence optics. In panel A, the particle exclusion assay is depicted of a low-density culture of chondrocytes. Panel B, represents the same field of view under fluorescence using a fluorescein filter. The three cells in Panel A that are devoid of pericellular matrices (arrows) are the same three GFP-positive cells highlighted by arrows in Panel B. Cells from a separate experiment, plated at higher density are shown in the insets. Again, the three encircled cells in Panel A inset that are devoid of pericellular matrices are the same three encircled GFP-positive cells highlighted by arrows in Panel B inset.
REFERENCES

1. Knudson, C. B. (1998) in The Chemistry, Biology and Medical Applications of Hyaluronan and its Derivatives (Laurent, T. C., ed), Portland Press, London
2. Knudson, C. B. (1993) J. Cell Biol. 120, 825-834
3. Knudson, W., Aguiar, D. J., Hua, Q., and Knudson, C. B. (1996) Exp. Cell Res. 228, 216-228
4. Chow, G., Knudson, C. B., Homandberg, G., and Knudson, W. (1995) J. Biol. Chem. 270, 27734-27741
5. Chow, G., Nietfeld, J., Knudson, C. B., and Knudson, W. (1998) Arthritis Rheum. 41, 1411-1419
6. Knudson, C. B., Nofal, G. A., Pamintuan, L., and Aguiar, D. J. (1999) Biochem. Soc. Trans. 27, 142-147
7. Knudson, C. B., and Knudson, W. (1993) FASEB J. 7, 1233-1241
8. Hua, Q., Knudson, C. B., and Knudson, W. (1993) J. Cell Sci. 106, 365-375
9. Nishida, Y., D'Souza, A. L., Thonar, J. M. A., and Knudson, W. (2000) Arthritis Rheum. 43, 1315-1316
10. Knudson, W., and Knudson, C. B. (1999) in Glycoforum Vol. http://www.glycoforum.gr.jp/science/hyaluronan/HA10/HA10E.html, pp. 1-30
11. Screaton, G. R., Bell, M. V., Jackson, D. G., Cornelis, F. B., Gerth, U., and Bell, J. I. (1992) Proc. Natl. Acad. Sci. USA 89, 12160-12164
12. Nishida, Y., Knudson, C. B., Nietfeld, J. J., Margulis, A., and Knudson, W. (1999) J. Biol. Chem. 274, 21893-21899
13. Jiang, H., Knudson, C. B., and Knudson, W. (2001) Arthritis Rheum. 44, 2599-2610
14. Kalomirisis, E. L., and Bourguignon, L. Y. W. (1988) J. Cell Biol. 106, 319-327
15. Pure, E., Camp, R. L., Peritt, D., Panettieri, R. A., Lazaar, A. L., and Nayak, S. (1995) J. Exp. Med. 181, 55-62
16. Knudson, W., Bartnik, E., and Knudson, C. B. (1993) Proc. Natl. Acad. Sci. USA 90, 4003-4007
17. Madry, H., and Trippel, S. B. (2000) *Gene Therapy* 7, 286-291
18. Goldstein, L. A., and Butcher, E. C. (1990) *Immunogenetics* 32, 389-397
19. Lesley, J., He, Q., Miyake, K., Hamann, A., Hyman, R., and Kincade, P. W. (1992) *J. Exp. Med.* 175, 257-266
20. Lesley, J., English, N., Charles, C., and Hyman, R. (2000) *Eur. J. Immunol.* 30, 245-253
21. Clark, R. A., Alon, R., and Springer, T. A. (1996) *J. Cell Biol.* 134, 1075-1087
22. Uff, C. R., Neame, S. J., and Isacke, C. M. (1995) *Eur. J. Immunol* 25, 1883-1887
23. Liu, D., Liu, T., and Sy, M. S. (1998) *Cell. Immunol.* 190, 132-140
24. Lokeshwar, V. B., Fregien, N., and Bourguignon, L. Y. W. (1994) *J. Cell Biol.* 126, 1099-1109
25. Perschl, A., Lesley, J., English, N., Trowbridge, I., and Hyman, R. (1995) *Eur. J. Immunol.* 25, 495-501
26. Legg, J. W., and Isacke, C. M. (1998) *Current Biol.* 8, 705-708
27. Liu, D., and Sy, M.-S. (1996) *J. Exp. Med.* 183, 1987-1994
28. Liu, D., and Sy, M. S. (1997) *J. Immunol.* 159, 2702-2711
29. Orkin, R. W., Underhill, C. B., and Toole, B. P. (1982) *J. Biol.Chem.* 257, 5821-5826
30. Culty, M., Nguyen, H. A., and Underhill, C. B. (1992) *J. Cell Biol.* 116, 1055-1062
31. Culty, M., Shizari, M., Thompson, E. W., and Underhill, C. B. (1994) *J. Cell. Physiol.* 160, 275-286
32. Kaya, G., Rodriguez, I., Jorcano, J. L., Vassalli, P., and Stamenkovic, I. (1997) *Genes & Develop.* 11, 996-1007
33. Tammi, R., Rilla, K., Pienimaki, J.-P., MacCallum, D. K., Hogg, M., Luukkonen, M., Hascall, V. C., and Tammi, M. (2001) *J. Biol. Chem.* 276, 35111-35122
34. Aguiar, D. J., Knudson, W., and Knudson, C. B. (1999) *Exp. Cell Res.* 252, 292-302
35. Zhou, B., Weigel, J. A., Fauss, L., and Weigel, P. H. (2000) *J. Biol. Chem.* 275, 37733-37741
36. Banerji, S., Ni, J., Wang, S.-X., Clasper, S., Su, J., Tammi, R., Jones, M., and Jackson, D. G. (1999) *J. Cell Biol.* 144, 789-801
37. Kirchhausen, T. (1999) *Annu. Rev. Cell Dev. Biol.* 15, 705-732
Table 1. Morphometric analysis of pericellular matrix size.

| D/d | pCDM8 (2µg) | pCD44HΔ67(2µg) | pCD44H(2µg) | pCD44H(2µg) | pCD44H(2µg) | pCD44H(2µg) |
|-----|-------------|----------------|-------------|-------------|-------------|-------------|
|     |             | -              | +           | +           | +           | +           |
| >2.0| 0%          | 0%             | 48.2% (±3.4%) | 30.1% (±2.0%) | 17.7% (±2.3%) | 9.5% (±1.2%) |
| 1.5-2.0| 0%       | 1.8% (±0.5%) | 12.3% (±1.5%) | 21.8% (±1.8%) | 27.7% (±3.1%) | 9.2% (±2.3%) |
| <1.5| 100%        | 98.2% (±1.5%) | 39.5% (±2.8%) | 48.1% (±3.7%) | 55.6% (±4.6%) | 71.3% (±5.6%) |

Morphometric analysis of changes in pericellular matrix size present on COS-7 cells transfected with CD44 constructs as described in Fig. 7. *D/d* is the ratio of the diameter of the pericellular matrix (D) divided by the diameter of the cell delineated by the plasma membrane (d). The data have been grouped into three ranges (>2.0, large matrices such as those depicted in Fig. 7A, 1.5-2.0, small-to-medium sized matrices such as those depicted in Fig. 7C and, <1.5, cells with small or no matrices). Data shown represent the average value of three separate experiments. In each experiment three random views of total 40 cells were analyzed.
A) CD44H (long tail)  

B) CD44 exon 19 (short tail)  

C) CD44HΔ67 (mock short tail)
Requirement for the CD44 cytoplasmic domain for Hyaluronan binding, pericellular matrix assembly, and receptor-mediated endocytosis in COS-7 cells
Hong Jiang, Richard S. Peterson, Weihua Wang, Eckart Bartnik, Cheryl B. Knudson and Warren Knudson

J. Biol. Chem. published online January 15, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M108654200

Alerts:
  • When this article is cited
  • When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts