Introduction: CD44 is the principle hyaluronan (HA) receptor, anchoring proteoglycans (i.e., aggrecan) to the chondrocyte cell surface via interaction of the HA with CD44. CD44-mediated cell interaction with HA has been implicated in a variety of physiological events, including cell-cellular matrix signaling, presentation of chemokines and growth factors, as well as HA uptake and degradation. Early osteoarthritis (OA) is characterized by loss of the proteoglycan (PG)-rich chondrocyte-associated matrix within cartilage tissue. In an effort to determine the biologic role of CD44 in events contributing to the onset of OA, we are examining the role of discrete CD44 cytoplasmic domains in regulating CD44 function. Our strategy is to transiently over-express full-length CD44, as well as carboxyl-terminal deletion constructs in non-CD44 expressing COS-7 cells. We have established that COS-7 cells transiently over-expressing hCD44HA67, (a ‘mimic’ of a naturally occurring alternatively spliced CD44 isoform which eliminates the carboxyl-terminal 67 amino acids (aa) contained within the 70 aa CD44 cytoplasmic tail), are incapable of anchoring an HA/PG cell-associated matrix, nor can the transfecants internalize fluorescein (Fl)-conjugated HA (1). We now report results obtained with the CD44 truncation-mutant expression-construct, hCD44HA54. hCD44HA54 deletes the carboxyl-terminal 54 aa, thus possessing the amino-proximal 16 aa contained within the CD44 cytoplasmic tail. Whereas hCD44HA67 exhibited none of the CD44-associated functional capacities, hCD44HA54 can both bind and internalize Fl-HA.

Materials and methods: Full-length hCD44H cDNA was cloned into the CMV expression vector, pCDM8 (Invitrogen). The hCD44H truncation mutants were created by the introduction of a stop codon employing PCR-mediated site-directed mutagenesis (Stratagene). COS-7 cells were transiently transfected with various CD44 constructs using LipofectAMINE 2000 (Gibco BRL). A constitutively expressing red fluorescent protein construct, PDsRed2-C1 (Clontech), was co-transfected in all experiments as a visual indicator of successful COS-7 transfection and, overall transfection efficiency. hCD44H and hCD44E CDNAs were subcloned into the pTRACER-EF/V5-His vector (Invitrogen) in-frame creating constructs hCD44H-EF/V5-His and hCD44E-EF/V5-His, respectively. Assays for fluorescein (Fl)-HA (Fl-HA) binding and internalization were performed as described previously (1). Western blot analysis was performed with a mouse anti-human CD44 monoclonal antibody, BU52 (The Binding Site), or the anti-V5 antibody (Invitrogen) that allows detection of recombinant proteins containing the V5 epitope.

Results: The Western depicted in Fig 1A demonstrates COS-7 transfecants expressing hCD44H, hCD44E and truncation derivative constructs. All of the signals are at the anticipated molecular weight for appropriately processed (e.g., glycosylated) translation products. Interestingly, lanes expressing hCD44Hwt, hCD44HΔ54, hCD44Ewt, and hCD44EA54 all demonstrate a high molecular weight, ‘shifted’, signal. The absence of the ‘shift’ in COS-7 transfecants expressing hCD44HΔ67 or hCD44EA67 correlates with the loss of CD44-associated biologic function(s). While this may represent CD44 oligomerization and/or association with an unknown protein, it is presently unknown. To further address this observation, hCD44H-EF/V5-His and hCD44E-EF/V5-His constructs were created for future immunoprecipitation experiments. Fig 1B depicts a Western that validates the expression of the recombinant CD44H- and E-V5/His proteins, (note the higher molecular weight signals attributed to the in-frame addition of 35 aa encoded by the V5/His ORF). Fig 2 demonstrates that both hCD44Hwt and hCD44HΔ54 both possess the ability to bind and internalize Fl-HA subsequent to a 4 Hr incubation with 50 ug/mL Fl-HA in complete medium. The right-side panels depict internalized Fl-HA subsequent to a 30-minute trypsin digestion to eliminate cell-surface binding.

Discussion: The results reported here indicate that at least a subset of CD44 biological function (i.e., stable HA cell-surface binding and retention, and HA internalization) require only the plasma membrane proximal 16 aa of the intact 70 aa CD44 cytoplasmic tail. Interestingly, hCD44EA54 disrupts a putative ankyrin-binding domain, yet possesses a plasma membrane proximal ERM family binding domain. Future experiments will include mutagenesis of 3 consecutive lysine residues, contained within the putative ERM domain, to further delineate cytoplasmic domain requirements for CD44 ligand HA binding, and internalization.

Acknowledgements: This work supported in part by NIH grants P50AR39239, RO1AR43384, RO1AR39507 and grants from the National Arthritis Foundation.