

The Liberation of CD44 Intracellular Domain Modulates Adenoviral Vector Transgene Expression^{*[5]}

Received for publication, January 30, 2012, and in revised form, July 18, 2012. Published, JBC Papers in Press, August 3, 2012, DOI 10.1074/jbc.M112.347369

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Background: Hyaluronan-CD44 interaction enhances expression of adenoviral vector transgenes.

Results: Inhibition of CD44 targeting proteases blocks enhancement of vector transgene expression and liberated intracellular domain enhances expression.

Conclusion: CD44 proteolysis plays a role in the modulation of adenoviral vector transgene expression.

Significance: Understanding the role of CD44 signaling in enhancement of transcription of adenoviral genes will be beneficial in viral gene transfer applications.

The success of gene therapy in the ocular environment is partly due to the presence of hyaluronan in vitreous. Here we explore the mechanism of hyaluronan-mediated enhancement of adenoviral vector transgene expression. Introduction of hyaluronan receptor CD44 into CD44-negative cells followed by transduction in the presence of vitreous with an adenoviral vector containing an IL-12-coding transgene increases IL-12 secretion. We demonstrate that sequential CD44 proteolysis is responsible for hyaluronan-mediated enhancement. Metalloproteinase or γ -secretase inhibitors decrease adenoviral-mediated transgene expression. Deletion of these proteolytic sites in CD44 also inhibits transgene expression. Expression of CD44 with a mutation to prevent phosphorylation of serine 325 inhibits the response to vitreous. Expression of the CD44 intracellular domain enhances transgene expression in the absence of vitreous. CD44-mediated enhancement of gene expression was observed with vectors using different promoters and appears because of an increase in mRNA production, not because of an increase in vector transduction as determined by quantitative RT-PCR and quantitative PCR, respectively. These data fit a model where the interaction of hyaluronan in vitreous and CD44 modulates transgene expression by initiating CD44 proteolysis and release of the cytoplasmic domain, resulting in increased transgene transcription.

Adenoviral (Ad)² vectors have been employed to deliver genes to treat retinal diseases and ocular malignancies such as

retinoblastoma (1, 2). When used within the intraocular environment, these and other viral vectors have demonstrated superior efficacy and safety when compared with systemic use (1–6). Although ocular immune privilege plays an important role in the success of ocular gene therapy (7), we sought to investigate what role the unique biochemical composition of the intraocular environment may play. In the eye, the vectors are exposed to the vitreous humor, the gelatinous fluid that fills the posterior cavity of the eye and whose major biochemical components are collagen and the nonsulfated glycosaminoglycan hyaluronan (HA). Our laboratory has shown that the interaction of HA with its cognate receptor CD44 can modulate Ad vector transgene expression both *in vitro* and *in vivo* (8). To further expand upon these observations, we studied signaling mechanisms of CD44 and their role in the modulation of Ad transgene expression in the presence of vitreous.

One mechanism of CD44 signaling involves sequential proteolysis and liberation of its intracellular domain (CD44ICD) (9), a process studied extensively in malignancies (10) and somatic cells (11). The first step in this process is the cleavage and shedding of the extracellular domain of CD44 by one of several matrix metalloproteases (MMPs) (12). The remaining CD44 peptide becomes the substrate of the γ -secretase complex. This enzymatic complex cleaves CD44 within its transmembrane domain and liberates the CD44ICD into the cellular cytoplasm (13). The CD44ICD then translocates to the nucleus where it can regulate gene expression (14). Additionally, CD44 is known to be phosphorylated at two serines in its intracellular domain at residues 291 and 325. These phosphorylations have been shown to potentially regulate the interaction of CD44 with cytoskeletal components (15). Phosphorylation at serine 325 has also been shown to be necessary for facilitating the interaction of CD44 with HA (16).

Multiple viral gene transfer strategies could potentially benefit from understanding the mechanism of increased transgene

^{*} This work was supported, in whole or in part, by National Institutes of Health Grants GM085793 (to C. J. I.), CA103698 (to R. L. H.), and CA97762 (to R. L. H.). This work was also supported by the Clayton Foundation for Research, the Retina Research Foundation, and Golfers Against Cancer and in part by a grant from the Howard Hughes Medical Institute's Med-Into-Grad Initiative.

[5] This article contains supplemental text and Figs. S1 and S2.

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² The abbreviations used are: Ad, adenovirus; Ad5, adenovirus serotype 5; CAR, coxsackie and adenovirus receptor; F35, adenovirus fiber protein

serotype 35, HA, hyaluronan; ICD, intracellular domain; LU, light unit; Luc, luciferase; MMP, matrix metalloprotease; MOI, multiplicity of infection; o-HA, oligosaccharides of hyaluronan.

expression through CD44-mediated signaling. Here we explore the potential of this approach to increase IL-12 production after gene transfer. IL-12 is a proinflammatory cytokine secreted by dendritic cells that, among other functions, promotes cytotoxic T cell and NK cell activity (17). The anti-tumor effects of IL-12 have been studied previously by administering recombinant IL-12 into a mouse model of neuroblastoma (18), and others have explored *ex vivo* modification of cells with Ad-IL12 vectors to induce an anti-tumor immune response after infusion into animal models of neuroblastoma (19) and glioblastoma (20). Although clinical application of IL-12 therapy has thus far not demonstrated robust efficacy (21), achieving higher levels of IL-12 expression in *ex vivo* modified immune cells or within the tumor itself could potentially enhance tumor killing *in vivo* using this strategy.

The studies reported here show that the vitreous-mediated enhancement of Ad transgene expression occurs under multiple promoters and is seen with Ad5 vectors that enter the cell via coxsackie and adenovirus receptor (CAR) or with Ad5F35 vectors that enter the cell via the Ad35 receptor CD46. These studies also demonstrate that the interaction of HA with CD44 plays a significant role in regulating vitreous-mediated enhancement of Ad transgene expression. This enhancement was found to result in an increase in transgene transcription without an increase in Ad vector transduction efficiency. We further demonstrate that the inhibition of MMPs or the γ -secretase complex by small molecule inhibitors significantly decreases Ad transgene expression *in vitro*. The role of these enzymes in the mechanism of CD44 signaling was confirmed by demonstrating that the deletion of the MMP or γ -secretase recognition sites on CD44 abrogated the vitreous-mediated enhancement of Ad transgene expression. Additionally, overexpression of the CD44ICD was shown to significantly increase the Ad transgene expression in a CD44-negative cell line. We also observed that the phosphorylation state of serine 325 in the intracellular domain of CD44 influences vitreous-mediated enhancement of Ad transgene expression.

EXPERIMENTAL PROCEDURES

Cell Cultures—Cell lines were grown as follows: Y79-Rb cells, 293T cells, and COS-7 cells were grown in DMEM (Mediatech, Manassas, VA) supplemented with 5% FBS (Gemini Bio-Products, Sacramento, CA) and 1% penicillin/streptomycin (Mediatech); HEK293 cells and Jurkat cells were grown in RPMI 1640 (Mediatech) supplemented with 5% FBS and 1% penicillin/streptomycin; and SK-N-DZ cells were grown in DMEM supplemented with 10% FBS, nonessential amino acids (Mediatech) and 1% penicillin/streptomycin. All of the cultures were maintained in a humidified incubator at 37 °C with 5% CO₂.

Antibodies and Chemicals—CD44 antibody (EPR1013Y) against the ectodomain was purchased from Abcam (Cambridge, MA); CD44 antibody against the intracellular domain was a gift from Drs. Osamu Nagano and Hideyuki Saya at Keio University School of Medicine, Tokyo, Japan; antibody against the V5 epitope, goat anti-mouse IgG Alexa-Fluor 488- and 555-conjugated secondary antibodies, and goat anti-rabbit IgG Alexa-Fluor 488- and 555-conjugated secondary antibodies were all purchased from Invitrogen; CD44 flow cytometry anti-

body conjugated to phycoerythrin (G44-26) was purchased from BD Pharmingen (San Diego, CA). The γ -secretase inhibitor DAPT, purified rooster comb hyaluronan and bovine testicular hyaluronidase type-IV were purchased from Sigma-Aldrich; MMP inhibitors TAPI-0 and TAPI-1 were purchased from EMD Millipore (San Diego, CA).

Adenoviral Vectors—The first generation Ad vector serotype 5 delivering a luciferase (Luc) reporter gene under the control of the CMV promoter (Ad5/CMV-Luc) was prepared and expanded by the Vector Development Laboratory at Baylor College of Medicine. The first generation Ad vector delivering the luciferase gene under the control of the SV40 promoter (Ad5/SV40-Luc) was obtained from Dr. Anja Ehrhardt from the Ludwig-Maximilians-Universität München (Munich, Germany) and expanded by the Vector Development Laboratory at Baylor College of Medicine. The Ad5F35/CMV-Flexi-IL-12 vector, a first generation Ad vector serotype 5 with a chimeric fiber protein of serotype 35, delivering a construct coding for the IL-12 heterodimer subunits p35 and p40 linked by a flexible linker peptide, was provided by Dr. Stephen Göttscalk at the Center for Cell and Gene Therapy at Baylor College of Medicine.

Preparation of Vitreous—Frozen bovine eyes were obtained from a local abattoir, immersed in 100% ethanol for 30 s to sterilize, and immersed in sterile PBS for 15 min to partially thaw (the sclera and surrounding tissues were thawed, but the vitreous sac was kept frozen to ease extraction). The cornea was removed using a scalpel, and the sclera was cut radially toward the posterior of the globe to liberate the vitreous sac. The vitreous sac was extracted and placed in a sterile 15-cm dish on ice. Once thawed, the hyaloid membrane and cortical vitreous were minced with a scalpel, and the vitreous was sheared using a 10-ml Luer-lock syringe with no needle, an 18-gauge needle, and a 21-gauge needle in succession until the viscosity of the vitreous was minimized at each step. The sheared vitreous was centrifuged at 4 °C at 5000 \times g for 10 min, and the supernatant was aliquoted and frozen.

Luciferase Assay—To assay luciferase activity, cells plated in a 96-well plate (2×10^4 cells/well) were washed once with PBS and lysed in 50 μ l/well reporter lysis buffer (Promega, Madison, WI). 5 μ l of cell lysate was added to 50 μ l of luciferase substrate (Promega) and mixed gently by flicking. Luminescence was averaged for 12 s using a luminometer. Counts per second were converted into light units (LU) by a standard curve using recombinant luciferase enzyme (Promega). Finally, LUs were divided by volume of lysate per reaction (LU/ μ l) and then standardized to LU/ μ g protein by dividing by total protein concentration.

Protein Determination—Protein concentration was determined using the Bradford method. Lysate samples in 200 μ l of Bradford reagent (Bio-Rad) were incubated in a 96-well plate at room temperature for 5 min, and the absorbance at 595 nm was determined in a microplate spectrophotometer using a BSA standard curve.

Generation of Small Oligosaccharides of Hyaluronan (o-HA)—Briefly, 20 mg of purified HA was dissolved in 4 ml of Buffer A (150 mM sodium chloride, 100 mM sodium acetate, 1 mM EDTA at pH 5.0). After dissolution, 200 units of hyaluron-

idase were added, and the reaction was incubated in a 37 °C water bath for 20 min. Protein was removed from the solution by the addition of 1 ml of 100% (w/v) TCA and centrifugation at >16,000g for 5 min. The supernatant was passed through a 5000 molecular weight cutoff centrifugal filter (Sartorius-Stedim, Bohemia, NY) to remove high molecular weight contaminants and HA fragments larger than 12 disaccharide units, after which the flow-through buffer was exchanged twice using a 1000 molecular weight cutoff dialysis membrane (G-Biosciences, Maryland Heights, MO) in 1 liter of PBS for 12 h each to remove the TCA. The solution was subsequently concentrated to ~500 μ l by placing the membrane in high molecular weight hygroscopic polymer (G-Biosciences). Purity of o-HA was verified by PAGE and staining using the Alcian blue-silver stain method (22). Briefly, the gel was stained by incubation with 0.05% (w/v) Alcian blue in deionized water for 30 min with gentle agitation followed by de-staining for 30 min in deionized water. The gel was then stained using the SilverQuest silver staining kit (Invitrogen) according to manufacturer's directions. The yield concentration of o-HA was quantified using a carbazole-based colorimetric assay (23). An aliquot of sample was placed in a borosilicate test tube. Next, 1.5 ml of acid-borate solution (30 mM borax, 80% (v/v) sulfuric acid in deionized water) was added to the sample and mixed, after which 50 μ l of 0.1% (w/v) carbazole solution (dissolved in 100% ethanol) was added and mixed. The samples were incubated at 60 °C for 1 h, and the absorbance of the samples was measured at 530 nm using a spectrophotometer.

Quantitative PCR—Quantitative PCR analysis of intracellular Ad genome copy number and quantitative RT-PCR analysis of IL-12 mRNA levels were performed as described in the [supplemental text](#).

Site-directed PCR Mutagenesis and Site-directed Ligase-independent Mutagenesis—Mutations within the γ -secretase cleavage recognition site of CD44 as well as point mutations of the serine residues 291 (S291G) and 325 (S325A) within the intracellular domain of CD44 were introduced by site-directed mutagenesis as described in the [supplemental text](#). Site-directed ligase-independent mutagenesis was performed as described by Chiu *et al.* (24) to delete amino acid residues 193–249 of CD44, the region containing all three recognition sites for metalloproteases as reported by Kajita *et al.* (25) and Murai *et al.* (26), as described in the [supplemental text](#).

Immunocytochemistry—The cells were grown at 2×10^5 cells/well in a 24-well culture plate. Growth media were removed, and the cells were washed once with PBS and then fixed in 4% formaldehyde in PBS at room temperature for 10 min. The cells were then washed twice with PBS. Quenching solution (0.1 M NH_4Cl in PBS) was added, and the cells were incubated at room temperature for 10 min and washed twice with PBS. The cells were permeabilized in 0.2% Triton X-100 in PBS for 30 min on ice and washed three times with PBS. The cells were blocked in 5% BSA solution in PBS at 37 °C for 20 min with constant agitation. The antibody at its proper dilution in 1% BSA in PBS was added for 1 h at 37 °C with constant agitation, followed by washing three times with PBS. Secondary antibody conjugated to Alexa-Fluor diluted 1:5000 in 1% BSA in PBS was added and incubated for 30 min at 37 °C with constant

agitation. The cells were then washed three times with PBS, counterstained using Hoechst 33342 stain (Sigma-Aldrich) and imaged with a fluorescent microscope. Mean nuclear fluorescence intensity was quantified using ImageJ software by using the DAPI channel to gate the nuclear compartments and quantifying pixel intensity within the nuclei.

Generation of Lentiviral Vectors and Stable Cell Lines—Wild-type standard isoform CD44 and its different mutants were cloned into pCDH-CMV-MCS-EF1-copGFP lentivector packaging plasmid (System Biosciences, Mountain View, CA). Positive plasmid clones were expanded and evaluated using a transient transfection into COS-7 cells (8×10^5 cells/well) using the Lipofectamine 2000 reagent (Invitrogen) in a 6-well plate as per the manufacturer's protocol. Transgene expression was determined by Western blot using SDS-PAGE (8% gel) and detection with an anti-CD44 antibody. To package the lentiviral vectors, 293T cells were seeded in 10 ml of DMEM supplemented with 5% FBS and 1% penicillin/streptomycin at 3×10^6 cells in a 10-cm dish and kept at 37 °C 5% CO_2 overnight. The media were removed and replaced with 9 ml of DMEM completed with 10% FBS without antibiotics. The pCDH plasmid containing the CD44 gene and the pPACKH1 packaging plasmid mix (System Biosciences) were transfected into the cells according to the manufacturer's directions. The plates were then kept at 37 °C and 5% CO_2 for 48 h. Supernatant containing the viral particles was harvested and filter-sterilized. To transduce cells, CD44-negative SK-N-DZ neuroblastoma cells were seeded in a 6-well plate at 3×10^5 cell/well in complete DMEM. After attachment to the plate, the media were removed and replaced with 2 ml of the lentiviral supernatant. The cells were transduced at 37 °C 5% CO_2 for 48 h. After transduction, the cells were subjected to FACS for high GFP expression.

Cloning the CD44 Intracellular Domain (CD44ICD)—The CD44ICD truncated gene was isolated and cloned into pcDNA6 plasmid to add a C terminus V5 epitope tag as described in the [supplemental text](#). The clones were sequenced to verify the correct mutation within the CD44 sequence. The clones were further tested by transient transfection into COS-7 cells, followed by Western blot against the V5 epitope to verify expression.

IL-12 ELISA Assay—Conditioned media from transduced cells were flash frozen using an ethanol/dry ice bath and stored at –80 °C until analysis. An IL-12 ELISA kit (Peprotech, Rocky Hill, NJ) was utilized per the manufacturer's instructions. 96-well microplates were coated with an IL-12 capture antibody overnight at room temperature, washed, and blocked. Either sample or recombinant IL-12 standard was added to each well, incubated at room temperature for 2 h, and removed. 100 μ l of IL-12 detection antibody was added to each well, incubated at room temperature for 2 h and removed. 100 μ l of diluted avidin-HRP conjugate was added followed by an incubation of 30 min at room temperature and removed. 150 μ l of ABTS liquid substrate (Thermo Fisher, Rockford, IL) was added to each well and incubated at room temperature for 15 min. Absorbance values were obtained using a 96-well plate reader at 405 nm and a reference wavelength at 650 nm.

Statistical Analysis—Quantitative data were analyzed using the Graph Pad Prism 5 software. For the comparison of more

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than two averages, an analysis of variance test was conducted followed by the Student-Neuman-Keuls or Dunnet multiple comparison post-test to detect significant differences between all groups or between a control group and experimental groups. Statistical significance was assumed at $p < 0.05$. Symbols denoting levels of statistical significance are as follows: *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$.

RESULTS

Vitreous Enhances Adenoviral Vector Transgene Expression by Increasing Transcription on Multiple Promoters—The enhancement of Ad transgene expression observed in the presence of vitreous is possibly due to an increase in the efficiency of Ad transduction. To determine the mechanism by which vitreous increases expression, the CD44-negative cell line SK-N-DZ was engineered to express CD44 by transducing the cells with a lentiviral vector delivering genes coding for the standard isoform of CD44 (RefSeq ID NM_001001391) and GFP. The cells were sorted for GFP expression by FACS and subsequently transduced with Ad5/CMV-Luc in medium with or without 5% vitreous for 18 h, after which DNA was isolated using TRIzol. The number of Ad genomes per cell was determined by quantitative real time PCR. The results show no significant difference in the amount of Ad genomes per cell in the presence or absence of 5% vitreous in either CD44-expressing or control cells (Fig. 1A), suggesting that vitreous does not increase internalization of the vector. To determine whether vitreous has a similar effect on Ad5F35 vectors (which bind and enter the cell via CD46 as opposed to CAR), Y79 cells were transduced with Ad5F35/CMV-Flexi-IL12 (the transgene of which codes for both proteins of the IL-12 heterodimer joined by a linker peptide) in medium with or without 5% vitreous for 18 h, after which DNA and RNA were isolated. The number of Ad genomes per cell was determined by quantitative real time PCR. The results also show no difference in the amount of Ad genomes per cell in the presence or absence of 5% vitreous (Fig. 1B), suggesting that vitreous has no effect on vector internalization regardless of its binding receptor. The effect of vitreous on transcription of the IL-12 transgene was then determined by quantitative RT-PCR assay measuring IL-12 transgene mRNA. Significant increases in mRNA levels (Fig. 1C) and protein levels (Fig. 1D) in the vitreous-treated cells were observed as compared with untreated controls. Total mRNA levels were not significantly changed after vitreous treatment (supplemental Fig. S1). These results suggest that vitreous enhances Ad transgene expression by increasing transcription without affecting viral vector internalization.

Previous work studying the effect of vitreous on Ad transgene expression was carried out using transgenes driven almost exclusively by the CMV promoter (8). To determine whether this enhancement of transgene expression is dependent on the CMV promoter that regulates the expression of the transgene, the effects of vitreous on Ad transgene expression with the vector using the CMV promoter were compared with the effects with a vector using the SV40 promoter. Multiplicities of infection (MOI) for each cell line and each promoter were determined empirically using the corresponding luciferase-expressing vector, choosing a vector dose that provided luciferase

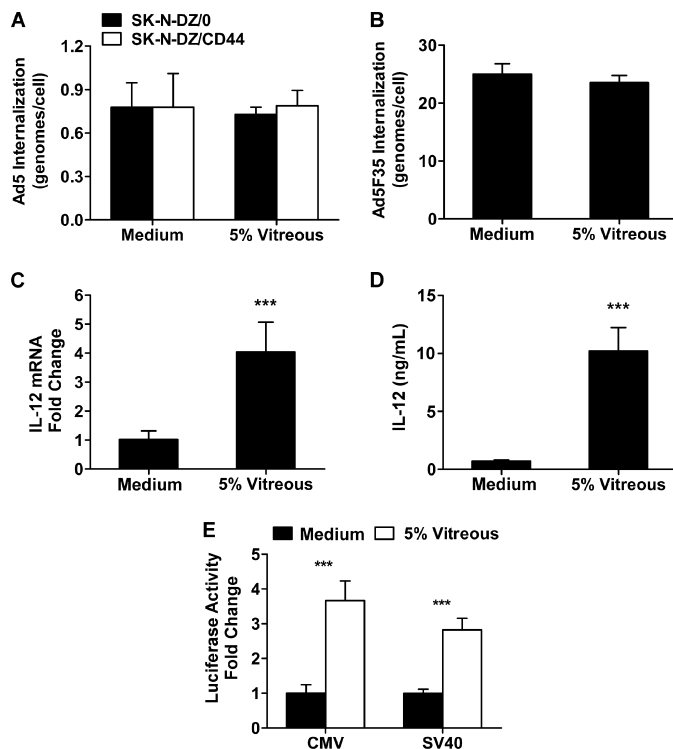


FIGURE 1. Vitreous enhances transcription of transgenes behind multiple promoters without increasing Ad vector internalization. A, SK-N-DZ cells were transduced with lentivector delivering standard CD44 or empty control as described under "Experimental Procedures." Selected cells (2×10^4 cells/well) were transduced with Ad5/CMV-Luc (MOI 50 pfu/cell) in the presence of 5% vitreous. After 18 h cells, the were harvested, and the total DNA was isolated. Vector genomes were quantified by quantitative PCR as described under "Experimental Procedures." No significant difference was detected in genomes per cell between CD44-positive or CD44-negative cells with or without 5% vitreous. B, Y79 cells were transduced with Ad5F35/CMV-Flexi-IL12 (MOI, 15 pfu/cell) in medium with or without 5% vitreous. After 18 h the cells were harvested, and total mRNA and DNA were isolated. Vector genomes were quantified by quantitative PCR as described under "Experimental Procedures." No significant difference was detected in genomes per cell between cells transduced in medium with or without 5% vitreous. C, total mRNA was extracted from Y79 cells transduced with Ad5F35/CMV-Flexi-IL12 (MOI, 15 pfu/cell) in medium with or without 5% vitreous for 18 h. Fold change of IL-12 mRNA was determined by SYBR Green quantitative RT-PCR. IL-12 values were standardized to GAPDH mRNA levels. D, supernatant from Y79 cells transduced with Ad5F35/CMV-Flexi-IL12 (MOI, 15 pfu/cell) in medium with or without 5% vitreous was harvested, and IL-12 levels from each sample were determined by sandwich ELISA. E, HEK293 cells (2×10^4 cells/well in 96-well plate) were transduced with Ad5/CMV-Luc (MOI 5 pfu/cell) or Ad5/SV40-Luc (MOI 50 pfu/cell) in medium with or without 5% vitreous for 16 h. Luciferase activity was determined and standardized as described under "Experimental Procedures." ***, $p < 0.001$.

reporter signals within the dynamic range of the quantitative assay used. All MOIs that provided an interpretable signal demonstrate increased expression in the presence of vitreous (data not shown). Although there are significant differences in the base-line levels of expression of transgene when comparing these promoters, the addition of vitreous to culture medium results in a significant increase of Ad transgene expression when either vector is used (Fig. 1E). Expression of transgenes under the control of either the keratin-18 promoter or CMV-chicken β -actin promoter (the chicken β -actin promoter with CMV early enhancer elements (27)) also increases in the presence of vitreous (data not shown). These results suggest that the vitreous-mediated enhancement of Ad transgene expression can be seen in both viral and eukaryotic promoters.

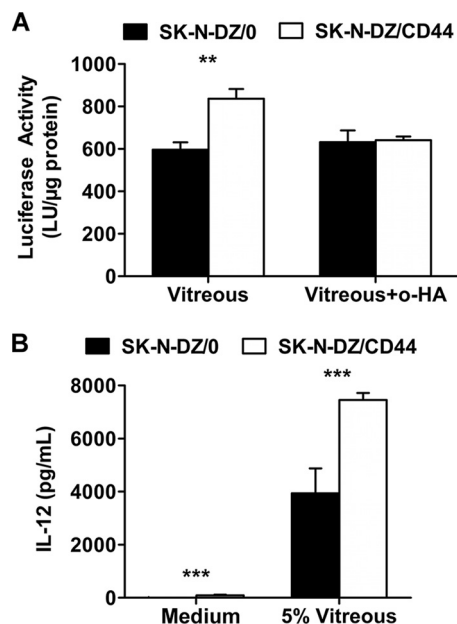


FIGURE 2. Hyaluronan in vitreous initiates signaling through CD44 that enhances Ad vector transgene expression. A, SK-N-DZ cells were transduced with lentivector delivering standard CD44 or empty control as described under "Experimental Procedures." Selected cells (2×10^4 cells/well) were transduced with Ad5/CMV-Luc (MOI, 50 pfu/cell) in the presence of 5% vitreous with or without o-HA (100 μ g/ml) for 16 h. Luciferase activity was determined and standardized as described under "Experimental Procedures." B, SK-N-DZ cells were transduced with lentiviral vectors delivering CD44 or empty control. Selected cells were seeded in a 24-well plate (2×10^5 cells/well) and transduced with Ad5F35/CMV-Flexi-IL-12 (MOI, 15 pfu/cell) in medium with or without 5% vitreous for 18 h. Supernatant was harvested, and the levels of secreted IL-12 were determined by sandwich ELISA. Even though vitreous significantly increased the levels of IL-12 in SK-N-DZ/0 cells, the addition of vitreous in SK-N-DZ/CD44 resulted in almost twice the levels of secreted IL-12 as compared with SK-N-DZ/0 cells. **, $p < 0.01$; ***, $p < 0.001$.

Interaction of HA and CD44 Contributes to Vitreous-mediated Enhancement of Ad Transgene Expression—Previous observations from our laboratory have shown that the digestion of vitreous with hyaluronidase decreases the enhancement of Ad transgene expression (8). Furthermore, the addition of the CD44-blocking antibody BRIC235 can inhibit the vitreous-mediated enhancement of Ad transgene expression (8). Other laboratories have reported that the presence of small o-HA can also antagonize the interaction of CD44 with high molecular weight HA (28). To further verify that vitreous is acting at least partially through the HA-CD44 interaction, the CD44-negative cell line SK-N-DZ was engineered to express CD44 as described above and subsequently transduced with Ad5/CMV-Luc in medium with or without 5% vitreous with or without the addition of o-HA. The data show that the addition of o-HA significantly decreased the vitreous-mediated enhancement of transgene expression without a significant effect on transgene expression in cells transduced with the empty control lentiviral vector (Fig. 2A). These results support the hypothesis that the HA-CD44 interaction that modulates transgene expression can be inhibited by o-HA and demonstrate that the HA-CD44 interaction plays a significant role in vitreous-mediated enhancement of Ad transgene expression.

To further confirm the role of CD44 signaling in modulating Ad transgene expression, we studied the effect of vitreous in the

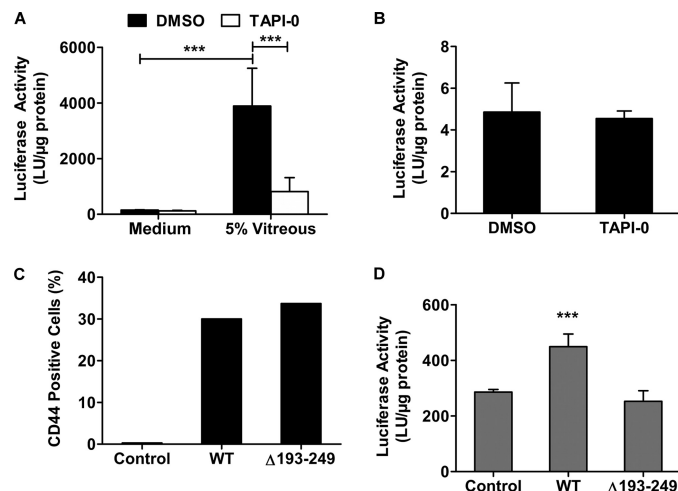


FIGURE 3. Matrix metalloprotease activity on CD44 is required for the vitreous-mediated enhancement of Ad transgene expression. A, Y79 cells (2×10^4 cells/well) were transduced with Ad5/CMV-Luc (MOI, 250 pfu/cell) in medium with or without different doses of vitreous with or without TAPI-0 (1 μ M) for 16 h. Luciferase activity was determined and standardized as described under "Experimental Procedures." B, Jurkat cells (CD44-negative, 2×10^4 cell/well) were transduced with Ad5/CMV-Luc (MOI 1000 pfu/cell) in the presence of 5% vitreous with or without TAPI-0 (1 μ M) for 16 h. Luciferase activity was determined and standardized as described under "Experimental Procedures." C, mutation of the recognition site of MMP in CD44 was created as described under "Experimental Procedures." SK-N-DZ cells were transduced with pCDH-CMV-MCS-EF1-copGFP with the respective gene of interest and sorted for GFP expression by FACS. CD44 expression was determined by flow cytometry using a phycoerythrin-conjugated anti-CD44 antibody and isotype control (data not shown). D, SK-N-DZ cells were transduced with lentiviral vectors delivering wild-type CD44, CD44:Δ193–249, or empty vector. Highly transduced cells were enriched by FACS sorting for GFP. The cells were then plated (2×10^4 cells/well) and transduced with Ad5/CMV-Luc (MOI, 250 pfu/cell) in the presence of 5% vitreous for 16 h. The cells were lysed, and the luciferase activity was determined as described under "Experimental Procedures." ***, $p < 0.001$.

presence or absence of CD44 on the secretion of IL-12 from the CD44-negative SK-N-DZ neuroblastoma cell line. SK-N-DZ cells were transduced with a lentiviral vector delivering CD44 and selected with puromycin (1 μ g/ml) for 2 weeks before being transduced with the Ad5F35/CMV-Flexi IL-12 vector in medium with or without 5% vitreous for 18 h (Fig. 2B). The addition of 5% vitreous significantly increased the levels of secreted IL-12, suggesting that the presence of CD44 plays a role in vitreous-mediated enhancement of Ad transgene expression.

Inhibition of MMP Activity or Deletion of the MMP Recognition Sequences on CD44 Abrogates the Vitreous-mediated Enhancement of Ad Transgene Expression—The hyaluronan receptor CD44 undergoes sequential proteolysis as part of its signaling mechanism. The first proteolytic step is carried out by the matrix metalloproteases MMP14, ADAM10, or ADAM17 (12). To determine whether the activity of MMPs is required for the vitreous-mediated enhancement of Ad transgene expression, the cells were transduced with Ad5/CMV-Luc in medium with or without 5% vitreous with or without the broad spectrum MMP inhibitor TAPI-0 (Fig. 3A). Whereas the addition of vitreous resulted in a significant increase in transgene expression, the effect of vitreous in the presence of TAPI-0 was significantly diminished. This inhibitor had no effect on transgene expression in the CD44-negative Jurkat cells (Fig. 3B). These

results suggest that MMP activity is necessary for CD44-mediated enhancement of Ad transgene expression.

MMPs have multiple protein substrates besides CD44. To determine whether the proteolysis of CD44 by MMP is required for the vitreous-mediated enhancement of Ad transgene expression, the reported MMP proteolytic sites between amino acids 193 and 249 on CD44 (12) were deleted using site-directed ligase-independent mutagenesis. The CD44: Δ 193–249 mutant showed a deletion of \sim 200 bp on a 0.8% agarose gel and was verified by DNA sequencing (data not shown). CD44-negative SK-N-DZ cells were engineered to express wild-type CD44 or CD44: Δ 193–249 using lentiviral vector gene transfer. The 193–249 deletion did not negatively affect the cell surface expression of the modified CD44 as determined by flow cytometry (Fig. 3C). The cells were then transduced with Ad5/CMV-Luc in medium with or without 5% vitreous. The results showed no significant difference on transgene expression in the absence of vitreous when standard CD44, CD44: Δ 193–249, and empty vector-transduced cells were compared (data not shown). However, a statistically significant increase of transgene expression was only observed on the standard CD44-transduced cells and not in the CD44: Δ 193–249-transduced group as compared with empty vector-transduced control cells (Fig. 3D). These results confirm that the proteolysis of CD44 by MMPs is an important step in CD44-mediated enhancement of Ad transgene expression.

Inhibition of the γ -Secretase Complex or the Deletion of Its Recognition Sequences on CD44 Abrogates the Vitreous-mediated Enhancement of Ad Transgene Expression—After the extracellular domain of CD44 is released by MMP, the remainder of the receptor associated with the cell membrane is recognized by the γ -secretase complex and proteolyzed within the transmembrane region. This proteolytic step releases the intracellular domain of CD44 (CD44ICD) (13). To determine whether the activity of this enzyme is required for the vitreous-mediated enhancement of transgene expression, cells were transduced with Ad5/CMV-Luc in medium with or without 5% vitreous with or without the γ -secretase inhibitor DAPT (Fig. 4A). The inhibition of γ -secretase significantly decreases the vitreous-mediated enhancement of transgene expression. Furthermore, this inhibitor has no effect on transgene expression in Jurkat cells (Fig. 4B). These results suggest that the γ -secretase activity is required for CD44-mediated enhancement of Ad transgene expression.

The γ -secretase complex is known to proteolyze CD44 and other type-I proteins such as amyloid precursor protein and Notch (29). To determine whether the effects of inhibiting the γ -secretase on the vitreous-mediated enhancement of Ad transgene expression are due to the proteolysis of CD44 and not another of its substrates, the proteolytic recognition site between amino acids 287 and 290 was deleted and replaced with a SacII restriction site using site-directed PCR mutagenesis as described by Okamoto *et al.* (9). CD44-negative SK-N-DZ cells were engineered to express wild-type CD44 or CD44: Δ 287–290 by lentiviral vector gene transfer. The 287–290 deletion did not negatively affect the cell surface expression of the modified CD44 as determined by flow cytometry (Fig. 4C). The cells were then transduced with Ad5/CMV-Luc in medium

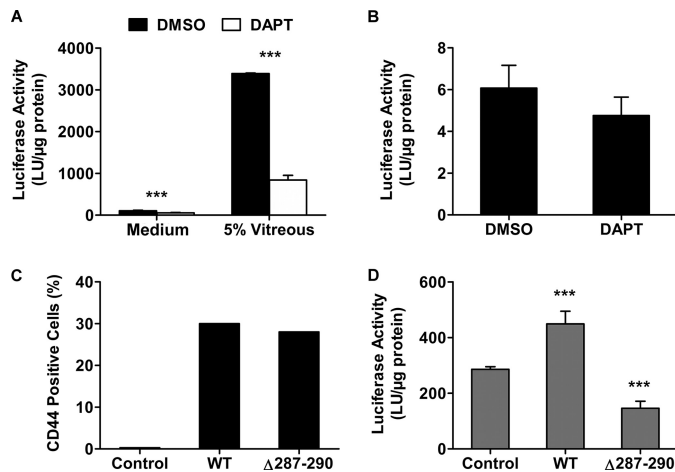


FIGURE 4. The activity of the γ -secretase complex on CD44 is required for the vitreous-mediated enhancement of Ad transgene expression. A, Y79 cells (2×10^4 cells/well) were transduced with Ad5/CMV-Luc (MOI, 250 pfu/cell) in medium with or without 5% vitreous with or without the γ -secretase inhibitor DAPT (500 nM) for 16 h. Luciferase activity was determined and standardized as described under "Experimental Procedures." The presence of DAPT significantly decreased the Ad transgene expression in medium with or without vitreous. B, Jurkat cells (2×10^4 cells/well) were transduced with Ad5/CMV-Luc (MOI, 1000 pfu/cell) in the presence or absence of DAPT (500 nM) for 16 h. Luciferase activity was determined and standardized as described under "Experimental Procedures." C, mutation of the recognition site of γ -secretase in CD44 was created as described under "Experimental Procedures." SK-N-DZ cells were transduced with pCDH-CMV-MCS-EF1-copGFP lentivectors with the respective gene of interest and sorted for GFP expression by FACS. CD44 expression was determined by flow cytometry using a phycoerythrin-conjugated anti-CD44 antibody and isotype control (data not shown). D, SK-N-DZ cells were transduced with lentiviral vectors delivering wild-type CD44, CD44: Δ 287–290, or empty vector. Highly transduced cells were enriched by FACS sorting for GFP. The cells were then plated (2×10^4 cells/well) and transduced with Ad5/CMV-Luc (MOI, 250 pfu/cell) in the presence of 5% vitreous for 16 h. The cells were lysed, and luciferase activity was determined as described under "Experimental Procedures." ***, $p < 0.001$.

with or without 5% vitreous. No significant difference in transgene expression in the absence of vitreous was observed when standard CD44, CD44: Δ 287–290, and empty vector-transduced cells were compared (data not shown). However, a statistically significant increase in transgene expression was only observed on the standard CD44-transduced cells and not in the CD44: Δ 287–290-transduced cells as compared with empty vector-transduced control cells (Fig. 4D). These results suggest that the proteolysis of CD44 by γ -secretase is required for the CD44-mediated enhancement of Ad transgene expression.

The Liberated CD44ICD Increases Ad Transgene Expression—According to our CD44 proteolysis model, the γ -secretase cleavage step liberates the CD44ICD into the cytoplasm, allowing its subsequent translocation into the nucleus. To study the effects of CD44 and CD44ICD, COS-7 cells were employed because of their previously published use in studying CD44 proteolysis (9), as well as their relatively low nucleus/cytoplasm ratio and ease of transfection. Based on our previous results, we hypothesized that the overexpression of the CD44ICD would result in a significant increase in Ad transgene expression in the absence of vitreous. To test this hypothesis, the CD44ICD sequence beginning at serine 291 was isolated from the standard isoform of CD44 and tagged with the V5 epitope at its C terminus. This truncated CD44 gene construct was transiently transfected into CD44-nega-

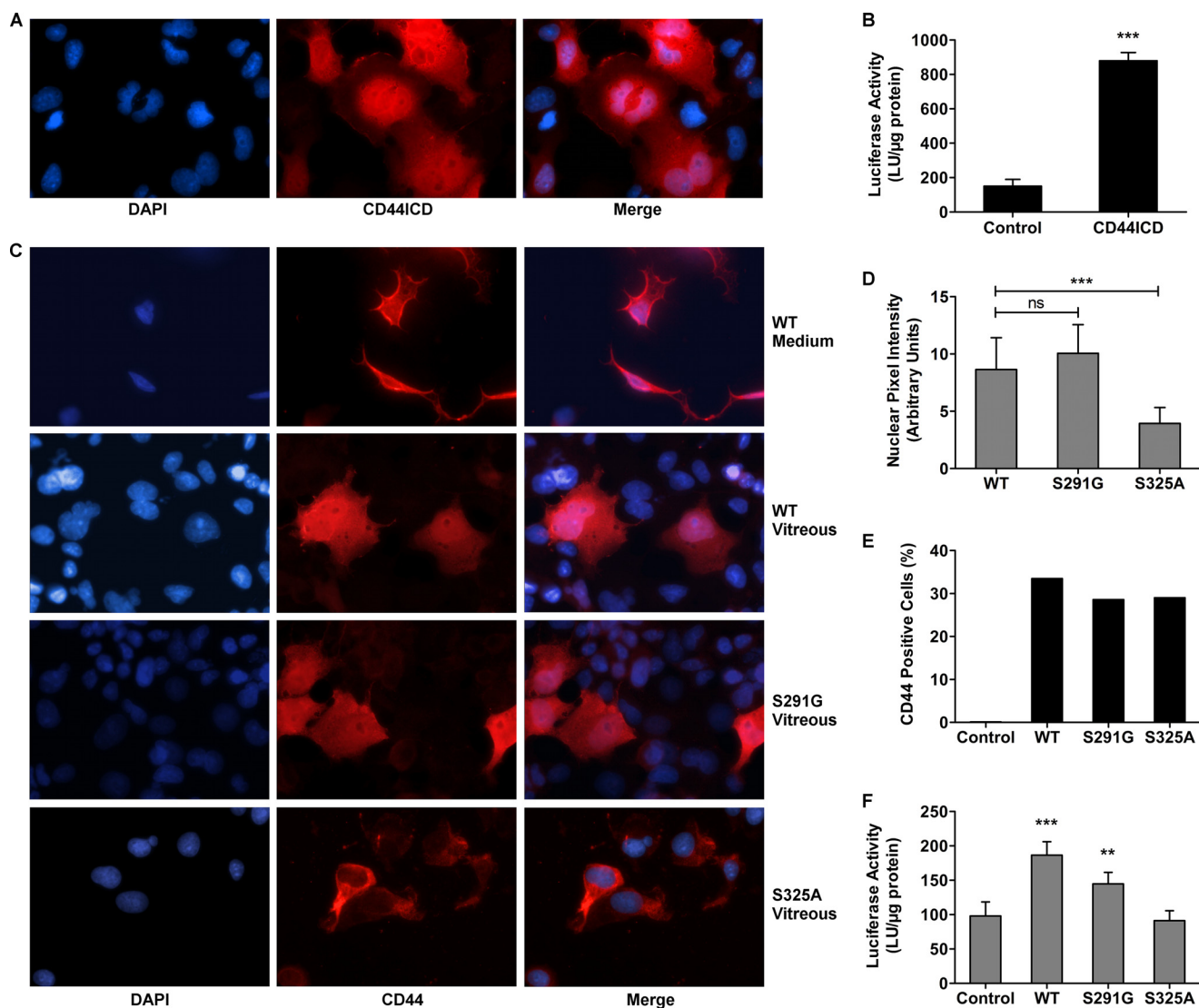


FIGURE 5. Liberation and nuclear translocation of CD44ICD enhances Ad transgene expression and is potentially affected by CD44 serine phosphorylation. *A*, COS-7 cells were transiently transfected with empty plasmid or plasmid delivering V5 epitope-tagged CD44ICD. The cells were fixed, and the localization of the CD44ICD peptide was determined by immunocytochemistry using an anti-V5 antibody as described under "Experimental Procedures." *B*, COS-7 cells transfected with pcDNA6 plasmid delivering V5 epitope-tagged CD44ICD (2×10^4 cell/well) were transduced with Ad5/CMV-Luc (MOI, 50 pfu/cell) for 16 h. Luciferase activity was determined as described under "Experimental Procedures." *C*, COS-7 cells were transfected with pcDNA3.1 plasmids delivering wild-type CD44 and CD44 serine mutants S291G and S325A and incubated in medium with or without 5% vitreous for 16 h. The cells were immunolabeled using an antibody against the CD44 intracellular domain as described under "Experimental Procedures." The cells were imaged via fluorescence microscopy. *D*, nuclear fluorescence pixel intensity of transfected cells ($n = 15$) was quantified after vitreous treatment using ImageJ software using DAPI-positive regions to gate the nuclear compartment. *E*, SK-N-DZ cells were transduced with pCDH-CMV-MCS-EF1-copGFP lentivectors delivering wild-type CD44 and CD44 serine mutants S291G and S325A and sorted for GFP expression by FACS. CD44 expression was determined by flow cytometry using a phycoerythrin-conjugated anti-CD44 antibody and isotype control (data not shown). *F*, SK-N-DZ cells were transduced with pCDH-CMV-MCS-EF1-copGFP lentivectors delivering wild-type CD44 and CD44 serine mutants S291G and S325A and sorted for GFP expression by FACS. The cells were then plated (2×10^4 cells/well) and transduced with Ad5/CMV-Luc (MOI, 250 pfu/cell) in medium supplemented with 5% vitreous for 16 h. The cells were lysed, and luciferase activity was determined as described under "Experimental Procedures." Scale bar, 50 μm. **, $p < 0.01$; ***, $p < 0.001$.

tive COS-7 cells. Transduced cells were fixed and immunolabeled using an anti-V5 antibody and then analyzed by fluorescence microscopy to verify the expression and nuclear translocation of the CD44ICD peptide (Fig. 5*A*). To determine whether the CD44ICD can modulate transgene expression, transfected cells were transduced with Ad5/CMV-Luc. The results show that the overexpression of the CD44ICD results in a significant increase of transgene expression compared with cells transfected with an empty plasmid control (Fig. 5*B*). Taken together, these results suggest that nuclear CD44ICD modulates Ad transgene expression.

Mutation of Phosphorylation Site Serine 325 in the Intracellular Domain of CD44 Alters Nuclear Localization of CD44ICD and Interferes with Vitreous-mediated Enhancement of Ad Transgene Expression—To determine the role of phosphorylation of the intracellular domain on nuclear localization of the CD44ICD, the serine residues 291 and 325 (known phosphorylation targets (15, 16)) were mutated using site-directed mutagenesis to glycine and alanine, respectively, to block phosphorylation at these residues. COS-7 cells were transfected with plasmids delivering wild-type CD44, CD44:S291G, or CD44:S325A. The cells were subsequently plated on poly-L-

CD44 Proteolysis Modulates Adenovector Transgene Expression

lysine-coated coverslips, treated with medium with or without 5% vitreous for 18 h, and labeled with either secondary antibody alone (supplemental Fig. S2) or an antibody specific to the intracellular domain of CD44. CD44ICD signal is present in the nucleus after vitreous treatment of wild-type CD44- and CD44:S291G-transfected cells (Fig. 5C), but nuclear signal intensity is reduced in the CD44:S325A-transfected cells after vitreous treatment (Fig. 5D). These observations suggest that phosphorylation of serine 325 of CD44 may be a mediating step in nuclear localization of CD44ICD.

To evaluate the role of CD44 phosphorylation in CD44-dependent modulation of Ad transgene expression, CD44-negative SK-N-DZ cells were engineered to express standard CD44, CD44:S291G, or CD44:S325A by lentiviral vector gene transfer. Expression of the constructs was confirmed by flow cytometric analysis (Fig. 5E). The cells were then transduced with Ad5/CMV-Luc in medium with or without 5% vitreous. The cells transduced to express the serine-325 mutant of CD44 show no statistically significant increase in transgene expression as compared with empty vector-transduced control cells, whereas mutation of serine 291 does show a significant increase (Fig. 5F). This observation suggests that phosphorylation of CD44 at serine 325 may facilitate CD44-dependent modulation of Ad transgene expression.

DISCUSSION

In this study we have shown that vitreous-mediated enhancement of Ad transgene expression, mediated at least in part by the interaction of HA with its receptor CD44, is the apparent result of an increase in transgene transcription and not an increase in vector internalization. Blocking the CD44-HA interaction and degradation of the HA in vitreous by hyaluronidase both decrease the effect of vitreous on transgene expression (8), supporting the conclusion that HA binding to CD44 causes enhancement of transgene expression. Ad vectors with either serotype 5 or serotype 35 fiber proteins (which initiate entry into the cell by binding to CAR or to CD46, respectively) both demonstrated an increase in transgene expression after vitreous treatment, suggesting that expression enhancement is independent of interaction of the virion with its receptor. Furthermore, the Ad genome internalization was not significantly changed following vitreous treatment with Ad5 and Ad5F35 vectors, also suggesting that the mechanism is independent of vector entry. This assertion is supported by previous observations by Chaudhuri *et al.* (8), who reported vitreous-mediated enhancement of transgenes delivered by Ad5F35, as well as Ad vectors containing mutations in hexon and fiber proteins, concluding that the mechanism of enhancement is independent of the binding mechanism involved.

Importantly, enhanced expression by CD44 signaling is seen with transgenes delivered by first generation adenoviral vectors and not seen with transgenes delivered by naked DNA, such as plasmid transfection, or with helper-dependent adenoviral vectors.³ First generation adenoviral vectors such as the ones used in this study have specific early gene regions deleted to render

the vector replication-incompetent, specifically the E1 and E3 regions. Although replication-incompetent, these vectors have been shown to drive expression of the remaining viral genes in the vector genome at low levels, which are believed to lead to low levels of viral replication and a subsequent cellular immune response by the host (30). The possibility may exist that expression of residual viral gene products in the vector genome facilitate the response of cells to vitreous and CD44 signaling to enhance transgene expression.

CD44 is widely expressed in many cells of the body and is principally thought to mediate cell-cell interactions as well as cell-extracellular matrix interactions. CD44 is a receptor for HA, which is present in the extracellular matrices of most tissues of the body. The concentration and molecular weight of CD44 can be modified by multiple physiological and pathophysiological processes (31). One physiologic compartment in which HA is present in high concentration is the vitreous humor of the mammalian eye (32). After HA interacts with CD44, signaling events have been reported to take place that regulate numerous cellular events ranging from cytoskeletal rearrangement to cell proliferation and regulation of apoptosis (33–37). In this study, vitreous was used as the source of HA. Because our study aims to model the application of viral vectors in the eye, the use of vitreous more precisely simulates the ligand-receptor interactions that are present in the intraocular environment. Additionally, whereas previous reports have established that purified HA enhances Ad transgene expression (8), the amount of vitreous needed to elicit an equivalent response to purified HA provides a much lower concentration of HA, suggesting the possibility that other components of vitreous are facilitating or otherwise increasing the efficiency of the CD44-HA interaction. Therefore, we postulate that use of vitreous more closely resembles the CD44-HA interaction that occurs *in vivo*.

We have observed that inhibitors of matrix metalloproteases and the γ -secretase complex inhibit vitreous-mediated enhancement of Ad transgene expression not only in SK-N-DZ cells expressing CD44 as shown here, but also in other cell lines such as Y79 (data not shown), suggesting that the mechanism of CD44 proteolysis is having similar effects across different cell lines. CD44 ectodomain shedding has been reported to occur in cancer cells (38, 39). This process is mediated by one of several matrix metalloproteinases that can cleave CD44 after it has bound to HA (25). The metalloproteinase inhibitor TAPI-1 inhibits the release of the CD44 ectodomain in HTB58 human squamous cell carcinoma cells (40). Inhibition of this cleavage has been shown to affect cancer cell migration, suggesting its importance in cell-extracellular matrix interactions. The γ -secretase complex initiates a second proteolytic cleavage of the CD44 molecule in the transmembrane region. High levels of the γ -secretase complex have been found in endocytic vesicles and could indicate their role in processing of proteins associated with endocytic pathways (41). Intramembranous proteolysis mediated by the γ -secretase complex is a common step in the degradation of many type-1 glycoproteins, with prior cleavage of the ectodomain also being required (29, 42). The intramembranous cleavage of CD44 generates a cytoplasmic fragment (CD44ICD) that subsequently translocates to the

³ C. J. Ildefonso, W. S. Bond, A. R. Al-Tawashi, M. Y. Hurwitz, and R. L. Hurwitz, unpublished observations.

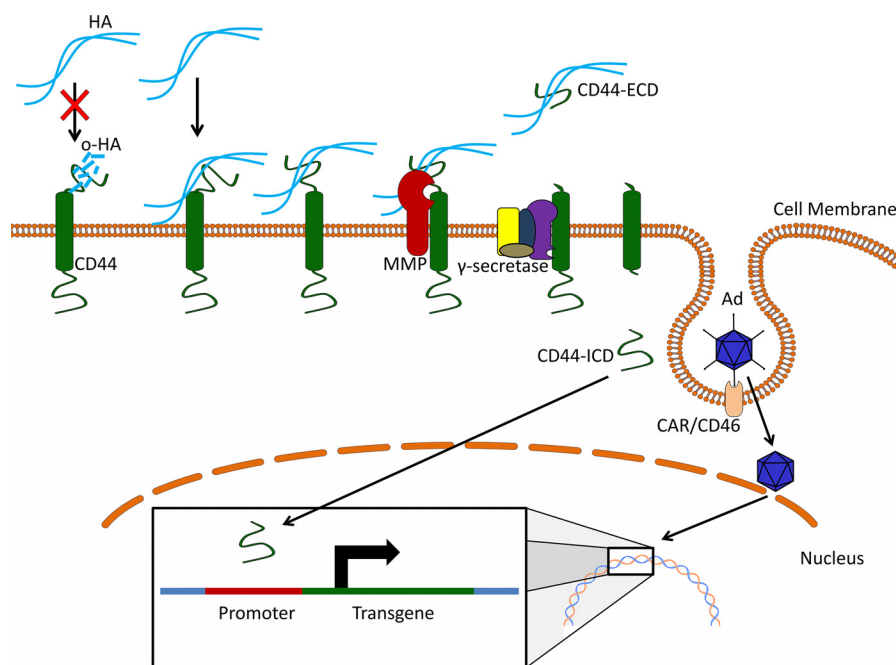


FIGURE 6. **Hypothetical model for CD44-mediated enhancement of Ad transgene expression.** The HA-CD44 interaction facilitates the cleavage of CD44 ectodomain by matrix metalloproteinases. o-HA may also bind CD44, preventing the interaction of high molecular weight HA and not facilitating MMP cleavage. The transmembrane domain of the remaining CD44 is subsequently proteolyzed by the γ -secretase complex. These proteolytic events liberate the CD44ICD, which translocates to the nucleus and modulates transgene expression of the Ad vector at the transcriptional level.

nucleus of the cell. Within the nucleus, this fragment has been shown to induce the transcription of genes under the control of tetradecanoylphorbol acetate-responsive elements by associating with the transcriptional machinery of the cell (43). Recently published research suggests that nuclear CD44ICD interacts with the transcription factor CREB and modulates its phosphorylation state (44), a finding that could provide a potential pathway by which CD44 proteolysis affects transcription of Ad-delivered transgenes. Another recent publication demonstrates that CD44ICD may modulate gene expression by directly binding to a novel DNA response element (45). Although it is unclear whether CD44ICD regulates transgene transcription directly or indirectly in this system, these previous observations of potential effects of free CD44ICD on transcriptional activity in the literature support a model of CD44ICD as a regulator of transcription. Whether other signaling pathways are involved or are necessary to facilitate the activity of CD44ICD in the nucleus remains an open question.

Phosphorylation of CD44 is known to occur at serine residues 291 and 325, both of which reside in the intracellular domain. These phosphorylations are thought to be regulated principally by PKC and calmodulin-dependent protein kinase II, respectively (15, 46). Phosphorylation of serine 325 appears to occur while in an inactivated state and has been reported to play a role in regulating the binding of CD44 to HA (16). It is not clear whether the phosphorylation state of CD44 specifically regulates sequential proteolysis and liberation of the CD44ICD. In this report, increased signal intensity of the intracellular domain of CD44 in cell nuclei after vitreous treatment is seen by immunocytochemistry in wild-type CD44-expressing cells. However, the increased nuclear signal in response to vitreous treatment appears reduced in cells expressing CD44 with a mutation blocking phosphorylation of serine 325, suggesting

that the phosphorylation state of CD44 may play a role in regulating either the sequential proteolysis of CD44 or the nuclear translocation of the CD44ICD.

Increasing production of IL-12 could improve the efficacy of IL-12 tumor immunotherapy, which have not proven to be very effective in previous clinical studies. We have shown that CD44 signaling can increase the secretion of IL-12 *in vitro*, an observation that can be generalized to suggest that, in addition to increasing production of transgene protein, secretion of the protein is also increased by this signaling pathway. Additionally, the CD44 mechanism reported here could play a role *in vivo* in modulating the expression of Ad vector transgene expression when delivered systemically. Given that the vitreous contains high concentrations of HA, the mechanism could be playing a significant role in enhancing the efficacy of ocular gene therapy compared with systemic use. Vitreous components such as HA, proteoglycans, and other trace proteins that may be playing a role in modulating transgene expression are also commonly found in extracellular matrix throughout the body. Moreover, by understanding the mechanism by which CD44-HA signaling modulates transgene expression, strategies can be devised to boost expression with systemically administered Ad vectors as well, such as the creation of a bicistronic Ad vector expressing a gene of interest behind a CD44ICD DNA response element as well as a truncated CD44ICD gene.

Our laboratory has previously shown that HA, including the HA in vitreous, can enhance expression of transgenes delivered by first generation Ad vectors through interaction with CD44, showing that antibodies that block HA binding to CD44 prevent Ad vector-mediated transgene expression both *in vitro* and *in vivo* (8). The results of this study suggest that CD44 degradation by matrix metalloproteinases and the γ -secretase

complex and the liberation of CD44ICD are important steps in the enhancement of Ad transgene expression by HA. Taken together, these results are consistent with a model that the sequential degradation of CD44, after binding of HA, by metalloproteinase and γ -secretase led to the liberation of the CD44ICD, which localizes to the nucleus and increases transcription of Ad vector transgene (Fig. 6). Considering that significant effects on CD44-negative cells were observed after treatment with vitreous, other mechanisms independent of CD44 are at work in this system, although the exact pathways and components involved are not yet elucidated. Although vitreous-stimulated CD44 signaling is only one of multiple attributes of the intraocular environment that aid in ocular viral gene therapy, the observations reported in this manuscript suggest a novel role of CD44 signaling in regulating Ad transgene expression that could be exploited to improve gene therapy strategies utilizing the Ad vector.

Acknowledgments—We thank Dr. Anja Ehrhardt of the Ludwig-Maximilians-Universität München for the generous gift of the SV40 promoter-driven adenovirus vector, Dr. Stephen Götttschalk at Baylor College of Medicine for providing the IL-12-expressing vector, and Drs. Osamu Nagano and Hideyuki Saya at Keio University School of Medicine for generously providing CD44 antibody.

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