Transportin Regulates Nuclear Import of CD44[S]

Michalina Janiszewska, Claudio De Vito, Marie-Aude Le Bitoux, Carlo Fusco, and Ivan Stamenkovic[1]

From the Division of Experimental Pathology, Institute of Pathology, Centre Hospitalier Universitaire Vaudois, University of Lausanne, Lausanne CH-1011, Switzerland

CD44 is a facultative cell surface proteoglycan that serves as the principal cell surface receptor for hyaluronan (HA). Studies have shown that in addition to participating in numerous signaling pathways, CD44 becomes internalized upon engagement by ligand and that a portion of its intracellular domain can translocate to the nucleus where it is believed to play a functional role in cell proliferation and survival. However, the mechanisms whereby fragments of CD44 enter the nucleus have not been elucidated. Here we show that CD44 interacts with two import receptors of the importin β superfamily, importin β itself and transportin. Inhibition of importin β-dependent transport failed to block CD44 accumulation in the nucleus. By contrast, inhibition of the transportin-dependent pathway abrogated CD44 import. Mutagenesis of the intracellular domain of CD44 revealed that the 20 membrane-proximal residues contain sequences required for transportin-mediated nuclear transport. Our observations provide evidence that CD44 interacts with importin family members and identify the transportin-dependent pathway as the mechanism whereby full-length CD44 enters the nucleus.

Proteins cross the double membrane of the nuclear envelope through nuclear pores. To traverse this barrier, proteins larger than 40 kDa must bind to soluble transport factors or carrier molecules that shuttle between the cytoplasm and the nucleus (1). Most of the nuclear transport factors belong to the β-karyopherin family and are classified, according to their function, as importins or exportins (2). In the cytoplasm, cargo proteins containing a nuclear localization signal (NLS)[2] are usually bound by the adaptor protein importin α, followed by the carrier importin β (2), although in some cases, importin α binding is not required (3). Importin-bound cargo is then translocated through the nuclear pore complex and is released in the nucleus following binding of importin β to RanGTP protein (2). The importin-Ran complex is exported to the cytoplasm where it dissociates upon RanGTP conversion to RanGDP. An analogous mechanism is used by mRNA-binding proteins, where cargo is recognized by the transportin1 carrier (2). Interestingly, it has also been shown that some proteins, including β-catenin, SMAD, and ERK2, can be imported into the nucleus without involvement of importins and RanGTP (4).

Nuclear export is analogous to the import mechanism. There is a single karyopherin protein, Crm1, that upon recognition of a nuclear export sequence binds the target protein in the nucleus and upon stimulation by Ran protein releases the cargo into the cytoplasm (2). The mechanisms described above are common for cytoplasmic proteins, but there is also some evidence that the same pathways might be used for nuclear translocation of the full-length transmembrane receptors (5, 6).

Growth factor receptor signaling proceeds as a cascade of intracellular events. Receptor engagement by ligand on the cell surface typically results in receptor clustering and, depending on the receptor type, phosphorylation of the intracellular domain. Recruitment of adaptor molecules ensues and an intracellular signal is generated and relayed by numerous proteins that constitute the cascade to finally target the gene transcription machinery and alter gene expression. In recent years, however, numerous reports have shown that transmembrane receptors themselves can play a more direct role in signal transmission to the nucleus (6).

CD44 is a polymorphic transmembrane glycoprotein that serves as the principal cell surface receptor for hyaluronan (HA) and can affect numerous cellular processes, including cell adhesion, proliferation, and migration. Based on its diverse functions, CD44 is implicated in a range of physiological and pathological processes including normal development, inflammation, and cancer progression (7). Because it resides in lipid rafts in the cell membrane, CD44 serves not only as a receptor for extracellular matrix components, including HA, but as a co-receptor for receptor tyrosine kinases of the ErbB family (8) and Met (9). CD44 also plays a role in anchorage of the actin cytoskeleton to the plasma membrane, which is important for cell motility, and interacts with the ezrin-radixin-moesin family of cytoskeletal adaptors (10), as well as merlin (11), through which much of its most relevant signaling for cell motility and survival is believed to occur. Upon engagement by HA or other ligands, CD44 is internalized and at least a fraction of the internalized pool is subjected to proteolytic cleavage. It has been shown that after sequential cleavage, the intracellular domain (ICD) of CD44 is translocated to the nucleus, where it is believed to participate in the regulation of gene transcription (12). However, the mechanism of CD44 ICD transport to the nucleus has not been elucidated.

In the present work, we show that the full-length CD44 protein localizes to the nucleus of several cell types. We also provide evidence that full-length CD44 nuclear shuttling is

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[1] To whom correspondence should be addressed: CHUV 25 Rue du Bugnon CH-1011 Lausanne, Switzerland. Tel.: 41-21-314-7136; Fax: 41-21-314-7110; E-mail: Ivan.Stamenkovic@chuv.ch.
[2] The abbreviations used are: NLS, nuclear localization signal; ICD, intracellular domain; PI, protease inhibitors; BrU, bromouridine; HA, hyaluronan.

dependent on transportin1 (TNPO1, karyopherinβ2) and exporin Crm1 and that it influences gene transcription.

EXPERIMENTAL PROCEDURES

Cell Culture

The MNNG/HOS sarcoma cell line was cultured in DMEM with 4.5 mg/ml of glucose (Invitrogen), supplemented with 10% fetal calf serum (Amimed), 1% non-essential amino acids, and 1% penicillin/streptomycin (10,000 units/10,000 μg/ml, Invitrogen). PC3 (prostate adenocarcinoma), MRC5 (fetal fibroblasts), and MC (melanoma) cells were cultured in DMEM with 4.5 mg/ml of glucose (Invitrogen), supplemented with 10% fetal calf serum and 1% penicillin/streptomycin (Invitrogen). MCF10A (mammary epithelial) cells were cultured in DMEM/ F-12 supplemented with 2% horse serum, 1% penicillin/streptomycin (Invitrogen), 2 mM L-glutamine, 20 ng/ml of EGF, 100 ng/ml of cholera toxin, 10 μg/ml of insulin, and 500 ng/ml of hydrocortisone.

Plasmid Constructs and Transfection

CD44 mutants were constructed by PCR using the standard isoform of CD44 as template. For the construction of the CD44 t-20 mutant two fragments were prepared using primers: F1, 5′-GAAGATCTTCGCCACCATGGACAAGTTTTGGTGG-CACGCAGCCTGGGGACT-3′; R1, 5′-GAGTCCACTTG-GCTTTCTGTCACTGTTGACTGCAATGCAAAC-3′; F2, 5′-GTTTGCATTGCAGTCAACAGTGACAGAAAGCCAA-GTGGACTC-3′; and R2, 5′-CCGCTCGAGCGGCTATTAC-ACCCCAATCTTCATGTC-3′. Both PCR products were simultaneously ligated into the pMSCV hygro vector (Clontech). For the construction of the CD44 t-60 mutant, primer F1 and primer R3, 5′-CCGCTCGAGCGGCTACAAATGCACC-ATTTCCTGAG-3′, were used. For the preparation of the CD44dt mutant primer F1 and R4, 5′-CCGCTCGAGCGGCT-AACTGTTGACTGCAATGCAAAC-3′, were used. For the construction of the CD44dsp mutant primer F3, 5′-GAAGA-TCTTCGCCACCATGCAGATCGATTTGAATATAACC-3′, and R2 were used. The CD44R41A mutant was prepared as described in Ref. 13. The CD44ICD was constructed with F4, 5′-CAGATCTGCCACCATGGCAGTCAACAGTCGAAGA-3′, and R2 primers.

MC cell infection was performed using a virus produced by GP2 cells transfected with the pMSCV_hygro vector constructs. Selection of infected MC cells was done by addition of 500 μg/ml of hygromycin to the culture medium. For over-expression in COS-7 cells pCDM8-based constructs were prepared by subcloning CD44 cytoplasmic tail mutants and amplifying TNPO1 with primers: F5, 5′-CGGGATCCCGCC-ACCATGGTGTGGGACCGGCAAAC-3′ and R5, 5′-CCCTC-GAGGGTTAAACACCATAAAAAGCTGCAAGAC-3′. Cells were transfected with FuGENE reagent (Roche) according to the manufacturer's protocol.

shRNA specific for CD44 was designed according to earlier reports (14). The sequences were cloned into the pSiren vector (Clontech), which was used to transfect the 293 cells. MNNG/ HOS cells were then infected and selected with 1 μg/ml of puromycin.

Immunofluorescence

Antibodies used in immunofluorescence experiments were: anti-CD44 N-terminal; Brick 223 and Brick 222 (1:100, 1 mg/ml, mouse monoclonal, kindly provided by Frances Spring from the International Blood Group Reference Laboratory, Bristol, UK), anti-BrdU (1:300, 0.5 mg/ml, mouse monoclonal, Pharmingen; 0.1 mg/ml, mouse monoclonal, Roche Applied Science), and donkey anti-mouse Alexa 488 (1:1300, Molecular Probes). For detection of the ICD domain, anti-CD44 C-terminal antibody was used (1:200, 0.5 mg/ml, rabbit polyclonal, Abcam). Specificity of each antibody was compared with the isotype-matched control antibody.

For immunofluorescence with CD44, cells were fixed with 4% paraformaldehyde, washed, permeabilized with 3% Triton X-100, stained with each antibody for 30 min, and mounted in 1:1000 DAPI in mounting medium (ThermoShandon). For BrU (bromouridine) incorporation immunofluorescence anti-BrdU antibodies from Pharmingen were used (1:300). All images were acquired with Leica SP5 AOBS confocal microscope at the Core Imaging Facility of the University of Lausanne. The acquisition was performed in sequential mode to avoid the dye crosstalk. CD44 localization was analyzed in *Z*-stacks with Imaris software. Colocalization percentage was calculated based on voxel colocalization within an image, normalized to cell number (Imaris and Image J softwares).

Immunoprecipitation

Immunoprecipitation from the Nuclear Fractions (Modified from Ref. 15)—Cells were cultured until 85–90% confluence, then scraped in PBS with protease inhibitors (PI), spun down, and lysed in a Dounce homogenizer with 3 volumes of the buffer A (10 mM Hepes, pH 7.9, 15 mM $MgCl_2$, 10 mM KCl, freshly added PI and 1 mM DTT). The number of strokes of the homogenizer was determined by checking the cell lysis under a light microscope. The lysate was then centrifuged at 3,500 × g for 5 min at 4 °C and the nuclear pellet was resuspended in 3 volumes of buffer C (20 mM Hepes, pH 7.9, 25% (v/v) glycerol, 420 mM KCl, 1.5 mM $MgCl_2$, 0.2 EDTA, freshly added PI, and 1 mM DTT). Nuclei were lysed for 30 min to 1 h on ice and the lysates were ultracentrifuged at 24,000 × g for 20 min at 4 °C. Nuclear supernatants were then collected and diluted 10 times with buffer D (20 mM Hepes, pH 7.9, 20% (v/v) glycerol, 0.2 EDTA, 0.2% Triton X-100, freshly added PI). To eliminate the precipitate, the sample was spun at 16,000 × g for 10 min at 4 °C. Preclearing with agarose-protein A beads (GE Healthcare) was then performed (the beads were previously washed with buffer D with 150 mM KCl). Next, the beads were spun down, discarded, and antibodies were added to the nuclear extract. Incubation with the antibodies was performed overnight at 4 °C. A fresh batch of pre-washed beads was then added to the extracts and incubated for 2–12 h at 4 °C. The beads were then spun down and washed 10 times with buffer D with 300 mM KCl. Finally, the beads were resuspended in reducing sample buffer and boiled for 15 min. The released proteins were analyzed by SDS-PAGE and Western blotting. For each precipitation experiment the nuclear extracts were divided into two

equal fractions, one of which was incubated with the unrelated, isotype-matched antibody to provide the control.

Immunoprecipitation for the Cytoplasmic Fraction—Cells were cultured until 85–90% confluence was reached, then scraped in PBS with protein inhibitors, spun down, and resuspended in 3 volumes of the modified RIPA lysis buffer (50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, PI). Lysis was performed for 30 min on ice, and the lysate was centrifuged at $3,500 \times g$ for 5 min at 4 °C. Agarose-protein A beads (previously washed with PBS) were added to the supernatants and discarded after 1 h of pre-clearing. Incubation with antibodies was preformed overnight at 4 °C, and incubation with the beads for 2 h. The beads were then washed 10 times with PBS, resuspended in sample buffer, and boiled for 15 min. The released proteins were analyzed by SDS-PAGE and Western blotting. Antibodies used in immunoprecipitation were anti-CD44 Brick 235 (kindly provided by Frances Spring), anti-transportin1 (mouse monoclonal, Abcam), mouse IgG2κb (BD Pharmingen), mouse IgG1 (Sigma), and anti-importin β (rabbit polyclonal, Abcam).

Western Blotting

Antibodies used in Western blotting were anti-CD44 KZ1 (1 μg/ml (16)), anti-importin β (rabbit polyclonal, Abcam), anti-transportin1 (mouse monoclonal, Abcam), exportin Crm1 (rabbit polyclonal, Abcam).

Biotinylation

MNNG/HOS cells were washed with cold PBS containing 1 mM Ca^{+2} and 1 mM Mg^{+2}. Biotinylation was performed for 15 min at 4 °C with 500 ng/ml of biotin in PBS containing 1 mM Ca^{+2} and 1 mM Mg^{+2}. Cells were then cultured in normal medium for 8 h, washed with PBS, scraped, and mechanically disrupted for immunoprecipitation.

Brefeldin A and Nocodazole Treatment

MNNG/HOS cells cultured on coverslips were incubated for 1 h with 5 μg/ml of brefeldin A (Sigma) or for 8 h with 100 ng/ml of nocodazole (Sigma). Cells were then fixed and stained for immunofluorescence.

Nuclear Import Assay (Modified from Refs. 3 and 17)

Cytosol Preparation for the Nuclear Import Assay—Cells were scraped and washed twice with cold PBS, then with washing buffer (10 mM Hepes, pH 7.3, 110 mM KOAc, 2 mM Mg[OAc]₂, 2 mM DTT) and resuspended in 1.5 volume of lysis buffer (5 mM Hepes, pH 7.3, 10 mM KOAc, 2 mM Mg[OAc]₂, 2 mM DTT, 20 μM cytochalasin B, freshly added protein inhibitors) and incubated on ice for 10 min. Lysis was performed with a Dounce homogenizer, and cell disruption was verified under a light microscope. Lysates were then subjected to centrifugation at $15,000 \times g$ for 20 min, and at $100,000 \times g$ for another 30 min. Cytosolic fractions were dialyzed overnight against the transport buffer (20 mM Hepes, pH 7.3, 110 mM KOAc, 5 mM NaOAc, 2 mM Mg[OAc]₂, 1 mM EGTA, 2 mM DTT, PI) and stored at −80 °C.

Importin β Depletion—To deplete importin β from the cytoplasmic fraction the protocol for immunoprecipitation was used. The supernatant remaining after the immunoprecipitation was then dialyzed against the transport buffer. Antibodies used in this assay were anti-importin β (rabbit polyclonal, Abcam) and rabbit IgG (Sigma).

Nuclear Import Assay—Cells grown on coverslips were washed with ice-cold transport buffer, permeabilized with 40 μg/ml of digitonin (Calbiochem) in ice-cold transport buffer for 5 min at room temperature, and washed with transport buffer. Coverslips were then blotted using 50 μl of complete transport mixture, containing 75% cytosol in transport buffer, 1 mM ATP, 5 mM creatine phosphate, 20 units/ml of creatine phosphokinase, and incubated at 30 °C for 6 h. Cells were then washed and stained. To block the nuclear transport, wheat germ agglutinin (Sigma) was added to the transport mixture to a final concentration of 0.1 mg/ml (18).

Leptomycin B Treatment—Cells were cultured on coverslips until 85% confluence. Leptomycin B (Sigma) was added to a final concentration of 20 ng/ml (19) and incubation was conducted for 2, 4, or 20 h at 37 °C. Cells were then prepared for immunofluorescence.

BrU Incorporation Assay (Modified from Ref. 20)

BrU Incorporation Immunofluorescence—Cells were cultured on coverslips until 85% confluence and incubated with 2 mM bromouridine (Sigma) for 3 h at 37 °C. Cells were then fixed in ice-cold methanol and incubated with anti-BrdU antibody (mouse monoclonal, Pharmingen; or mouse monoclonal, Roche Applied Science) and secondary goat anti-mouse Alexa 488 antibody (Molecular Probes).

BrU Incorporation Multiwell Test—Cells cultured in a 96-well plate were incubated for 3 h with 2 mM BrU, fixed with ice-cold methanol, and incubated with anti-BrdU antibody (mouse monoclonal, Pharmingen). The cells were then incubated with secondary antibody, washed, and incubated with the substrate according to the Cell Proliferation ELISA BrdU colorimetric assay (Roche Applied Science).

Mouse Fibroblast Isolation—Pups of B6 or B6/J129 CD44KO mice were euthanized and rinsed in Betadine and PBS. After removal of the limbs, the skin was peeled off, flattened on a Petri dish and dried for about 30 min. Trypsinization was performed overnight at 4 °C with 0.25% trypsin (Sigma) in PBS with antibiotic/antimycotic (1:100, Invitrogen). Next, the epidermis was peeled off and the dermis was incubated for 1 h at 37 °C in 0.33 mg/ml of collagenase II and IV (Sigma) with 250 μg/ml of glucose (Sigma). The sample was then filtered and cells were spun down, resuspended, and plated at 2×10^6 cells/dish in DMEM with 10% FCS, antibiotic/antimycotic.

Statistical Methods—p values were established by Student's t test with equal sample size and unpaired equal variances. The error bars on the graphs represent S.D. The variances were analyzed with the analysis of variance test.

RESULTS

Nuclear Localization of CD44—It has been shown that the intracellular domain of CD44 can be translocated to the nucleus (12) and that engagement of HA triggers endocytosis of the mature CD44 protein (8, 21). To address the intracellular fate of CD44, we used MNNG/HOS osteosarcoma cells that

A

B
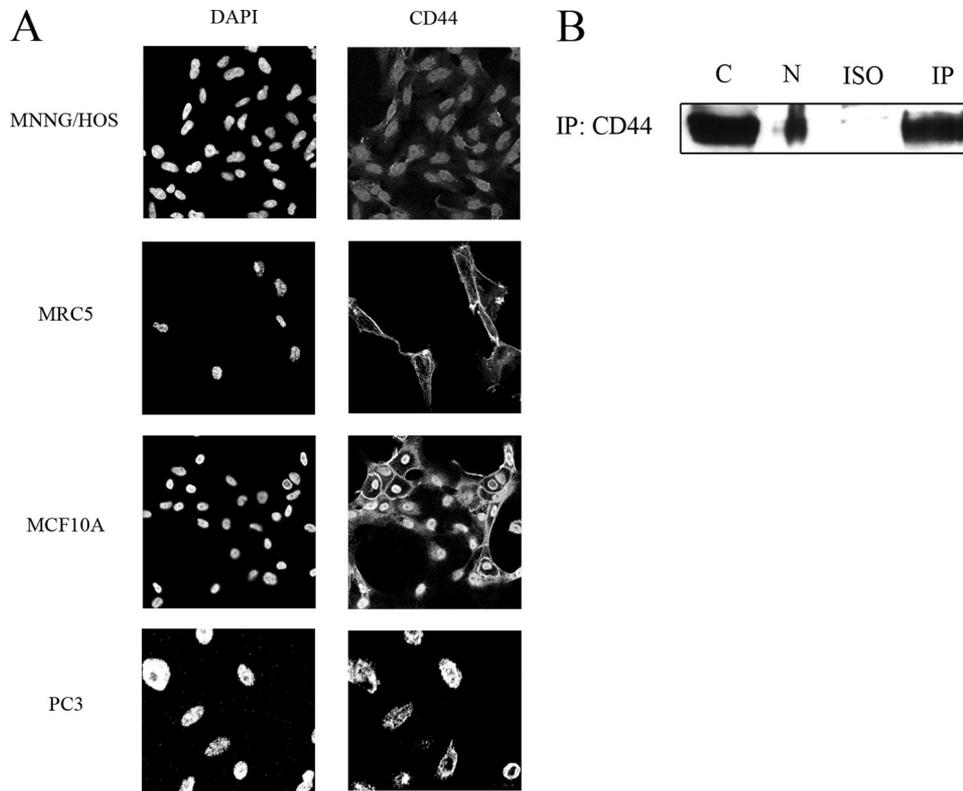
FIGURE 1. **Nuclear localization of CD44.** *A,* immunofluorescence staining of CD44 in the indicated cell types; nuclei stained with DAPI; *B,* immunoprecipitation of CD44 from the nuclear fraction of MNNG/HOS cells; immunoprecipitation was performed with anti-CD44 Brick 235 mAb, and the Western blot with anti-CD44 KZ1 mAb. *C,* cytosolic fraction; *N,* nuclear fraction; *IP,* fraction immunoprecipitated with anti-CD44 mAb; *ISO,* fraction immunoprecipitated with unrelated isotype-matched immunoglobulin.

Confocal microscopy revealed that CD44 colocalization with DAPI, which is a hallmark of nuclear localization, decreased after nocodazol treatment. This result supports the role of endocytic trafficking in nuclear transport of CD44.

Previous work (22) has shown that ligation of cell surface CD44 by antibody or osteopontin can enhance its nuclear translocation. We therefore asked whether hyaluronic acid binding could play the same role *in vivo*. To answer this question we used the CD44 R41A mutant, which is unable to bind HA (13), the dsp mutant lacking the signal peptide (amino acids 1–20), and the intracellular domain of CD44 alone (ICD, starting with A669) (12). pMSCVhygro plasmids containing mutant CD44 were expressed in human melanoma MC cells, which have negligible levels of endogenous HA receptor (Fig. 2*C*). We then performed immunofluorescence monitoring of the nuclear localization of CD44 by assessing its colocalization with DAPI (Fig. 2*D*). We observed that the HA-binding

constitutively express high levels of the standard 85–90-kDa CD44 isoform (CD44H). Immunofluorescence microscopy of MNNG/HOS cells using an antibody to the extracellular domain of CD44 revealed the presence of epitopes recognized by the antibody in the cell membrane, cytoplasm, but also in the nucleus (Fig. 1*A*). Staining of different cell lines, including MNNG/HOS, the immortalized fibroblast MRC5, and breast epithelial MCF10A, cell lines, and the prostate carcinoma cell line PC3, with anti-CD44 antibody revealed that the presence of the extracellular domain of CD44 in the nucleus was a common feature, irrespective of cell type. Those results were confirmed with another anti-CD44 antibody (data not shown).

To ensure that nuclear localization that we observed was due to translocation of the full-length receptor and not just an extracellular fragment, we performed immunoprecipitation of CD44 from the nuclear fraction of MNNG/HOS cells. Western blot analysis of the nuclear fraction using anti-CD44 antibody confirmed the presence of an 85–90-kDa CD44 species that corresponds to full-length CD44H in the nucleus of MNNG/HOS cells (Fig. 1*B*).

Membrane Origin of Nuclear CD44—To confirm the findings of Lee *et al.* (22), who showed that nuclear full-length CD44 originates from the cell membrane, we determined whether biotinylated CD44 could be immunoprecipitated from the nuclear fraction of MNNG/HOS cells (Fig. 2*A*). The potential involvement of the Golgi apparatus and endocytic pathway were assessed by fluorescent antibody staining of CD44 in cells treated with brefeldin A or nocodazole, respectively (Fig. 2*B*).

mutant is transported to the nucleus as efficiently as the full-length protein. However, deletion of the signal peptide that results in a mutant that cannot be incorporated into the cell membrane significantly impaired nuclear localization. The above results are therefore consistent with previous findings and show that membrane localization of CD44 is necessary for its nuclear translocation via the endosomal pathway.

CD44 Interacts with Proteins from the Importin/Exportin Family—CD44 has been suggested to mediate signal transduction and to participate in the modulation of numerous cell signaling pathways (7). However, the mechanisms whereby the HA receptor regulates cell function and behavior remain to be fully elucidated. To provide insight into CD44 protein-protein interactions that might be relevant to CD44 transport and intracellular signaling, we performed a pull-down assay, a large scale immunoprecipitation followed by a mass spectrometric analysis, from MNNG/HOS cell lysates using anti-CD44 antibody. Mass spectrometric analysis revealed that the full-length CD44 protein present in the cytoplasmic fraction of MNNG/HOS cells co-precipitates with several karyopherins that mediate nuclear-cytoplasmic shuttling (Fig. 3*A*). Immunoprecipitation and Western blot analysis confirmed the interaction of CD44 with importin β, transportin 1, and exportin Crm1 (Fig. 3*B*).

Nuclear Import of CD44 Is Transportin1-dependent—After confirming the interaction of CD44 with importin β and transportin1, we assessed the relevance of the two proteins to nuclear import of the HA receptor by performing a series of
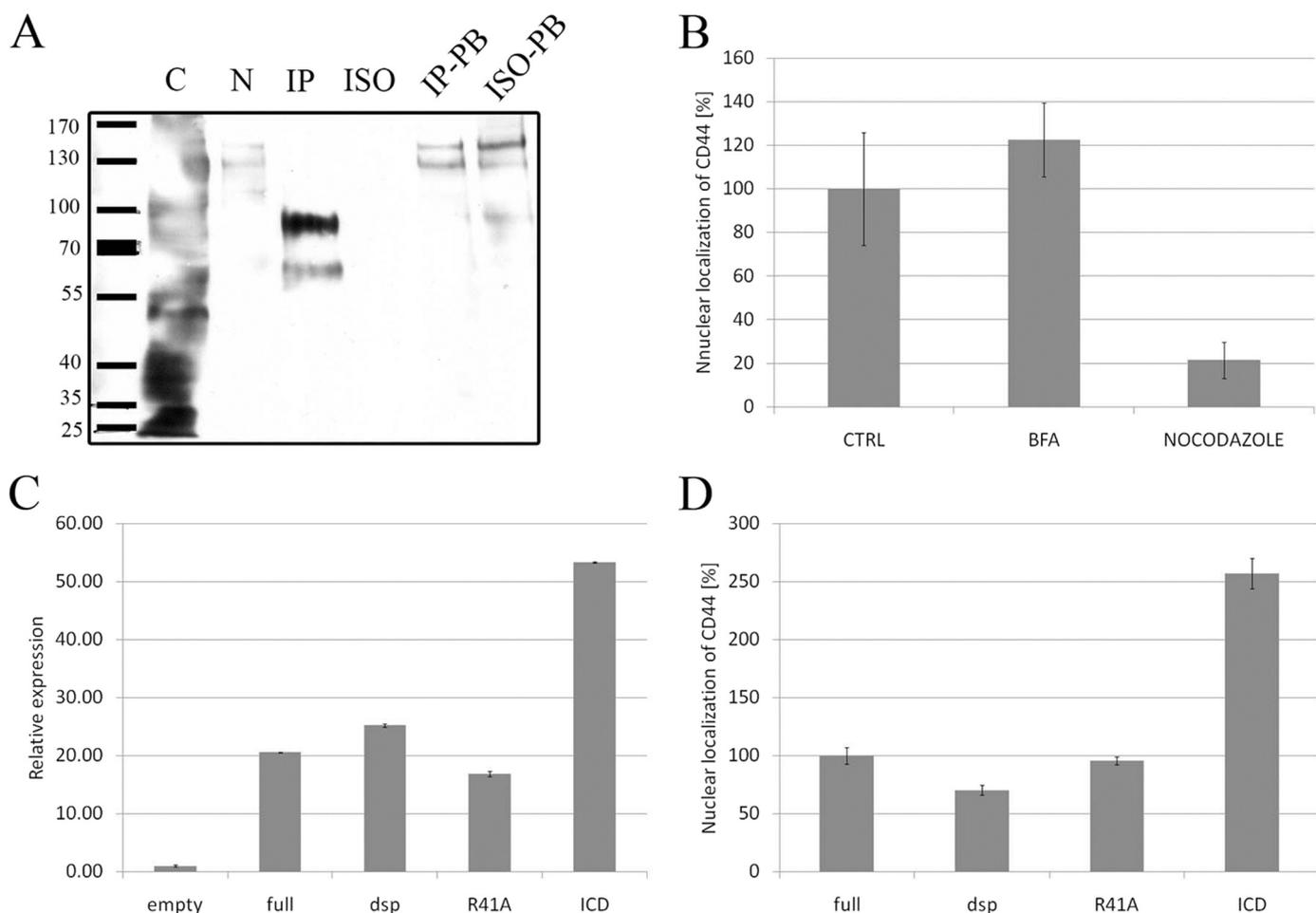
FIGURE 2. **Cell membrane origin of nuclear CD44.** *A,* CD44 immunoprecipitation from the nuclear fraction of surface-biotinylated MNNG/HOS cells. *C,* cytosolic fraction; *N,* nuclear fraction; *IP,* fraction immunoprecipitated with anti-CD44 mAb; *ISO,* fraction immunoprecipitated with unrelated isotype-matched immunoglobulin; *PB,* post-immunoprecipitation fractions. *B,* inhibition of microtubule-dependent endocytosis impairs nuclear localization of CD44. *BFA,* brefeldin A. *C,* overexpression of CD44 mutants, real-time PCR with F4 and R2 primers. *D,* nuclear localization of CD44 mutants. *B* and *D,* nuclear localization of CD44 was observed upon its colocalization with DAPI and measured with Imaris software. Means of at least 5 confocal images are shown.

nuclear transport assays, as described previously (17). Briefly, MNNG/HOS cells cultured on coverslips were permeabilized with digitonin and incubated with cytosol in the presence or absence of transport inhibitors or with cytosol depleted of importin β. After 6 h of incubation at 30 °C, the cells were fixed, stained, and examined by immunofluorescence microscopy. Localization of CD44 was assessed by confocal microscopy and a colocalization channel for CD44 and DAPI was built. Importin β depletion from the cytosolic fraction was not sufficient to inhibit CD44 import to the nucleus (Fig. 4*A*). However, when M9M-MBP, a specific inhibitor of transportin1 (kindly provided by Dr. Zi Chao Zhang) (23), was added to the cytosol, CD44 import was impaired to the same degree as when wheat germ agglutinin, a blocker of nuclear pores (18), was used. Quantification of the colocalization of CD44 and DAPI showed a nearly 75% decrease of nuclear CD44 in the presence of M9M peptide (Fig. 4*A*), supporting the notion that transportin1 is required for nuclear import of CD44.

CD44 Is Exported by Exportin CRM1—In the exportin family of proteins the one with the broadest spectrum of cargo proteins is exportin Crm1 (2). The possible involvement of Crm1 in nuclear export of CD44 was therefore tested. Leptomycin B, an

inhibitor of Crm1, was added to MNNG/HOS cells cultured on coverslips and after 2, 4, and 20 h, localization of CD44 was assessed by immunofluorescence. Strong nuclear accumulation of the HA receptor after 4 h of leptomycin treatment was observed (Fig. 4*B*), supporting the notion that CD44 shuttles between the cytoplasm and nucleus.

The First 20 Residues of the Cytoplasmic Tail Contain Sequences Necessary for Nuclear Translocation of CD44—Because both mature CD44 and its intracellular domain can translocate to the nucleus, we assumed that CD44 sequences that constitute the NLS are located within its cytoplasmic domain. To identify the putative CD44 NLS, we constructed three mutants, each lacking a portion of the cytoplasmic domain (Fig. 5*A*). pMSCVhygro plasmids containing mutant CD44 were expressed in human melanoma MC cells, which lack constitutive HA receptor expression (24). We expected that the mutant lacking the first 20 residues of the cytoplasmic domain would display impaired import to the nucleus based on the similarity between the 20-amino acid sequence and the predicted transportin1 recognition sequence (Fig. 5*B*) (25). Protein expression of the CD44 mutants was assessed by fluorescence-activated cell sorting (FACS) (Fig. 5*C*), and revealed only small variations

A

	Accession number	Protein mol. weight	Peptides in IP C	Peptides ISO
importin exportin family				
Importin subunit beta-3 - Homo sapiens (Human)	IMB3_HUMAN,Q5T578_HUMAN	123'614.2	62	0
Importin subunit beta-1 - Homo sapiens (Human)	IMB1_HUMAN,Q53XN2_HUMAN	97'153.3	54	4
Transportin-1 - Homo sapiens (Human)	TNPO1_HUMAN	101'295.6	39	1
cDNA FLJ78427, highly similar to Homo sapiens exportin 1	A8K1K5_HUMAN,XPO1_HUMAN	123'370.9	23	0
Exportin-2 - Homo sapiens (Human)	XPO2_HUMAN	110'403.9	15	0
Importin-4 - Homo sapiens (Human)	IPO4_HUMAN,Q59FI4_HUMAN	118'700.7	12	0
cDNA FLJ78655, highly similar to Homo sapiens exportin 5	A8K5Y7_HUMAN,XPO5_HUMAN	136'295.9	7	0
Transportin-3 - Homo sapiens (Human)	TNPO3_HUMAN	104'186.3	3	0
TNPO2 variant protein - Homo sapiens (Human)	Q4LE60_HUMAN,Q6IN77_HUMAN	117'662.9	2	0

B
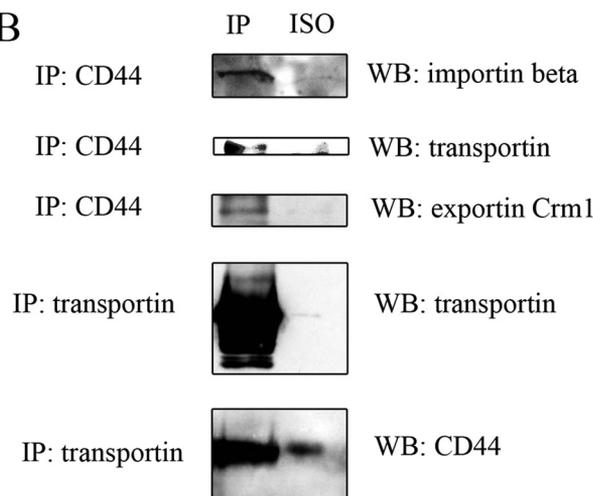
FIGURE 3. **CD44 interacts with karyopherins.** *A*, CD44 interactors identified in the pulldown assay belong to importin/exportin family of proteins. *B*, immunoprecipitation (*IP*) and Western blot (*WB*) analysis confirm CD44 interaction with importin β, transportin, and exportin. ISO, unrelated isotype-matched immunoglobulin.

in fluorescence intensity among MC cells expressing the different mutants.

Confocal microscopy revealed clear differences in nuclear localization of mutant CD44, shown here as the colocalization between CD44 and DAPI staining (Fig. 5*C*). Mutants lacking the putative transportin1 recognition sequence displayed strongly reduced nuclear localization, whereas their cell surface expression level was comparable with that of WT CD44 (Fig. 5*C*). These observations are consistent with the involvement of transportin1 in nuclear import of CD44.

Transportin1 Binding to the Cytoplasmic Tail of CD44—To assess the binding of transportin1 to the CD44 cytoplasmic domain mutants, we subcloned the constructs into the pCDM8 vector and co-expressed the CD44 mutants and transportin in COS7 cells. Immunoprecipitation of the CD44 mutants from the cytosolic fraction was performed and the presence of TNPO1 was assessed by Western blotting (Fig. 5*D*). Deletion of the cytosolic domain (dt mutant) disrupted the interaction between CD44 and TNPO1. However, the CD44 mutant lacking the first 20 amino acids of the cytoplasmic domain could still bind transportin, even though the major portion of the transportin recognition sequence was missing (Fig. 5, *A* and *B*). This observation shows that removal of the first 20 residues of the cytoplasmic domain is not sufficient to abrogate binding of TNPO1 to CD44, but as we have shown above, the observed residual binding is insufficient for nuclear translocation of the mutant.

Nuclear CD44 Influences RNA Polymerization—In searching for the potential function of CD44 in the nucleus we tested its effect on RNA polymerization. Incorporation of BrU into nascent RNA reflects the transcriptional activity of cells and can be readily measured by immunofluorescence or colorimetric assays. For the purpose of this assay, MNNG/HOS cells were stably transfected with pSiren vector containing CD44 shRNA (14). Although the knock-down was efficient with both shRNA sequences (Fig. 6*A*), sequence 1 was chosen for further experiments. MNNG/HOS expressing CD44 shRNA or control sequence were then incubated for 3 h with BrU and stained with anti-bromodeoxyuridine antibody. Analysis by immunofluorescence microscopy indicated a decrease of BrU incorporation close to 60% in knock-down cells (Fig. 6*B*). The same result was obtained with the colorimetric BrU incorporation assay (based on Cell Proliferation ELISA BrdU kit, Roche) (Fig. 6*C*). An analogous experiment was performed on fibroblasts from CD44-deficient mice. BrU incorporation was decreased in cells lacking HA receptor expression in comparison to wild-type cells (Fig. 6*D*).

Because these observations suggest the implication of CD44 in RNA polymerization, we asked whether the polymerization is linked to the presence of full-length CD44 protein in the nucleus. We therefore performed a BrU incorporation assay on MC cells expressing CD44 extracellular or cytoplasmic domain mutants (Fig. 6, *E* and *F*). MC cells showed increased BrU incorporation when expressing wild-type CD44 compared with the empty vector, whereas CD44 with impaired nuclear localization (dsp, t-20, dt mutants) was not able to induce the same effect. These results demonstrate that nuclear localization of CD44 influences RNA polymerization, and is hence involved in the regulation of gene transcription. Interestingly, it seems that the HA-binding domain is partially responsible for this effect, as the R41A mutant failed to increase BrU incorporation even though it localized to the nucleus (Fig. 2*D*). Expression of the ICD of CD44 alone was sufficient to raise the level of BrU incorporation to that attained as a result of full-length receptor expression. It is conceivable that conformational changes in the intracellular domain of the R41A mutant impair its influence on RNA polymerization. These results demonstrate that nuclear localization of CD44 influences RNA polymerization, and is hence involved in the regulation of gene transcription.

DISCUSSION

In this work we have uncovered the mechanism whereby CD44, a transmembrane extracellular matrix receptor protein, translocates to the nucleus. Previously, the cytoplasmic domain of CD44 had been detected in the nucleus (12), but the possi-
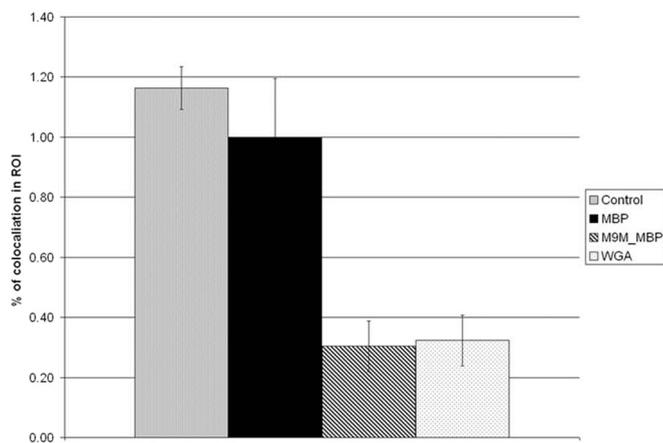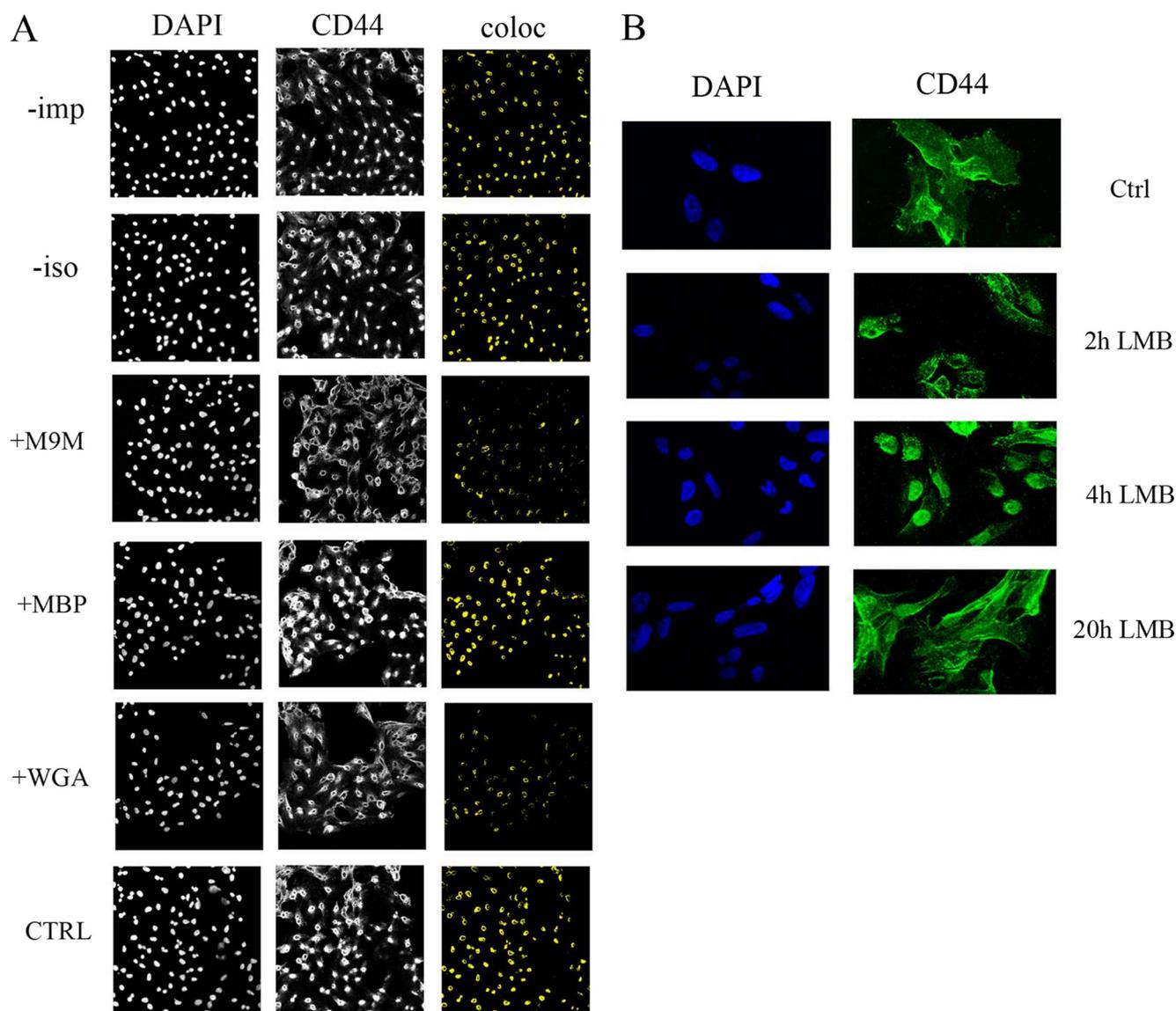
FIGURE 4. **Nuclear translocation of CD44 depends on transportin1 and exportin Crm1 but not on importin β.** *A*, nuclear import assay; nuclear CD44 was monitored according to its colocalization with DAPI staining; importin β depletion has no effect on CD44 nuclear localization; transportin1 inhibitor M9M peptide fused to maltose-binding protein (*MBP*) has the same effect as the nulcear pore blocker wheat germ agglutinin (*WGA*) (*p* values < 0. 005); the effect is quantified by Imaris software as the percentage of colocalization in the region of interest (*ROI*); 250–300 cells analyzed per condition. *B*, leptomycin B (*LMB*) treatment for 2, 4, and 20 h; 4 h incubation with LMB triggers nuclear accumulation of CD44. All the experiments have been done in triplicate.
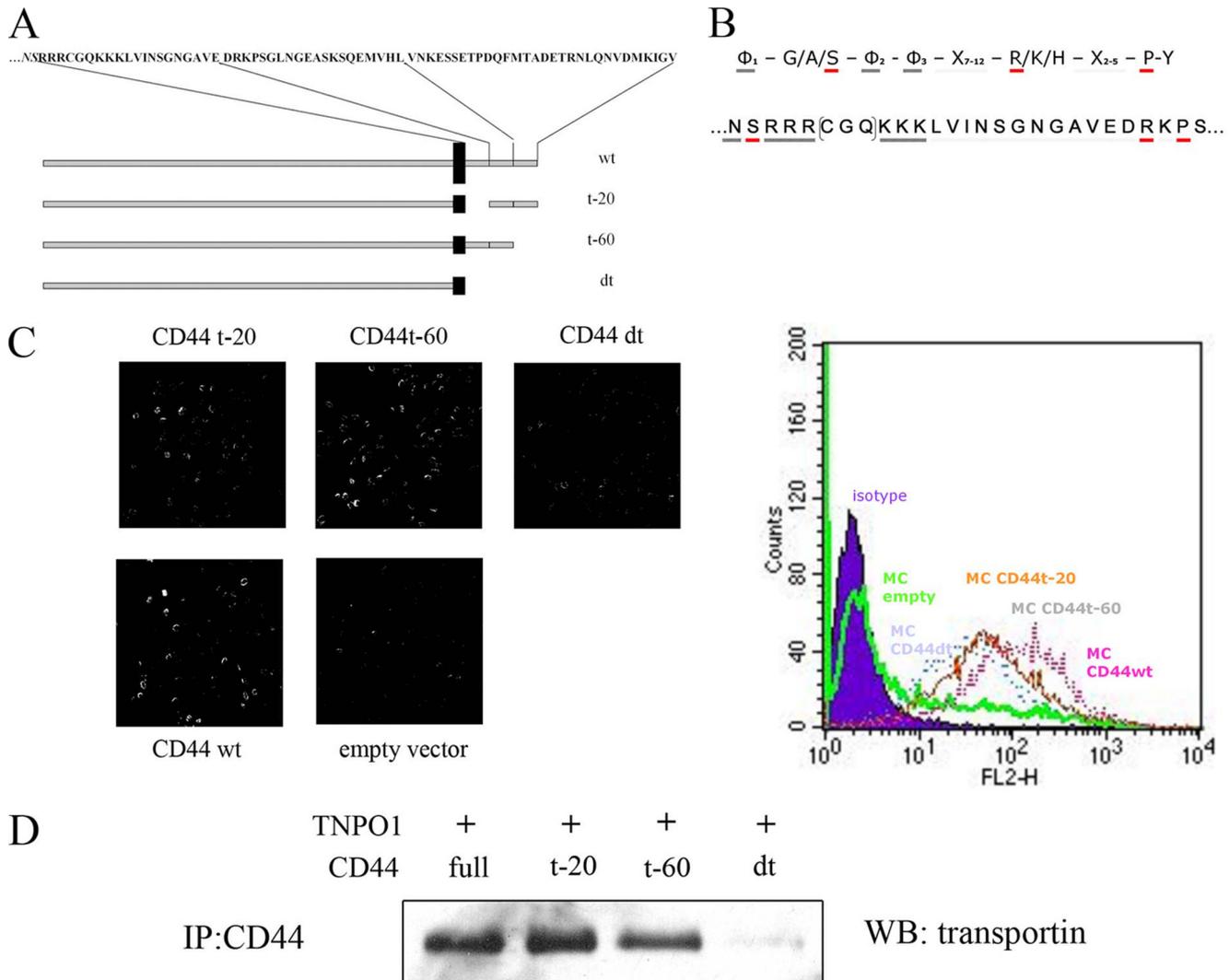
FIGURE 5. **CD44 contains a transportin1-specific NLS.** *A*, construction of CD44 cytoplasmic tail mutants. *B*, predicted sequence of transportin1 recognized NLS (*upper line*) (23) corresponds to sequences deleted in CD44t-20 mutant (*lower line*). *C*, CD44 tail mutant expression (*right panel*) and localization (*left panel*) in MC melanoma cells; nuclear CD44 localization in MC cells is presented as colocalization channel of CD44 immunofluorescent staining with DAPI. FACS analysis shows similar levels of expression of all CD44 constructs in MC cells. *D*, co-immunoprecipitation of transportin1 with CD44 cytoplasmic tail mutants. COS7 cells overexpressing TNPO1 together with one of the CD44 tail mutants were subjected to CD44 immunoprecipitation with Brick 235 mAb, and Western blot (*WB*) analysis revealed the presence of transportin in immunoprecipitated fractions. Input controls and isotype-matched controls have been done (data not shown).

bility that the full-length HA receptor might also be present in the nucleus had not been ruled out. Recently, Lee *et al.* (22) have shown nuclear localization of the full-length CD44 in the H1299 gastric cancer cell line. We performed immunofluorescent staining of the HA receptor in several cell types, noting the presence of full-length CD44 in the nuclei. In the human MNNG/HOS osteosarcoma cell line this localization was confirmed by immunoprecipitation of the receptor from the nuclear fraction. In their work, Lee *et al.* (22) postulated the internalization of CD44 via the endosomal sorting as the pathway responsible for the nuclear trafficking of the receptor. Our results are consistent with this notion, as we were able to precipitate a biotinylated CD44 from the nuclear fraction of the MNNG/HOS cells and as blocking of the endocytosis, but not the Golgi apparatus, was sufficient to inhibit nuclear localization of the receptor. We also show that mutant CD44, lacking the signal peptide required for membrane localization, has a

diminished ability to localize to the nucleus. Ligation of CD44 by osteopontin or Hermes-3 antibody promotes its nuclear localization (22). We show here that hyaluronic acid binding does not influence nuclear localization, as nuclear import of the R41A mutant is not impaired.

The mechanism of CD44 import into the nucleus had until now not been resolved. Our pulldown assays of CD44 revealed that its intracellular interactors are predominantly members of the karyopherin family consistent with the notion that CD44 may shuttle between the cytoplasm and nucleus. Interactions between CD44 and transportin1, importin β, and exportin Crm1 were confirmed by co-immunoprecipitation experiments. Functional studies using a combination of CD44 mutagenesis and selective transport inhibitors revealed that transportin may be the key player in nuclear transport of CD44. Selective inhibitors of nuclear shuttling pathways, including the M9M peptide, which is an inhibitor of transportin1 (23), are

A

sh1 sh2 shCTRL

CD44

tubulin

B

MNNG/HOS
shCTRL

MNNG/HOS
shCD44

C

D

E

F
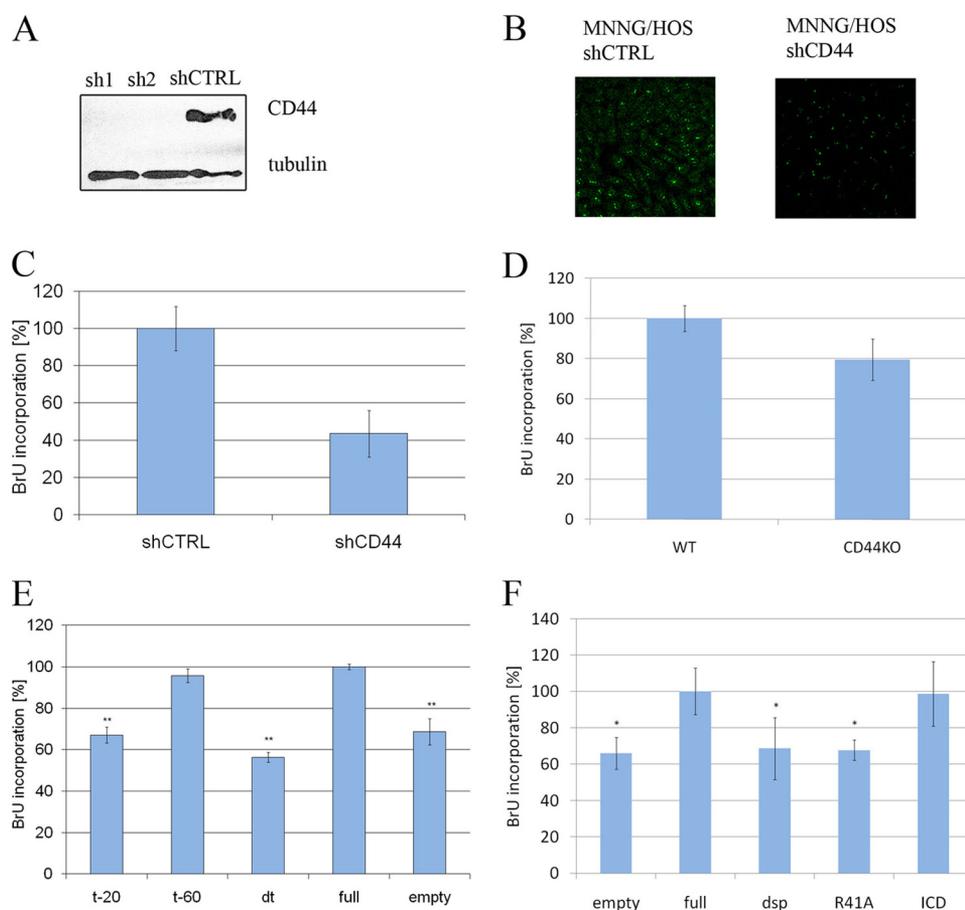
FIGURE 6. **Nuclear CD44 enhances RNA synthesis.** *A*, shRNA down-regulation of CD44 expression. Immuno-fluorescence (*B*) and a colorimetric assay (*C*) show weaker BrU incorporation in cells depleted of CD44 compared with control cells (mean of three experiments, *p* value: * <0.05). *D*, a similar effect is observed in colorimetric assays with $CD44^{-/-}$ mouse fibroblasts compared with the wild-type cells (mean of three experiments, *p* value: * <0.05). *E*, MC CD44 t-20 mutant incorporates less BrU that the wild type and the CD44 t-60 mutant as assessed by the colorimetric assay (mean of three experiments, *t* test comparison of mutants to WT, *p* values: * <0.05; analysis of variance *p* value is 0.005593). *F*, the role of the extracellular domain in BrU incorporation. Lack of the signal peptide (*dsp*) or the R41A hyaluronan-binding site mutation decreases BrU incorporation (representative experiment, *t* test comparison of mutants to WT, *p* values: * <0.05; analysis of variance *p* value is 0.025).

for the interaction (26). Site A, at the C-terminal end of the NLS, is the primary, high affinity binding site. The localization of this site corresponds to residues DRKPS, which belong to the predicted NLS, but were not deleted in our CD44t-20 mutant. TNPO1 binding at site B, localized at the N terminus of the NLS, is important for conformational changes in transportin1 and for dissociation of the cargo protein in the nucleus (26). Our results show that site A binding is sufficient for interaction of CD44 with TNPO1, but the functional nuclear import of CD44 cannot occur without the first membrane-proximal 20 amino acids of the cytoplasmic tail, which contain TNPO1 binding site B.

This observation supports the notion that transportin1 is responsible for nuclear import of CD44. The fact that CD44 binds several proteins from the importin family, but that only one of them is sufficient for nuclear translocation is not surprising. It has been shown that the transcription factor c-Fos binds several importins, including importin β and transportin1 (27). Detailed analysis showed that transportin1 plays the key role in the import of c-Fos.

In the present work we also investigated the nuclear export of CD44. The majority of transcription factors are exported from the nucleus

valuable tools for the study of nuclear import and export. The sequence of the M9M peptide corresponds to the predicted NLS recognized by transportin1. Thus M9M is a competitive inhibitor of cargoes shuttled by transportin1. Nuclear localization of CD44 was drastically decreased when the M9M peptide was added to the transport mixture. The same effect was observed upon treatment with wheat germ agglutinin, a compound that blocks the nuclear pores and disables molecule exchange between the nucleus and cytoplasm. Conversely, depletion of another CD44-interacting import protein, importin β, from the transport machinery did not cause impairment of CD44 nuclear localization. Taken together, these results are consistent with a functional interaction between CD44 and transportin1. Furthermore, the intracellular domain of CD44 contains a sequence that bears homology to a predicted NLS recognized by transportin1 (25). Mutant CD44 protein, which lacks the 20-aa fragment, was no longer found in the nucleus. Interestingly, deletion of the $Arg^{673}-Glu^{692}$ was not sufficient to prevent binding of transportin1 to the mutant CD44. Crystal structures of TNPO1 with its several cargo proteins reveal that the NLS of the protein contains two sites that are responsible

by exportin Crm1 (2). Interaction between CD44 and exportin Crm1 was confirmed by co-immunoprecipitation. To study the involvement of Crm1 in the export of nuclear proteins leptomycin B, a selective inhibitor of this exportin, was used (2, 19). Incubation of the cells with leptomycin B resulted in accumulation of nuclear CD44, thus providing support for the function of Crm1 in CD44 export.

It has been previously shown that the intracellular domain of CD44 regulates transcription through CBP/p300 responsive elements (12). Upon the discovery of the full-length CD44 in the nucleus we also speculated that it could take part in the gene expression process. To target this question we performed BrU incorporation assays. Bromouridine is taken up by the cells, and after phosphorylation it is incorporated in the nascent RNA (20). BrU incorporation can be detected with anti-BrdU antibodies and the amount of labeling reflects the RNA synthesis levels in the cell. Decreased BrU incorporation was observed in human MNNG/HOS cells depleted of CD44 and in mouse fibroblasts derived from HA-receptor knock-out animals. The decrease in RNA polymerization appears to depend on nuclear localization of CD44. It was true for cells expressing the mutant

lacking the signal peptide that cannot follow the membrane-endosome-nucleus pathway, as well as for cells expressing the mutant lacking the NLS recognized by transportin1. Interestingly, the R41A mutation, which disables hyaluronic acid binding, also decreased incorporation of BrU. To determine, whether HA bound to CD44 at the cell membrane and imported into the nucleus or whether the R41A mutant is causing a conformational change that impairs the protein machinery responsible for RNA polymerization will require further studies. The experiments based on overexpression of the ICD domain alone allow us to speculate that it is the domain responsible for recruiting the nuclear proteins that induce RNA polymerization. Interestingly, in MNNG/HOS cells we did not find significant amounts of nuclear ICD. Attempts to induce cleavage of CD44 with 12-*O*-tetradecanoylphorbol-13-acetate were also not successful (supplemental Fig. S1). This could mean that in some cell types, where cleavage of the intracellular domain of CD44 is not efficient, nuclear translocation of full-length protein is required.

The present work provides new insight into the processing and function of CD44. The full-length protein is shown to be imported from the cytoplasm to the nucleus by transportin1 where it induces gene transcription. Functional interaction with transportin requires the membrane-proximal 20 amino acids of the cytoplasmic tail of CD44 that contain the transportin1-specific NLS. From the nucleus, full-length CD44 shuttles back to the cytoplasm upon Crm1 exportin binding. This hitherto unrecognized mechanism of CD44 translocation can help uncover new molecular pathways whereby it participates in the regulation of various cellular functions.

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