Hyaluronan Cell Surface Binding Is Induced by Type I Collagen and Regulated by Caveolae in Glioma Cells*

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Hyaluronan (HA) is a component of the brain extracellular matrix environment that is synthesized and secreted by glioma cells. The primary cell surface receptor for HA is CD44, a membrane glycoprotein that is functionally regulated by a membrane type 1 matrix metalloproteinase (MT1-MMP). Both CD44 and MT1-MMP are partially located in Triton X-100-insoluble domains, but no functional link has yet been established between them. In the present study, we studied the regulation of HA cell surface binding in U-87 glioma cells. We show that an MMP-dependent mechanism regulates the intrinsic cell surface binding of HA as ilomastat, a broad MMP inhibitor, increased HA binding to glioma cells. HA binding was also rapidly and specifically up-regulated by 3-fold by type I collagen in U-87 cells, which also induced a significant morphological reorganization associated with the activation of a latent form of MMP-2 through a MT1-MMP-mediated mechanism. Interestingly, caveolae depletion with a cell surface cholesterol-depleting agent β-cyclodextrin triggered an additional increase (9-fold) in the binding of HA, in synergy with type I collagen. On the other hand, HA cell surface binding was diminished by the MEK inhibitor PD98059 and by the overexpression of a recombinant, wild type MT1-MMP, whereas its cytoplasmic-deleted form had no effect. Taken together, our results suggest that MT1-MMP regulates, through its cytoplasmic domain, the cell surface functions of CD44 in a collagen-rich pericellular environment. Additionally, we describe a new molecular mechanism regulating the invasive potential of glioma cells involving a MT1-MMP/CD44/caveolin interaction, which could represent a potential target for anti-cancer therapies.

The principal molecules that have been identified in the normal brain extracellular matrix (ECM)[1] are hyaluronan (HA; also known as hyaluronic acid, hyaluronate) and chondroitin sulfate (1, 2). HA is an important glycosaminoglycan constituent believed to be implicated in angiogenesis, the formation of new blood vessels from preexisting vasculature. Although the serum level of HA is already used as an indicator of progressive malignant disease (3), its effects on *in vivo* angiogenesis and endothelial cell (EC) function are complex and have been reported to depend on HA concentration and molecular size (4, 5). Accordingly, whereas high molecular weight HA was shown to inhibit EC functions (6), low molecular weight HA stimulated EC proliferation, tubulogenesis (6, 7), and neovascularization (8). Moreover, small HA polymers efficiently regulated CD44 cell surface functional expression and promoted tumor cell migration (9).

Astrocytic tumors of the central nervous system express CD44 among other cell adhesion receptors of the integrin or immunoglobulin superfamily. Although HA is the principal ligand of CD44, other CD44 ligands include the ECM components collagen, fibronectin, laminin, and chondroitin sulfate, whereas mucosal addressin, serglycin, osteopontin, and the class II invariant chain represent ECM-unrelated ligands of the molecule. CD44 is also implicated in the promotion of tumor growth, invasiveness, and metastatic potential in experimental and human cancers (10, 11). Recent findings suggest that CD44 provides a docking site for matrix metalloproteinase (MMP)-9 on the surface of melanoma and carcinoma cells and thus can indirectly contribute to pericellular proteolysis of types IV and V but not type I collagen (12). Although several other cell surface receptors for HA have been reported, it has been shown that gliomas express significant levels of CD44 and that CD44 expression could be relevant in determining their highly invasive behavior (13, 14).

Several studies have revealed different molecular and cellular mechanisms regulating CD44-mediated processes. The most recent studies evaluated the role of CD44 in the rolling interaction of lymphoid cells with HA (15), the role of E-cadherin in CD44-mediated tumor invasion (16), and the role of intracellular Rho-mediated signaling leading to cytokine production and breast tumor progression (17). Moreover, molecular paradigms of cell migration that may be involved in tumor invasion and metastasis have also recently been highlighted by the common cell surface localization of CD44 with a membrane type (MT) 1-MMP at the leading edge of motile cells (lamellipodia) (18, 19). These studies elegantly demonstrated that CD44 directed MT1-MMP to lamellipodia by associating with

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[1] The abbreviations used are: ECM, extracellular matrix; EC, endothelial cell; EGCg, epigallocatechin-(3)-gallate; HA, hyaluronan (hyaluronic acid, hyaluronate); MMP, matrix metalloproteinase; MT1-MMP, membrane type 1 matrix metalloproteinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; MAPK, mitogen-activated protein kinase; PBS, phosphate-buffered saline; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; Wt, wild type; TBST, Tris-buffered saline plus Tween 20; ERK, extracellular signal-regulated kinase; Mes, 4-morpholineethanesulfonic acid.

its hemopexin-like domain, and that cell surface MT1-MMP-mediated cleavage of CD44 subsequently played a critical role in promoting tumor cell migration. In addition, one other common feature between MT1-MMP and CD44 is that both partially localize within Triton X-100-insoluble and cholesterol-enriched membrane domains (20–23). Accordingly, the caveolar location of MT1-MMP has been suggested to provide a regulatory mechanism of glioma cells invasiveness by caveolin-1. Whether MT1-MMP, aside from its classical roles in cell migration, tubulogenesis, and activation of proMMP-2, also regulates CD44-mediated binding to HA is unknown. Moreover, very little is known about the common MT1-MMP/CD44 caveolar regulation of ECM protein recognition.

In the present study, we investigated the mechanisms involved in the regulation of CD44 function in cells derived from a highly aggressive and vascularized brain tumor glioblastoma. Specifically, we addressed the importance of caveolae with respect to the potential MT1-MMP-dependent functional regulation of HA recognition/binding at the cell surface of glioma cells. More importantly, we provide the first evidence of a new regulatory cell surface functional cross-talk between MT1-MMP and CD44 that impacts on the ability of gliomas to bind HA. Finally, we show that type I collagen triggers an increased cell surface binding to HA in glioma cells, and that the cytoplasmic domain and caveolar location of MT1-MMP may in part regulate such effect through MAPK-dependent intracellular signaling. Collectively, our results demonstrate a new potential MT1-MMP/CD44/caveolin cross-talk that could regulate the invasive potential of glioma cells through their interaction with the brain ECM environment and that could represent a new potential target for anti-cancer therapies.

EXPERIMENTAL PROCEDURES

Reagents—Agarose, (−)-epigallocatechin 3-gallate (EGCg), sodium dodecyl sulfate (SDS), gelatin, β-cyclodextrin, bovine serum albumin (BSA), laminin-1, and Triton X-100 were purchased from Sigma (Oakville, Ontario, Canada). ilomastat (GM-6001) was from BIOMOL (Plymouth Meeting, PA), TRIzol reagent was from Invitrogen. Fu-GENE-6 transfection reagent and fibronectin were from Roche Diagnostics Canada (Laval, Quebec, Canada). Type I collagen was extracted from rat tail tendon according to classical protocols (24). The anti-CD44 R-phycoerythrin-conjugated mouse anti-human monoclonal antibody (G44–26) and mouse IgG2bκ (clone 27–35) were from BD Pharmingen (Franklin Lakes, NJ). The anti-MT1-MMP polyclonal antibody AB-815, the anti-caveolin-1 monoclonal antibody, the anti-ERK and anti-phospho-ERK (Thr[202]/Tyr[204]) antibodies were from Chemicon (Temecula, CA). Fluorescein isothiocyanate-labeled hyaluronic acid (HA-FITC) and hyaluronic acid sodium salt were from CarboMer (San Diego, CA). The MEK inhibitor PD98059 was from Calbiochem (La Jolla, CA).

Cell Culture and cDNA Transfection Method—The U-87 glioma cell line was purchased from American Type Culture Collection and maintained in Eagle's minimum essential medium containing 10% (v/v) fetal bovine serum (HyClone Laboratories, Logan, UT), 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and were cultured at 37 °C under a humidified atmosphere containing 5% CO_2. The MT1-MMP cDNA constructs were previously generated and validated by us (25) as follows. Wt encodes the full-length MT1-MMP protein (Met[1]–Val[582]); Δ1 encodes a protein, which lacks the entire C-terminal 20-amino acid cytoplasmic domain (Met[1]–Phe[562]); ΔTM encodes a soluble secreted form of MT1-MMP, which lacks the entire transmembrane and cytoplasmic domain (Met[1]–Cys[508]). U-87 cells were transiently transfected with cDNA constructs using the nonliposomal formulation FuGENE-6 transfection reagent. Transfection efficiency was confirmed by Western blotting and zymography. All experiments involving these cells were performed 36 h after transfection. Mock transfections of U-87 cultures with pcDNA (3.1+) expression vector alone were used as controls.

Total RNA Isolation and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analysis—Total RNA was extracted from monolayers of cultured U-87 cells using TRIzol reagent. One microgram of total RNA was used for first strand cDNA synthesis, followed by specific gene product amplification with the One-Step RT-PCR kit (Invitrogen). Primers for CD44s (forward, 5′-TTTGCCTCTTACAGTTGAGCCTG-3′; reverse, 5′-GGTGCCATCACGGTTGACAATAG-3′) were derived from
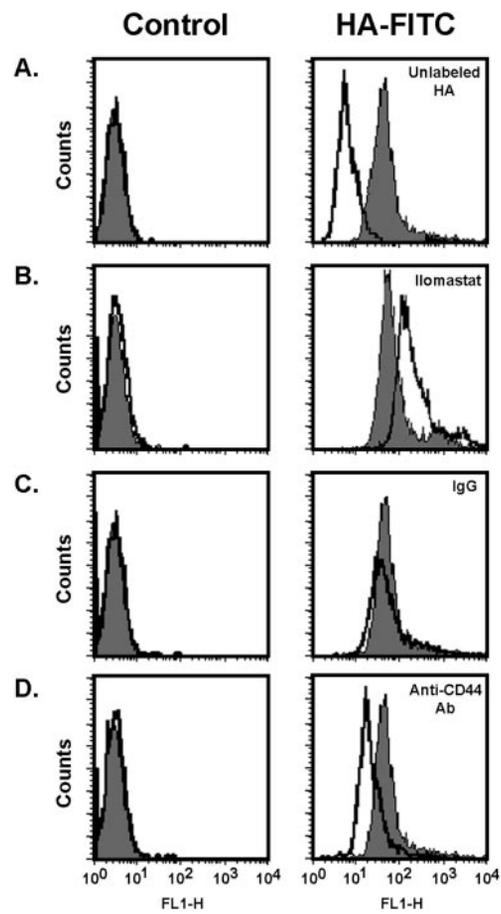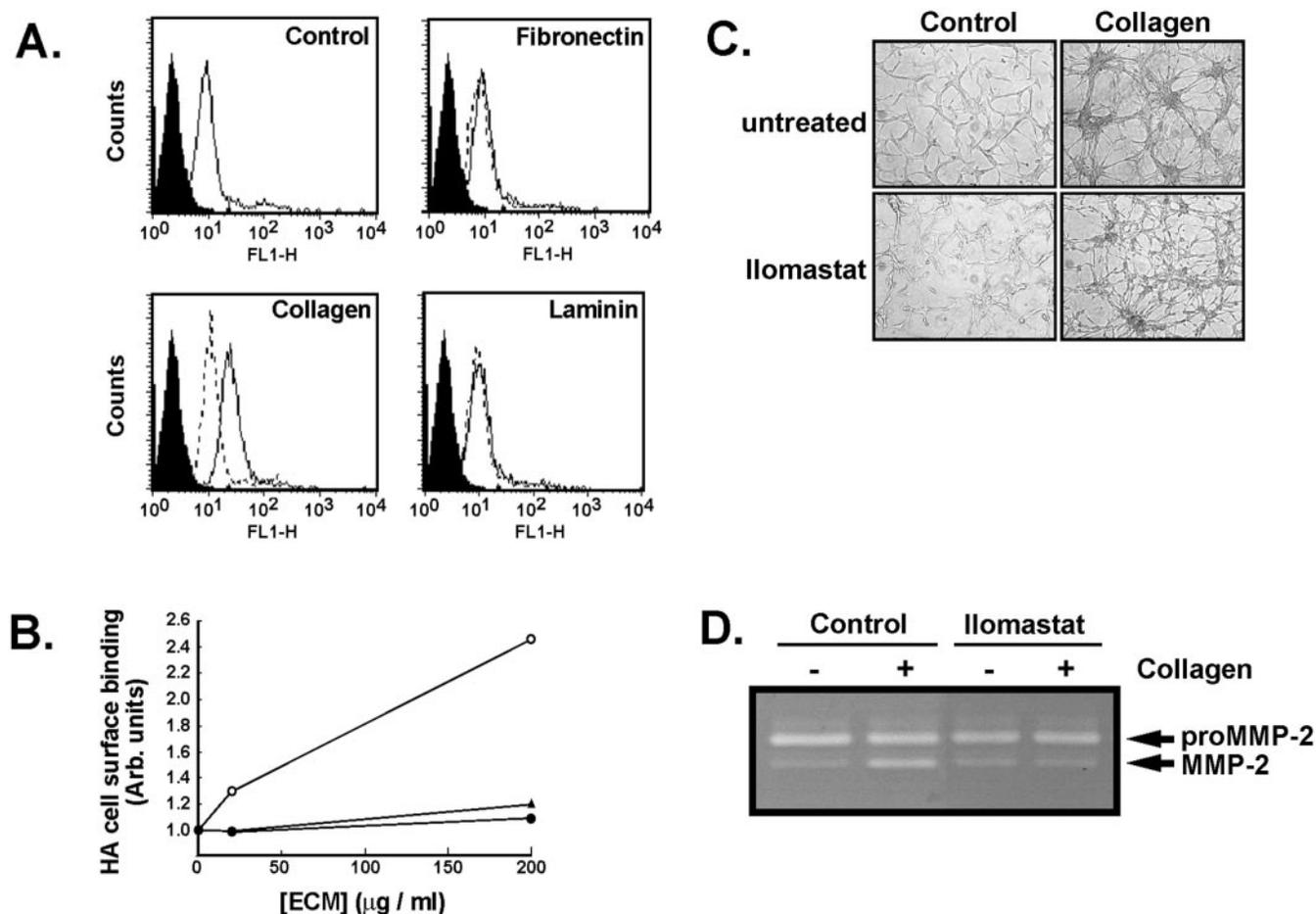

FIG. 1. **HA binding to U-87 glioma cell surface is regulated through an MMP-dependent mechanism.** Control U-87 glioma cells (*left panels*) were resuspended and incubated for 1 h at 4 °C as described under "Experimental Procedures" with 1 mg/ml unlabeled HA (*A*), IgG (*C*), or anti-CD44 antibody (*D*). U-87 cells were also incubated for 18 h at 37 °C with 20 μM ilomastat (*B*). After the respective treatments, cells were analyzed by flow cytometry for their ability to bind 20 μg/ml FITC-labeled HA (*right panels*). *Filled tracings* represent either the intrinsic cell fluorescence (*left panels*) or the cell surface fluorescence in the presence of HA-FITC (*right panels*). *Bold line tracings* represent the treatments described above in the absence (control) or presence of HA-FITC. The results are representative of three independent experiments.

human sequences, and PCR conditions optimized so that the gene products were in the exponential phase of the amplification (94 °C for 2 min; then run for 30 cycles at 94 °C for 30 s, 55 °C for 1 min, and 72 °C for 1 min; followed by a 7-min final elongation at 72 °C). PCR products were resolved on 1.5% agarose gels containing 1 μg/ml ethidium bromide.

Immunoblotting Procedures—Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE). After electrophoresis, proteins were electrotransferred to polyvinylidene difluoride membranes, which were then blocked overnight at 4 °C with 5% nonfat dry milk in Tris-buffered saline (150 mM Tris, 20 mM Tris-HCl, pH 7.5) containing 0.3% Tween 20 (TBST). Membranes were further washed in TBST and incubated with primary antibodies (1/1,000 dilution) in TBST containing 3% bovine serum albumin, washed again in TBST, and followed by a 1-h incubation with horseradish peroxidase-conjugated anti-rabbit IgG (1/10,000 dilution) in TBST containing 5% nonfat dry milk. Immunoreactive material was visualized by enhanced chemiluminescence (Amersham Biosciences, Baie d'Urfé, Quebec, Canada). Note that immunodetection of the recombinant MT1-MMP proteins was performed after a 30-s exposure to ECL, whereas that of the endogenous MT1-MMP required at least 2 min to obtain a significant signal.

Gelatin Zymography—To assess the extent of functional recombinant MT1-MMP expression in transfected U-87 cells, we measured the activation of a latent, exogenous source of proMMP-2 by gelatin zymography as described previously (20, 26). Briefly, an aliquot (20 μl) of the culture medium was subjected to SDS-PAGE in a gel containing 0.1

FIG. 2. **Type I collagen specifically triggers HA binding to the cell surface of U-87 glioma cells.** *A*, U-87 glioma cells were trypsinized and co-incubated for 1 h at 4 °C with HA-FITC in the presence of increasing concentrations of fibronectin, type I collagen, or laminin (no ECM protein, control (*shaded plot*), 20 μg/ml ECM proteins (*dashed line*), 200 μg/ml ECM proteins (*full lines*)). HA binding was monitored by flow cytometry as described under "Experimental Procedures," and the quantified data are shown in *B* (*open circles*, collagen; *closed circles*, fibronectin; *closed triangles*, laminin). Cell morphology of adherent U-87 glioma cells was observed under visible light in response to type I collagen, ilomastat, or combined collagen/ilomastat treatment (*C*). The heavy network correlated with type I collagen-induced proMMP-2 activation (*D*), as assessed by gelatin zymography.

mg/ml gelatin. The gels were then incubated in 2.5% Triton X-100 and rinsed in double-distilled H_2O. Gels were further incubated at 37 °C for 20 h in 20 mM NaCl, 5 mM $CaCl_2$, 0.02% Brij-35, 50 mM Tris-HCl buffer, pH 7.6, then stained with 0.1% Coomassie Brilliant Blue R-250 and destained in 10% acetic acid, 30% methanol in H_2O. Gelatinolytic activity was detected as unstained bands on a blue background. All experiments were carried out with cells that had been serum-deprived by overnight incubation.

Flow Cytometry Analysis and Fluorescein Isothiocyanate-labeled HA Binding Assay—Serum-deprived cells were preincubated for 1 h at 37 °C under a humidified atmosphere containing 5% CO_2 with or without 5 mM β-cyclodextrin, 20 μM ilomastat, and 10 μM EGCg. For overnight treatments, serum-deprived cells were preincubated for 18 h at 37 °C under a humidified atmosphere containing 5% CO_2 with 20 μM ilomastat. Cells were dislodged after brief trypsinization, washed extensively, resuspended in 10% fetal bovine serum/Dulbecco's modified Eagle's medium at a concentration of 10^5 cells/ml, washed once with 0.1% PBS plus 0.1% BSA, and then incubated with 100 μg/ml HA-FITC for 1 h on ice with or without 100 μg/ml type I collagen. After washing with PBS/BSA, the cells were suspended in 1 ml of PBS/BSA, and analyzed on a FACSCalibur flow cytometer with the CellQuestPro software (BD Biosciences, Mississauga, Ontario, Canada). For assessment of cell surface CD44 expression, cells were detached from plates as described above and resuspended in 10% fetal bovine serum/Dulbecco's modified Eagle's medium at a concentration of 10^6 cells/ml, washed two times, and blocked for 15 min at room temperature in PBS containing 5% inactivated fetal calf serum (PBS/FCS). The cells were then incubated in PBS plus 0.5% FCS with 0.5 μg/ml CD44 monoclonal antibody or mouse IgG2bκ at room temperature for 30 min, washed once, and resuspended in PBS plus 0.5% FCS. Results are expressed as the ratio of relative geometric mean

values from the HA-treated cells to their untreated controls and are representative of three independent experiments.

Extraction of Caveolae-enriched Membrane Fractions with Alkaline Carbonate, and Purification—Carbonate extraction was performed as described previously (27). In brief, cells were grown to confluence in a F-75 dish and were washed twice in cold PBS. After aspiration of the PBS solution, 2 ml of 500 mM Na_2CO_3, pH 11.0, was used to scrape the cells off the dish. The sample was transferred to a 5-ml polycarbonate tube and was homogenized with a Polytron instrument, followed by sonication. The resulting homogenate was mixed with an equal volume of 90% (w/v) sucrose prepared in 25 mM Mes, 0.15 M NaCl (pH 6.5) (MBS). The sample was then transferred to a 12-ml ultracentrifuge tube and overlaid with a discontinuous sucrose gradient (4 ml of 35% (w/v) sucrose, 4 ml of 5% (w/v) sucrose, both prepared in MBS lacking detergent). The samples were centrifuged at $200,000 \times g$ (39,000 rpm in a Beckman SWT-1 rotor) for 18 h at 4 °C. A light-scattering band was observed at the 5–35% sucrose interface. Twelve 1-ml fractions were collected, and 20-μl aliquots from fractions 2–10 were subjected to SDS-PAGE and immunoblot analysis.

Statistical Data Analysis—Data are representative of three or more independent experiments. Statistical significance was assessed using Student's unpaired *t* test and was used to compare the relative HA cell surface binding of transfected or treated cells to untreated (mock or control) U-87 cells. Probability values of less than 0.05 were considered significant, and an *asterisk* (*) identifies such significance in each figure.

RESULTS

HA Binding to U-87 Glioma Cells Is an MMP-regulated Event—Using flow cytometry, we first evaluated whether HA
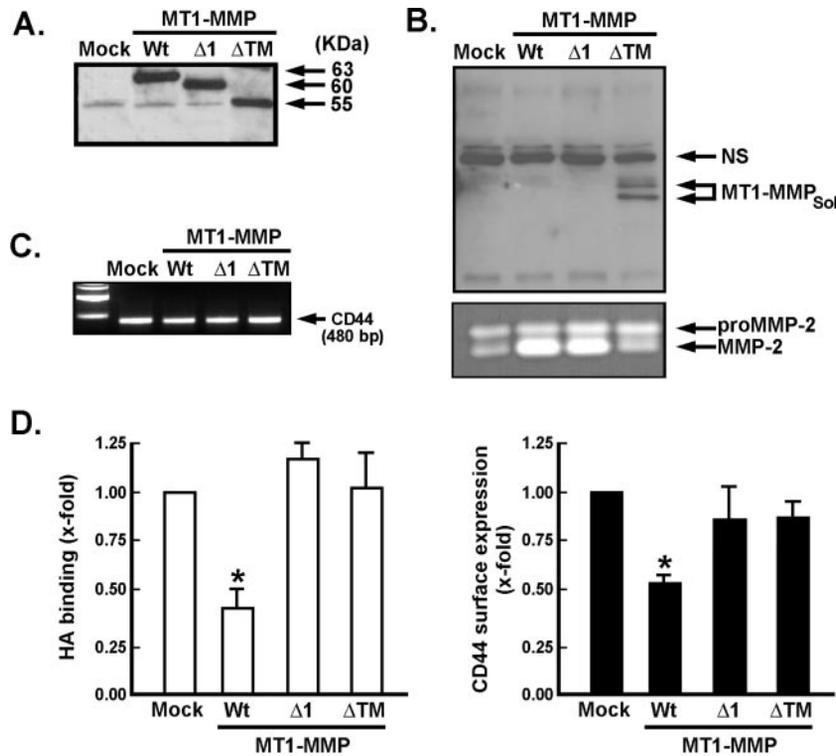
FIG. 3. **The cytoplasmic domain of MT1-MMP regulates the cell surface expression level of CD44 and the HA binding function.** *A*, U-87 glioblastoma cells were transfected with cDNA plasmids encoding the full-length (*Wt*) MT1-MMP, cytoplasmic domain-deleted (*Δ1*) MT1-MMP, and soluble (*ΔTM*) MT1-MMP recombinant proteins (25) as described under "Experimental Procedures." Cell lysates (20 μg) were migrated through 9% SDS-PAGE gels followed by Western blotting and immunodetection with anti-MT1-MMP antibody as described under "Experimental Procedures." *B*, the recombinant soluble MT1-MMP secretion was monitored in the conditioned media of transfected U-87 glioblastoma cells. Conditioned media were resolved on 9% SDS-PAGE and immunoblotted for MT1-MMP. *NS* represents nonspecific immunoreactive proteins. Conditioned medium was also used to monitor membrane-bound MT1-MMP-dependent proMMP-2 activation by gelatin zymography as described under "Experimental Procedures." *C*, total RNA was isolated from U-87 glioblastoma cells transfected (or not) with the different MT1-MMP cDNAs as described under "Experimental Procedures." RT-PCR was performed to amplify a 480 bp DNA fragment for CD44. *D*, flow cytometry was used to monitor HA-FITC cell surface binding in transfected cells as well as CD44 cell surface protein expression as described under "Experimental Procedures."

binding to U-87 glioma cells could be assessed. Because HA uptake/degradation was shown to occur at higher temperatures, we performed the FITC-labeled HA (HA-FITC) binding assay at 4 °C, at which no active internalization occurs (28, 29). HA-FITC bound to the cell surface of glioma cells detached by trypsinization, as was demonstrated by the shift in fluorescence intensity (Fig. 1, *right panels*, *shaded plots*). Similar levels of HA binding were obtained with cells detached by treatment with EDTA (data not shown). Excess nonfluorescent unlabeled HA is shown to compete for cell surface HA binding sites, as the shift in fluorescence is significantly diminished (Fig. 1*A*). Interestingly, an overnight (18 h) incubation of the cells with ilomastat, a broad spectrum MMP inhibitor reported to block the activation of MT1-MMP (30), and to tightly bind the recombinant catalytic domain of MT1-MMP (31, 32), resulted in an increase in basal HA binding (Fig. 1*B*). This observation suggests that a membrane-anchored MMP activity is involved in the long term regulation of the functional cell surface binding of HA because this effect could not be observed at a shorter time (1 h, see Fig. 5). Because such MMP-mediated regulation of HA binding has recently been shown to involve CD44 (19), we pre-incubated some U-87 cells with control IgG (Fig. 1*C*) or a blocking anti-CD44 antibody (Fig. 1*D*). The latter specific antibody inhibited CD44-mediated cell surface HA binding, but suggests that other alternate HA-binding cell surface molecules could also be involved because only partial inhibition was observed. Interestingly, whereas IgG had no effect on the ilomastat-induced HA cell surface binding, the anti-CD44 blocking antibody antagonized that increase in HA binding (data not shown). This further supports the cross-talk that

links membrane-bound MMP activity to CD44 functions in binding HA.

Type I Collagen Specifically Triggers HA Binding to the Cell Surface of U-87 Glioma Cells—To examine the effect and specificity of several matrix molecules on the binding of HA, we incubated glioma cells in the presence of several ECM proteins. Incubation of the cells with type I collagen triggered a significant, 2.5-fold increase in cell surface-associated HA binding (Fig. 2*A*), whereas neither fibronectin nor laminin had such an effect (Fig. 2*B*). Type I collagen treatment of U-87 cells also induced profound morphological changes (Fig. 2*C*). This cell morphology perturbation was accompanied by activation of a latent, secreted, soluble form of proMMP-2 into its active MMP-2 form, as assessed by gelatin zymography, and which activation was inhibited by ilomastat (Fig. 2*D*). Such activation by type I collagen was recently suggested to be mediated through an MT1-MMP process in cardiac fibroblasts (33). Whether a similar MT1-MMP-dependent mechanism could functionally regulate HA cell surface binding in glioma cells was next investigated.

MT1-MMP Overexpression Antagonizes HA Binding through CD44 Cell Surface Down-regulation—Aside from its well documented effect on proMMP-2 activation, MT1-MMP is also thought to be involved in CD44 regulation at the cell surface. However, the structure-function relationships of MT1-MMP to cellular HA binding are unknown. We transfected U-87 cells with cDNA constructs to overexpress three MT1-MMP recombinant forms. These were the full-length Wt MT1-MMP, the cytoplasmic-truncated domain (Δ1) MT1-MMP, and a complete cytoplasmic/transmembrane-domain truncated (ΔTM) MT1-
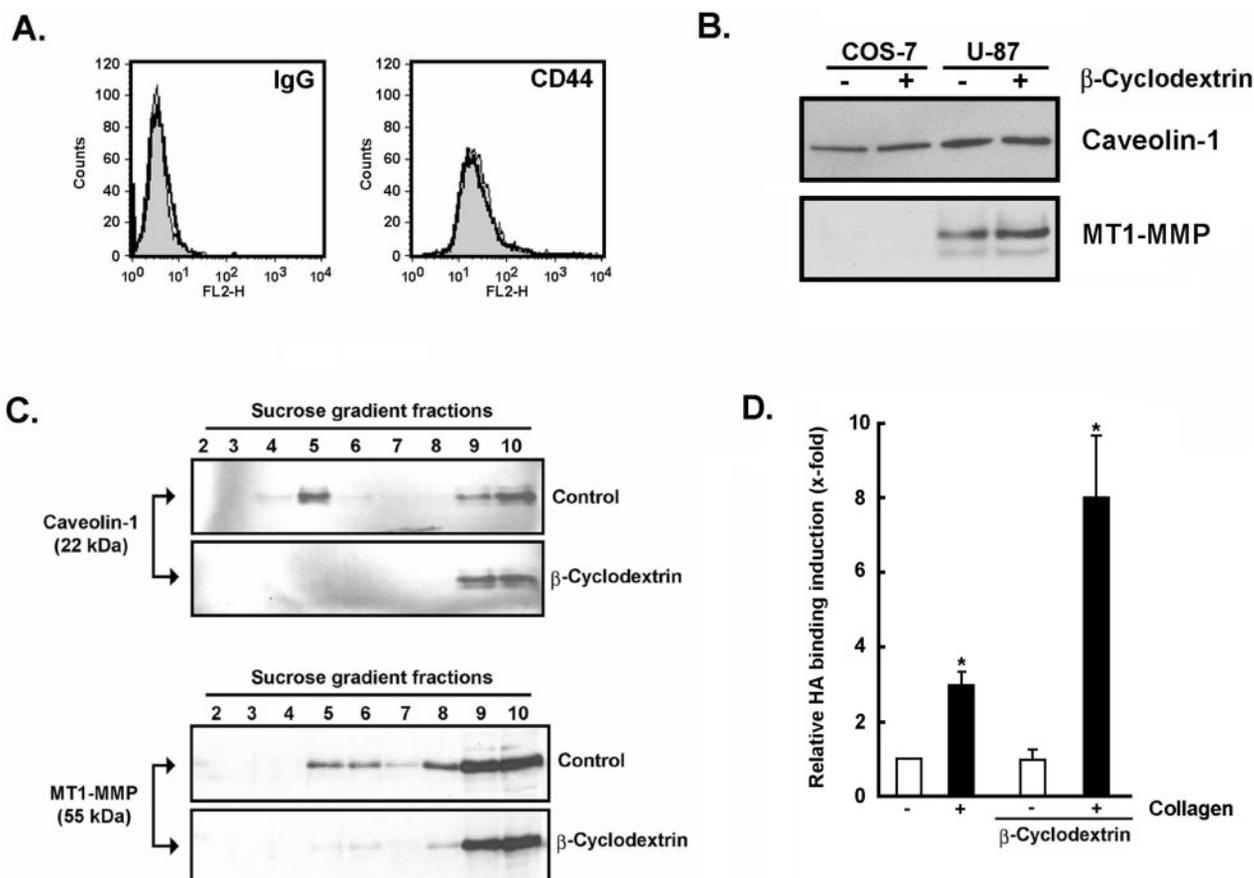
FIG. 4. **Cholesterol depletion increases the cell surface binding of HA to U-87 glioma cells in synergy with collagen.** Cell surface cholesterol depletion using 5 mM β-cyclodextrin was performed on serum-deprived cells as described under "Experimental Procedures." *A*, flow cytometry was used to assess the cell surface CD44 protein levels in untreated (*filled tracings*) or β-cyclodextrin-treated U-87 cells (*bold lines*). *B*, cholesterol depletion effects were monitored in total membrane preparations from COS-7 and U-87 cells. Twenty micrograms of protein/well were resolved by SDS-PAGE. Immunoblotting for caveolin-1 and MT1-MMP protein expression in total membrane preparations are shown. *C*, carbonate extraction was performed as described under "Experimental Procedures"; lysates were fractionated in a discontinuous sucrose density gradient. *Fractions 2–10* were analyzed for caveolin-1 and MT1-MMP immunoreactivity in untreated or β-cyclodextrin-treated U-87 glioma cells. *D*, the effect of caveolae depletion (5 mM β-cyclodextrin) was evaluated on cell surface HA binding in untreated (*open bars*) or type I collagen-treated (*closed bars*) glioma cells.
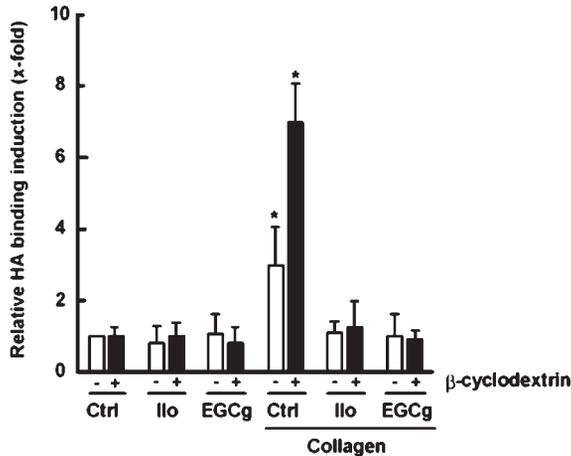
MMP. All the MT1-MMP recombinant forms were overexpressed in cell lysates (Fig. 3*A*), but only the soluble ΔTM was secreted into the conditioned media (Fig. 3*B*). Moreover, only the plasma membrane-anchored Wt and Δ1 recombinant MT1-MMP forms were able to activate proMMP-2 (Fig. 3*B*), confirming that the cytoplasmic domain of MT1-MMP is not required for this function, as previously documented by us (25, 34). Interestingly, whereas CD44 gene expression was not affected by overexpression of any of the recombinant MT1-MMP forms (Fig. 3*C*), only the Wt MT1-MMP recombinant protein was capable of down-regulating both the cell surface binding of HA as well as the CD44 cell surface protein expression. This was assessed by flow cytometry, quantified, and expressed as the ratio of relative geometric mean values from the respective cell conditions (Fig. 3*D*). Although total CD44 protein expression was not assessed in cell lysates, Wt-MT1-MMP expression did trigger, as previously demonstrated (18, 19), CD44 shedding into the conditioned media of transfected U-87 cells (data not shown). The sum of these observations definitively proves that the intracellular portion of MT1-MMP, although not required for proMMP-2 activation, plays a crucial role in mediating the intracellular signaling necessary for CD44 cell surface expression and function.

Cholesterol Depletion Increases the Cell Surface Binding of HA to U-87 Glioma Cells in Synergy with Type I Collagen—The recent finding that MT1-MMP and CD44 were partially localized within cholesterol-enriched plasma membrane domains

(20, 22) prompted us to evaluate the importance of this localization. Using β-cyclodextrin, we first showed that cholesterol depletion from U-87 cell plasma membranes did not modulate cell surface CD44 expression, as assessed by flow cytometry (Fig. 4*A*). Caveolae depletion of the cells did not modulate the expression of total plasma membrane-associated caveolin-1 in either Cos-7 epithelial or U-87 glioma cells, nor did it modulate total membrane-associated MT1-MMP in glioma cells (Fig. 4*B*). Cos-7 cells do not express endogenous MT1-MMP, and CD44 gene expression was not affected by either type I collagen or β-cyclodextrin treatments of U-87 cells (data not shown). A discontinuous sucrose gradient fractionation further confirmed that, although total caveolin-1 or MT1-MMP protein expression was unaffected by a β-cyclodextrin treatment, cholesterol depletion triggered a redistribution of both MT1-MMP and caveolin-1 to the higher density fractions associated with low cholesterol content (Fig. 4*C*). More importantly, subsequent type I collagen treatment of caveolae-depleted glioma cells resulted in increased cell surface HA binding (Fig. 4*D*).

The Type I Collagen-mediated Increase in HA Cell Surface Binding Is Regulated through a Caveolar Cell Surface-associated MMP Activity—We next evaluated whether the rapid, type I collagen-induced HA cell surface binding in glioma cells depleted of caveolae involved any caveolar cell surface-associated MMP activity. Glioma cells were thus co-incubated with either ilomastat or EGCg, a naturally occurring green tea catechin for which we have recently documented several crucial
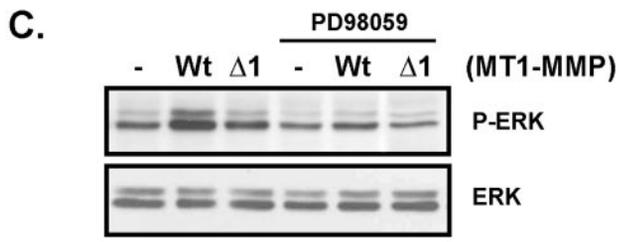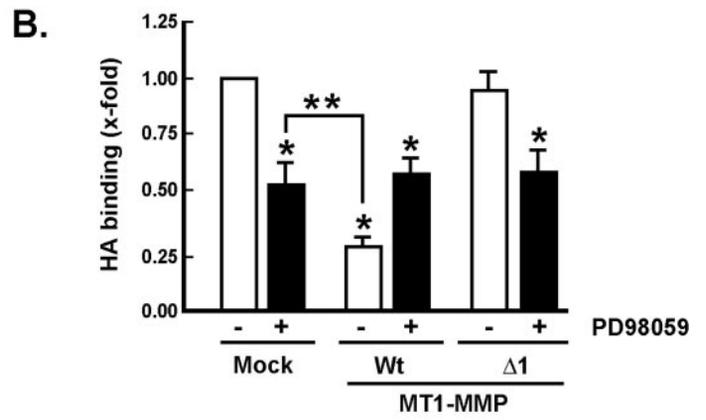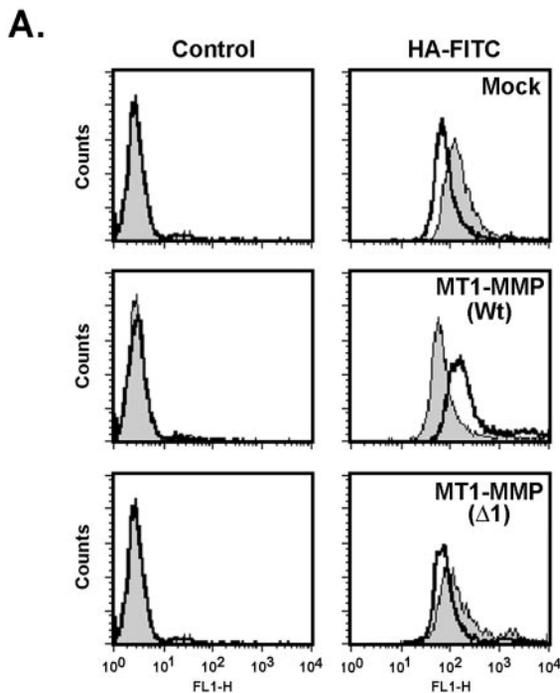
anti-angiogenic and anti-MMP inhibitory activities (35–37). We demonstrated that HA cell surface binding in control glioma cells treated for 1 h with either ilomastat or EGCg had no effect (Fig. 5). However, whereas a 1-h treatment with type I collagen had no effect on MT1-MMP gene expression (data not shown), the type I collagen-induced HA binding was found increased in β-cyclodextrin-treated cells and both type I collagen stimulation and β-cyclodextrin stimulation were inhibited

by ilomastat and EGCg (Fig. 5). These observations confirm that some caveolae-associated protein location potentially down-regulates HA cell surface binding, and that depletion of the cholesterol-enriched plasma membrane domains releases cryptic sites that become accessible to the subsequent rapid action of type I collagen.

The Cytoplasmic Domain of MT1-MMP Regulates HA Binding through the Activation of the Extracellular Signal-regulated Protein Kinase ERK Cascade—We have previously reported that MT1-MMP triggered cellular signal transduction events leading to cell migration, and that these signals originated from its cytoplasmic domain (34). We now investigated whether any signaling from the MAPK pathway would regulate HA cell surface binding. Mock-, Wt-, or Δ1-MT1-MMP-transfected U-87 cells were treated with PD98059, a selective and cell-permeable inhibitor of MAPK kinase (MEK) (Fig. 6*A*, *bold line tracings*). We show that basal HA cell surface binding was diminished by ~50% in PD98059-treated mock-transfected cells (Fig. 6, *A* (*upper right panel*) and *B*), suggesting that the MAPK pathway regulated HA cell surface binding in resting cells. When U-87 cells overexpressed recombinant Wt-MT1-MMP, HA binding accordingly diminished by 74% (Fig. 6*A*, *shaded plot* in *middle right panel*) and *B*) as reported in Fig. 3*D*. That reduced HA binding was, however, partially reversed by the addition of the MEK inhibitor (Fig. 6, *A* (*bold line tracing* in *middle right panel*) and *B*) to levels still lower than untreated mock cells but comparable with that of the PD98059-treated mock cells. The overexpression of the recombinant cytoplasmic truncated domain-(Δ1)-MT1-MMP had, however, no effect on HA cell surface binding, which was accordingly inhibited by PD98059 to the same level as the control (Fig. 6*A*, compare the *shaded plot* of the *lower right panel* with that of the *upper right panel*) suggesting that crucial MT1-MMP-mediated intracellu-

FIG. 5. **MMP-dependent mechanisms regulate the cholesterol depletion-induced up-regulation of HA cell surface binding in U-87 glioma cells.** Serum-deprived U-87 glioma cells were preincubated for 1 h at 37 °C with or without 5 mM β-cyclodextrin to depleted plasma membrane cholesterol. Then 20 μM ilomastat or 10 μM EGCg were added to the cells in the presence or absence of 100 μg/ml type I collagen. Cells were then trypsinized and 10^6 cells incubated for 1 h at 4 °C with HA-FITC. Flow cytometry was used to assess HA cell surface binding. The results are representative of three independent experiments.

FIG. 6. **The cytoplasmic domain of MT1-MMP regulates HA cell surface binding through the MAPK pathway.** *A*, U-87 cells were transfected with cDNA vector alone (*Mock*, *upper panels*), or cDNA encoding Wt-MT1-MMP (*middle panels*), or cytoplasmic domain-truncated (Δ1)-MT1-MMP (*bottom panels*). *Left panels* represent cell autofluorescence, whereas *right panels* reflect the extent of HA-FITC cell surface binding. In addition, the effect of the MEK inhibitor PD98059 (50 μM, 30 min at 30 °C) (*bold line tracings*) was also assessed on the capacity of the different cell lines to bind HA-FITC in comparison to untreated cells (*shaded plots*) as described under "Experimental Procedures," and quantified in *B*. The *double asterisk* (**) identifies a statistical significant difference ($p < 0.05$) between Wt-MT1-MMP and PD98059 treatment of U-87 cells. The respective cell lysates were then further analyzed for the extent of ERK phosphorylation. Twenty μg were loaded on a 9% SDS-PAGE and the activation of ERK visualized using anti-phosphospecific ERK antibodies (*upper panel*), whereas total ERK was detected using a monoclonal ERK antibody (*lower panel*).
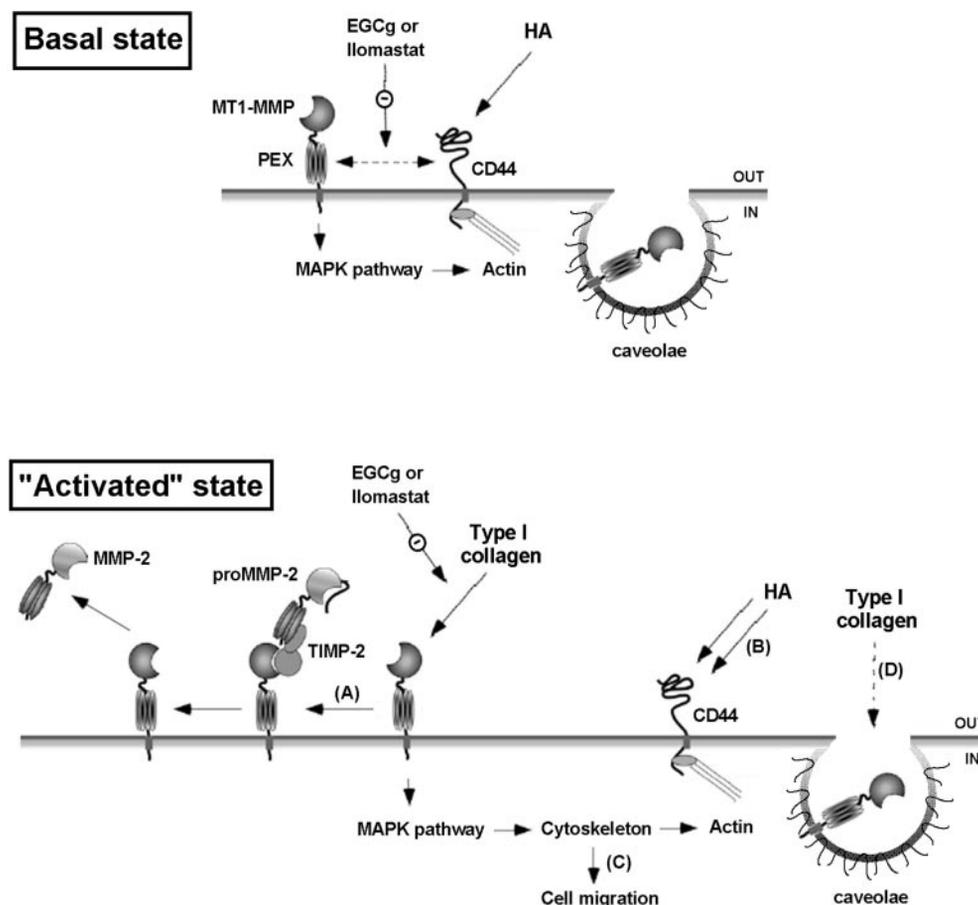
FIG. 7. **MT1-MMP and its caveolar location may regulate the CD44-mediated HA cell surface binding in glioma cells.** MT1-MMP is a multifunctional protein that regulates several pericellular processes at the cell surface of cancer cells. In the basal state, MT1-MMP regulates CD44 cell surface expression through a MAPK-dependent pathway, and this is antagonized by two inhibitors of MT1-MMP functions ilomastat and EGCg, a green tea catechin with anticancer and anti-angiogenic properties (37). In the "activated" state, type I collagen triggers proMMP-2 activation through the formation of a trimolecular complex involving TIMP-2, which subsequently releases an active form of MMP-2 (*A*) (73). The profound cytoskeletal reorganization induced by type I collagen signals an increase in HA cell surface binding to CD44 (*double arrow*, *B*). Although the intracellular domain of MT1-MMP is unnecessary for proMMP-2 processing, it was crucial for MT1-MMP cell migration and activation of the MAPK pathway, as well as CD44 cell surface functional expression (*C*) (74). Down-regulation of HA binding activity by inhibition of the MAPK cascade further suggests that an MT1-MMP/CD44/ERK-dependent regulatory signaling regulates HA cell surface binding in glioma cells. Cholesterol depletion may also trigger a further increase in type I collagen interaction with caveolae-associated proteins such as caveolar MT1-MMP (*D*), but this needs to be confirmed. Overall, these mechanisms provide new means of regulating the metastatic processes involved in the interaction of glioma cells with their brain ECM environment.

lar signaling regulates HA cell surface binding. These effects were all quantified in Fig. 6*B*. Furthermore, we show that the cytoplasmic domain of MT1-MMP clearly transduces HA cell surface binding signaling through the MAPK pathway. Indeed, ERK phosphorylation is increased by the overexpression of the full-length Wt-MT1-MMP protein and is reflected by the decrease in HA cell surface binding. In contrast, the overexpression of the cytoplasmic truncated domain-(Δ1)-MT1-MMP protein did not trigger ERK phosphorylation and did not modulate HA cell surface binding differently than in mock cells. Total ERK expression remained unaffected (Fig. 6c). Finally, the inhibition of the MAPK pathway by PD98059 significantly reduced Wt-MT1-MMP-induced ERK phosphorylation, which subsequent effect was to bring back HA cell surface binding to levels similar to that of PD98059-treated mock cells (Fig. 6, *B* and *C*). Collectively, this MT1-MMP-mediated regulation of the MAPK pathway correlates with the subsequent effects leading to HA cell surface binding.

DISCUSSION

Malignant gliomas are the most common and aggressive primary tumors of the adult central nervous system. The interaction between glioma and brain ECM components is, however, extremely complex and remains poorly understood. The recent finding that the expression of CD44 directly correlates with the highly invasive behavior of gliomas (14) establishes CD44 protein expression and functions as potential central nervous system tumor markers (38, 39). Accordingly, CD44-mediated HA binding at the cell surface of gliomas is thought to involve multivalent binding events affected by the size of the HA ligand, the quantity and density of cell surface CD44, and the activation state of CD44 (40). Moreover, high grade gliomas in the brain become more aggressive when they co-express HA synthase and hyaluronidases, further emphasizing the crucial implication of HA cell surface recognition and subsequent metabolism in the growing tumor (41).

HA, which represents ~35% of the total glycosaminoglycans in the adult mammalian brain (42), was recently found endocytosed in membrane regions termed lipid rafts (43), in which a significant portion of one of its receptor CD44 molecules were identified (22, 44). Because specialized plasma membrane domains such as lamellipodia and caveolae dictate some of the characteristics of glioma invasion (45, 46), the identification and functional characterization of specific molecular players located within these domains should provide further insight into their role in interacting with ECM proteins. For instance, it has been hypothesized that lipids or accessory proteins as-

sociated within such lipidic bilayer may significantly modulate the functional activity of CD44 (47, 48). However, the biological significance of such regulation in Triton X-100-insoluble plasma membrane domains still remains unknown. These observations strongly suggest that caveolae-associated proteins must regulate HA binding to the cell surface, and that one such candidate could be caveolar MT1-MMP. Accordingly, a very recent study provides supportive evidence toward the new functional role that would be attributable to caveolar MT1-MMP (49). That study suggests that caveolin-enriched lipid rafts represent a new modality in regulating the functional activity of MT1-MMP in malignant cells, and that the yet-to-be identified mechanisms involving the cytoplasmic tail peptide sequence are likely to facilitate the enzyme translocation to that distinct membrane compartment.

Our study supports the fact that MT1-MMP-mediated CD44 cell surface cleavage may indeed play a critical role in efficient cell detachment from HA substrate and could consequently promote glioma cell migration. Structure-function studies have demonstrated that the PEX domain of MT1-MMP was responsible for the formation of a complex with CD44 (50), whereas its short 20-amino acid cytoplasmic tail was found to mediate internalization of the enzyme (51, 52). Interestingly, a complete deletion or point mutation within the MT1-MMP cytoplasmic domain retains its proteolytic activity on the cell surface at a level comparable with that of the Wt MT1-MMP (25). Our study provides new evidence that the MT1-MMP cytoplasmic domain further regulates cell surface CD44 function of binding HA through intracellular signaling that involves the MAPK pathway (summarized in Fig. 7). The activation of this pathway has already been shown to regulate MT1-MMP-mediated cell invasion processes (34). Whether that regulatory CD44/MT1-MMP-dependent functions could affect other documented processes involving MT1-MMP, such as its potent fibrinolytic activity or its role in endothelial cell tubulogenesis, is currently under investigation. Interestingly, a very recent study showed for the first time that a new 19-kDa protein, which has been identified as MTCBP-1 and which belongs to the newly proposed Cupin superfamily, associated with the cytoplasmic domain of MT1-MMP and regulated its function in cell invasion and ECM recognition (53). Whether this new intracellular protein regulates other MT1-MMP-mediated signaling and/or functions remains to be investigated. Because for most cancers the precise role of CD44 in the diffusion of tumors is still unclear, CD44 interaction with MT1-MMP, HA, and other ECM molecules thus becomes obviously relevant to various processes in metastasis, a complex multistep process that is known to vary from tumor to tumor and site to site.

CD44 is also known to bind other ECM components. The sole effect of type I collagen that we report on cell surface HA binding, combined with the lack of effect of the other ECM proteins tested in the present study, thus strongly suggests the requirement for new cell surface mechanisms regulating CD44-mediated HA binding function. However, the diversity of responses that has been reported from CD44 ligation indicates that downstream events following ligand binding by CD44 may vary depending on the cell type expressing CD44 and on the environment of that cell (54). Among the intracellular pathways, CD44 mediates phosphorylation of ZAP-70 and activation of phospholipase Cγ, RAS, protein kinase Cζ, and NF-κB binding activity (55). Low molecular mass HA fragments, on the other hand, enhance tumor cell migration (56) and activate the Ras-mitogen-activated protein kinase pathway as well as the phosphoinositide 3-kinase pathway (57). One effect of CD44 "outside-in" signaling is up-regulation and activation of integrins (58) and the expression of MMP (59). It is possible that

CD44 thus works in concert with another cell surface molecule to efficiently bind to type I collagen and to subsequently up-regulate cell signaling pathways. In fact, one candidate that we cannot exclude at this point is β_1 integrin, which has been reported to mediate type I collagen induction of proMMP-2 processing in normal fibroblasts (60), and to affect the expression, processing, and activity of MT1-MMP in ovarian carcinoma cells (61). One common intracellular pathway may also involve Ca^{2+}/calmodulin-dependent signaling, which has been shown to regulate MT1-MMP cell surface processing (25), as well as the phosphorylation of CD44 required for cell migration on HA (62). Finally, we also report that EGCg, an effective inhibitor of MT1-MMP-mediated functions (36, 63), also antagonized MT1-MMP-mediated cell surface HA-binding.

CD44 and several isoforms have been characterized on a variety of tumor cell surfaces and have been suggested to be prognostic indicators of malignant melanoma (64, 65). However, although CD44 binds to types I, IV, VI, and XIV collagen, it is not a primary receptor for cell adhesion to collagen (66–69). Thus, other HA receptors, such as the receptor for HA-mediated motility and brain-enriched HA binding/brevican, could also play a role in glioma cell migration (70–72). These could also be involved in the metastatic processes through interaction with, and movement through, collagen, most often type I and/or basement membrane (type IV) collagen. Our study thus provides a new look at a fundamental mechanism of glioma invasion that depicts a unique interaction between collagen and HA cell surface binding. Manipulation of this interaction could ultimately serve as a target in the development of new anticancer therapeutic strategy.

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