An Alternative Processing of Integrin $\alpha_v$ Subunit in Tumor Cells by Membrane Type-1 Matrix Metalloproteinase*

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Membrane type-1 matrix metalloproteinase (MT1-MMP) and $\alpha_v\beta_3$ integrin are both essential to cell invasion. Maturation of integrin pro-$\alpha_v$ chain (pro-$\alpha_v$) involves its cleavage by proprotein convertases (PC) to form the disulfide-bonded 125-kDa heavy and 25-kDa light $\alpha_v$ chains. Our report presents evidence of an alternative pathway of pro-$\alpha_v$ processing involving MT1-MMP. In breast carcinoma MCF7 cells deficient in MT1-MMP, pro-$\alpha_v$ is processed by a conventional furin-like PC, and the mature $\alpha_v$ integrin subunit is represented by the 125-kDa heavy chain and the 25-kDa light chain commencing from the N-terminal Asp<sup>891</sup>. In contrast, in cells co-expressing $\alpha_v\beta_3$ and MT1-MMP, MT1-MMP functions as an integrin convertase. MT1-MMP specifically cleaves pro-$\alpha_v$, generating a 115-kDa heavy chain with the truncated C terminus and a 25-kDa light chain commencing from the N-terminal Leu<sup>852</sup>. PC-cleavable $\alpha_3$ and $\alpha_5$ but not the PC-resistant $\alpha_2$ integrin subunit are also susceptible to MT1-MMP cleavage. These novel mechanisms involved in the processing of integrin $\alpha$ subunits underscore the significance and complexity of interactions between MT1-MMP and adhesion receptors and suggest that regulation of integrin functionality may be an important role of MT1-MMP in migrating tumor cells.

The integrins are a family of heterodimeric transmembrane receptors that mediate dynamic interactions between the extracellular matrix (ECM) and the cytoskeleton (1–3). By linking the ECM with the cytoskeleton, integrins regulate cell adhesion, motility, contractility, and invasion (4, 5). The integrins are composed of noncovalently associated $\alpha$ and $\beta$ subunits that combine to form about 25 different receptors. Among all known integrins, $\alpha_v\beta_3$ plays a unique functional role in tumor angiogenesis and metastasis (6).

It has been established that certain mature integrin subunits including $\alpha_3$, $\alpha_4$, $\alpha_5$, $\alpha_6$, $\alpha_7$, $\alpha_8$, $\alpha_9$, $\alpha_c$, $\alpha_{10}$, and $\alpha_g$ are generated by post-translational endoproteolytic cleavage of the respective precursors. The cleavage at the highly conserved pairs of basic amino acids is a redundant function of proprotein convertases (PC) from the subtilisin/kexin family. PC cleavage converts single chain $\alpha$ precursors into respective mature subunits consisting of an N-terminal heavy chain and a C-terminal light chain connected by a disulfide bridge (7). Furin, a member of the PC family, has been specifically implicated in the cleavage of pro-$\alpha_{50}$, $\alpha_6$, and $\alpha_c$ chains (7, 8). The role of this cleavage in integrin function is unclear. Arguably, post-translational maturation by proteolytic cleavage of the $\alpha$ chain is not required for ligand binding and cell adhesion but is essential to “outside-in” signal transduction by integrins (9, 10). Thus, inhibition of pro-$\alpha_v$ cleavage by overexpression of $\alpha_1$-antitrypsin Portland, a furin inhibitor, impaired integrin $\alpha_v\beta_3$-mediated signal transduction and spreading in adenocarcinoma HT29-D4 cells (9).

In addition to integrins, tumor cells are believed to exploit matrix metalloproteinases (MMPs) to cross the ECM barriers. MMPs are a family of zinc-dependent endopeptidases shown to degrade the ECM (11). MT1-MMP belongs to a subfamily of MMPs, which are distinguished by a transmembrane domain that anchors the molecule to the plasma membrane. It has been suggested that the principal function of MT1-MMP is to mediate the activation pathway of soluble MMPs including MMP-2 and MMP-13 (12–14). Although MT1-MMP is detectable in normal tissue, elevated functional activity of MT1-MMP is associated with malignant and metastatic tumors (11, 15, 16).

Up-regulation of MMPs in invasive cells has frequently been associated with integrin expression (17–22). There is increasing evidence that integrins interact with MMPs on the surface of normal and tumor cells (23). Thus, $\alpha_v\beta_3$ integrin has been demonstrated to bind and localize activated MMP-2 to discrete regions of invasive melanoma cells (24). In agreement, expression of integrin $\alpha_v\beta_3$ was repeatedly linked to the activation of MMP-2 in multiple tumor cell types (25–27). In human glioma U251 and melanoma BML cells, $\alpha_v\beta_3$ was shown to co-localize and interact with MT1-MMP (16, 27).

Our study demonstrated that in addition to the breakdown of the ECM and activation of soluble MMPs, MT1-MMP functions as an integrin convertase. We show that in breast carcinoma MCF7 cells co-expressing $\alpha_v\beta_3$ integrin and MT1-MMP, the protease is directly involved in the endoproteolytic cleavage of pro-$\alpha_v$. This alternative processing of pro-$\alpha_v$ generates $\alpha_v\beta_3$ integrin that is superior relative to conventional $\alpha_v\beta_3$ integrin.

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† The abbreviations used are: ECM, extracellular matrix; dec-RVKR-cmk, decanoyl-Arg-Val-Lys-Arg-chloromethylketone; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; BSA, bovine serum albumin; DPBS, Dulbecco’s PBS; Tw, Tween 20; HRP, horseradish peroxidase; MMP, matrix metalloproteinase; MT1-MMP, membrane type-1 MMP; MT-E240A, a catalytically inactive MT1-MMP mutant; PC, proprotein convertase; rMT-cat, a recombinant catalytic domain of MT1-MMP; WT, wild type; mAb, monoclonal antibody.
in promoting cell adhesion, migration, and focal adhesion kinase phosphorylation (43). Furthermore, other integrin α chains, which undergo PC-mediated maturation, were also sensitive to cleavage by MT1-MMP. Our results suggest that MT1-MMP-mediated integrin processing may be a general mechanism by which cells selectively regulate the functionality of integrins. This novel regulatory mechanism of integrin function underscores the significance and complexity of interactions between MT1-MMP and cell adhesion receptors in tumor cells.

MATERIALS AND METHODS

**Antibodies and Reagents**—Rabbit antibodies AB815 specific to a hinge region of MT1-MMP and integrin-specific antibodies, including murine mAbs LM609 (α3), VNR139 and AV1 (both α5), and rabbit antibodies AB1936 (α1), AB1992 (α2), AB1949 (α3), AB1932 (β3), and AB1890 (the cytoplasmic tail of the α3 integrin chain) were from Chemicon International (Temecula, CA). mAb L230 against the α3 integrin subunit was purified from the medium conditioned by the hybridoma cells (ATCC). A recombinant catalytic domain of MT1-MMP (rMT-cat) was purified and refolded as reported earlier (28). Fab-9 was purified as described (29). The purified human placental α3β3 integrin was from Chemicon. N-terminal peptides were synthesized and were biotinylated by the manufacturer. mAbs L230 and LM609 were from Becton Dickinson. Anti-integrin and anti-MT1-MMP antibodies in ice-cold Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FCS (DMEM/FCS) with MCF7 cells expressing MT-WT or MT-E240A alone.

To generate double transfectants, the parental MCF7 cells were first transfected with the full-length MMP mutant (MT-E240A) was generated by replacing the Glu240 of the enzyme’s active site with Ala (31). Cell transfections were performed using LipofectAMINE (Invitrogen) according to the manufacturer’s instructions. To generate double transfectants, the parental MCF7 cells were first transfected with the full-length β3 integrin chain cDNA in the pcDNA.3-zeo plasmid carrying MT-WT or mutant MT-E240A. Routinely, doubly transfectants (MCF7/MA1003/MT-WT and MCF7/MA1003/MT-E240A) were used in these studies. Pro-MMP-2 was purified from medium conditioned by p2AHT2A72 cells (a derivative of HT-1080 fibrosarcoma cell line doubly transfected with E1A and pro-MMP-2) (12). Protease inhibitors (phenylmethylsulfonyl fluoride, leupeptin, pepstatin, and aprotinin) sodium fluoride, sodium pyrophosphate, and sodium orthovanadate were from Sigma.

**Cell Culture**—MCF7 breast carcinoma cells (ATCC) were cultured in DMEM supplemented with 10% FCS (DMEM/FCS) and 10 μg/ml penicillin and streptomycin. Where indicated, cells were incubated for 12 h at 14°C. These cells were washed with DPBS and lysed with 50 mM N-ethyl-p-glycyranside (Amresco, Solon, OH) in Tris-buffered saline supplemented with 0.1 mM CaCl2, 1 mM MgCl2, 1 mM EDTA, and protease inhibitors containing 1 mM phenylmethylsulfonyl fluoride and 2 μg/ml each of aprotinin, pepstatin, and leupeptin (OG buffer). The lysates were precleared with Pansorbin (Calbiochem). The samples of cell lysates each containing 1.0 mg of protein were mixed with 2–3 μg of anti-integrin or anti-MT1-MMP antibodies and Protein G agarose beads (Pierce) with DPBS supplemented with 0.05% Tween (200 μl DPBS/Tw) and 0.5 mM NaCl and with DPBS/Tw. Immune complexes were released by boiling the beads for 5 min in 2× SDS sample buffer (20 μl). Where indicated, the samples were washed with 50 mM dithiothreitol. After centrifugation, solubilized proteins were subjected to SDS-PAGE. Separated proteins were transferred to an Immobilon P membrane (Millipore Corp., Bedford, MA). Following blocking with 1% casein in DPBS/Tw, the membrane was probed with Extravidin-HRP (Sigma), and the bound HRP activity was visualized with TMB/M substrate (Chemicon).

To identify the MT1-MMP-processed forms of α3 and α5 integrin subunits, the lysates of cells surface labeled with biotin were adsorbed on avidin–agarose beads and were boiled in SDS-PAGE buffer and separated on an 8% SDS-PAGE gel followed by transfer to a membrane. Integrin chains were visualized by Western blotting.

**Purification and the NH2-terminal Sequencing of α3β3 Integrin**—Integrin α3β3 was purified from β3/zeo, β3/MT-WT and β3/MT-E240A cells on a mAb LM609 column as previously reported (29). Briefly, the OG lysate of 1.6 × 107 cells was passed over the column. After washing the column with the OG buffer, adsorbed α3β3 integrin was eluted with 0.05% trifluoroacetic acid, pH 2.5, in 50 mM N-ethyl-p-glycyranside. The samples were quickly neutralized, dialyzed against 5 mM Tris-HCl, pH 7.5, containing 0.1% SDS, concentrated 20-fold in a Speed Vac concentrator, separated by SDS-PAGE, and transferred to an Immobilon membrane. After staining with Coomassie Blue, the integrin bands were excised and subjected to the NH2-terminal microsequencing.

To analyze whether MT1-MMP co-purifies with α3β3 integrin, the samples of purified α3β3 integrin were separated by SDS-PAGE and transferred to a membrane. Immunoblotting was performed using rabbit antibody AB1932 against β3 integrins and antibody AB815 against MT1-MMP. Bound primary antibodies were visualized with the HRP-conjugated secondary antibody and the TMB/M substrate.

**Digestion of α3β3 by rMT-cat—Placentation α3β3 integrin (2 μg; 8 pmol) was mixed with rMT-cat (0.1 μg; 5 pmol) (28) in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 50 mM ZnCl2, 5 mM CaCl2. After incubation for 24 h at 37 °C, samples were boiled for 5 min in an equal volume of 2× SDS-PAGE sample buffer. The samples were resolved by SDS-PAGE, and the gels were stained with Coomassie Blue R-250. The α3β3 integrin chains, which undergo PC-mediated maturation, were also subjected to the NH2-terminal microsequencing.

**Confocal Microscopy**—Cells were plated at 1–1.5 × 104 cells/well of an eight-well LabTek II glass chamber (Nalge Nunc, Naperville, IL). Following incubation for 48 h, cells were washed with PBS and fixed for 20 min with an ice-cold, 1:1 methanol/aceton mixture. Nonspecific binding was blocked by incubation for 30 min at room temperature in DPBS containing 10% goat serum and 5% BSA. Cells were washed with 10 μg/ml mAb LM609 or mAb L230 and further with 20 μg/ml goat anti-mouse IgG conjugated with Alexa 568 ( Molecular Probes, Inc., Eugene, OR). Next, cells were stained with 10 μg/ml rabbit anti-MT1-MMP antibodies and 20 μg/ml goat anti-rabbit IgG conjugated with Alexa 488 ( Molecular Probes). After washing in DPBS, cells were embedded into Vectashield (Vector, Burlingame, CA) and examined using a scanning confocal microscope (MRC-1024; Bio-Rad). Acquisition and processing of images were performed with the Lasersharp (Bio-Rad) and AdobePhotoshop software.

**Gelatin Zymography**—Cells were plated in DMEM/FCS with or without increasing concentrations of Ilomastat. After incubation for 24 h, cells were washed and further incubated for 24 h with 50 ng/ml MMP-2 in serum-free DMEM supplemented with the same concentration of Ilomastat. Medium was mixed with an equal volume of 2× SDS sample

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buffer. Aliquots (10 μl) were run on gelatin-gels (Novex, Carlsbad, CA). To visualize gelatinolytic bands, the gels were processed as reported (16).

RESULTS

Expression of αβ3 Integrin and MT1-MMP in MCF7 Cells—We specifically selected breast carcinoma MCF7 cells for our studies, since the parental cell line was deficient in MT1-MMP, MMP-2, and the β3 integrin subunit but expressed αv integrins. Previously, we have characterized MCF7 cells stably transfected with αvβ3 integrin subunit (αvβ3 cells) and wild type MT1-MMP (MT-WT cells) (16). To extend our studies, we generated MCF7 cell lines co-expressing αvβ3 integrin with either the wild type or the enzymatically inactive mutant MT1-MMP (MT-E240A), in which the essential Glu240 of the catalytic center was replaced by the Ala residue (MT-E240A cells). To generate αvβ3/zeo control cells, αvβ3 cells were additionally transfected with the original pcDNA-zeo plasmid. The αvβ3/zeo, αvβ3/MT-WT, and αvβ3/MT-E240A cells were characterized by flow cytometry, gelatin zymography, immunoprecipitation, and Western blotting.

Staining with specific antibodies followed by flow cytometry revealed similarly high levels of MT1-MMP in αvβ3/MT-WT and αvβ3/MT-E240A cells. No MT1-MMP was detected in αvβ3/zeo cells (Fig. 1, right). All cell lines, αvβ3/zeo, αvβ3/MT-WT, and αvβ3/MT-E240A, demonstrated high levels of αvβ3 integrin (Fig. 1, left). In agreement with our earlier report (31), MT1-MMP was self-degraded in αvβ3/MT-WT cells and existed as the 42- and 39-kDa autolytic stable forms. Since inactive MT-E240A is incapable of self-proteolysis, the individual 60-kDa full-length MT1-MMP form has been found in αvβ3/MT-E240A cells. Incubation for 48 h with AG3340, a hydroxamate inhibitor of MMPs, completely blocked the proteolysis of MT1-MMP in αvβ3/MT-WT cells. Consequently, the 60-kDa form of MT1-MMP was the major species observed in the cells co-incubated with the inhibitor (Fig. 2A).

To further prove that MT-E240A has no enzymatic activity, we used gelatin zymography to analyze the ability of cells to activate exogenous pro-MMP-2 (Fig. 2B). The αvβ3/zeo and αvβ3/
Precipitation (A and C) and gelatin zymography (B). A, the lysates of surface biotinylated cells were precipitated with rabbit anti-MT1-MMP antibody. Precipitates were analyzed by reducing SDS-PAGE in 10% gels, followed by Western blotting with Extravidin-HRP. B, cells were preincubated for 24 h in serum-free DMEM supplemented with 50 ng/ml pro-MMP-2 in the presence or absence of 10 μM AG3340. Medium was analyzed on gelatin-gels to follow pro-MMP-2 activation and the conversion of the 68-kDa pro-MMP-2 into the 64-kDa intermediate and was analyzed on gelatin-gels to follow pro-MMP-2 activation and the conversion of the 68-kDa pro-MMP-2 into the 64-kDa intermediate and was analyzed on gelatin-gels to follow pro-MMP-2 activation and the conversion of the 68-kDa pro-MMP-2 into the 64-kDa intermediate and was analyzed on gelatin-gels to follow pro-MMP-2 activation and the conversion of the 68-kDa pro-MMP-2 into the 64-kDa intermediate.

To confirm a correlation between the MT1-MMP activity and the presence of the 115-kDa αv form, we inhibited the MMP proteolytic activity by incubating β3/zeo (control) and β3/MT-WT cells for 48 h with increasing concentrations of Ilomastat, a potent hydroxamate inhibitor of MMPs. Following incubation, the cells were surface-labeled with biotin and lysed, and the lysates were immunoprecipitated with anti-αvβ3 mAb LM609 followed by Western blotting. The inhibitor did not affect the composition of integrin αvβ3 in β3/zeo cells (Fig. 3A). On the contrary, Ilomastat at increasing concentrations gradually diminished the amounts of the 115-kDa αv heavy chain and reciprocally increased the levels of the 150-kDa pro-αv in β3/MT-WT cells (Fig. 3B, upper panel). At the 10–50 μM range of Ilomastat concentrations, the status of αvβ3 integrin in β3/MT-WT cells was similar to that observed in β3/zeo and β3/MT-E240A cells. The inhibition of the pro-αv processing by Ilomastat correlated well with the inactivation of the autolytic activity and the ability of MT1-MMP to activate exogenous pro-MMP-2 (Fig. 3B, middle and lower panels, respectively). Inhibitors of serine, cysteine, and aspartic proteases failed to affect either the pro-αv processing or self-degradation of MT1-MMP in β3/MT-WT cells (data not shown), making the involvement of these proteases unlikely.

To further elaborate on these observations, we evaluated the turnover rates of MT1-MMP and αvβ3 integrin. For these purposes, β3/MT-WT cells were incubated for 12–72 h with 10 μM AG3340 followed by biotinylation and immunoprecipitation of αvβ3 integrin and MT1-MMP. In cells incubated with the inhibitor for 12 h, the 60-kDa MT1-MMP form fully replaced the 150-kDa pro-αv form with an unusually low molecular weight (115 kDa), and the 105-kDa β3 chain. No pro-αv was observed in β3/MT-WT cells (Fig. 2C). These data strongly suggested a correlation between the presence of the 115-kDa αv form and MT1-MMP activity in β3/MT-WT cells. In addition, our results indicated that the light αv chain was still disulfide-bonded to the heavy αv chain in β3/MT-WT cells.

MT-E240A cells both failed to activate MMP-2, while β3/MT-WT cells activated the 68-kDa pro-MMP-2 to the 62-kDa mature enzyme via the 64-kDa intermediate. AG3340 completely inhibited the activation of pro-MMP-2 by β3/MT-WT cells (Fig. 2B). These findings confirmed that MT-E240A was catalytically inactive. The MT1-MMP-dependent Processing of αvβ3 Integrin—Co-expression of MT1-MMP and αvβ3 integrin enhanced migration of MCF7 cells on vitronectin, suggesting specific interactions between the protease and the integrin (16). To evaluate this possibility, we immunoprecipitated αvβ3 integrin from cells surface labeled with biotin and analyzed the precipitates by Western blotting. In MT1-MMP-deficient cells (β3/zeo) and in cells expressing the mutant (β3/MT-E240A) or the wild type enzyme inhibited by the hydroxamate inhibitor AG3340 (β3/MT-WT), αvβ3 integrin consisted of the 150-kDa αv and the 90-kDa β3 chains (nonreducing conditions). Under reducing conditions, the 150 kDa pro-αv, the 125-kDa heavy αv chain, and the 105-kDa β3 chain were observed in these cell lines. Nonreduced αvβ3 integrin from β3/MT-WT cells had an additional 140-kDa αv band. Reduced αvβ3 integrin from β3/MT-WT cells consisted of the 125-kDa heavy αv chain, an αv heavy chain with an unusually low molecular weight (115 kDa), and the 105-kDa β3 chain. No pro-αv was observed in β3/MT-WT cells (Fig. 2C). These data strongly suggested a correlation between the presence of the 115-kDa αv form and MT1-MMP activity in β3/MT-WT cells. In addition, our results indicated that the light αv chain was still disulfide-bonded to the heavy αv chain in β3/MT-WT cells.

Further, we used pro-αv integrins purified from furin-deficient colon carcinoma LoVo cells and mature αvβ3 integrin isolated from human placenta to reproduce in vitro the effects...
of MT1-MMP observed in cells. For these purposes, both integrin samples were digested with rMT-cat, the recombinant catalytic domain of MT1-MMP. Although rMT-cat was highly active and readily degraded α3-antitrypsin, a known substrate of MT1-MMP (data not shown), no cleavage of the placental αv heavy chain was observed (Fig. 4A). In turn, rMT-cat digested LoVo pro-αv and generated a 16-kDa C-terminal fragment recognizable on Western blots by antibody AB1930 specific to αv and the 25-kDa light chain that is one residue shorter from its N terminus relative to the PC-processed αv892LAL chain.

MT1-MMP Cleaves α3 and α5 but Not α2—Since MT1-MMP cleavage of pro-α3 occurs within a region of high homology with other α integrin chains, we examined if other α subunits could also be processed by MT1-MMP. For these purposes, cells were surface-labeled with biotin, and biotin-labeled proteins were isolated with avidin-agarose beads and analyzed by Western blotting using mAb LM609 or anti-integrin mAbs (Fig. 4C). Both α3 and α5 integrin chains appeared to be processed by MT1-MMP in β3/MT-WT cells but not in β3/zeo cells. In contrast, the pattern of the α2 integrin subunit, known to be resistant to furin-like convertases, was highly similar in β3/MT-WT and β3/zeo cells. These observations suggest that MT1-MMP is likely to be capable of proteolytic modification of integrin α subunits, which mature by PC cleavage.

Proximity of α3, β3 Integrin and MT1-MMP on the Cell Surface—To confirm that pro-α3 is accessible to MT1-MMP cleavage, we investigated whether the proteinase and the recombinant catalytic domain of MT1-MMP was clearly seen at multiple cell surface sites. Staining with anti-MT1-MMP antibodies and anti-α3 mAb L230. Co-localization of integrins α3 and β3 anti-integrin mAbs (Fig. 4B). Both α3 and α5 integrin chains appeared to be processed by MT1-MMP in β3/MT-WT cells but not in β3/zeo cells. In contrast, the pattern of the α2 integrin subunit, known to be resistant to furin-like convertases, was highly similar in β3/MT-WT and β3/zeo cells. These observations suggest that MT1-MMP is likely to be capable of proteolytic modification of integrin α subunits, which mature by PC cleavage.

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Fig. 4. Processing of α integrins by MT1-MMP. A, placental αβ3 integrin (2 μg) was incubated for 16 h alone or with rMT-cat (0.1 μg). The samples were separated by reducing SDS-PAGE and stained with Coomassie Blue. Positions of molecular weight markers are indicated on the left. B, purified LoVo pro-α (0.2 μg) was incubated alone or with rMT-cat (0.1 μg) and AG3340 (1 μM). The reduced samples were separated by SDS-PAGE in a 4–20% gradient gel followed by Western blotting with antibody AB1930 specific to the cytoplasmic tail of αv integrin and a secondary HRP-conjugated antibody. C, β3/zeo and β3/MT-WT cells were surface labeled with biotin and lysed. The biotinylated proteins were precipitated from the cell lysates with avidin-agarose and separated by SDS-PAGE under nonreducing conditions. Integrin αv, α2, α3, and α5 subunits were identified by Western blotting using mAb AV1, AB1936, AB1920, and AB1949, respectively. D, schematic representation of pro-αv processing by MT1-MMP. The cleavage site is enlarged. The first MT1-MMP cleavage generates the 25-kDa light chain that is one residue shorter from its N terminus relative to the PC-processed α chain. The C-terminal truncation distinguishes the 115-kDa heavy chain of the 140-kDa αv subunit from the conventional 125-kDa heavy αv chain of the 150-kDa αv subunit. The putative second MT1-MMP cleavage site is localized downstream from the Cys892. Glycosylation of the Cys892–Cys904 loop is depicted by an asterisk.
β3/MT-WT and β3/MT-E240A cells. Thus, the samples of affinity-purified αβ3 integrin from β3/zeo, β3/MT-WT, and β3/MT-E240A cells were studied by immunoblotting employing the β3-specific and MT1-MMP-specific antibodies. The use of anti-β3 confirmed the presence of αβ3 integrin in all samples (Fig. 6B, upper panel), whereas anti-MT1-MMP visualized the characteristic 42- and 60-kDa species of the protease in αβ3 integrin from β3/MT-WT and β3/MT-E240A cells, respectively.

Finally, co-cultivation of cells expressing either MT1-MMP or αβ3 integrin failed to promote the cleavage of pro-αv. Thus, the β3/zeo cells were co-cultured for 48 h with the β3-deficient MT-WT or MT-E240A cells. Then αβ3 integrin was precipitated from the cell lysates and evaluated by immunoblotting (Fig. 6C). Evidently, co-cultivation did not affect the status of αβ3 integrin in mixed cultures. It appears that simultaneous expression of both molecules in the same cell is required for the processing of pro-αv by MT1-MMP.

To study the functional implications of our findings, we analyzed whether the RGD ligand binding of αβ3 integrin was affected in cells expressing MT1-MMP. For these purposes, αβ3 integrin expressed by β3/zeo, MT-WT, and MT-E240A cells was immunoprecipitated using a recombinant Fab fragment, Fab-9, designed as an RGD ligand highly specific for β3 integrins (29, 35). As shown in Fig. 7, this chimeric ligand was similarly efficient relative to anti-αβ3 mAb LM609 in precipitating all αβ3 species.

Our further studies demonstrated that the alternative processing of pro-αv by MT1-MMP resulted in αβ3 integrin that was superior relative to conventional αβ3 in promoting cell adhesion, migration, and focal adhesion kinase phosphorylation (43).

**DISCUSSION**

Emerging evidence indicates that membrane-tethered MMPs such as MT1-MMP are directly involved in endoproteolytic modifications of cell surface receptors, including CD44, tissue transglutaminase, and αβ3 integrin (16, 36–38), enabling cells to adjust the receptor profile in the continually changing ECM environment. Our work identifies a novel functional link between MT1-MMP and αβ3 integrin and demonstrates for the first time that, similarly to furin, MT1-MMP is able to specifically cleave pro-αv in tumor cells. This cleavage appears to occur at two distinct sites localized within a loop between the disulfide-bonded Cy852 and Cy904 of pro-αv. Cleavage at these sites was found to generate a 115-kDa heavy chain and a light chain commencing from the N-terminal Leu892. Relative to the PC-processed αv subunit species, the MT1-MMP-processed heavy αv chain is C-terminally truncated, and the light αv chain is one residue shorter from its N terminus. Accordingly, PC processing of pro-αv at Asp891 should prevent MT1-MMP cleavage at Leu892 and vice versa. The loop bordered by Cy852 and Cy904 contains a putative glycosylation site at the Asn674. The loss of the glycosyl component in addition to the deletion of the C-terminal peptide sequence can explain the 10-kDa size difference between the 115-kDa heavy chain processed by MT1-MMP and the 125-kDa PC-processed heavy chain of the αv integrin subunit.

It appears that MT1-MMP is capable of processing other PC-cleavable α integrin subunits such as α3 and α5. The α chains that are resistant to PC cleavage (such as the α6 integrin chain) are also resistant to MT1-MMP (Table I). Relatively broad cleavage specificity of MT1-MMP is largely defined by the presence of a hydrophobic residue at the P1′ position of the scissile bond (39). This may explain the ability of MT1-MMP to hydrolyze those multiple α integrin chains that bear a hydrophobic residue at the P1′ position. Processing of α integrin subunits by MT1-MMP observed in MCF7 cells was confirmed in glioma U251 cells co-expressing αβ3 integrin and MT1-MMP.
The processing of pro-αv by MT1-MMP did not affect the ability of αvβ3 integrin to efficiently bind the RGD ligand. Our findings support the hypothesis that in migrating aggressive tumor cells overexpressing MT1-MMP novel, additional portions of the mature αv integrins generated from pro-αv, via the MT1-MMP pathway will co-localize with this proteinase, apparently, at the invasive cell front and the cell protrusions, the right place for a functionally promoting cell invasiveness.

Adhesive function of integrins has been associated with the transduction of biochemical signals into the interior of the cell (40). Integrin ligation normally induces outside-in signaling and phosphorylation of cytoplasmic tyrosine kinases, including focal adhesion kinase (41) that specifically modulates integrin-mediated cell migration (42). In agreement with these observations, MT1-MMP-mediated processing of pro-αv-stimulated

The putative cleavage site of MT1-MMP in α integrins

| Cleavage site | Integrin |
|---------------|----------|
| KRD | αv |
| RRQ | α3 |
| KRE | αPS |
| KRE | α |
| KRE | αL |
| KRD | αV |
| RKV | αDC |
| KRE | αQV |

outside-in signal transduction through focal adhesion kinase (43). Accordingly, co-expression of MT1-MMP and αvβ3 integrin promoted cell adhesion and migration on vitronectin.

Overall, our findings identified a novel, MT1-MMP-dependent pathway of αvβ3 integrin maturation. This pathway is likely to be functionally important in aggressive tumor cells overexpressing MT1-MMP. The data also suggest that matrix breakdown may not be the primary function of MT1-MMP in tumor cells. In turn, localized proteolytic control of cell receptors is unlikely for soluble MMPs, while the proteolytic regulation of cell receptors including integrins may be an important function of MT1-MMP in migrating cells. This hypothesis may partially explain a unique functional role of MT1-MMP in tumor cell migration and invasion (15). However, since the available cell systems naturally express relatively low levels of MT1-MMP activity, pro-αv, or both, the question as to whether
the cleavage of pro-αv occurs in untransfected cells remains to be answered. To specifically address this question, the experiments employing transfection of cells with αv-antitrypsin
Portland that inhibits processing of precursors mediated by proprotein convertases are currently under way in our laboratory.

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Processing of Integirn αv Subunit by MT1-MMP

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