RGD-dependent Binding of Procathepsin X to Integrin αvβ3 Mediates Cell-adhesive Properties*

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Secreted lysosomal cysteine proteases (cathepsins) are involved in degradation and remodeling of the extracellular matrix, thus contributing to cell adhesion and migration. Among the eleven human lysosomal cysteine proteases, only procathepsin X contains an RGD motif located in a highly exposed region of the propeptide, which may allow binding of procathepsin X to integrin αvβ3. We have tested procathepsin X for cell-adhesive properties and found that it supports integrin αvβ3-dependent attachment and spreading of human umbilical vein endothelial cells. Using site-directed mutants of procathepsin X, we proved that this effect is mediated by the RGD sequence within the proregion of the protease. Endogenous procathepsin X is transported to the plasma membrane, accumulates in vesicles at lamellipodia of the human umbilical vein endothelial cell, and is partly associated with the cell surface, as shown by immunofluorescence. In addition, procathepsin X is partly co-localized with integrin β3, as detected by immunogold electron microscopy. A direct interaction between endogenous procathepsin X and αvβ3 was demonstrated by co-immunoprecipitation. Moreover, surface plasmon resonance analysis revealed significant and RGD-dependent binding of procathepsin X to integrin αvβ3. Our results provide for the first time evidence that the extracellular function of cathepsin X may include binding to integrins thereby modulating the attachment of migrating cells to ECM components.

Cellular migration through extracellular matrix (ECM) requires both cell adhesion to and proteolysis of ECM components. A large number of proteases, including matrix metalloproteases, serine proteases, but also lysosomal cysteine proteases are known to be responsible for ECM breakdown, particularly during tumor invasion (1–3). Cell adhesion to ECM on the other hand is largely mediated by heterodimeric cell surface receptors of the integrin family. Integrins are composed of noncovalently associated α and β chains that form various heterodimers with distinct adhesive specificities (4, 5).

Several proteases have been shown to mediate adhesion and migration of cells through interaction with integrins. Thus, prothrombin, the zymogen of a multifunctional serine protease of the blood coagulation system, interacts directly with integrins via its RGD sequence, a motif involved in integrin binding (6–8). Although the RGD sequence is partly buried in the native conformation (9), prothrombin can bind in an RGD-dependent fashion to integrins αIIbβ3 of platelets and αvβ3 of cultured human endothelial cells (10–12). There is increasing evidence that interactions between prothrombin and β3 integrins play important roles in the regulation of hemostatic and vascular functions. Furthermore, the urokinase-type plasminogen activator/urokinase receptor (uPA/uPAR) system not only plays a key role in fibrinolysis but also regulates cell adhesion, cell migration, and cell proliferation through interaction with integrins (for review, see Refs. 13 and 14). In this case, it is not the protease but its receptor uPAR that binds to β3 integrins and localizes urokinase to sites of cell attachment to ECM.

Besides serine proteases, lysosomal cysteine proteases are also secreted from cells, particularly during tumor invasion and are known to degrade ECM (for review, see Ref. 15). An essential requirement for efficient degradation of ECM is the endopeptolytic activity displayed by most of the lysosomal cathepsins. Cathepsin X, however, a cysteine protease highly expressed in prostate cancer and gastric cancer (intestinal type) is a strict carboxypeptidase and therefore unlikely to participate in ECM degradation to a significant extent (16–18). Indeed, the inhibition of cathepsin X activity with anti-catalytic antibodies did not result in impaired migration of breast cancer cells (19). Yet, as we have recently shown, inhibition of cathepsin X at the mRNA level leads to decreased migration of gastric epithelial cancer cells (18). This apparent contradiction led us to speculate that the enzyme may be involved in mechanisms of adhesion, migration, or invasion, which are not related to proteolysis.

Interestingly, the crystal structure of procathepsin X reveals that the prosegment and its mode of inactivation of the protease are strikingly different when compared with the proforms of other cathepsins (20, 21). The inhibition of the proteolytic
activity of the enzyme by the proregion is achieved through a disulfide bond between the cysteine residue in the proregion and the active site cysteine residue. The RGD sequence in the proregion is unique among lysosomal cysteine proteases and forms a surface bulge with no contacts to the enzyme. Therefore, we hypothesized that this solvent-exposed motif may be important for the interaction of procathepsin X with cell-adhesion integrins. With respect to cathepsin X orthologs, the RGD motif is conserved in murine procathepsin X but not in the enzyme from lower animals such as nematodes.

Because procathepsin X is released in large amounts into the circulation (22), it was tempting to speculate that procathepsin X can serve as an extracellular ligand for integrins. In the present study, we have demonstrated the ability of procathepsin X to interact with integrin \( \alpha_\beta_3 \) through its RGD motif. To examine whether procathepsin X could modulate cell-adhesive properties, attachment assays were performed with human umbilical vein endothelial cells (HUVECs). These cells were chosen because they express a wide range of extracellular matrix receptors including integrin \( \alpha_\beta_3 \). Our data show that specific binding to integrin \( \alpha_\beta_3 \) via the RGD-containing propeptide of cathepsin X can indeed mediate adhesive properties of HUVECs. Moreover, procathepsin X accumulates at lamellipodia of endothelial cells and is partly co-localized with \( \alpha_\beta_3 \). We also demonstrate a direct interaction between procathepsin X and integrin \( \alpha_\beta_3 \) by co-immunoprecipitation of the endogenous proteins from HUVECs and by surface plasmon resonance. Thus, we provide first evidence that the extracellular function of cathepsin X may include modulation of cell attachment to ECM components through binding to integrins.

**EXPERIMENTAL PROCEDURES**

**Materials**—Integrins \( \alpha_\beta_3 \), \( \alpha_\beta_1 \), and monoclonal antibodies anti-\( \alpha_\beta_3 \) (clone LM 609) and anti-\( \alpha_\beta_3 \) (clone JBS 5) were purchased from Chemicon (Temecula, CA). Integrin \( \alpha_\mu_\beta_3 \) was obtained from Haemochrom Diagnostica (Essen, Germany). Human vitronectin was purchased from Promega (Madison, WI) and BSA from Sigma-Aldrich. The antibody against \( \beta_3 \) was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The two cyclic peptides Cyclo(-Arg-Gly-Asp-D-Phe-Val) (RGD peptide) and Cyclo(-Arg-Ala-Asp-D-Phe-Val) (RAD peptide) were from Bachem (Bubendorf, Switzerland). The antibody against procathepsin X was produced as described previously (17). Accutase was obtained from PAA Laboratories (Pasching, Austria). Further materials are mentioned in the following sections.

**Mutagenesis and Expression/Purification**—Human procathepsin X was expressed and purified as described previously (20). Mutagenesis using the QuikChange® site-directed mutagenesis kit (Stratagene, La Jolla, CA) was carried out on the plasmid harboring the procathepsin X sequence. The primers for the RAD mutant and the AAA mutant of procathepsin X were 5'-CTA CCG GCC TCT GCC CGG AGA CGG GCT AGC TCC GCT GG-3' and 5'-CTA CCG GCC TCT GCC TGC AGC CGG GCT AGC TCC GCT GG-3', respectively. Mutations were confirmed by DNA sequencing, and the mutants were expressed and purified as described for the wild type (20).

**Cell Culture**—HUVECs were purchased from Cambrex (East Rutherford, NJ). The cells were cultivated according to the supplier’s recommendations in endothelial growth medium 2 (Cambrex) at 37 °C in a humidified 5% CO\(_2\) incubator. Experiments were performed with cells in passage 3–7. All plastic flasks and well plates were from Nalge Nunc (Rochester, NY). Viable cells (as determined by trypan blue exclusion) were counted in a hemocytometer (Neubauer chamber).

**Attachment Assay**—Attachment of HUVECs was assayed in flat-bottomed 96-well plates (MaxiSorp, Nalge Nunc, Rochester, NY) coated overnight at 4 °C with 50 µl/well of different proteins in PBS. These proteins were vitronectin (1 µg/ml), procathepsin X (wild type, RGD mutant, RAD mutant, 5 µg/ml) or BSA (5 µg/ml) as a control. The wells were blocked with 7.5% BSA for 4 h at 4 °C. Subconfluent HUVECs were detached from the culture flask with accutase, resuspended at 4 x 10\(^5\) cells/ml in DMEM containing 1% BSA and 0.5 mM MnCl\(_2\), and 100 µl of cells were added to each well. Inhibition studies were performed by pre-incubating cells with various concentrations of cyclic RGD and RAD peptides (0.5, 1 and 10 µM) or 5–10 µg/ml of function-blocking antibodies against \( \alpha_\beta_3 \) or \( \alpha_\beta_1 \) for 15 min at 25 °C before addition to the wells. The plates were incubated for 90 min at 37 °C in 5% CO\(_2\). After washing with DMEM containing 0.2 mM MnCl\(_2\) to remove the unattached cells, the adherent cells were fixed with 1% glutaraldehyde for 10 min at room temperature, stained with 0.1% crystal violet for 20 min, and washed with water. The cells were lysed with 0.5% Triton X-100 and the absorbance (595 nm) was measured in a multiwell plate reader (Tecan Systems, Inc., San Jose, CA). Furthermore, adherent cells were routinely examined microscopically to assess the extent of cell attachment and spreading. Mean and standard deviations of all cell-based experiments were determined. Each point represents the average of triplicate samples, and each experiment was performed at least three times yielding comparable results.

**Binding of Procathepsin X to the Surface of Endothelial Cells and Detection of Remaining Fraction by Enzyme-linked Immunosorbent Assay**—Subconfluent HUVECs were starved in serum-free medium (DMEM) for 3 h at 37 °C. After detachment with accutase, 1.5 x 10\(^5\) cells in suspension were incubated in 125 µl of DMEM containing 10% fetal calf serum and procathepsin X (450 ng/ml) at 25 °C for 30 min. After centrifugation at 800 x g for 10 min, the supernatant was removed and the concentration of unbound procathepsin X was determined by enzyme-linked immunosorbent assay as described previously (22). The incubation of the procathepsin X-containing medium was performed in the presence or absence of cells as well as in the presence or absence of the function-blocking antibody against integrin \( \alpha_\beta_3 \) (LM 609, 10 µg/ml).

**Immunofluorescence**—HUVECs were isolated and cultivated as described previously (23). Briefly, cells were cultured on 8-chamber µ-slides (Ibidi, Munich, Germany). The freshly adherent cells were incubated for 1 h at 37 °C (5% CO\(_2\)) with primary antibodies against human procathepsin X (1:100, (17)) and/or against integrin \( \alpha_\beta_3 \) (1:100, monoclonal, Chemicon, clone LM 609). After washing with PBS (three times), cells were fixed with paraformaldehyde (3.7%), washed again with PBS, and incubated with secondary antibodies (goat anti-rabbit...
labeled with Alexa 488, or goat anti-mouse with Alexa 633, respectively, both from Invitrogen. This procedure was used to detect exclusively extracellular proteins. Alternatively, cells were fixed for 15 min with 3.7% paraformaldehyde and permeabilized for 2 min with 0.2% Triton X-100 followed by washing with PBS, incubation with primary antibodies, and subsequently with secondary antibodies. After the final washing, cells were covered with PermaFluor mounting medium (Thermo Shandon, Pittsburgh, PA). Immunostained cells were viewed in a confocal microscope (LSM 510 invert, Zeiss, Jena, Germany).

**Immunoelectron Microscopy**—Subconfluent HUVECs were detached from the culture flask with accutase (PAA Laboratoires, re-suspended at 4 × 10⁵ cells/ml in endothelial growth medium 2 containing 0.5 mM MnCl₂. The cell suspension was applied to polyvinyl formal carbon-coated nickel grids and allowed to adhere for 2 h. Immunogold labeling was performed essentially as described previously (24). Briefly, the grids were passed over successive drops of the following solutions (50 μl for every incubation step). After washing with PBS (containing 0.5 mM MnCl₂), the attached cells were fixed with 4% paraformaldehyde and 0.5% glutaraldehyde in Soerensen buffer (0.1 M phosphate, pH 7.4) for 20 min and then washed again with PBS. After treatment with 50 mM glycine for 15 min and washing with PBS, the cells were blocked with PBS containing 2.5% BSA and 2.5% goat serum for 30 min. Double immunolabeling was carried out by incubating the cells simultaneously with antibodies against procathepsin X (1:10) and integrin β₃ (1:5) for 1 h at room temperature, overnight at 4 °C, and again for 1 h at room temperature. It should be noted that the monoclonal antibody against αvβ₃ (clone LM 609) was less suitable for electron microscopy and was therefore replaced by an antibody directed against the integrin β₃ subunit. Grids were washed with PBS and incubated with the secondary gold-conjugated antibody against the integrin heterodimer and/or its complex with procathepsin X during the purification procedure.

**Surface Plasmon Resonance**—A BIACORE 2000 surface plasmon resonance-based biosensor (Biacore AB, Uppsala, Sweden) was used to measure binding and kinetic parameters for the interaction between different integrins (analytes) and immobilized vitronectin, procathepsin X, or a mutant of procathepsin X with its RGD sequence exchanged to AAA (ligands). Each ligand was immobilized to the sensor chip (CM5) surface by the amine coupling method according to the manufacturer’s suggestion. It should be noted that initial experiments with immobilized integrins coupled to the CM5 chip failed, most likely due to a strained (inactive) conformation of the integrin heterodimers on the chip surface. Integrin solutions were injected into the flow cells using the KINJECT command specifying a 40-μl analyte volume and a 600-s dissociation time. Each assay cycle was performed with a constant flow of 8 μl/min. All experiments were carried out in 25 mM Tris buffer, pH 7.4, containing 150 mM NaCl, 10 mM octyl-β-D-glucopyranoside (Sigma-Aldrich), 1 mM MgCl₂, 0.1 mM CaCl₂, and 0.5 mM MnCl₂ at 25 °C. Analyte solutions were dialyzed against running buffer prior to injection. Between cycles, the immobilized ligands were regenerated by injecting 5 μl of 4 mM guanidine hydrochloride. For each immobilized protein, a complete set of sensorgrams was recorded at five different analyte concentrations in the range between 50 and 500 nM. This set of sensorgrams was recorded at five different analyte concentrations. For data analysis, a control sensorgram with BSA was subtracted from the experimental sensorgrams to eliminate nonspecific binding and refractive index changes due to buffers. Both an association rate constant k₁ (M⁻¹ s⁻¹) and a dissociation rate constant k₂ (s⁻¹) were obtained from the entire data set (global fit). The overall affinity constant K_d was derived from k_d/k_a. All kinetic data were fit most adequately by assuming a bimolecular model for interaction between soluble analyte and
immobilized ligand, equivalent to the Langmuir isotherm for adsorption to a surface.

**Structural Models**—Coordinates obtained from the Protein Data Bank (1DEU and 1L5G) were overlaid and visualized using the free programs Deep View (25) and ViewerLite (Accelrys).

**Statistical Analysis**—Statistical significance was assessed by comparing the mean values (±S.D.) using Student’s *t* test for independent groups. Differences were scored as statistically significant for *p* < 0.05. All statistical analyses were carried out using the GraphPad Prism software.

**RESULTS**

**Adhesive Properties of Endothelial Cells Mediated by Integrin α̂β₂β₃ Interaction with Procathepsin X**—To support our assumption that procathepsin X released into the circulation may serve as an extracellular ligand for integrins and to examine its adhesive properties, we performed attachment assays with HUVECs. Plates were coated overnight with procathepsin X, and cells were added to each well for 90 min. As shown in Fig. 1A, the cells readily attached to procathepsin X (control). This attachment could be blocked with a cyclic RGD peptide (commonly used as an antagonist), whereas the control cyclic RAD peptide had no effect at the same concentration (0.5 μM). Similar results were obtained with a monoclonal antibody against α̂β₂ (clone LM 609), which selectively blocked binding to procathepsin X. Moreover, a function-blocking antibody against integrin α̂β₁ (clone JBS 5) did not inhibit the adhesive interaction, indicating that procathepsin X does not bind to integrin α̂β₁. This antibody, however, was able to prevent binding of HUVECs to fibronectin, a matrix protein commonly recognized by α̂β₁ (data not shown). To further exclude unspecific binding, plates were coated with procathepsin X mutants with the RGD motif mutated to RAD or AAA, processed cathepsin X (mature enzyme lacking the RGD containing propeptide), or BSA. None of these proteins mediated attachment of HUVECs (Fig. 1B). After removal of nonadherent cells, all plates were routinely observed microscopically to assess the extent of cell attachment and spreading. Following a 90-min incubation of HUVECs in plates coated with procathepsin X, most cells attached and started to spread (Fig. 1C), whereas only a few round cells were occasionally seen on plates coated with mutant procathepsin X lacking the RGD sequence (Fig. 1D).

**α̂β₂β₃-Dependent Binding of Procathepsin X to the Surface of Endothelial Cells**—α̂β₂β₃-Dependent binding of procathepsin X to the surface of HUVECs was also shown with cells in suspension. Cells were incubated for 30 min in medium containing procathepsin X (450 ng/ml). The concentration of added procathepsin X was chosen to reflect pathophysiological concentrations. We have previously determined procathepsin X concentrations in plasma of healthy volunteers to be in the range of 31–165 ng/ml. During severe inflammation, however, the procathepsin X levels are significantly higher (increased up to 8-fold of normal, (22)). After removal of cells by centrifugation, the unbound fraction of procathepsin X was assayed in the medium by enzyme-linked immunosorbent assay (Fig. 2). In the presence of the function-blocking antibody against integrin α̂β₃ (LM 609), the concentration of procathepsin X in the medium remained unchanged, whereas in the absence of the antibody, the concentration of procathepsin X was significantly reduced (*p* < 0.05).

**Subcellular Localization of procathepsin X and Integrin α̂β₃**—We also examined whether HUVECs themselves could be a source of procathepsin X, as we have previously detected the
enzyme in adult vascular endothelial cells of different tissue samples. To determine the subcellular distribution of procathepsin X and a possible co-localization with integrin αvβ3, subconfluent HUVECs were stained for both procathepsin X and αvβ3 and examined by immunofluorescence. Membrane association of procathepsin X was clearly observed regardless of the labeling procedure. To detect exclusively extracellular proteins, cells were incubated with primary antibodies prior to fixation (Fig. 3A). Immunofluorescence was not detected on the abluminal side of nonpermeabilized cells, most likely because of accessibility for antibodies. Permeabilization of the cells with Triton X-100 again revealed membrane association of procathepsin X and partial co-localization with αvβ3, particularly at lamellipodia (Fig. 3, B and C). It should be noted that co-localization of procathepsin X and αvβ3 is restricted to vesicles associated with the plasma membrane, indicating that the intracellular transport does not occur within the same vesicles (Fig. 3C) (vesicles stained green do not co-localize with vesicles stained red, except for those associated with the plasma membrane). The nuclear staining observed with permeabilized cells appears to be due to an unspecific reaction observed quite often with secondary antibodies (data not shown). Cells stained with only one of the antibodies against procathepsin X or αvβ3 did not reveal any spillover of fluorescence into the channel used for detection of the other antigen (data not shown).

To analyze in more detail the localization of procathepsin X and integrin αvβ3 on the surface of endothelial cells, double immunogold labeling studies were performed (Fig. 4). To detect exclusively extracellular proteins, HUVEC cells were not permeabilized before incubation with antibodies. Whole mounts of HUVECs were contrasted with uranyl acetate and studied using transmission electron microscopy. Double immunogold labeling revealed a partial co-localization of procathepsin X and β3 particularly at lamellipodia of HUVECs.

Clusters of 10-nm gold particles corresponding to anti-procathepsin X co-existed on the cell surface with the smaller size 6-nm gold particles representing immunoreactive integrin subunit β3 (Fig. 4, B and C). Unspecific staining was excluded in control experiments without primary antibodies (data not shown).
Co-Immunoprecipitation of Procathepsin X and Integrin αβ₃—To address whether endothelial procathepsin X is physically associated with endogenous integrin αβ₃, we immunoprecipitated endogenous αβ₃ from HUVECs and probed the precipitate by Western blotting for procathepsin X. Indeed, endogenous procathepsin X co-immunoprecipitated from extracts of HUVECs (Fig. 5A). The cell lysate contained several molecular forms that showed immunoreactivity with anti-procathepsin X antibodies with the main form being the processed enzyme corresponding to a molecular mass of 37 kDa (Fig. 5A, lane 1). In the immunoprecipitate (lane 5), however, the higher molecular mass form corresponding to the proenzyme (39 kDa) was detected. This indicates the specificity of the precipitation; because the RGD sequence is located within the proenzyme (39 kDa) was detected. This indicates the specificity of the precipitation.

Co-immunoprecipitation of procathepsin X from HUVECs. A, detection of procathepsin X and integrin β₃ with specific antibodies (Western blotting) before and after immunoprecipitation of cell extracts with anti-αβ₃ (LM 609). Lane 1, cell lysate before immunoprecipitation; lanes 2–4, consecutive wash fractions; lane 5, eluate (IP). In the eluate containing the immunoprecipitated proteins, the 39-kDa band corresponding to procathepsin X is detected. The lower band at 37 kDa corresponds to mature cathepsin X (lacking the RGD sequence). Arrows indicate procathepsin X (upper panel) and β₃ (lower panel). B, detection of recombinant procathepsin X and mutants lacking the RGD motif by Western blotting before and after co-immunoprecipitation with anti-αβ₃. Lane 1, before immunoprecipitation; lanes 2–6, consecutive washes; lane 7, eluate. Only wild-type procathepsin X co-precipitated with αβ₃ and is detected in the eluate.

Binding of Procathepsin X to Purified Integrin αβ₃—To further characterize the procathepsin X-integrin interaction, the binding of procathepsin X to integrins in comparison to other ligands was examined by surface plasmon resonance. The four ligands, procathepsin X, a mutant of procathepsin X with its RGD sequence exchanged to AAA, vitronectin, and BSA were immobilized on a CM5 chip. Subsequently, the chip was used to measure binding and kinetic parameters for the interaction with different integrins (analytes). Both procathepsin X and vitronectin bound to αβ₃ in a dose-dependent manner with dissociation constants in the order of 100 nM (Fig. 6, A and C). The obtained data were fitted to a simple 1:1 binding model (Langmuir) as described under “Experimental Procedures.” In contrast to the wild type, different concentrations of the procathepsin X mutant lacking the RGD sequence exhibited significantly lower binding (Fig. 6B). The interaction of procathepsin X or vitronectin with αβ₃ could be blocked with the cyclic RGD peptide, whereas no influence on the low interaction between the procathepsin X mutant and αβ₃ was observed (Fig. 7).

Remarkably, the association of αβ₃ to procathepsin X or vitronectin as well as the dissociation were highly dependent on the concentration of divalent cations, particularly Mn²⁺ (data not shown). Optimal sensograms were obtained at 0.5 mM Mn²⁺.

The same chip was also used to compare the binding of the different ligands to αβ₃ with that to two other integrins, α₁β₃ (a vitronectin-recognizing integrin) and α₃β₁ (a receptor for fibronectin but not for vitronectin) (Fig. 8). With regard to α₁β₃, we detected binding of both procathepsin X and vitronectin, whereas virtually no binding was seen with the mutant lacking the RGD sequence (Fig. 8B). Only “background” binding of vitronectin and procathepsin X to integrin α₁β₁ was observed (Fig. 8C).

DISCUSSION

Cell-adhesive processes and proteolytic mechanisms function in a coordinated manner to provide directed cell migration and are critical during normal and pathological processes such as development, wound repair, inflammation, and tumor cell invasion (26–29). At the molecular level, adhesion receptors (integrins) are displayed on the cell surface and bind to ECM proteins, whereas proteases (e.g. matrix metalloproteases, cysteine proteases) modify or degrade components of the ECM. However, some proteases have also been proposed to interfere with cell adhesion and/or migration in a nonproteolytic fashion (8, 13, 30).

In this study, we have presented several lines of evidence that the cysteine protease precursor procathepsin X is capable of interacting with integrin αβ₃ through an RGD-dependent mechanism. First, we have shown that purified procathepsin X is able to support the cell-adhesive properties of HUVECs, which could be blocked specifically with antibodies to αβ₃ but not with antibodies to α₁β₃. Moreover, procathepsin X at pathophysiological concentrations is also able to bind to endothelial cells in suspension in an integrin αβ₃-dependent manner. Second, using immunofluorescence and immunogold electron microscopy, we have demonstrated membrane-associated
Localization of procathepsin X and partial co-localization with αvβ3 specifically at the lamellipodia of HUVECs. Third, co-immunoprecipitation of procathepsin X and αvβ3 from HUVECs indicates a physical association of the two molecules in a cellular environment. Finally, using surface plasmon resonance, we have shown that purified procathepsin X is able to bind directly to integrin αvβ3. Taken together, our in vitro results demonstrate that procathepsin X is able to form a complex with integrin αvβ3, and we hypothesize that this interaction may take place in vivo as well, thereby mediating cell adhesion and presumably also migration processes.

In this context, using specific silencing of gene expression, we have shown recently that transmigration of human gastric epithelial tumor cells (AGS) through ECM can indeed be reduced if the cells are transfected with cathepsin X antisense oligonucleotides (18). Similarly, transmigration of HUVECs through human ECM is also reduced after transfection with small interfering RNAs targeted against cathepsin X (data not shown). On the other hand, a report of Kos et al. (19) showed that inhibition of the proteolytic activity of cathepsin X by neutralizing antibodies does not affect transmigration. This apparent discrepancy may be clarified by our newest results. Taking into account that cathepsin X is a carboxypeptidase, the enzyme cannot be expected to participate in migration processes through ECM degradation to a significant extent. The reduced transmigration after knockdown of cathepsin X at the mRNA level may, however, be explained by nonproteolytic functions of procathepsin X related to integrin-mediated adhesion as a prerequisite of the transmigration process.

HUVECs turned out to be a reliable model for proving our hypothesis, particularly because they express a large number of integrins including αvβ3. The binding of ligands to the extracellular part of integrins leads to conformational changes and to integrin clustering. Such “activated” integrins preferentially localize to protrusions (lamellipodia), where new adhesions are formed (31).

Interestingly, as demonstrated here, procathepsin X accumulates in vesicles near the membrane at such protrusions. We speculate that procathepsin X is transported through the constitutive and/or regulated secretory pathway toward the plasma membrane of endothelial cells and possibly also other cells, such as monocytes or tumor cells, and is secreted to some extent. In cell culture, procathepsin X is secreted into the...
medium, and an equilibrium seems to exist between free (soluble) and membrane-bound enzymes. Before secretion and/or transport to lysosomes, however, procathepsin X is retarded within the endoplasmic reticulum/Golgi compartment. This finding is in good agreement with a report that identified the lectin ERGIC-53 as a cargo transport receptor for procathepsin X (32). It should be noted that other lysosomal cysteine proteases, e.g. cathepsins B and L, can also be routed to secretory vesicles (33, 34). Procathepsin X is released into the circulation by endothelial cells as well as by monocytes and macrophages (22). As we have shown by immunogold electron microscopy, clusters of immunoreactive procathepsin X and integrin β3 are indeed associated with the cell surface. However, both immunofluorescence as well as immunogold electron microscopy suggest that a second mode of procathepsin X association with the plasma membrane may exist.

Such an alternative way, however, not evaluated by us may be the binding of procathepsin X to cell surface heparan sulfate proteoglycans as suggested by a recent paper (35). Because integrin activation depends on chemokine action, which is modulated by heparan sulfate (36), procathepsin X interaction with integrins may be either direct binding through its RGD sequence or indirect action through its binding to heparan sulfate proteoglycans. This alternative binding mode may account for the free (unbound) procathepsin X in the first wash fraction (Fig. 5A) as well as for the portion of surface-associated procathepsin X that is not co-localized with the integrin as shown by immunofluorescence (Fig. 3A) or immunogold electron microscopy (Fig. 4). Because these mechanisms do not involve covalent binding, cell treatment (in particular detachment) may also remove procathepsin X from the cell surface to some extent. However, further studies will have to clarify the mechanisms involved and the signals and signaling pathways required for trafficking and secretion of procathepsin X.

Using co-immunoprecipitation and real-time surface plasmon resonance, we were able to show direct binding of procathepsin X to integrin αvβ3 in an RGD-dependent fashion. The RGD motif of procathepsin X is highly exposed within a surface bulge of the proregion (20, 21). Its backbone conformation is strikingly similar to that of the cyclic RGD peptide complexed with the extracellular segment of integrin αvβ3 (37) and would allow a comparable binding mode (Fig. 9). Remarkably, the same cyclic peptide was able to compete with procathepsin X in our surface plasmon resonance experiments (see Fig. 7). This is additional evidence for the mechanism of the interaction between procathepsin X and integrins. Binding clearly occurs through the RGD sequence present in the proregion. It seems conceivable, however, that binding of the RGD motif to the integrin could affect the stability of the Cys-10P–Cys-31 disulfide bond (see Fig. 9).

Remarkably, the interaction between procathepsin X and integrin αvβ3 is highly dependent on the concentration of divalent cations, in particular, of Mn²⁺. It is a well known fact that integrins are regulated by divalent cations to adopt an active conformation that enables their binding to adhesive proteins (38). Interestingly, we could demonstrate that both vitronectin and procathepsin X require similar concentrations of Mn²⁺ ions for their RGD-dependent interaction with integrins. At concentrations exceeding 0.5 mM Mn²⁺, the complex formation was fast, but dissociation was extremely slow (data not shown). It should also be noted that the selectivity of vitronectin for certain integrins observed by surface plasmon resonance
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FIGURE 9. Overlay of the RGD loop of procathepsin X and a cyclic RGD peptide complexed with the extracellular segment of α₃β₃. The corresponding α carbons of the RGD motifs in procathepsin X (Protein Data Bank (PDB) code 1DEU; gray) and the peptide-integrin complex (PDB code 1L5G; green) were fitted with Deep View, and a section of the resulting structures was visualized with ViewerLite. The disulfide bond linking the proregion with the active site Cys-31 of cathepsin X is highlighted.

is consistent with previous reports indicating a higher preference for α₃β₃ and α₃β₃ (39).

With respect to the physiological significance of our in vitro findings, one should keep in mind that pathophysiological concentrations of procathepsin X in human plasma range between 1 and 10 nM (22). Therefore, the dissociation constant calculated for the interaction of procathepsin X with integrin α₃β₃ in the order of 100 nM would account for a fraction of 1–10% proenzyme bound to α₃β₃. This interaction may well be of pathophysiological importance, particularly at sites of inflammation or at the invasive edge of a tumor, where local concentrations of procathepsin X may be considerably increased. In such an environment, procathepsin X may be able to compete with vitronectin for the binding to integrin α₃β₃.

As we have previously shown, procathepsin X is highly overexpressed in both early and late stages of prostate cancer (17). On the other hand, integrin α₃β₃ has been implicated in tumor-induced angiogenesis and is also expressed in several primary and metastatic tumors, including prostate cancer, where it plays a critical role in tumor cell growth and metastasis (40–42). Antagonists to α₃β₃ induce apoptosis specifically in angiogenic endothelial cells, thereby facilitating regression in several tumors (for review, see Refs. 43 and 44). Therefore, additional studies are required to further define the significance of procathepsin X and its interaction with integrins during tumor invasion and metastasis and/or tumor-induced angiogenesis.

In conclusion, our results provide first evidence that the RGD motif in the proregion of the lysosomal cysteine protease cathepsin X can mediate adhesion and possibly also migration processes via binding to integrins. However, this property still remains to be fully elucidated. In particular, further investigations of the downstream signal transduction pathways may help to explain the precise role of procathepsin X at the molecular level.

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