Regulation of Fibroblast Migration on Collagenous Matrix by a Cell Surface Peptidase Complex*

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The invasion of migratory cells through connective tissues involves metallo- and serine types of cell surface proteases. We show that formation of a novel protease complex, consisting of the membrane-bound prolyl peptidases seprase and dipeptidyl peptidase IV (DPPIV), at invadopodia of migratory fibroblasts is a prerequisite for cell invasion and migration on a collagenous matrix. Seprase and DPPIV form a complex on the cell surface that elicits both gelatin binding and gelatinase activities located at invadopodia of cells migrating on collagenous fibers. The protease complex participates in the binding to gelatin and localized gelatin degradation, cellular migration, and monolayer wound closure. Serine protease inhibitors can block the gelatinase activity and the localized gelatin degradation by cells. Antibodies to the gelatin-binding domain of DPPIV reduce the cellular abilities of the proteases to degrade gelatin but do not affect cellular adhesion or spreading on type I collagen. Furthermore, expression of the seprase-DPPIV complex is restricted to migratory cells involved in wound closure in vitro and in connective tissue cells during closure of gingival wounds but not in differentiated tissue cells. Thus, we have identified cell surface proteolytic activities, which are non-metalloproteases, seprase and DPPIV, that are responsible for the tissue-invasive phenotype.

Tissue repair requires remodeling of the extracellular matrix (ECM) by migratory cells (1). In cancer invasion such cellular activities occur on membrane protrusions, invadopodia (2), which exhibit dynamic membrane mobility, ECM adhesion, and degradation. Whether the invasive phenotype arises physiologically during tissue repair is not known. However, metallo-collagenolytic activities were found to be involved in these processes (3). Among major metalloproteases, type I collagenase activity is rapidly induced in human skin at the wound edge after acute injury, and its activity persists during healing and stops at wound closure (4, 5). Other proteases, including matrix metalloproteases (6), serine proteases, and neutrophil elastase (7), may also participate in the cell surface proteolysis.

Among the membrane proteases that may coordinate with interstitial collagenase in tissue remodeling are a group of serine prolyl peptidases, including dipeptidyl peptide IV (DPPIV)/CD26 that is known to process peptide hormones and chemokines (8) and seprase/FAP to degrade large molecules such as denatured collagens (9–12). DPPIV and seprase are type II transmembrane proteins, with cytoplasmic tails that contain 6 amino acids (aa) followed by a 20- (seprase) or 22-aa (DPPIV) transmembrane domain at the N terminus and a stretch of 200 aa at the C terminus that constitutes a catalytic region with the catalytic serine in a non-classical orientation as compared with serine proteinases such as trypsin and chymotrypsin (13, 14). DPPIV has binding capability for collagen (15–17) and fibronectin (18–20). In addition, a recent report (21) showed that DPPIV also possesses a seprase-like gelatinase activity and therefore endopeptidase activity, suggesting its involvement in gelatin degradation. Seprase, originally identified as a 170-kDa membrane gelatinase, is expressed on invadopodia of highly aggressive melanoma LOX cells (22–24). The active enzyme is a homodimer of 97-kDa subunits (14). Analysis of the deduced amino acid sequence from a cDNA that encodes the 97-kDa subunits (14) revealed that it is homologous to DPPIV and is essentially identical to FAP (25), which is expressed on reactive stromal fibroblasts of epithelial cancers and healing wounds but not in epithelial or endothelial cells (26). Recently, the gelatinase and prolyl peptidase activities of FAPo have been demonstrated (11, 12), further confirming that seprase and FAPo are identical proteins.

Here, we report the coordinated involvement of two prolyl peptidases, DPPIV and seprase, in the degradation of denatured collagens by migratory cells. We have investigated the functional expression of seprase and DPPIV on surfaces of migratory connective tissue cells. We have also identified an invadopodia-specific protease complex consisting of seprase and DPPIV, which elicits both gelatinase and prolyl peptidase activities and is activated on human connective tissue cells in response to wounding. The protease complex contributes to cell migration on gelatinous matrix that is necessary for the formation of repair tissue.

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EXPERIMENTAL PROCEDURES

Materials—The human amelanotic melanoma cell line LOX was obtained from Professor Oystein Fodstad, Institute for Cancer Research, The Norwegian Radium Hospital, Oslo, Norway. The human embryonic lung fibroblast line WI-38, MDA-MB-436 human breast carcinoma cells, and COS-1 cell line ATCC CRL-1650 (kidney, SV40-transformed, African green monkey) were obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in a 1:3 mixture of Dulbecco’s modified Eagle’s medium (DMEM) and RPMI1640 supplemented with 10% calf serum, 5% Nu-serum (Collaborative Research, Inc., Bedford, MA), 2 mM L-glutamine, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 1 unit/mL penicillin, and 10 μg/mL streptomycin.

Supernatant was used for immunoprecipitation reactions.°

addition of 2M Trizma (Tris base). To determine the subunit composition of isolated protein complexes, immunoprecipitates of the surface-associated DPPIV, rat mAbs D8 and D28 against human placental seprase (13, 14), rat mAb C37 against cell surface glycoprotein (gp)-90 (23). The seprase-DPPIV complex was isolated from human supernatant, and antibodies were produced as described (14). Monoclonal antibodies E26, E19, E3, and F4 belong to IgG2a subclass, and they react with DPPIV but not seprase of the seprase-DPPIV complex. Mouse anti-αv, and anti-β3 mAbs were from American Type Culture Collection (clone 12G0, catalog number H8448 and clone AP-3, catalog number HB242, respectively).

The prolyl dipeptidase substrate, Gly-Pro-p-nitroanilide p-toluene-sulfonate salt (Gly-Pro-pNA) was purchased from Sigma. Rabbit anti-rat mAbs were purchased from Rockland and streptavidin-HRP conjugates from Dako, and HRP substrate 2,2′-azino-bis(3-ethylenbenzthiazoline-6-sulfonic acid) diammonium salt was from Amersham Biosciences. Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS in 50 mM Tris acidified water (pH 2.0), but could be readily polymerized back to collagen fibers as a protease substrate, 100 ng of seprase-DPPIV complex isolated from WI38 RIPA solution (1 mg/ml) was mixed with 100 μl of Gly-Pro-pNA (2 μg/ml), or control antibody in PBS and incubated at 37 °C for 2 h. Membrane extracts or WGA-enriched protease supernatants were added and incubated for 1 h at 4 °C. The unbound material was removed; wells were washed three times with wash buffer, and prolyl peptide activity was assayed using 100 μl of Gly-Pro-pNA (2 μg/ml) and HRP substrate 2,2′-azino-bis(3-ethylenbenzthiazoline-6-sulfonic acid) diammonium salt was from Amersham Biosciences. Colorimetric and fluorescent measurements were performed using Sulfo-NSH-biotin (Pierce) according to the manufacturer’s instructions. Briefly, the cell layer and buffer were scraped and transferred to a 50-ml conical tube and incubated for 30 min at 4 °C with end-over-end agitation. The extraction was clarified by centrifugation at 10,000 × g for 20 min at 4 °C, and the supernatant was used for immunoprecipitation reactions.

Isolation of Seprase-DPPIV Complex—Cells were seeded onto hydrated type I collagen films (rat tail type I collagen at 0.2 mg/ml, Collaborative Biomedical Products, BD PharMingen) and cultured until 90% confluent. Surface biotinylation of WI38 monolayers was performed using Sulfo-NSH-biotin (Pierce) according to the manufacturer’s instructions. To harvest lysates, each culture plate was washed three times with PBS, followed by a 50-ml PBS washing for 2 days and a 50-ml distilled water wash for another 2 days. Labeled samples were recovered using 50 mM Tris-HCl, pH 7.4, containing 3% Triton X-100 (1 ha t3 7°C to allow gel formation. The gel was washed with 30 μM of Gly-Pro-pNA (2 μg/ml), or control antibody in PBS, pH 7.4 with or without inhibitors (200 μM) or specific mAbs used for capture of antigens (10 μg/ml). The absorbance was read at a wavelength of 405 nm using a Microplate Spectrophotometer System (Molecular Devices).

Labeling of Collagen Fibers—Collagen was polymerized prior to bioin, fluorescein, or rhodamine labeling so that sites of polymerization were not perturbed. Labeled collagen fibers were then solubilized in acidified water (pH 2.0), but could be readily polymerized back to collagen fibers under experimental conditions. Specifically, 10 ml of type I collagen solution (rat tail type I collagen, 4.66 mg/ml, Collaborative Biomedical Products, BD PharMingen) was mixed with 10 ml of DMEM at 4 °C. The mixture was incubated for 30 min at 37 °C to allow polymerization of the collagen fibers (gel). The gel was washed with 30 ml of 50 μM of borate buffer, pH 9.3 (Sigma), for 30 min, and then incubated with 30 ml of borate buffer containing 3 mg of Sulfo-NHS-Biotin (Pierce), fluorescein isothiocyanate I hydrochloride (FITC), or tetramethylrhodamine isothiocyanate (TRITC) (Research Organics Inc, Cleveland, OH) at 25 °C on a shaker. Conjugation was stopped by washing three times with PBS, followed by a 50-ml PBS washing for 2 days and a 50-ml distilled water wash for another 2 days. Labeled collagen was then solubilized in acidified water (pH 2.0), but could be readily polymerized back to collagen fibers as a protease substrate, 100 ng of seprase-DPPIV complex isolated from WI38 RIPA solution (1 mg/ml) was mixed with 10 μl of p-puffer and incubated for 1 h at 37 °C to allow gel formation. The gel was washed with PBS, and ~50 ng of seprase-DPPIV complex isolated from WI38 RIPA extracts were added. Bacterial gelatinase, purified seprase (derived from LOX cells), MMP-2 (recombinant form isolated from COS-1 MMP-2 transfectant), and proteins associated with β3 or β1 integrins were also applied at the concentration of 50 ng/ml and used as controls for biotinylated peptide release. The reaction was performed at 37 °C for 8 h, during which time the collagen gels in the controls (Fig. 4, lanes marked Control and Ip β3 integrin) remained gelled and partially degraded; the reaction was performed at 37 °C for 24 h. The supernatant could contain native collagenase peptides that were not accessible to MMP-2 digestion (Fig. 4; and data not shown). It showed obviously denatured collagen; thus, the collagen gel in this study is referred as “gelatin” throughout the text. The reaction was stopped by low speed centrifugation (2,000 rpm) at 4 °C for 10 min. Resulting supernatants containing biotinylated gelatin peptides were solubilized with 2× SDS sample buffer and analyzed by SDS-PAGE (7.5% gel).
Blots containing biotinylated gelatin peptides were stained with HRP-conjugated streptavidin and the ECL system (Amersham Biosciences).

The gelatinase activity on migratory cells was measured by growing sparse cultures of WI38 cells in collagen gels. Briefly, in a 96-well tissue culture plate (Nunc, Rochester, NY), 50 μL/well of TRITC/gelatin solution (600 μg/ml) was first loaded and the solution allowed to gel in an incubator at 37 °C for 30 min. The TRITC/gelatin gel was then overlaid with 50 μL/well of the TRITC/gelatin solution containing 10² cells and mAbs (0.01-50 μg/ml), and the culture was allowed to gel in a CO₂ incubator for 30 min at 37 °C. The culture was then supplemented with 150 μL of fresh complete media per well. All media were prepared free of phenol red. At times 100 μL of culture media from each well was removed to measure the release of TRITC/gelatin peptides using a fluorescent microplate reader with excitation at 544 nm and emission at 590 nm (Molecular Devices fMax Fluorescence Microplate Reader). Thymidine and leucine incorporations were used to determine metabolic activities of cells under culture conditions, in which 150 μL/well of media containing 2 μCi/ml [³H]Thymidine or [³H]Leucine were added into the culture, and the cell-collagen layers were solubilized in 5 ml of scintillation fluid and counted in a scintillation counter (Beckman LS-7500).

Cell Migration and Collagen Removal Assays—Fluorescent collagen fibers overlaying a monolayer wound culture were used to examine cell migration in collagen gels during wound closure. WI38 cells were grown in 2-well chambered coverglasses (Lab-Tek, Rochester, NY) to confluence. The monolayer was scratched with a pipette tip to generate well-defined wound edges. Culture media were then replaced with TRITC/collagen in DMEM (600 μg/ml; 50 μL/well), and the culture was allowed to gel in a CO₂ incubator for 30 min at 37 °C. Media containing control proteins or inhibitory mAbs (300 μL/well) were then added, and their effects on cell migration in real time were observed using phase-contrast and fluorescence microscopy (Nikon Inverted Microscope). Cell migration and collagen removal were quantified by measuring the areas of cell outgrowth and fluorescent collagen removal by migratory cells using NIH Image 1.62b/fat analysis program.

Cell Attachment and Spreading Assays—Attachment and spreading of WI38 human embryonic fibroblasts were assayed in 96-well plates (Nunc Inc., Naperville, IL) coated with type I collagen fibers and were performed as described (27). Briefly, cells, 5 × 10⁴ cells/well, were seeded on plastic surfaces in a 96-well plate that were coated with type I collagen fibers at the concentration of 600 μg/ml, in the presence of mAbs at the concentrations indicated in Fig. 5. Cells were incubated in a CO₂ incubator at 37 °C for 60 min and fixed for cell counting. The effects of antibodies directed against gp90, DPPIV, or β₁ integrins were determined by counting the number of cells in the area that remained either attached or spread on 9 × 10⁴ μm² areas of the collagen-coated wells. The number of cells in parallel wells that did not contain antibody was used as standard for comparisons.

Immunofluorescent Labeling of Seprase and DPPIV—WI38 cells were cultured in gelatin gel, fixed, and immunolabeled in a single step using rhodamine-conjugated mAb D28 against seprase and the fluorescently labeled mAb E26 against DPPIV (23). Stained samples were photographed using the Planapo 25/1.2 or 63/1.4 objective on a Zeiss Photomicroscope III (Carl Zeiss, Inc.) under epifluorescence.

Human Gingival Wounds—Human gingival biopsies were derived from the University of Turku, Finland. Full thickness wounds of oral mucosa were made from two healthy volunteers, and biopsies were collected after 3, 7, 14, and 28 days of wounding. Immediately after biopsy, fresh tissue blocks were mounted in Historep® (Fisher) and snap-frozen in liquid nitrogen. Frozen sections (6 μm) were cut, fixed with acetone at −20 °C for 5 min, and stored at −70 °C. For routine histology, the sections were stained with hematoxylin and eosin. For immunohistochemical staining, sections were washed with PBS containing 0.1% bovine serum albumin (BSA; Sigma) and incubated with rhodamine-conjugated mAb D28 against seprase or mAb E19 against DPPIV in PBS/BSA in humid chamber at 4 °C for 16 h. The sections were then washed with PBS/BSA and water, briefly air-dried, and mounted using cyanoacrylate glue (Krazy Glue, Borden Co., Ltd.). The staining was examined using a Zeiss Axioskop 20 light, fluorescence and confocal microscopy, and photographed using MC 80 Zeiss microscope camera. Control staining was performed with rhodamine-conjugated secondary antibody and showed no specific stain. DPPIV and Seprase staining was examined using a Zeiss Axioskop 20 light, fluorescence and confocal microscopy, and photographed using MC 80 Zeiss microscope camera. Control staining was performed with rhodamine-conjugated secondary antibody and showed no specific stain.

Preparation of Digital Images—Gel and photographic images of immunoblotting, gelatin zymograms, and Gly-Pro-ABC overlay results were acquired using HP ScanJet 4c scanner (Hewlett-Packard) customized to 400 dpi or QuickScan slide scanner (Minolta) setting on resolution 2. Breast cancer sections were taken with Digital Photo Camera DKC-5000 3CCD (Sony). Digital images were processed with Corel Photo-Paint 9, Corel Draw 9, Microsoft PowerPoint Office 2000 or Adobe PhotoShop 5 programs.

RESULTS

Purification of the Seprase-DPPIV Complex—We found that like seprase in LOX human malignant melanoma cells (14), the majority of seprase and DPPIV in WI38 human embryonic fibroblasts was present as a >400-kDa complex in nonionic detergents including Triton X-100 and Triton X-114, in RIPA buffer containing 0.1% SDS, and octyl glucoside, and in WGA-agarose affinity-purified material. The >400-kDa complex was eluted in the void volume fractions on Sephacryl S-200 gel filtration chromatography (data not shown). Isolation of WGA-purified material followed by Superose 12 gel filtration liquid chromatography (Fig. 1a) exhibited major forms of about 820 (fraction 13), 750 (fraction 14), and 480 kDa (fraction 16). As seprase contains a 97-kDa subunit and DPPIV a 110-kDa monomer, the gel filtration data suggest the presence of the seprase-DPPIV complex at 480–820-kDa sizes (Fig. 1b).

Immunoprecipitation of cell surface proteins using mAbs D8 or D28 (against seprase) and mAbs E19 or E26 (against DPPIV) identified two major bands in the WI38 cell extract that was surface-biotinylated (Fig. 2a). The two bands, co-immunoprecipitated by mAb D28 or E19, indicate seprase and DPPIV dimers, respectively, that form the protease complex on the cell surface (Fig. 2a, left two lanes). In such SDS gels when samples were solubilized in 1% SDS and not boiled, the top or slower band at 200-kDa was identified by immunoblotting as DPPIV dimer and the lower or faster band migrating at 170-kDa as seprase dimer, respectively (Fig. 2b). The large heteromeric aggregates shown in nonionic detergents (Fig. 1) were not detected in SDS gels following SDS solubilization of the samples (Fig. 2, a and b), suggesting that the heteromeric aggregate dissociated into two stable dimers of 200-kDa DPPIV and 170-kDa seprase, respectively, in SDS buffers. In three independent experiments involving RIPA (containing low concentration of SDS) cell extracts, a stable association of seprase and DPPIV was detected using mAbs against seprase and DPPIV but not those against β₁ and β₃ integrins (Fig. 2, a and b).

Such a heteromeric complex was also demonstrated by the proteolytic activities of the immuno-isolated proteases of the protease complex. Antigens were isolated from WI38 nonionic detergent extracts by affinity purification using either mAb D28 or mAb E19 that recognize seprase or DPPIV, respectively. Gelatin zymography detected a 170-kDa gelatinase activity in immunoprecipitates with anti-seprase mAb D28 (Fig. 2c, IP, seprase), anti-DPPIV mAb E19 (Fig. 2c, IP, DPPIV), or purified seprase in the presence of 2 mM EDTA, an inhibitor of metalloproteinases (Fig. 2c, +EDTA). The gelatinase activity of the seprase-DPPIV complex was lost in the presence of 2 mM PMSF, an inhibitor of serine proteinases (Fig. 2c, +PMSF). In addition, proteins associated with β₁ integrin did not show the 170-kDa gelatinase activity (Fig. 2c). Similarly, prolyl peptidase substrate overlay assay detected a 200-kDa prolyl peptidase activity in immunoprecipitates of anti-seprase mAb D28 (Fig. 2d, IP, seprase) or anti-DPPIV mAb E19 (Fig. 2d, IP, DPPIV). However, no prolyl peptidase activity could be observed for β₁ integrin (Fig. 2d, IP, β₁ integrin) and control seprase immunoprecipitate showed little detectable prolyl peptidase activity on the substrate overlay membranes (Fig. 2d, IP, control). These results suggest a 170-kDa gelatinase activity for seprase, and a 200-kDa prolyl peptidase activity for DPPIV in the protease complex. However, zymography and substrate overlay assays were less sensitive for detecting seprase and DPPIV than soluble gelatin and prolyl dipeptide assays (10, 11), and lack of detectable activities by these assays could not indicate the absence of proteases in immunoprecipitated...
tates. Overall, gelatin zymography and the substrate overlay assay confirmed the above purification studies that, in nonionic detergents, a stable association between seprase and DPPIV occurred.

Prolyl Peptidase Activity of the Protease Complex—A classical color substrate used to measure DPPIV-like activity, Gly-Pro-pNA, can react effectively with catalytic domains of purified DPPIV, seprase, and the seprase-DPPIV complex (Fig. 3). The DPPIV-seprase complex (Fig. 3A, red bars in panels indicated as E3, E19, E26, D8, and D28; proteins derived from MDA-MB-436 human breast carcinoma cells), seprase (Fig. 3A, purple bars in panels indicated as E3, E19, E26, D8, and D28; protein derived from LOX cells), and recombinant DPPIV (Fig. 3A, red bars in panels indicated as E3, E19, and E26; protein derived from a COS-1 DPPIV transfectant) exhibited the peptidase activity against Gly-Pro-pNA. There was no DPPIV activity detectable in LOX cells (Fig. 3A, purple bars in the panels marked as E3, E19, and E26), confirming the lack of DPPIV in LOX cells. The mAbs D8 and D28 (against seprase) could bind both free seprase and DPPIV-seprase complex present in the MDA-MB-436 cells, which exhibited ~50% of prolyl peptidase activity of free DPPIV and DPPIV-seprase complex immobilized by mAbs E3, E19, and E26 (Fig. 3A, red bars). The effect of various mAbs against DPPIV (E3, E19, and E26) and seprase (D8 and D28) on the catalytic activity was also examined using the same mAbs that captured antigens as potential inhibitors. None of these monoclonal antibodies blocked the peptidase activity against Gly-Pro-pNA (Fig. 3, A and B). All mAbs tested, including mAb E19 and E26, do not inhibit the prolyl peptidase activity of the DPPIV-seprase complex (Fig. 3A, red bars in panels indicated as E3, E19, E26, D8, and D28), seprase (Fig. 3A, purple bars in panels indicated as D8 and D28), and recombinant DPPIV as indicated by the absence of Gly-Pro-pNA cleavage.

Fig. 1. Gel filtration column chromatography and immunodot blotting for seprase and DPPIV. a, WGA-purified, detergent-soluble proteins derived from WI38 cells were separated by a gel filtration column of Superose 12 (Amersham Biosciences). The column was equilibrated with 10% glycerol, 1% SDS in PBS and performed as described (14). Protein standards were used to calibrate the column; they were thyroglobulin (669-kDa, labeled 1), ferritin (440-kDa, labeled 2), catalase (232-kDa, labeled 3), and aldolase (158-kDa, labeled 4). WI38 cells were surface cross-linked with irreversible cross-linker BS3 and extracted with RIPA buffer. The extract was then affinity-purified on WGA columns. The void volume was collected in 0.5-ml fractions, and the remaining samples were collected in 0.2-ml fractions as indicated. b, fractions were analyzed by immunodot blotting using mAbs against seprase and DPPIV. Seprase and DPPIV were found in 820- (fraction 13), 750- (fraction 14), and 480-kDa (fraction 16) ranges, suggesting the presence of the seprase-DPPIV complex at 480–820-kDa sizes.
binant DPPIV (Fig. 3B, red bars in panels indicated as E3, E19, and E26). However, small reversible DPPIV inhibitors, H-Ile-Thia (K_i = 8 × 10^{-8}) and H-Glu(Gly-5)-Thia (K_i = 8 × 10^{-8}) (28), could block DPPIV and seprase catalytic activities against Gly-Pro-pNA (Fig. 3A, red, blue, and green bars in panels indicated by E3, E19, E26, D8, and D28). Interestingly, these DPPIV inhibitors inhibited most peptidase activity of the DPPIV-seprase complex and free DPPIV immobilized by mAbs E3, E19, and E26 to background levels (Fig. 3A, red, blue, and green bars in panels indicated by E3, E19, E26, D8, and D28), and they only blocked ~50% of prolyl peptidase activity of seprase isolated from LOX cells by mAbs D8 and D28 (Fig. 3A, purple, brown, and yellow bars in panels indicated by D8 and D28).

**Antibody Inhibition of DPPIV Binding to Gelatin—**To map possible epitopes of mAbs E19 and E26 at the binding sites for gelatin, a competition assay was performed (Fig. 3, C–F). Although mAbs E19 and E26 do not inhibit the peptidase activity described above, both of them inhibit the binding of gelatin to the DPPIV-seprase complex (Fig. 3, C and E) and recombinant DPPIV (Fig. 3, G and I) in comparison with control class-matched, anti-DPPIV mAb E3 (red curves in Fig. 3, C, E, G, and I). The inhibition profile was shown in the Lineweaver-Burk plot in small inserts (Fig. 3, D, F, H, and J). It appears that mAbs E19 and E26 recognize the gelatin-binding site and prevent the macromolecular substrate from entering the catalytic center of the complex.

**Gelatinase Activity of the Protease Complex—**Gelatin degrading activity of isolated seprase-DPPIV complex was determined by the release of peptide fragments from biotinylated type I
FIG. 3. Prolyl peptidase activities and DPPIV binding to gelatin. A and B, enzymatic assays for prolyl peptidase activities of immunoprecipitated seprase and DPPIV. mAb E3, E19, E26 (against DPPIV), D8, and D28 (against seprase) are class-matched (IgG2a) and can immunoprecipitate the DPPIV-seprase complex (A, red bars in all panels indicated by mAbs; proteins derived from MDA-MB-436 human breast carcinoma cells), seprase (A, purple bars in all panels indicated by mAbs; proteins derived from LOX human amelanotic melanoma cells) and recombinant DPPIV (B, red bars in panels indicated as E3, E19, and E26; proteins derived from a COS-1 DPPIV transfectant) that exhibit the peptidase activity against Gly-Pro-pNA. These mAbs could not react with cell extracts from pcDNA3.1 vector control (B, blue bars proteins derived from a COS-1 vector transfectant). The dashed lines in A and B showed the background level. Micromole amount of released p-nitroaniline per min was defined as 1 activity unit. Reversible DPPIV inhibitors, inhib 1 (H-Ile-Thia, $K_i = 8 \times 10^{-8}$) and inhib 2 (H-Glu(Gly-5)-Thia, $K_i = 8 \times 10^{-8}$) could block catalytic activities against Gly-Pro-pNA of the DPPIV-seprase complex (A, red, blue, and green bars in panels indicated by E3, E19, E26, D8, and D28) to the background level and these of seprase (A, purple, brown, and yellow bars in panels indicated by D8 and D28) to approximate 50% level. Histogram bars represent the mean of four independent experiments. C–J, antibody perturbation of gelatin binding to DPPIV. 96-Well microtiter plates were coated with rabbit anti-rat antibodies (20 μg/ml) and incubated at 37 °C for 2 h. Wells were rinsed with wash buffer (PBS, 0.1% Tween 20) and blocked with blocking buffer (2% bovine serum albumin in PBS) overnight at 4 °C. The wells were then coated with rat anti-DPPIV mAb E3 (non-inhibitory mAb) at 25 μg/ml and incubated at 37 °C for 2 h. The cell lysates containing the seprase-DPPIV complex (C–F) or recombinant DPPIV (G–J) were added and incubated at 37 °C for 2 h. Biotinylated gelatin, 1–128 μg/ml, was added in the presence of different concentrations (red, 0 μg/ml; blue, 6.25 μg/ml; pink, 12.5 μg/ml and green, 25.0 μg/ml) of mAb E19 (shown in C, D, G, and H) and E26 (shown in E, F, I, and J) in PBS, pH 7.4. Following incubation and washing, streptavidin HRP conjugates (Dako, 1:1000 dilution in PBS, pH 7.4) were added and kept at room temperature for 1 h. The HRP substrate, (2',2'-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid) diammomium salt was added and allowed color development at room temperature for 30 min. The collagen binding activity was determined by the absorbance at the wavelength of 405 nm. The color curves represent the mean of three independent experiments, mean ± S.E. (n = 3 or 4).
collagen gel by immuno-isolated proteases or the protease complex. Fig. 4 shows the peptides released from the gel in the presence of the protease complex and potential inhibitors. The seprase-DPPIV complex, immuno-isolated by anti-seprase or DPPIV antibodies, exhibited a gelatinolytic activity that released multiple peptide fragments from the collagen gel in a stepladder pattern (Fig. 4). The collagen peptide pattern generated by the seprase-DPPIV complex can be blocked by the mAbs E19 and E26 and also by the serine proteinase inhibitor AEBSF but not by the metalloproteinase inhibitor CG1847 (Fig. 4). Furthermore, proteins associated with β1 integrin or control immuno-isolates do not release the gelatin peptides. Taken together, E19 and E26 inhibition of the gelatin degradation may result from antibody effects on binding of gelatin substrates to the enzymes.

**Role in Cell Adhesion**—As DPPIV was shown to be an adhesion receptor for collagen (15–17) or fibronectin (18–20), we have examined whether the inhibitory effect of mAb E19 (against DPPIV) on cellular migration in collagen gels and on gelatin degradation by migratory cells was due to its influence on adhesion activity. In a parallel comparison to integrin adhesion to collagen fibers, whereas mAb E19 (against DPPIV) inhibits cellular migration in collagen gels and gelatin degradation by migratory cells, it does not affect WI38 cell spreading on type I collagen substratum and attachment to collagen substratum (data not shown). However, mAb C27 (against β1 integrins) inhibits WI38 cell spreading on and adhesion to collagen substratum, but mAb E19 (against DPPIV) or mAb C37 (anti-gp-90) does not (data not shown). These data support the notion that β1 integrins may be the primary collagen receptors on WI38 cells responsible for the cellular adhesion to collagen substratum.

**Role in Cell Migration and Localized Collagen Degradation**—To determine the role of the seprase-DPPIV complex in cell migration and in localized collagen degradation, we developed a monolayer wound closure assay using type I collagen gel. A WI38 cell monolayer wound model was overlaid with a thin layer of fluorescent type I collagen gel for morphological examination of cell migration and collagen removal by cells (Fig. 5, A–E). Cell migration and local collagen removal by cells were measured by counting the area of cell migration or collagen removal using image analysis in conjunction with phase contrast and fluorescence microscopy (Fig. 5, A–E). The fluorescent collagen gel, without cells and with the serum proteinase inhibitor AEBSF and the metalloproteinase inhibitor CG1847, released very little fluorescent peptides into the medium during the first 24 h of incubation. In a collagen gel, WI38 cells at the wound edge migrate into the gel and close the wound within 2 days. An evenly intense red fluorescent collagen that covered the cell layer and glass surface was seen at the beginning; however, local collagen removal and extensive cell migration occurred from the wound edge within 18 h (Fig. 5, A–D). Importantly, mAb E19 (against DPPIV) and the serine proteinase inhibitor AEBSF blocked cell migration (Fig. 5, B and C) as well as local collagen removal by cells (Fig. 5D), whereas that of a class-matched mAb (IgG 2a) did not (Fig. 5, B–D). There was an increase in inhibition with increasing amounts of mAb E19 and AEBSF (Fig. 5B), and the antibody inhibitory effect could be reversed by removal of E19 in comparison to that of AEBSF, suggesting that the mAb inhibitors were less toxic (Fig. 5C). Consistently, there was no significant alteration in [3H]thymidine uptake in cell cultures under different conditions (Fig. 5E). The cell-associated, collagen degradation appears to be due in small part to cell proliferation, as collagen removal by migratory cells increased to 120% at day 2 from day 1 and to 153% at day 3 but cell proliferation only increased to 21% at day 2 from day 1 and to 47% at day 3 (Fig. 5, D and E).

The involvement of active recombinant MMP-2 that exhibited gelatin degrading activity by zymography was also accessed by incubation of the collagen gel model with active MMP-2 (Fig. 5, D and E). MMP-2 enhanced collagen removal by cells within 1 day, and its inhibitor CG1847 completely blocked collagen removal by the cells (Fig. 5, B–E). WI38 cells produce constitutively MMP-2 (data not shown) and seprase or DPPIV in culture. Both the serine protease inhibitor AEBSF and the MMP inhibitor CG1847 are highly effective in blocking cellular migration (Fig. 5B), collagen removal (Fig. 5D), and gelatin degradation (Fig. 5F), suggesting the inhibition of endogenous cellular proteases. To test this hypothesis, 0.5 μg/ml of mAb E19 was combined with increasing concentrations of CG1847 and added into the monolayer wound model (Fig. 5B). The combined DPPIV and MMP inhibitors exhibited strongest inhibition on cellular migration (Fig. 5B). These results suggest that localized collagen removal by the cells may be due to the cooperative and
Fig. 5. Cell migration in collagen gel and the localized gelatin degradation by WI38 fibroblasts. A, morphology of WI38 at 1 (a and b) and 18 h (c and d) after wounding of the cell monolayer (photographed while cells were alive). a and c, phase contrast of WI38 at the interface between the wound edge and cell-free glass surface, showing that spindle-shaped cells migrated on collagen fibers at 1 (a) and at 18 h (c). b and d, fluorescent collagen gels in same fields shown in a and c, respectively. Uniform layer of TRITC-labeled collagen is seen at 1 h (b) but local removal
synergistic action of both the seprase-DPPIV complex and MMPs including interstitial collagenases and MMP-2.

To measure the cell-associated gelatin degradation, a microtiter plate format of the cell-gelatin degradation assay was developed. WI38 cells were cultured in fluorescent collagen gels in 96-well microtiter plates for 4–48 h, and their gelatin degrading activities were measured by the release of fluorescent collagen peptides from the collagen gel using spectrofluorometry (Fig. 5F). Cells in sparse culture (10^5 cells per well) are known to be migratory and active due to less “contact inhibition of migration” (29). Active WI38 cells showed time-dependent gelatin degradation from 4 to 48 h in culture (Fig. 5F). The cell-associated gelatin degradation was inhibited completely by AEBSF (20 μM) and mAb E19 (against DPPIV, 5 μg/ml) but not by the control mAb C37 (anti-gp-90, 5 μg/ml), within a 24-h incubation (Fig. 5F). Cell metabolic activity remained unaltered in the presence of AEBSF, mAb E19, and control mAb C37 as indicated by [3H]leucine uptake by WI38 cells (Fig. 5G). These data confirm the involvement of the seprase-DPPIV complex in the cell-associated, gelatin degradation shown as morphological data above.

**Immunofluorescent Localization**—To confirm that seprase and DPPIV are associated in invadopodia of migratory fibroblasts in a collagenous gel, double label immunofluorescence experiments were performed (Fig. 6). We found that invadopodia of a spindle-shaped cell migrating in the gel (Fig. 6A, a and a') were stained positively with TRITC-mAb D28 against seprase (Fig. 6A, b and b') and FITC-mAb E26 against DPPIV (Fig. 6A, c and c'). The superimposed image also shows that seprase and DPPIV co-localize at the invadopodia (Fig. 6A, d'). Furthermore, specific invadopodia labelings of seprase and DPPIV could be competed away by unlabeled mAbs D28 or E26 (data not shown). To further control for the stickiness of rat mAbs to the membrane protrusions, mAb C27 against β1 integrins was replaced to mAb E26 (against DPPIV) in a similar double labeling experiment (Fig. 6B). In this case, seprase was found to similarly localize at invadopodia of a spindle-shaped fibroblast (Fig. 6B, b and b') and β1 integrins distributed in other membrane sites in addition to invadopodia (Fig. 6B, c'). Among 114 cells migrating in the collagenous gel that have a spindle-shaped morphology and form multiple membrane protrusions, 87 cells (73%) exhibit seprase-DPPIV co-localized invadopodia; 12 cells (10%) have seprase-containing protrusions; and 15 cells (12%) show DPPIV-containing protrusions. We therefore suggest that seprase-DPPIV association at invadopodia may participate in the localized gelatin degradation process necessary for extension of a migratory cell in the collagenous gel (Figs. 5 and 6).

**Protein Distribution in Vivo**—Unlike human umbilical cord smooth muscle cells in culture (31), both seprase and DPPIV preferentially distribute among mesenchymal cells but not differentiated muscle and endothelial cells of large vessels in human embryonic tissues, including placenta and umbilical cord (not shown). To determine whether seprase and DPPIV expression in stromal fibroblasts is induced during wound closure in vivo, we investigated the immunohistochemistry of human gingival mucosa wound closure (Fig. 7). A strong expression of seprase and DPPIV was seen in connective tissue cells at day 3 after wounding (Figs. 7, b and f). Later, at day 7 after wounding, only a few cells in the middle of the granulation tissue were reactive with the anti-seprase antibody (Fig. 7c) but not with the anti-DPPIV antibody (Fig. 7g). Seprase and DPPIV staining disappeared from connective tissue cells after 1 week, and cells of normal mucosa adjacent to the wounds also did not react with the antibodies against seprase (Fig. 7d) and DPPIV (Fig. 7h). Furthermore, no specific reaction was seen in the fibrin clot area and epithelium. We therefore suggest that seprase and DPPIV are activation proteases that are expressed on fibroblastic cells and that they may participate in the local gelatin degradation necessary for cellular migration.

**DISCUSSION**

In this paper, we provide several lines of biochemical evidence supporting the role of the seprase-DPPIV complex in the localized gelatin degradation occurring during cell migration. We showed that seprase and DPPIV formed a membrane complex on the cell surface of migratory fibroblasts that could elicit an efficient gelatin degrading activity. The collagen peptide pattern generated by the seprase-DPPIV complex appears to be unique, as it is blocked by anti-DPPIV mAbs E19 and E26 and the serine protease inhibitor AEBSF but not by the metallo-protease inhibitor CG1847 (Fig. 4). In some studies on the chemokine truncation by DPPIV (30) and gelatin degradation by seprase (10, 11), the cleavage of prolyl dipeptides required a large amount of enzyme and inordinately long incubation times. This raises the possibility that formation of the seprase-DPPIV complex enhanced gelatin degradation by migratory cells.

On the other hand, major matrix metalloproteases including interstitial collagenases and MMP-2 could be involved in the initial denaturing process of collagen to provide a better ECM substrate for the seprase-DPPIV complex. Membrane type 1 matrix metalloprotease and active MMP-2 can degrade cell-associated fibronectin and collagens immediately adjacent to invadopodia, and MMP inhibitors block ECM degrading activity (31). Other proteolytic enzymes, in turn, may act directly on the ECM itself or activate or release growth factors from the mesenchymal tissue. Our investigation has demonstrated through the use of antibodies or inhibitors on [3H]leucine uptake by WI38 cells in 4–48 h cultures using the 96-well tissue culture plate method that gelatin degradation was measured by the release of fluorescent collagen peptides from a collagen gel by WI38 cells. Bacterial gelatinase (50 ng/ml) was used as a positive control for overall peptide release. Controls for the cell-associated, gelatin degradation include the inhibition of fluorescent collagen peptide release by AEBSF (20 μM) and CG1847 (50 μM) and by mAb E19 (against DPPIV, 5 μg/ml) but not by control mAb C37 (anti-gp-90, 5 μg/ml). G, histograms showing the effects of antibodies or inhibitors on [3H]leucine uptake by WI38 cells in 4–48 h cultures using the 96-well tissue culture plate method as F above. Cell metabolic activity was indicated by [3H]leucine uptake by WI38 cells in the presence of AEBSF (20 μM), CG1847 (50 μM), mAb E19 (against DPPIV, 5 μg/ml), and control mAb C37 (anti-gp-90, 5 μg/ml). No significant alteration in [3H]leucine uptake was detected in cell cultures under different conditions.
ECM, thereby facilitating cell invasion. As described above, DPPIV and seprase exhibit both prolyl peptidase activity and gelatinase activity and when they form a complex are capable of degrading denatured collagens or other ECM components that allow cells to migrate in the connective tissue.

This paper also reports an interesting protein expression profile of seprase and DPPIV; they form a protease complex at invadopodia of migrating cells (Fig. 6), and they are expressed at noticeable levels during closure of gingival wounds (Fig. 7). Seprase and DPPIV exhibit transient expression in adult tissues; in serial tissue sections both enzymes were induced in connective tissue cells during initial closure of gingival wounds but not in differentiated tissue cells. Invadopodial localization of the seprase-DPPIV complex may provide a driving force for cell migration occurring during wound closure. These results strongly suggest that the seprase-DPPIV complex could be inducible to proteases responsive to wound or inflammatory stimuli. However, the inducible seprase and DPPIV expression in vivo could not be recapitulated in the wound monolayer experiments shown in Fig. 5, as WI38 cells produced seprase and DPPIV constitutively in culture. Interestingly, primary cultures of human endothelial cells exhibited such inducible expression of seprase and DPPIV in monolayer wound model.2 However, the molecular inducers for the expression of the seprase-DPPIV complex in these tissue cells remain to be elucidated.

Previously, we suggested that seprase was a biomarker for melanoma cell invasiveness and the invadopodia of tumor cells

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2 G. Ghersi and W.-T. Chen, unpublished data.
(23, 24). Other studies also demonstrated the involvement of seprase and matrix metalloproteases in breast cancer cell invasion (32, 33). A retrospective study on follicular thyroid carcinoma supports the proposal that DPPIV could be a marker for cell invasiveness (34). Here we show that the seprase-DPPIV complex is also a marker for connective tissue cell invasiveness and the invadopodia of migrating fibroblasts.

It has also been suggested that FAPα/seprase might be a key cell surface protease involved in promoting ECM degradation, tissue remodeling, and fibrosis. FAPα was shown to be expressed at sites of liver tissue remodeling, e.g., stellate cells in cirrhotic human liver (11). FAPα immuno-reactivity was most intense on perisinusoidal cells of the periseptal regions within regenerative nodules (15 of 15 cases); this pattern coincides with the tissue remodeling interface. The pattern of DPPIV expression is altered in cirrhotic human liver, with normal liver showing DPPIV expression in the bile canalicular domain of hepatocytes, whereas cirrhotic liver shows a loss of zonal expression, and DPPIV is redistributed on proliferating bile ductules, ductocytes, and the basolateral domain of hepatocytes (11). Studies with Fap−/− LacZ mice showed that mice express β-galactosidase at regions of active tissue remodeling during embryogenesis including somites and perichondrial mesenchyme from primordial cartilage (35).

Consistent with the potential prolyl peptidease activity toward macromolecules, it is conceivable that seprase or DPPIV may form transient, adhesive bonds with collagen and other ECM components. The cysteine-rich domain is believed to be responsible for DPPIV binding to collagen I and fibronectin (17). In vitro binding assays, DPPIV binds to collagens, preferentially to the collagens I and III, which are both characterized by the formation of large triple-helical domains. Within collagen I, the α1(I) chain was found to be the most prominent binding ligand of DPPIV. A monoclonal anti-DPPIV antibody (13.4) specifically inhibited the interaction of DPPIV with collagen I. In this study, mAbs E19 and E26 could block the binding of DPPIV or the protease complex to denatured type I collagen (Fig. 3), also gelatin degradation (Fig. 4) and cell migration (Fig. 5) could not block cleavage of prolyl dipeptidases (Fig. 3) and cell attachment and spreading on collagen substrate (data not shown). Taken together, there are dual functions of the seprase-DPPIV complex, i.e., substrate binding and gelatin degradation. This report, however, did not confirm DPPIV function in cell adhesion as suggested in earlier studies. For example, outside-out luminal membrane vesicles isolated from rat lung microvascular endothelial by in situ perfusion with a low strength paraformaldehyde solution were shown to bind in significantly larger numbers to lung metastatic than to non-metastatic rat breast carcinoma cells (18, 19). The mAb 6A3 generated against lung-derived endothelial cell membrane vesicles was shown to be reactive to DPPIV and to inhibit specific adhesion of lung endothelial vesicles to lung metastatic breast cancer cells. The DPPIV ligand was identified as tumor cell surface-associated fibronectin, and concomitantly, a correlation between the level of fibronectin expression and the ability of the tumor cells to bind to DPPIV on endothelial cells and to metastasize to the lungs was demonstrated (18).

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