Identification of the 170-kDa Melanoma Membrane-bound Gelatinase (Seprase) as a Serine Integral Membrane Protease*

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The 170-kDa membrane-bound gelatinase, seprase, is a cell surface protease, the expression of which correlates with the invasive phenotype of human melanoma and carcinoma cells. We have isolated seprase from cell membranes and shed vesicles of LOX human melanoma cells. The active enzyme is a dimer of N-glycosylated 97-kDa subunits. Sequence analysis of three internal proteolytic fragments of the 97-kDa polypeptide revealed up to 87.5% identity to the 95-kDa fibroblast activation protein α (FAPα), the function of which is unknown. Thus, we used reverse transcription-polymerase chain reaction to generate a 2.4-kilobase cDNA from LOX mRNA with FAPα primers. COS-7 cells transfected with this cDNA expressed a 170-kDa gelatinase that is recognized by monoclonal antibodies directed against seprase. Sequence analysis also showed similarities to the 110-kDa subunit of dipeptidyl peptidase IV (DPPIV). Like DPPIV, the gelatinase activity of seprase was completely blocked by serine-protease inhibitors, including diisopropyl fluorophosphate. Seprase could be affinity-labeled by [3H]diisopropyl fluorophosphate, but the proteolytically inactive 97-kDa subunit could not, confirming the existence of a serine protease active site on the dimeric form. Proteolytic activity is lost upon dissociation into its 97-kDa subunit following treatment with acid, heat, or cysteine and histidine-modifying agents. We conclude that seprase, FAPα, and DPPIV are related serine integral membrane proteases and that seprase is similar to DPPIV, the proteolytic activities of which are dependent upon subunit association.

Seprase was originally identified from a human malignant melanoma cell line LOX, which exhibited aggressive behavior in experimental metastasis (1, 2). It is a membrane glycoprotein with gelatinase activity that is expressed and localized at the invasion front during invasion into the ECM1 by human melanoma, breast carcinoma cells, and chicken embryo fibroblasts transformed by Rous sarcoma virus (3–6). The protease is a hydrophobic glycoprotein soluble in Triton X-100 and in SDS (3). Seprase shows gelatinolytic activity as demonstrated by gelatin zymography (3, 4). The enzyme maintains maximal activity at neutral pH, which can be further enhanced by SDS buffer, EDTA, the cysteine protease inhibitor trans-epoxysuccinyl-L-leucylamido(4-guanidino)-butane and dithiothreitol. However, seprase has a peculiar protease inhibitor profile; it is inhibited by the protease inhibitors PMSF and NEM (3).

A major problem in purifying seprase was the apparent low level of this protein in cultured cell lines. Because LOX cells produced a large quantity of membrane vesicles that were enriched in seprase, we isolated seprase from the cell membranes and shed vesicles in LOX conditioned medium and characterized its subunit composition and its enzymatic activity. We found that the protease had an apparent molecular mass of 170 kDa composed of proteolytically inactive 97-kDa subunits. Amino acid analysis of internal proteolytic fragments of the 97-kDa polypeptide revealed similarity to the 95-kDa FAPα (7) and the 110-kDa subunit of human DPPIV (8–10). RT-PCR analysis suggests an identical size for the mRNA encoding the 97-kDa seprase subunit from LOX cells as well as the 95-kDa FAPα from WI-38 fibroblasts. When a 2.4-kilobase cDNA amplicon generated by RT-PCR from LOX mRNA using FAPα primers was expressed in COS-7 cells, it produced a 170-kDa gelatinase that was recognized by mAbs directed against seprase. In addition, this report demonstrates that like DPPIV (11, 12), seprase requires dimerization for its gelatinase activity. This gelatinase activity was completely blocked by the serine-protease inhibitors, DFP, PMSF, AEBSF, and APSF. Dimeric seprase could be affinity-labeled by [3H]DFP, but the proteolytically inactive 97-kDa subunit could not. These data demonstrate structural regulation of protease activity by the formation of a serine protease active site upon association of its subunits. Based on its protease characteristics and on the deduced amino acid sequence (GenBank accession number U76833) from its cDNA, we suggest that seprase and DPPIV represent novel serine integral membrane proteases, the proteolytic activity of which is regulated by subunit association.

EXPERIMENTAL PROCEDURES

Materials—The human amelanotic melanoma cell line LOX was obtained from Professor Oystein Fostad, Institute for Cancer Research, The Norwegian Radium Hospital, Oslo, Norway. The human embryonic lung fibroblast line WI-38 and African Green monkey kidney fibroblast line COS-7 were obtained from the American Type Culture Collection (Rockville, MD). Cell culture materials and most protease

fluoride hydrochloride; APSF, 4-amidino phenylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; TBS, Tris-HCl buffered saline; HPLC, high-performance liquid chromatography; WGA, wheat germ agglutinin; DEPC, diethyl pyrocarbonate.
inhibitors were obtained as described (3). [3H]DFP was purchased from DuPont NEN (Boston, MA). 38S-labeled Tran label, DFP, and ABEFS were obtained from ICN Biomedicals, and APSF was obtained from Dr. Jorg Sturzebecher (Erfurt, Germany). Three hybridoma cell lines, D8, D28, and D43, which secreted monoclonal IgG (all class 2a) antibodies, were obtained from the Salk Institute. For purification of rat seprase, cells of Sprague-Dawley rats that were immunized for four-week intervals with 50 μg of partially purified seprase derived from human placenta.

RT-PCR, DNA Cloning, and Expression in COS-7 Cells—Total RNA was isolated from LOX and WI-38 cells using the RNA Stat-60 kit (Tel-Test “B”, Inc.). Reverse transcription was carried out with 6 μg of total RNA using oligo(dT) 12–18 as the primer. The reaction was catalyzed by SuperScript II RNase H–reverse transcriptase, as directed by the manufacturer (Life Technologies, Inc.). Two oligonucleotide primers were synthesized which correspond to a sense sequence within the 5′ untranslated region (FAP 6) of the published FAP sequence (9). A –2.4-kilobase ampiclon generated with either LOX RNA or WI-38 RNA and the FAP 1 (5′-CCAGCGTCTGCTACCGAATTT-3′ (no. 161–181)) and FAP 6 (5′-TCAGATCTGCTATACGGC-3′ (no. 2523–2505)) primers using the Expand Long Template PCR System (Boehringer Mannheim) and a Perkin Elmer GeneAmp 9600 cycler was isolated from a 1% agarose gel using a QiaGen gel extraction kit. Purified cDNA (40 ng of each) was ligated into the vector, vector, transformation, and selection of recombinant clones were carried out using the Eukaryotic TA cloning kit (Invitrogen). A recombinant plasmid, clone pA15, was purified using the Qiagen Plasmid Maxi kit. Transfection of COS-7 cells was carried out by electroporation using a Bio-Rad Gene Pulser II system (Conditions: 0.3 kV and 950 μF). Each electro-transformation was carried out on ∼5 × 106 cells in a volume of 0.5 ml using 20 μg of plasmid. Transfection efficiency was ∼20%, as determined by immunofluorescence. To enrich for cells that express seprase, transfected cells were immunoselected using a panning procedure (13) with the mAbs D8 and D28.

Immunofluorescence Staining and Confocal Microscopy—LOX and transfected COS-7 cells were fixed with 3% paraformaldehyde in PBS for 15 min and stained with mAbs D8 or D28 for confocal microscopic analysis as described previously (4).

Immunoprecipitation of 170-kDa Gelatinase and Its 97-kDa Subunit—Protein A-Sepharose beads (15 μl; Pierce) were coated with rabbit anti-rat IgG (24 μg; Amersham Corp.), and rat mAbs D43 or D28 (25 μg each) prior to incubation with cell detergent extract. Alternatively, mAbs D8, D28, and D43 were coupled to CNBr-Sepharose 4B (Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions. Protease-pretreated preparations were incubated with the antibody-coated beads for 2 h at 4 °C. The antigen-antibody complex-coated beads were collected by centrifugation at low speed (1300 × g), supernatants were removed, and the beads were washed four times with 1% Triton X-100 and 1 mM EDTA in TBS, pH 8.0, at 4 °C (buffer A). The beads were then washed with 1.0% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS in TBS (buffer B) and incubated with a cell extract (buffer C) at 4 °C. The sample buffer containing 0.1 μl diithiithreitol at 37 °C or 80 °C for 10 min, and the SDS-solubilized samples were analyzed by gelatin zymography, SDS-PAGE autoradiography, or immunoblotting. For immunoprecipitations requiring metabolic labeling, cells were incubated overnight with 0.025 μCi/ml 3H-labeled Tran label (ICN Biomedical) in methionine-free Dulbecco's modified Eagle's medium, 10% fetal bovine serum, 1% complete Dulbecco's modified Eagle's medium, 2 mM glutamine, and 1% penicillin/streptomycin. For assaying the gelatin-degrading activity of seprase, heat-denatured rat tail type I collagen (gelatin) was incubated at 37 °C for 45 h in TBS, in the presence of seprase immobilized on mAbs D8, D28, and D43 coupled to Sepharose beads. Following digestion, the supernatants were collected, and the digested gelatin was resolved by SDS-PAGE (7.5% acrylamide gel). The gels were stained by Coomassie Brilliant Blue.

Immunofluorescence Staining—We isolated seprase from 100 liters of LOX cell-conditioned media and 15 ml of LOX cell pellet using WGA and organomercurial chromatography as described (3). Alternatively, seprase was enriched by 40% ammonium sulfate cut of LOX cell RIPA lysates. Protein suspensions were spun at 10,000 × g for 30 min, and the sediment was washed in triplicate with TBS containing 1% octylglucoside. Resuspended proteins were dialyzed against 2 × 500 μl of 1% octylglucoside/TBS. The ammonium sulfate concentrated cell lystate was cleared by centrifugation at 10,000 × g for 30 min at 4 °C. The cleared lysate was loaded onto a BSA precolumn and then onto a mAb D28 affinity column. Column chromatography was carried out at 4 °C. The D28 mAb column was washed with five bed volumes of buffer A and five bed volumes of RIPA buffer. Seprase was eluted with 50 mM glycine buffer, pH 2.4, containing 1% octylglucoside, and fractions (1.5 ml) were immediately neutralized with 1.5 μl Tris buffer, pH 8.8. Fractions were analyzed by SDS-PAGE, and protein bands were visualized using silver staining.

Biochemical Analysis and Sequence Determination—Amino acid sequence analysis was performed as described by Matsudaira (14). The immunofluorescence purified 97-kDa subunit was reduced by 20 mM dithiothreitol and then alkylated by 200 mM NEM. About 10 μg of the alkylated subunit was resolved on SDS-PAGE and then transferred to a polyvinylidene difluoride membrane (Boehringer Mannheim; sequencing grade) and exposed to different pH buffers, or different temperatures. 

Proteases were electrofocused on SDS-PAGE gels and electrophoresed on SDS gels and analyzed on a substrate overlay membrane (Enzyme Systems Products) previously equilibrated with 10% glycerol and 1% Triton X-100 in PBS. The sample was loaded and eluted at a flow rate of 0.05 ml/min and 0.1 ml/min, respectively. The void volume was collected in 0.5-ml fractions, and the remaining samples were collected in 0.2-ml fractions.

Serine Protease Inhibitor-Affinity Ligation—The 170-kDa form of seprase isolated by isoelectric focusing, the 97-kDa subunit obtained from mAb D28-affinity chromatography, or seprase from LOX cell lysate immobilized on mAbs D8, D28, and D43 beads was incubated in the presence of 33 μM [3H]DFP (10 μCi/mmol; DuPont NEN) in 0.1% Tween 20/TBS (TBS-T), pH 7.5, for 1 h at room temperature. The free [3H]DFP was washed out with TBS by consecutive rounds of dialysis/concentration using a Centricon concentrator (Amicon). The DFP-labeled seprase was resolved by SDS-PAGE under nonboiling/nonreducing conditions. [3H]Seprase immobilized on antibody beads was washed with TBS-T and eluted with SDS by reducing and boiling. The eluate was then subjected to SDS-PAGE. Proteins were fixed with 40% methanol/10% acetic acid for 30 min. The gel was incubated with Amplify solution (Amersham Corp.) for 20 min at room temperature and dried. Autoradiograms were exposed for 3, 10, or 20 days at ∼80 °C using Hyperfilm (Amersham Corp.).

DPPIV Substrate Membrane Overlay Assay—Proteases were electrophoresed on SDS gels and analyzed on a substrate overlay membrane (Enzyme Systems Products) coupled with the fluorescent substrate Alas-Pro-7-amino-4-trifluoromethyl coumarin (16). The membrane was moistened in 0.5 μl Tris-HCl, pH 7.8, placed against the gel, and incubated at 37 °C in a humidified chamber. The membrane was then removed from the gel and air dried. The DPPIV activity of individual protease was monitored by detecting 7-amino-4-trifluoromethyl coumarin released from the substrate using a long wave length ultraviolet lamp.

Gelatin Zymography and Immunoblotting of Seprase in the Presence of Protease Inhibitors, Buffers at Different pH, and Different Temperatures—Seprase isolated from LOX lysates was treated with inhibitors for various classes of proteases, exposed to different pH buffers, or incubated at temperatures of 40 °C, 50 °C, or 60 °C. To prepare the protease for exposure to different buffers, seprase in 1% Triton X-114 was partitioned three times using saline to replace the Tris buffer. The resulting detergent phase was incubated with buffers at pH 4 (0.06 M
RESULTS

Localization and Isolation of Seprase and Its 97-kDa Subunit—Seprase was detectable by immunofluorescence with mAbs D8, D28, and D43 on cell surface extensions and membrane vesicles of LOX cells (Fig. 1, A–E). Confocal microscopic analysis of cells cultured on glass coverslips and stained with mAb D28 shows intense seprase localization on membrane extensions at the leading edge, lamellipodia, of the cell (Fig. 1, A–C). When cells were cultured on fibronectin-coated cross-linked gelatin films and stained with mAb D28, seprase became concentrated in invadopodia, specialized protrusions of the ventral membrane, that contacted the film (Fig. 1D). In addition, seprase could be observed on shed vesicles and on surface extensions in the cell-gelatin film interface (Fig. 1E). These results support the observation that seprase can be localized to the cell surface lamellipodia, invadopodia, and on shed vesicles.

Immunoblotting analysis showed that mAbs D8 and D28 recognized seprase (170 kDa) and its 97-kDa subunit, whereas mAb D43 labeled only dimeric seprase (Fig. 1F). Immunoprecipitation of metabolically labeled cells resulted in elution of a major 97-kDa protein band following washing with both 1% Triton X-100/TBS and RIPA buffer (Fig. 2A, lane 1), but multiple components were eluted when only 1% Triton X-100/TBS was used for washing (Fig. 2A, lane 2). Monoclonal antibody D28 was used for affinity purification of seprase from LOX cell membranes and shed vesicles. Fractions (1–3) were analyzed by silver staining (Fig. 2B, lanes 1–3) and immunoblotting with mAb D8 (Fig. 2B, lanes 4–6). The conditions used to elute seprase from the mAb column (glycine buffer, pH 2.4) resulted in elution of the proteolytically inactive 97-kDa subunit (Fig. 2B). Analysis of seprase to determine the extent of its glycosylation indicated that the 97-kDa subunit undergoes a reduction of apparent molecular mass of approximately 20 kDa after exposure to N-glycosidase F (Fig. 2C, lanes 1 and 2). D8 (data not shown) and D28 mAbs recognized the deglycosylated 97-kDa subunit (Fig. 2C, lanes 3 and 4), indicating that their epitopes did not include the N-linked side chains. These results show that seprase is composed of monomeric, N-glycosylated 97-kDa subunits.

Partial Amino Acid Sequence Analysis of the 97-kDa Protease Subunit—Initial attempts to obtain protein sequence data from the 97-kDa subunit were not successful, suggesting that its N terminus may be blocked. Three internal proteolytic fragments were generated from the 97-kDa subunit by Lys-C digestion, purified by HPLC, and subjected to microsequencing. Comparison of the HPLC patterns from the proteolytic digest of the 110-kDa subunit of human placental DPPIV and the 97-kDa subunit of seprase revealed differences (data not shown), suggesting that seprase was distinct from DPPIV. Sequence analysis of the three peptides, which were 10, 12, and 8 amino acids in length, exhibited 80, 66.7, and 87.5% identities, respectively, with the corresponding deduced sequences of FAPs. In addition, the 10- and 12-mers exhibited 70 and 33.3% identities with that of DPPIV, respectively (Fig. 3). These results suggest that the 97-kDa subunit of seprase may be highly homologous to the 95-kDa FAPα and related to the 110-kDa subunit of DPPIV.

Cloning and Expression of Seprase—We carried out RT-PCR...
of LOX total RNA using oligonucleotide primers that corre-
sponded to the 5′ untranslated region (FAP 1) and the 3′
untranslated region (FAP 6) of the FAPα cDNA (7). The result-
ant ~2.4-kilobase amplicon was subcloned into the pCR3.1
mammalian expression vector (clone pA15). To confirm that
this amplicon encoded the 97-kDa subunit of seprase, we trans-
fected COS-7 cells with pA15 or the vector alone (pA11). Two mAbs,
D8 and D28, which had been shown previously (4) to
recognize epitopes on both seprase and its monomeric 97-kDa
subunit, were used for the detection of surface expression,
Western blot analysis, and gelatinolytic activity upshift. As can
be seen in Fig. 4, mAb D8 specifically stained cells that had
been transfected with pA15 (Fig. 4A), whereas only background
staining was observed for those cells transfected with vector
alone (Fig. 4B). In a functional assay, detergent extracts of
immunoselected pA15- and pA11 (vector alone)-transfected
COS-7 cells and LOX cells were assayed for proteolytic activity
by gelatin zymography. The pA15-transfected cells gave rise to
a gelatinolytic band at ~170 kDa (Fig. 4C, lane 3) that corre-
sponds with the region of lysis produced by the LOX cell deter-
genic 97-kDa subunit. Intense staining can be observed in those cells
transfected with pA15 (A), and only background staining is observed in
pA11-transfected cells (B). In C, detergent extracts from pA11 vector
and pA15-transfected COS-7 cells and WGA-purified LOX cell extract
were assayed for proteolytic activity by gelatin zymography. Lane 1,
vector-transfected COS-7 cells (20 μg). Lane 2, vector-transfected cells
that were panned with anti-seprase mAb D28 (<1 μg). Lane 3, pA15-
transfected COS-7 cells panned with D28 (~5 μg). Lane 4, LOX cell
detergent extract purified by WGA chromatography (~30 μg). Lane 5,
lane 4 plus 5 μl of E19, a class-matched IgG2a, negative control mAb
hybridoma supernatant. Lanes 6 and 7, lane 4 plus 5 μl of anti-seprase
D8 or D28, respectively. Lanes 8 and 9, lane 3 plus 5 μl of D8 or D28,
respectively. Hybridoma supernatants were incubated with extracts for
2 h at 4 °C. The results in lanes 5–9 demonstrate that anti-seprase
mAbs specifically form complexes with and upshift the gelatinolytic
activity. D, Western blot analysis using the anti-seprase mAb D8 of
detergent extracts of mock- and pA15-transfected COS-7 cells and LOX
cell extract that was purified by WGA column chromatography. Lane 1,
24 μg of mock cell extract. Lane 2, 22 μg of WGA-purified LOX extract.
Lane 3, 12 μg of pA15 cell extract.

WGA-purified seprase by isoelectric focusing (at pI 5), followed
by Superose 12 gel filtration liquid chromatography, exhibited
major forms of about 200 and 230–490 kDa (Fig. 5A). Seprase,
170 kDa (Fig. 5A, insert, lane 1), when subjected to gelatin
zymography under nonboiled conditions showed a diffuse clear
zone between 150 and 200 kDa (Fig. 5A, insert, lane 3). Gelatin
zymography of the 97-kDa subunit (Fig. 5A, insert, lane 2) did
not show any activity, indicating that it is proteolytically inac-
tive (Fig. 5A, insert, lane 4).

Substrate specificity studies using zymography have shown that
seprase degrades gelatin but not laminin, fibronectin, fibrin,
or casein (3). Thus, we examined possible ECM sub-
substrates, including type I collagen, type IV collagen, laminin,
and fibronectin, using a soluble proteolytic assay. Seprase was
immobilized on mAbs D8-, D28-, and D43-Sepharose beads.
Native type I and type IV collagens, when incubated with the
immobilized protease, were apparently not digested (data not
shown), but thermally denatured type I (Fig. 5B, lanes 2–4) and
type IV collagens (data not shown) incubated at 37 °C were
digested into smaller polypeptides. Seprase did not degrade
fibronectin and laminin under these conditions (data not
shown). Also, based on its protein sequence similarity with
DPPIV, we investigated whether seprase possessed DPPIV
proteolytic activity using DPPIV derived from placental tissue
as a positive control (Fig. 5C, lane 2). We did not detect any
DPPIV activity for seprase (Fig. 5C, lane 1)

Identification of Seprase as a Serine Protease—Initial classi-

| DPP IV | Seprase | FAPα |
|--------|---------|------|
| 222    | F L A Y A Q F N D T | 231  |
|        | F L A Y A E F R D Y |      |
| 470    | Y Y Q L R C S G P G L P | 481 |
| 461    | A V A L V C Y A P W I P |      |
|        | Y Y A L V C Y G P G I P | 472 |
| 516    | F I I L N E T K      | 523  |
|        | K L E V D E I R      |      |
| 511    | K L E V D E I T      | 518  |

FIG. 3. Partial amino acid sequences of the 97-kDa subunit of
seprase. Amino acids are identified with one-letter code. Peptide se-
quences from seprase were aligned with portions of FAPα (accession no.
U09278) and DPPIV (accession no. M47777). The numbers represent
the amino acid positions of the proteins encoded by these cDNAs, and
the bars indicate positions of identity. Three fragments of 10, 12, and 8
residues from a Lys-C digest of the 97-kDa subunit have 80, 66.7, and
87.5% identities to three corresponding sequences of FAPα, whereas the 10-
and 12-amino acid fragments show 70 and 33.3% identities with
different DPPIV, respectively.

FIG. 4. COS-7 cells overexpressing seprase. A and B, immuno-
fluorescent labeling of pA15 (full-length) and pA11 (vector alone) trans-
fected COS-7 cells using mAb D8 that recognizes seprase and its mo-
monic 97-kDa subunit. Intense staining can be observed in those cells
transfected with pA15 (A), and only background staining is observed in
pA11-transfected cells (B). In C, detergent extracts from pA11 vector
and pA15-transfected COS-7 cells and WGA-purified LOX cell extract
were assayed for proteolytic activity by gelatin zymography. Lane 1,
vector-transfected COS-7 cells (20 μg). Lane 2, vector-transfected cells
that were panned with anti-seprase mAb D28 (<1 μg). Lane 3, pA15-
transfected COS-7 cells panned with D28 (~5 μg). Lane 4, LOX cell

detergent extract purified by WGA chromatography (~30 μg). Lane 5,
lane 4 plus 5 μl of E19, a class-matched IgG2a, negative control mAb
hybridoma supernatant. Lanes 6 and 7, lane 4 plus 5 μl of anti-seprase
D8 or D28, respectively. Lanes 8 and 9, lane 3 plus 5 μl of D8 or D28,
respectively. Hybridoma supernatants were incubated with extracts for
2 h at 4 °C. The results in lanes 5–9 demonstrate that anti-seprase
mAbs specifically form complexes with and upshift the gelatinolytic
activity. D, Western blot analysis using the anti-seprase mAb D8 of
detergent extracts of mock- and pA15-transfected COS-7 cells and LOX
cell extract that was purified by WGA column chromatography. Lane 1,
24 μg of mock cell extract. Lane 2, 22 μg of WGA-purified LOX extract.
Lane 3, 12 μg of pA15 cell extract.

| DPP IV | Seprase | FAPα |
|--------|---------|------|
|        | F L A Y A | Q F N D T | 231  |
|        | F L A Y A E | F R D Y |      |
| 470    | Y Y Q L R | C S G P | G L P | 481 |
| 461    | A V A L | V C Y A | P W I P |      |
|        | Y Y A L | V C Y G | P G I | 472 |
| 516    | F I I L | N E T K | 523  |
|        | K L E V | D E I R |      |
| 511    | K L E V | D E I T | 518  |
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FIG. 5. Gel filtration column chromatography and proteolytic assays for seprase. In A, isoelectric focusing-purified material (pl 5) was separated by a gel filtration column of Superose 12 (Pharmacia Biotech Inc.). The column was equilibrated with 10% glycerol/1% Triton X-100 in PBS. Protein standards used to calibrate the column were vitatn B₆ (1.35 kDa), myoglobin (17 kDa), ovalbumin (44 kDa), gamma globulin (158 kDa), aldolase (158 kDa), catalase (232 kDa), ferritin (440 kDa), and thyroglobulin (670 kDa). Fractions were analyzed by enzyme-linked immunosorbent assay using mAb D8 to capture the antigen and biotinylated D43 to detect seprase. Insert: lanes 1 and 2, immunoblotting with mAb D8 of purified 170-kDa seprase (lane 1) and the 97-kDa subunit (lane 2). Lanes 3 and 4, gelatin zymogram of the 170-kDa gelatinase (lane 3) and the 97-kDa monomer (lane 4). B, degradation of soluble heat-denatured type I collagen by seprase immobilized on mAbs D8 (lane 2), D28 (lane 3), and D43 (lane 4) beads. Heat-denatured rat tail type I collagen was incubated with seprase immobilized on mAbs D8, D28, and D43 precoated beads at 37 °C for 45 h in TBS. Lane 1 is the control BSA-coated beads without enzyme. C, detection of DPPIV activity using the fluorescent Ala-Pro-7-amino-4-trifluoromethyl coumarin substrate overlay assay. No activity could be observed for WGA-purified seprase (lane 1) as compared with placental DPPIV used as the positive control (lane 2).

Identification studies suggested that seprase is a serine protease sensitive to PMSF and the sulphydryl-modifying agent NEM (3). To further classify the enzyme, we examined the effects of various inhibitors specific for serine proteases by gelatin zymography (Fig. 6A). In addition to inhibition of the 170-kDa gelatinase by PMSF, other serine-protease inhibitors, AEBSF (5 mM), APSF (0.05 mM), and DFP (0.005 mM), were found to completely inhibit the 170-kDa gelatinase, whereas the cysteine protease inhibitor trans-epoxysuccinyl-l-leucylamido(4-guanidino)-butane enhanced it (Fig. 6A). These inhibitors did not dissociate seprase into its 97-kDa subunit at the concentrations used to inhibit its enzymatic activity (data not shown). Other inhibitors of aspartate, serine, or metalloproteases, including pepstatin (6 mM), benzamidine (10 mM), EDTA (5 mM), and 1,10-phenanthroline (2 mM), and cysteine protease inhibitors, including leupeptin (0.1 mM), iodoacetic acid (1 mM), iodoacetamide (1 mM), and trans-epoxysuccinyl-l-leucylamido(4-guanidino)-butane (5 mM), had no inhibitory effect on the activity of the 170-kDa gelatinase. The inhibition study suggests that seprase contains a catalytically active serine residue(s).

[3H]DFP affinity labeling experiments showed that the label was associated with both the 170-kDa form (Fig. 6B, lanes 1 and 3) and the larger forms (Fig. 6B, lanes 4–7) but not with the 97-kDa monomer (Fig. 6B, lane 2). When labeling was performed on seprase immunoabsorbed to mAbs D8 (Fig. 6B, lanes 3, 4, and 7), D28 (Fig. 6B, lane 5), and D43 (Fig. 6B, lane 6), the radioactive label was detected on the 97-kDa subunit because seprase was dissociated upon elution by boiling. The active site affinity labeling is specific because [3H]DFP labeling was abolished when seprase was preincubated with cold DFP (Fig. 6B, lanes 7 and 8). [3H]DFP labeling of seprase appeared to alter mobility of the enzyme on SDS gels (Fig. 6B, lane 1). In addition, the isolated 97-kDa subunit could not be labeled with [3H]DFP (Fig. 6B, lane 2) and failed to degrade gelatin (Fig. 5A, insert, lane 4). Furthermore, seprase could not be affinity labeled by the cysteine protease inhibitors biotin-Phe-Ala-CH₂N₂ or biotin-Phe-CH₂Cl (data not shown). We have, therefore, demonstrated that seprase, like DPPIV, is a serine protease and that its 97-kDa subunit is not active.

Dimerization Is an Essential Structural Requirement for Generation of the Seprase Serine Protease Active Site(s)—The effects of acid, heat, and amino acid-modifying agents, such as NEM and DEPC, in inhibiting seprase gelatinase activity have been investigated. The sensitivity of the seprase dimeric structure and its gelatinase activity to buffers at different pHs (Fig. 7A), incubation at different temperatures (Fig. 7B), and treatment with the histidine-modifying agent DEPC (Fig. 7C) was investigated using gelatin zymography and immunoblotting. Gelatinase activity was measured by zymography, and changes in subunit association were detected by immunoblotting with mAb D8 that recognizes both seprase and its 97-kDa subunit and with mAb D43 that recognizes only seprase. At pH lower than 5 (Fig. 7A), incubation at temperatures above 50 °C (Fig. 7B), or DEPC treatment at concentrations over 5 mM (Fig. 7C),
Gelatinase activity was inhibited at pH lower than 5 and by temperature. Type I collagen is a substrate for seprase (Fig. 5A). We have isolated seprase using isoelectric focusing and have determined that it is gelatinase from LOX cell membranes. We have also isolated a 97-kDa subunit of seprase using immunoblotting with mAb D8 that recognized both seprase and the 97-kDa subunit (left panels). The protein antigen was analyzed by immunoblotting with mAb D8 that recognized both seprase and the 97-kDa subunit (center panels) and with mAb D43, recognizing only dimeric seprase (right panels). A, sensitivity to pH was determined by incubating WGA-purified seprase with citric acid/phosphate buffers at pH 4, 5, and 6. In B, the effect of temperature was measured by incubation of the enzyme at 40 °C, 50 °C, and 60 °C. In C, the histidine-modifying agent DEPC was incubated with the enzyme at 0, 1, and 5 mM concentrations. The gelatinase activity was inhibited at pH lower than 5 and by temperatures above 50 °C. DEPC was inhibitory at a concentration of 5 mM. Under these conditions, loss of gelatinase activity was always accompanied by the disappearance of the 170-kDa form and the appearance of the 97-kDa subunit.

Loss of gelatinase activity was always accompanied by the disappearance of the 170-kDa form and the appearance of the 97-kDa subunit. A similar effect to DEPC was observed for the cysteine-modifying agent NEM but not for the serine protease inhibitors PMSF, AEBSF, APSF, and DFP at the concentrations that inhibited the gelatinase activity (data not shown). Thus, acidic pH, heat, NEM, and DEPC treatment inhibited the seprase gelatinase by altering its subunit association, which is necessary for its catalytic activity.

**DISCUSSION**

This study focused on the characterization of melanoma seprase in terms of its amino acid sequence, expression of its cDNA, classification of its proteolytic activity, and structure/assembly relationships. Seprase is localized at cell surface invadopodia, lamellipodia, and vesicles. These results, together with the previous observation that LOX cells with higher levels of seprase display a more invasive phenotype than those with lower levels (4), make it important to identify and characterize this protease.

Previously, seprase was defined by its gelatinolytic activity rather than by its isolation (3, 4). Here we have used nondenaturing chromatographic techniques followed by D28 immunoaffinity chromatography to purify a Triton X-100-soluble gelatinase from LOX cell membranes. We have also isolated seprase using isoelectric focusing and have determined that it has an isoelectric point of 5. We showed here that heat-denatured type I collagen is a substrate for seprase (Fig. 5B) but not native type I collagen, supporting the notion that it is a gelatinolytic endopeptidase. Also, we showed that seprase is a serine protease by virtue of its inhibition by the serine protease inhibitors DFP, PMSF, APSF, and AEBSF and by affinity-labeling with [3H]DFP. Consistent with these results, the deduced amino acid sequence of a cDNA clone that encodes the 97-kDa subunit of seprase (GenBank accession number U76833) is highly homologous to those of the nonclassical serine protease DPPIV (8–10) and the putative nonclassical serine protease FAPα (7) in their catalytic regions.

The affinity-purified material gave a single band at 97-kDa by silver staining and was recognized by mAbs D8 and D28. This 97-kDa subunit was devoid of proteolytic activity, as determined by gelatin zymography, a soluble gelatin degradation assay, and DFP affinity labeling. Lack of [3H]DFP labeling of the 97-kDa form could be due to the fact that serine proteases need to be in an active conformation to accept phosphorylation of the OH group of their catalytic site serine (18). However, we do know that each 97-kDa subunit of seprase contains the consensus motif of serine proteases, GXXSG, as well as the other residues that make up the catalytic triad (GenBank accession number U76833).

Most secreted ECM-degrading enzymes are proteolytically activated from their zymogen precursors; however, formation of a dimeric structure appears to be necessary for expression of the high molecular mass dipeptidase activity of DPPIV (11, 12, 19). We have shown that like DPPIV (20, 21), seprase required dimerization to exhibit its gelatinase activity, which was completely blocked by the serine-protease inhibitors DFP, PMSF, AEBSF, and APSF. In addition, seprase could be affinity-labeled by [3H]DFP, but the proteolytically inactive 97-kDa subunit could not. These results are further confirmed by our COS-7 cell expression experiments in which the expressed 97-kDa subunit dimerized to produce the active form of seprase. These data demonstrate that there is structural regulation of the proteolytic activity of seprase; the formation of the serine protease active site(s) is dependent on the association of two subunits. However, the mechanisms by which seprase subunit association is regulated are not yet understood.

We speculate that this structural switch for proteolytic activity may be a common mechanism for the regulation of serine integral membrane proteases, including seprase and DPPIV. The requirement for a dimeric structure for seprase gelatinase activity is supported by our studies showing a correlation between structure stability and gelatinase activity at various pH, temperatures, and in the presence of the amino acid-modifying agents NEM and DEPC. We have shown that seprase was not active when samples were exposed to acidic conditions (pH < 5), to high temperatures (>50 °C), or to 5 mM NEM or DEPC. In all cases, protease inactivation was accompanied by protease dissociation into the 97-kDa subunits. Similarly, the dimeric 150–220-kDa DPPIV has been reported to be active and accessible to DFP, but the 110-kDa monomeric DPPIV was not active (11, 22). These properties of membrane-bound enzymes point to the possibility that subunits may assemble to form active enzymes at sites of protease action, i.e., the cell surface. Furthermore, the fact that seprase is sensitive to an acidic environment suggests that seprase may be inactivated in an endocytic compartment. Thus, increasing or decreasing the stability of an oligomeric complex may be a means for cells to regulate the proteolytic activity of membrane proteases.

**Acknowledgments**—We are most grateful to Susette C. Mueller for critical reviews of this work, to Steve Akiyama and Scott W. Argraves for help with the initial stage of this study, to Jörg Stürzebecher for providing APSF and several newly synthesized serine protease inhibitors, and to Tom Winters for help with the gel filtration experiment.
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