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mp170 seprase is a homodimeric 170 kDa membrane-bound gelatinase whose expression correlates with the invasiveness of the human melanoma cell line LOX. We have isolated a cDNA clone, pA15, that encodes its 97 kDa subunit. COS-7 cells transfected with pA15 express a 170 kDa gelatinase which is recognized by mAbs directed against mp170 seprase. The deduced amino acid sequence predicts a type II integral membrane protein of 760 amino acids. And its carboxyl terminus contains a putative catalytic region which is homologous to the nonclassical serine protease subfamily represented by the ectopeptidase Dipetidyl Peptidase IV (DPPIV). However, mp170 seprase exhibits its highest identity(94%) with the integral membrane protein Fibroblast Activation Protein Alpha (FAP) that is expressed in vivo in carcinomas and sarcomas but whose function is unknown. Unlike FAP, we have isolated cDNA clones that encode mp170 seprase in the human breast carcinoma cell line MDA-MB-436. Preliminary sequence analysis confirms the sequence obtained for LOX mp170 seprase.

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Leslie Goldstein 8/29/96
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INTRODUCTION

Background

One of the fundamental requirements for the metastatic behavior of cancer is the ability of tumor cells to invade the extracellular matrix (ECM). This capability allows for the subsequent dissemination of tumor cells to secondary sites in the body via the circulation and lymphatics (1,2). Indeed, in advanced breast cancer metastases to the lung, liver, lymphatic organs and bone etc. are common. And it is well established that a variety of secreted proteases of the serine(3,4), cysteine(5,6), metalloproteinase(7,8,9) and aspartic acid (10,11) classes which can also be localized to the cell membrane (12,13,14) are involved in this process.

Our laboratory has shown that transformed cells elaborate extracellular ventral membrane protrusions termed “invadopodia” at the cell-ECM interface (12). These specialized structures exhibit cell surface proteolytic activities that allow metastatic cells to dissolve the surrounding matrix. Furthermore, by utilizing an in vitro assay system that consists of growing cells on fluorescence labeled or radiolabeled ECM components that are covalently crosslinked to gelatin film, we can detect the elaboration of invadopodia by human cancer cell lines (15). As a result of this ECM invasion/degredation assay our laboratory identified a highly invasive melanoma cell line, LOX, from over 32 human tumor cell lines (16). This cell line had previously been shown to have a high incidence of lung metastasis after intravenous injection into athymic mice (17). Recently, two additional cell lines were identified, another melanoma line RPMI7951 and a breast carcinoma cell line MDA-MB-436 that exhibited the invasive phenotype.

LOX cells express a 170 kDa membrane-bound gelatinase, mp170 seprase, that has been reported to be localized on invadopodia and whose expression correlates with LOX invasiveness in the in vitro ECM degradation/invasion assay (18). It is a homodimer consisting of 97 kDa subunits that are proteolytically inactive(19). Recently, mp170 seprase was classified as a serine protease whose active site is generated upon subunit association (19). Preliminary protein sequence data obtained from 3 internal peptides that were generated by digesting the 97 kDa subunit with the endopeptidase Lys C indicated that mp170 seprase was homologous to fibroblast activation protein α (FAPα) and to dipeptidyl peptidase IV (DPPIV) (19). Fibroblast activation protein α is a 95 kDa type II integral membrane protein of unknown function that is homologous to DPPIV (48% amino acid identity). It is selectively expressed in vivo on reactive stromal fibroblasts of carcinomas and healing wounds and on sarcoma tumor tissues; in vitro it is expressed on fibroblasts and some tumor cell lines but not on carcinoma cell lines (20,21,22). Dipeptidyl peptidase IV is a multifunctional protein that has wide tissue distribution and is a member of a new subfamily of nonclassical serine hydrolases(23,24,25). Like mp170 seprase its protease activity is dependent on the association of its 110 kDa subunits (26,27).
Purpose of present work

We initially proposed to determine the role of mp170 seprase in the metastasis of breast cancer. The \textit{in vitro} breast carcinoma line MDA-MB-436 which is known to express mp170 seprase would appear to be a good candidate for transfection experiments with sense and antisense cDNA constructs of mp170 seprase. Using the \textit{in vitro} ECM invasion/degradation assay we could monitor the effects of the overexpression and underexpression of this gelatinase on the invasive phenotype of MDA-MB-436 as well as other cell lines (MDA-MB-231 etc.) at various stages of neoplastic development. Therefore, our first priority is to isolate a cDNA clone that encodes the entire open reading frame (ORF) of mp170 seprase.

Methods of approach

The results presented above, namely, the sequence homology of internal peptides, subunit size similarity and the ability of the mp170 seprase and FAP\(\alpha\) to associate with DPPIV and form a heteromeric complex (20) suggested that these two proteins may be highly homologous. Thus, oligonucleotide primers based on the FAP\(\alpha\) cDNA sequence (20) could be used to synthesize a cDNA probe from LOX mRNA by reverse transcriptase-polymerase chain reaction (RT-PCR) to screen a LOX cDNA library. Confirmation of a putative positive clone(s) would be accomplished by subcloning the cDNA insert in a mammalian expression vector and transfecting COS-7 cells. Identification of mp170 seprase would be accomplished with anti-mp170 seprase mAbs and in a functional assay by performing gelatin zymography on transfected COS-7 cell detergent extracts. As will be described in the Body section of this report we abandoned screening of a cDNA library and utilized RT-PCR to generate a cDNA clone that encodes the 97 kDa subunit of mp170 seprase.
Screening of LOX cDNA library

In the previous annual report we isolated two putative mp170 seprase clones from a LOX lambda gt11 cDNA library. These clones were isolated using an amplicon (0.8 kb) that was generated by RT-PCR of LOX mRNA using primers that corresponded to the FAPα cDNA sequence [sense: 5'-CCAGCAATGATAGCCTCAA-3' (#1055-1073); antisense: 5'-ACAGACCTTACACTCTGAC-3'(#1863-1845)]. The two putative mp170 seprase clones lambda 50A2 (1.8 kb) and lambda 30B1 (1.4 kb) appeared to overlap each other and encode the entire open reading frame predicted by the FAPα cDNA sequence (2277 bp). This conclusion was based on restriction endonuclease digests of these clones using 9 enzymes and a computer generated restriction map of the FAPα cDNA. However, sequence analysis of both clones revealed that although they exhibited regions of identity with the FAPα cDNA sequence both clones contained unrelated sequence inserts in their putative overlap region. Specifically, lambda 50A2 showed identity with the FAPα cDNA corresponding to nucleotide positions #161 to 1610 (the ORF begins at # 209); it contained an insert at its 3' end of ~250 bp which when translated would cause premature termination of mp170 seprase 8 residues downstream of the insertion point. Likewise, lambda 30B1 which encoded the 3' region of the ORF, showed identity with the FAPα cDNA from nucleotide positions # 1531 to 2523 (termination of the ORF at #2486); however, it contained an insertion of 120 bp between nucleotide positions #1610 and 1611 which would prematurely terminate translation 2 residues downstream from its insertion point. It also contained a deletion of 170 bp between positions # 1658 and 1828 which would terminate translation 9 residues downstream of the deletion. Analysis of the sequences surrounding the regions of insertions and deletions did not indicate that they were exon-intron junctions nor did the inserts appear to be Alu sequences. As a result of these findings and similar findings with other clones from this library we decided to abandon the library screening procedure and attempt to generate a cDNA clone that encoded the complete ORF of mp170 seprase by RT-PCR.

Isolation of mp170 seprase cDNA

Using oligonucleotide primers that correspond to the 5' untranslated region (UTR) and the 3' UTR of the FAPα cDNA [sense: 5'-CCACGCTCTGAAGACAGAATT-3' (#161-181); antisense: 5'-TCAGATTTCTGATACGGCT-3' (#2523-2505)] we carried out RT-PCR of LOX total RNA. The resultant ~ 2.4 kb amplicon was subcloned into the mammalian expression vector pCR3.1 (Invitrogen). We transfected COS-7 cells with a recombinant plasmid, pA15, that contained the ~ 2.4 kb insert and also carried out mock transfections with the ligated expression vector, pA11. To determine if this amplicon
encoded mp170 seprase we utilized two monoclonal antibodies (mAbs), D8 and D28, which recognize epitopes on both dimeric mp170 seprase and its 97 kDa subunit (18). Both mAbs stained cells that had been transfected with pA15 but did not specifically stain cells transfected with pA11. In addition, we utilized a class matched negative control mAb(IgG2a) that did not specifically stain cells transfected with pA15. Western blot analysis of detergent extracts of pA15 and pA11 transfected COS-7 cells using mAbs D8 and D28 confirmed the cell staining experiments. Both mAbs detected a band at ~ 170 kDa in pA15 extracts which comigrated with the band detected from LOX extract. No corresponding band was detected in pA11 extracts. Similarly, when mp170 seprase was dissociated by heat into its 97 kDa subunit in the pA15 and LOX detergent extracts it was recognized by mAb D8 but not in the pA11 extracts. Furthermore, in a functional assay to detect proteolytic activity by gelatin zymography (Fig. 1) the pA15 extract gave rise to a gelatinolytic band at ~ 170 kDa that comigrated with the region of lysis produced by the LOX extract. No gelatinolytic band was detected in the mock transfected COS-7 cells. We also confirmed the identity of the gelatinolytic bands observed with the pA15 and LOX extracts by forming mp170 seprase-mAb complexes using the mAbs D8 and D28. Complex formation upshifted the proteolytic activity bands in these extracts but a class matched negative control mAb did not. Thus, we feel that the results from immunostaining of transfected COS-7 cells coupled with Western blot analysis and gelatin zymography of transfected COS-7 cell detergent extracts confirm that pA15 encodes mp170 seprase.

**Sequence analysis of pA15 cDNA insert**

Both strands of the cDNA were sequenced using sense and antisense oligonucleotide primers that generated overlapping sequence data on each strand. Sequence analysis of the cDNA insert of pA15 revealed an ORF of 2280 bp which encodes a polypeptide of 760 amino acids with a M, 87,722 (Fig.2). The cDNA sequence predicts a type II integral membrane protein with a short cytoplasmic tail (6 amino acids) followed by a hydrophobic transmembrane domain (20 amino acids) and a relatively large extracellular domain composed of 734 amino acids. There are 5 potential N-glycosylation sites: 4 of the sites are clustered in a membrane proximal region extending from Asn49 to Asn314 with the fifth site Asn679 located in the putative catalytic region (see below). There are 12 cysteine residues: 7 of these residues are clustered in a region that extends from Cys305 to Cys448. Located at the carboxyl terminus is a putative catalytic region consisting of ~240 amino acids that extends from Tyr520 to Asp760. Within this region is the catalytic triad of residues Ser624, Asp702 and His734 which are in a nonclassical sequence orientation (23,24,25). The consensus motif Gly-X-Ser-X-Gly which is characteristic of serine proteases is conserved around Ser624.
Sequence comparison of pA15 and FAPα cDNA coding regions

A comparison of the nucleotide sequence of the pA15 cDNA to that for FAPα (20) indicated a difference of 8 base pairs in the ORF of their respective cDNAs (Fig.2). There are 5 base substitutions: 3 give rise to nonconservative amino acid substitutions at residues #207 (Pro to Ala), #229 (Lys to Thr) and #354 (Arg to Thr), respectively. The fourth and fifth are silent substitutions corresponding to nucleotide positions #252 (A to G) and #2124 (A to G), respectively. The remaining difference of 3 bp is due to the insertion of 3 guanine nucleotides at positions #1876 or 1877, 1879 or 1880 and 2010 in the pA15 cDNA. The nucleotide sequence from nucleotide #1876-G to #2010-G encodes a contiguous sequence of 45 amino acids extending from Gly<sup>626</sup> to Lys<sup>670</sup> in the putative catalytic region. This sequence overlaps the serine protease conserved sequence motif Gly-X-Ser-X-Gly which is not conserved in FAPα. Thus the difference of 8 bp between the mp170 and FAPα cDNAs gives rise to a total difference of 48 amino acids in their deduced polypeptide sequences or an amino acid identity of ~94%.

We reported last year that we had detected the presence of mp170seprase/FAPα mRNA in the breast carcinoma cell line MDA-MB-436 using FAPα sense and antisense primers by RT-PCR. After determining the sequence of pA15 we carried out RT-PCR on MDA-MB-436 total RNA with the same sense and antisense primers used to generate pA15. Preliminary sequence analysis (1 strand) of cDNA clones from MDA-MB-436 found a difference of 4 bp with pA15 in the coding region: two base substitutions give rise to nonconservative amino acid substitutions at position #133 (Tyr to His) and position #279 (Glu to Gly). The remaining two substitutions at nucleotide positions #252 (G to A) and #2124 (G to A) are silent. Importantly, the insertion of 3 G nucleotides in the sequence encoding the catalytic region of pA15 is confirmed in MDA-MB-436. This result was also observed for mp170 seprase in the invasive melanoma cell line RPMI7951.
CONCLUSIONS

Isolation and identification of a cDNA clone (pA15) that encodes mp170 seprase confirms the previously obtained peptide sequence data from the its 97 kDa subunit (19). Namely, that mp170 seprase exhibits highest homology with human FAPα. There is also a striking sequence identity with the ectoenzyme DPPIV (28,29,30). The amino acid identity between mp170 seprase and DPPIV is 52% but increases to 68% in the catalytic region. The high extent of sequence identity between the pA15 and FAPα cDNAs indicates that either both encode the same protein or virtually identical proteins. The significance of the 3 nonconservative amino acid substitutions remains to be determined. However, the 3 guanine nucleotide insertions in the pA15 cDNA result in a divergence of the amino acid sequence (45 contiguous residues) in the putative catalytic region of this protein (Fig.2). Importantly, the consensus sequence motif Gly-X-Ser-X-Gly which is identical in mp170 seprase and the related mammalian protease DPPIV (Gly-Trp-Ser-Tyr-Gly; see below) is not conserved in FAPα (Gly-Trp-Ser-Tyr-Glu). The substitution of Gly with Glu in FAPα could be significant since it has been reported that substitution of this Gly residue in rat DPPIV results in complete loss of proteolytic activity (31) and no proteolytic activity has yet been ascribed to FAPα. Thus if the divergence in the nucleotide sequence encoding this region in FAPα is not an artifact, then there may be important differences in the biological activities of these two proteins. Also, multiple nonproteolytic functions have been ascribed to DPPIV (32-35). For example, it has been reported to bind the ECM components fibronectin and collagen (32,33). It is interesting to speculate whether these DPPIV structurally related proteins, mp170 seprase and FAPα, may function in roles other than or in addition to integral membrane proteases. Indeed, the fact that mp170 seprase is expressed on invasive melanoma cell lines (16,17,18) while FAPα can be expressed on “reactive” fibroblasts of carcinomas (21,22) could mean that these very similar proteins may have different roles when they are expressed on cells with different metastatic potentials.

Our next priority will be to transfect human breast carcinoma cell lines such as MDA-MB 436 or MDA-MB-231 (negative for mp170 seprase by RT-PCR analysis) etc. as well as normal epithelial cell lines with sense and/or antisense constructs of mp170 seprase. We will use constructs encoding DPPIV that are generated by RT-PCR and subcloned in the pCR3.1 vector in sense and antisense orientations as one of our controls. In addition, since it is known that mp170 seprase and FAPα (20) form heteromeric complexes with DPPIV, we will also do cotransfections with constructs for these homologous proteins. It should be noted that their proteolytic substrate specificities are distinct (19). We will then attempt to detect possible phenotypic changes in our transfected cell lines as a result of increased expression or inhibition of expression of mp170 seprase using the in vitro invasion/degradation assay etc. If phenotypic changes are detected we will begin to pursue the underlying biochemical and cell biological mechanisms that are involved.
REFERENCES


APPENDIX

Figure Legends

Fig. 1. **pA15 encodes functional mp170 seprase.**

Detergent extracts from mock and pA15 transfected COS-7 cells and WGA purified LOX cell extract (19) were assayed for proteolytic activity by gelatin zymography (Lanes 1-9). Lanes 5-9 demonstrate that anti-mp170 seprase mAbs specifically form complexes with the gelatinolytic activity. *Lane* 1, mock transfected (pA11) COS-7 cells (20 μg). *Lane* 2, mock transfected cells that were panned with anti-mp170 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, same as *lane* 4 + 5 μl of B5 - a class matched (IgG2a) negative control mAb hybridoma supernatant. *Lane* 6, same as *lane* 4 + 5 μl of anti-mp170 seprase D8. *Lane* 7, same as *lane* 4 + 5 μl of anti-mp170 seprase D28. *Lane* 8, same as *lane* 3 + 5 μl D8. *Lane* 9, same as *lane* 3 + 5 μl of D28. Hybridoma supernatants were incubated with extracts for 2 h at 4 °C.

Fig. 2. **Nucleotide and deduced amino acid sequences for the coding region of pA15.**

Nucleotide and amino acid sequence numbers are shown to the right. The putative transmembrane region is represented in bold characters. Potential N-glycosylation sites are shown as bold *italicized* characters. Nucleotide substitutions and insertions relative to the FAPα cDNA sequence are underlined. Amino acid substitutions relative to the deduced amino acid sequence of the FAPα cDNA are represented by underlined characters. The putative catalytic triad of mp170 seprase (Ser624, Asp702, His734) is represented by bold underlined characters.
Figure 1.

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