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13. ABSTRACT *(Maximum 200 words)*

This report documents progress made in the second year of DAMD17-96-1-6097 "Role of seprase in breast cancer invasion". The goals are to clone a full length cDNA encoding seprase and then produce cells that overexpress seprase to investigate its role in tumor cell invasion. This year an apparent full-length seprase cDNA has been amplified by RT-PCR from MDA-MB-436 human breast cancer cells. Moreover, 0.78 and 1.5 kb partial chicken embryo seprase cDNAs have been cloned and characterized. Peptides based on the deduced C-terminal 341 amino acids of chicken embryo seprase have been produced and used to immunize mice. An enzyme linked immunosorbent assay (ELISA) has been established for seprase-specific peptides. This ELISA will be used to screen hybridoma supernatants to identify hybridomas that produce seprase-specific antibodies. Two papers and a submitted manuscript describing new results and interpretations of various aspects of this project were completed this year. One of these papers was presented at the DoD Breast Cancer Research Program meeting "Era of Hope". The research progress closely approximates the time line proposed in the original statement of work, thus we anticipate successful completion of the proposed research within the 4 year project period.

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Thomas J. Kelly, Jr. 9/25/98

PI - Signature Date

Table of Contents

Introduction

This report describes progress made in the second year of a four year project entitled "Role of seprase in breast cancer invasion". Seprase is a serine integral membrane protease (1) first identified in human melanoma cells (2) and chicken embryo fibroblasts transformed by Rous sarcoma virus (3). In 1997, a cDNA encoding human melanoma seprase was cloned and sequenced by others (1). The sequence reveals that seprase is a member of a family serine integral membrane proteases that includes fibroblast activation protein-α (FAP-α) (4), and dipeptidyl peptidase IV (DPPIV) (5, 6). Seprase is known to degrade gelatin (1-3) and is thought to facilitate erosion of the extracellular matrix thereby promoting invasion of malignant cells. Supporting this concept is the association of seprase overexpression with the invasive phenotype and its concentration on the invadopodial membranes of human melanoma cells (2, 7). Moreover, accumulation of seprase on the surface of invadopodial membranes is stimulated by ligation of $\alpha_6\beta_1$ integrin on the melanoma cell surface (8). The localization of seprase to invadopodia supports a role for seprase in degrading extracellular matrix and facilitating tumor cell invasion because invadopodia are specialized protrusions of the plasma membranes of invasive cells that contact and degrade extracellular matrix (9, 10). Invadopodia can cause proteolysis of intact fibroblast extracellular matrices, type I collagen, type IV collagen, laminin, and fibronectin (3). Several malignant cell types are known to use invadopodia to degrade extracellular matrix including, human melanoma cells (2, 7, 10), Rous sarcoma virus-transformed chicken embryo fibroblasts (3, 9, 11) and human breast cancer cells (12-14). The fact that malignant human breast cells degrade extracellular matrix with invadopodia suggested that seprase might have a role in promoting the invasive spread of human breast cancer.

Seprase is overexpressed by invasive human breast cancer (15). This conclusion is based on three lines of evidence that have been either published or submitted for publication as the direct result of the funding of this project. Specifically, a seprase-specific polyclonal antibody produced using affinity-purified chicken embryo seprase intensely labels malignant breast cells but not normal breast epithelia or stromal cells (15). In addition, seprase activity was detected by zymography in extracts of human breast cancer tumors and the seprase activity was five times greater than that of chicken embryo seprase as determined using a ^{3}H-gelatin substrate (16). Finally, seprase activity was detected by zymography in extracts of human breast cancer cell lines but not in extracts of a normal human breast cell line. Thus we believe that seprase has an important role in breast cancer cell invasion. To investigate the role of seprase in breast cancer cell invasion we proposed these specific aims:

1) Identify and characterize a full-length cDNA for human breast cancer seprase.
2) Investigate the contribution of seprase to the metastatic potential of breast cells.

Progress made towards completing these objectives is described in the body of this report in relation to the statement of work submitted with the original proposal. Our research suggests that the seprase species identified in chicken embryos and human breast cancers are related to but distinct from the seprase identified in human melanoma cells. The major areas of progress towards the first specific objective include our identification, isolation and characterization of two chicken embryo seprase-specific cDNA clones using a reverse transcriptase-polymerase chain reaction (RT-PCR) strategy. In addition, an apparently full length seprase cDNA has been amplified by RT-PCR from human breast cancer cell mRNA. In terms of monoclonal antibody production, further analysis of the antibodies elicited against partially purified seprase revealed that we were unsuccessful in our first attempt to produce seprase-specific monoclonal antibodies. A promising new approach for producing monoclonal antibodies has been taken that takes advantage of the nucleic acid sequence analysis of the chicken embryo seprase cDNAs that we have identified and characterized.

Three papers directly related to and supported by this project have been produced this year. Reprint, preprint, and manuscript are provided in the appendix

Kelly, T., S. Kechelava, T.L. Rozypal, K.W. West, and S. Korourian (1998). Seprase, a membrane-bound protease, is overexpressed by invasive ductal carcinoma cells of human breast cancers. *Mod. Pathol.* 11: 855-863.

Kelly, T., Y. Yan, R.L. Osborne, A.B. Athota, T.L. Rozypal, J.C. Colclasure, and W.S. Chu (1998). Proteolysis of extracellular matrix by invadopodia facilitates human breast cancer cell invasion and is mediated by matrix metalloproteinases. Clin. Exp. Metastasis. *In press.*

Kelly, T. (1998). Evaluation of seprase activity. *Submitted.*

Body

Personnel change:

Varsha Kaushal, Ph.D., Research Assistant Professor left the laboratory effective July, 1, 1998. Dr. Kaushal is responsible for the cloning the chicken embryo cDNAs described in this report. She also performed the nucleic acid sequencing and sequence analyses of the chicken embryo cDNAs. Dr. Kaushal produced the chicken embryo cDNA libraries described last year. Dr. Kaushal's salary was not supported by the DoD monies.

Other personnel:

Hailing (Helen) Zhang, is a graduate student in the Department of Pathology who continues to lead the effort to produce monoclonal antibodies directed against seprase. Ms. Zhang's stipend is supported completely by the Department of Pathology at UAMS.

Tricia Rozypal is a Research Technologist II who devotes 100 % of her effort towards this project. Ms. Rozypal is leading the seprase cDNA cloning effort and has successfully amplified an apparently full length cDNA for seprase from human breast cancer cells by RT-PCR. Ms. Rozypal's entire salary and benefits are provided by the DoD monies.

Thomas Kelly, Ph.D., is the PI on the project and continues to devote 60 % of his time towards data analysis and interpretation, deciding experimental strategies, writing and publishing the findings, and performing experiments pertinent to this project. The DoD monies provide 60 % of Dr. Kelly's salary and fringe benefits.

Progress in year 2

SOW Task 1. Months 1-3: Produce cDNA expression library with mRNA purified from human breast tumors.

As discussed in the previous report, we have already produced high quality chicken embryo cDNA expression libraries. Obtaining quality mRNA from human tumor samples has remained a persistent problem. Nevertheless, we believe immediate access to a limited number of human breast tumors will occur shortly. In the mean time, we have focused our efforts on RT-PCR cloning of the human breast cancer seprase cDNA from the breast cancer cell line MDA-MB-436 as described below.

SOW Task 2. Months 1-36: Produce additional seprase-specific probes for screening the cDNA expression library.

I) **Oligonucleotide probes based on seprase, fibroblast activating protein-α (FAP-α) and dipeptidyl peptidase IV (DPPIV) sequences.** Last year, another group produced a 2.4 kb full length seprase cDNA from LOX mRNA using FAP-α oligonucleotide primers and RT-PCR (1, 17). This work has shown that the proteolytically active 170 kDa form of human melanoma seprase is a dimer of the 97 kDa subunit (1).

The seprase that is expressed by its chicken embryos and is overexpressed by breast cancer cells in human tumors may represent a novel member(s) of the seprase, FAP-α, DPPIV family of integral membrane proteases. Supporting this notion is the fact that FAP-α oligonucleotide primers failed to amplify a cDNA from chicken embryo RNA.

Late last year, an alternative RT-PCR based cloning strategy was initiated after comparing the sequences of other seprase-family proteases. As reported last year, RT-PCR with degenerate oligonucleotide primers based on conserved sequences in the catalytic sites of these enzymes successfully amplified a 250 bp cDNA from chicken embryo mRNA. The 250 bp cDNA was subcloned and sequenced. Sequence analysis revealed 85 % homology to known serine integral membrane proteases, including seprase.

This year we amplified, cloned and characterized 780 bp chicken embryo seprase partial cDNA. Specific oligonucleotide primers were produced for the regions for which we had precise nucleic acid sequence information (3' end) obtained from the 250 bp catalytic domain cDNA amplified earlier. Degenerate oligonucleotide primers were produced for conserved regions further out towards the 5' end of the seprase cDNA. In this way we were able to amplify by RT-PCR, clone, and characterize the 780 bp cDNA that encodes most of the catalytic domain of chicken embryo seprase (appendix, Fig. 1). This 780 bp cDNA was used to probe the λZAP chicken embryo cDNA library. A 1.5 kb cDNA was identified, cloned and characterized. This cDNA contains the entire 780 bp nucleic acid sequence, an additional 220 bp extension of the 5' end of the coding region, and the complete catalytic domain as well as approximately 500 bp of the 3' untranslated region. The 1.5 kb cDNA encodes the C-terminal 341 amino acids of chicken embryo seprase (see deduced amino acid sequence data, appendix, Fig. 2). Thus far, a full length chicken embryo seprase cDNA has not been identified by probing the chicken embryo λZAP cDNA library. Currently we are employing a RACE, PCR strategy to extend the 5' end of the seprase cDNA using large oligonucleotide primers for the 5'-most region that we have sequenced.

These seprase-specific partial cDNAs from chicken embryos will also be used as probes to investigate expression of seprase by normal and malignant cells. In addition, these probes will be used to investigate the expression of seprase by control

[redacted]

transfectants and seprase-transfected cells. In future experiments, these probes will be useful to investigate the mechanism of seprase overexpression in human breast cancers.

II) **Production of monoclonal antibodies directed against seprase.**

Last year we reported that two hybridoma subclones 56.E6 and 8D2.E2 were identified that precipitated seprase activity in more than one immunoprecipitation assay. At that time we believed we had produced antibodies to seprase. This was an overly optimistic conclusion. Though reproduced in a few separate trials early on, the precipitation of seprase activity by these antibodies was always weak and never strong or convincing. Upon expanding these clones, the ability to precipitate seprase activity was completely lost. The final summary of this fusion is provided in the appendix (Fig. 3).

A major obstacle towards obtaining monoclonal antibodies to seprase has been the lack of pure antigen to inject. Strategies using partially purified native chicken embryo seprase of increasing purity have been unsuccessful three different times (twice before this project and once last year). Thus, we have switched our effort from using partially purified seprase to using pure seprase-specific peptides as the antigen.

Leading this effort is Hai Ling Zhang, a graduate student in the Department of Pathology.

Choosing peptides for use as antigens: We determined the amino acid sequence of the C-terminal 341 amino acids for chicken embryo seprase using the 1.5 kb seprase specific cDNA that we isolated and characterized (Appendix, Fig. 2). This stretch corresponds to amino acids 419-760 of human melanoma seprase and it is exposed to the extracellular environment (Appendix, Fig. 1A). To identify regions likely to elicit an immune response, the amino acid sequence we determined for chicken embryo seprase was analyzed using the Mac Vector 3.5 version 6.0 software (kindly supervised by Dr. Xuming Zhang, Department of Microbiology & Immunology, UAMS). This analysis predicted the hydrophilicity (Kyte-Doolittle), surface probability, flexibility, antigenic index, amphiphilic helix, amphiphilic sheet, and secondary structure of this 341 amino acid polypeptide (Appendix Figs. 4 & 5). Based on this information, two regions of the seprase polypeptide with high antigenic index were identified and selected for synthesis. Peptide 1 (LRKERCQYYTARFSERSK) is 18 amino acids in length and corresponds to amino acids 443-461 of human melanoma seprase (17) (Appendix, Figs. 1A, 6 & 7). Peptide 2 (YGGPCSQNVKHTFS) is 14 amino acids long and corresponds to amino acids 543-556 of human melanoma seprase (17) see (Appendix, Figs. 1A, 8 & 9). These peptides were synthesized and purified by Bio Synthesis, Inc.

[redacted]

Immunization of mice with seprase specific peptides. Peptides to be used as immunogens were covalently coupled to keyhole limpet hemocyanin (KLH) with glutaraldehyde as described by others (18). Female Balb c mice were injected IP with KLH-coupled peptide 1 (3 mice) or KLH-coupled peptide 2 (4 mice) emulsified 1:1 in Freund's complete adjuvant. Approximately 10 μg of peptide was injected into each mouse. At 3 weeks after the initial injection, mice were boosted with 10 μg of peptide by IP injections of the appropriate KLH-coupled peptide emulsified 1:1 in Freund's incomplete adjuvant. Ten days after the boost, approximately 200 μl of sera was collected from each mouse for testing by enzyme linked imunosorbent assay (ELISA) and immunoblotting. Two additional boosts were administered three weeks apart as described. Ten days following each boost, 200 μl of sera was collected and tested.

ELISA procedure and results: An ELISA for peptides 1 and 2 has been established to detect those hybridomas secreting antibody directed against these seprase specific peptides (Appendix, Figs. 10, 11 & 12). The ELISA will be useful as a sensitive and rapid, high through-put initial screen of hybridoma supernatants. Sera from bleeds of mice immunized with KLH-coupled seprase-specific peptides have been used to establish the ELISA as well as to identify mice to be sacrificed for monoclonal antibody production. Results from these experiments reveal that the ELISA is sensitive and specific for seprase peptides 1 and 2 (Appendix, Figs. 10, 11 & 12).

The ELISAs were performed as described in this section. In the first example shown each row (12 wells across) of a 96-well ELISA plate was coated with serial dilutions (1:5 to 1:10,240) of peptide overnight at 4°C (Appendix, Figs. 10 & 11). The 1:5 dilution was in column "1" and the 1:10.240 dilution in column 12. In the second example, wells in rows A, B, E & F are coated with the 1:200 dilution of appropriate peptide but wells in rows C,D, G & H are with a 1:500 dilution of the appropriate peptide (Appendix, Fig. 12). After the overnight incubation, the peptide solutions were removed and discarded. The wells were washed three times with phosphate buffered saline (PBS) containing 0.1% Tween-20. Free protein-binding sites were blocked by incubating the wells at 22° C for 2 h with 1 % bovine serum albumin (BSA) in PBS. The blocking solution was discarded and the wells washed three times with PBS, 0.1 % Tween-20. Duplicate rows were treated with identical dilutions of the antisera to be tested (for example 1:50 or 1:100). The antisera (100μl/well) were incubated with the immobilized peptides for 2 h at 22° C. The antisera were discarded and the wells washed three times with PBS, 0.1% Tween-20. Goat anti-mouse IgG coupled to alkaline phosphatase was diluted to 1:2000, added to each well (100μl/well), and incubated for 2 h at 22° C. This secondary antibody was discarded and the wells washed three times with PBS, 0.1% Tween-20. The colorimetric substrate (1 mg/ml p-nitrophenyl phosphate in 1 M diethanolamine, 0.5 mM MgCl2, pH 9.8) was added to each well (100μl/well) and incubated for 20 min at 22° C. Reactions were stopped with 25 μl of 3N NaOH. The absorbances at 405 nm

determined (Appendix, Figs. 11 & 12). Negative control wells include those i) coated with BSA instead of peptide, ii) those coated with peptide but substituting normal mouse serum instead of the experimental sera, and iii) those coated with peptide, incubated with antisera, but not incubated with the secondary antibody (Fig. 12).

After the initial injection and first boost, all but one mouse produced a antisera with titer against the peptide antigen that was detected by the ELISA. Currently all mice have produced sera that recognizes the peptide used for immunization. However, only the most recent bleeds of several, but not all of the mice, react weakly with protein bands at approximately 160 kDa and 97 kDa on immunoblots of partially purified chicken embryo seprase (not shown). Sera from these most promising mice are being tested for ability to immunoprecipitate the gelatinase activity of seprase.

When the mice have titer to native seprase is sufficient to convincingly immunoprecipitate seprase activity then they will be used for monoclonal antibody production.

SOW Task 3. Months 3-18: Identify clones with full-length seprase cDNA inserts.

Human breast cancer seprase: We have amplified an approximately 2.4 kb cDNA by RT-PCR with RNA isolated from human MDA-MB-436 invasive breast cancer cells (Fig. 13). This cDNA was amplified using the same FAP-α oligonucleotide primers (FAP1 and FAP6) used to clone seprase from human melanoma cells (1, 17). Thus, we believe that the 2.4 kb cDNA amplicon is a full length cDNA encoding human breast cancer seprase. In addition, an approximately 1 kb amplification product of unknown identity was also produced with the seprase/ FAP-α specific primers (Fig. 13). To date we have not cloned either the 2.4 kb or 1 kb cDNA amplicons. Because it is critical for us to clone the full length cDNA as quickly as possible, we have arranged to get help from Dr. Barbara L. Parsons at the National Center for Toxicological Research with cloning this putative full length seprase cDNA. Dr. Parsons' is an experienced molecular biologist who was listed as a consultant on the original grant application. Ms. Rozypal and Dr. Kelly will travel to Dr. Parsons' laboratory to clone the 2.4 kb cDNA RT-PCR amplicon encoding human breast cancer seprase.

Chicken embryo seprase: Partial cDNAs for chicken embryo seprase have been cloned and characterized as described above. Obtaining the 5' end of the cDNA has been troublesome. We suspect that the nucleic acid sequence in this region of chicken embryo seprase which encodes the cell-proximal carbohydrate rich domain, membrane spanning domain, and cytoplasmic domain is more divergent from the published seprase sequence and possible GC rich regions that are difficult to denature and amplify. Effort to obtain the full length chicken embryo cDNA will continue as described earlier.

SOW Task 4 Months 4-24: Confirm full-length seprase cDNA clones.

This task has not been accomplished because we still lack a full length cDNA clone for seprase. However, we are not far behind schedule because we have been able to produce a putative full length cDNA for human breast cancer seprase by RT-PCR. Thus, we anticipate cloning this cDNA will be relatively straight-forward and expect to it to be completed by early November, 1998. Upon cloning the 2.4 kb RT-PCR amplification product, we will immediately proceed with confirming the clones by nucleic acid sequencing, expression of the protein, and other procedures detailed in the original proposal.

SOW Task 5 Months 18-36: Produce stable transfectants of malignant and normal breast cells that overexpress assembled, active seprase to the cell surface. Task 5 months 18-36

Task 5 requires an isolated, characterized and confirmed full-length seprase cDNA. No progress has been made on this task yet. However, we are still within the anticipated time frame for this task.

Preparations for future completion of SOW Tasks 6 & 7.
Task 6. Months 24-48: Determine effects of seprase overexpression on cell-mediated matrix proteolysis.
Task 7. Months 30-48: Determine effects of seprase overexpression on breast cell invasion of extracellular matrix.

An immunohistochemical study documenting the overexpression of seprase by invasive ductal carcinoma cells of human breast cancers was published this year. This study was begun prior to submitting the original research proposal to the US Army BCRP. Completion and publication of this study provides peer-reviewed support for the hypothesis that seprase has a role in the malignant progression of human breast cancer. (A reprint is included in the appendix).

Last year we reported submitting a manuscript describing a completed study evaluating human breast cancer cells for invadopodial proteolysis of extracellular matrix and invasiveness. This study is now in press and demonstrates the assays that will be used to determine proteolysis of matrix and invasiveness (Page proofs included in the appendix).

We have also completed and submitted a study documenting a method for quantifying the proteolytic activity of seprase. This seprase-activity assay will be an important method for measuring increased seprase activity in cells genetically engineered to

overexpress seprase. Such determinations will help establish that the seprase overexpressed in transfected cells is functionally active (The submitted manuscript is included in the appendix).

Conclusions:

This past year has been successful and much progress has been made towards completing the goals of the project. Specifically, production of a full length human breast cancer seprase cDNA by RT-PCR ensures that the goals of the project will be completed before the end of the four study period. Obtaining the full length seprase cDNA was the most time consuming and riskiest part of this project. Now that the cDNA has been produced, it is quite likely that all of the proposed experiments will be completed in a timely fashion. Secondly, three manuscripts pertaining to the project have been prepared and submitted. Of these, one is published, one is in press, and the third is in the peer-review process. Thus, this research continues to be productive, providing new information about seprase and breast cancer cell invasion.

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Appendix

Role of seprase in breast cancer invasion
Second year progress report
September 29, 1998

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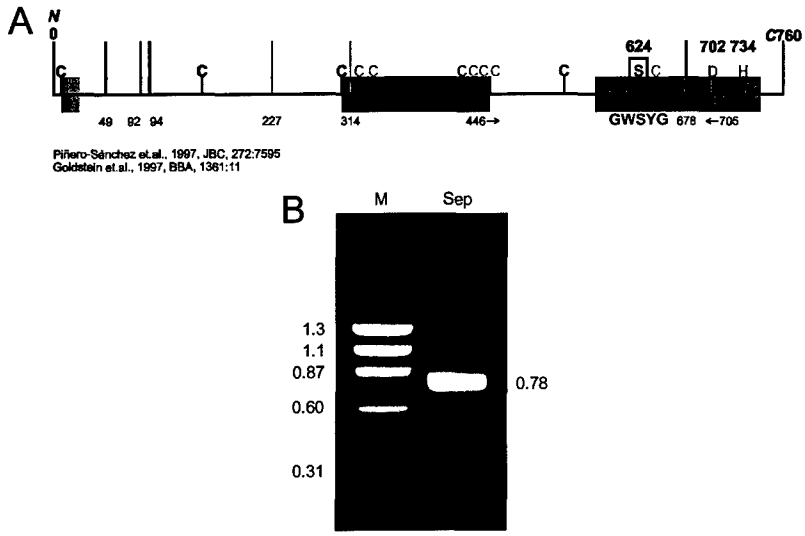

Figure 1. RT-PCR cloning of a partial seprase cDNA from chicken embryo RNA

A) The deduced cytoplasmic (black), membrane-spanning (blue), glycosylation-rich (yellow), cysteine-rich (pink) and catalytic (green) domains of human melanoma seprase. Putative glycosylation sites (|), cysteine residues (C), and catalytic residues (S, D, H, & GWSYG) that are conserved between all members of the integral membrane serine protease family are noted in blue, those conserved between seprase and DPPIV are shown in pink, and those that are not conserved are noted in black. Arrows denote the location of the RT-PCR primers used to amplify chicken embryo seprase.

B) Agarose gel electrophoresis showing the 780 bp RT-PCR amplification product produced from a chicken embryo cDNA library (Sep). Molecular sizes of the MX174 Hae III digest fragments (M) are given on the left.

Figure 2. Sequence of the C-terminal 341 amino acids of chicken embryo seprase deduced from the sequence of the 1.5 kb chicken embryo seprase cDNA.

1 SARGNIYKIS IGSKPIRKLC ITCNLRKERC QYYTARFSER SKYYALICYG

51 PGIPISTLFE NESDRELRIL EDNQELQSAL QEIILPKEEI NKLEVDGITL

101 WYKMLIPPQF DRSKKYPLLI QVYGGPCSQN VKHTFSISWI TYLASKEGII

151 VALVDGRGTA YQGDKILHAV YRRLGVYEVE DQISAVKKFI EMGFIDEKRI

201 AIWGWSYGGY VTSLALGSGS GVFKCGIAVA PVSSWEYYAS IYTERFMGLP

251 VESDNLEHYK NSTVMARAKN FQNVEYLLIH GTADDNVHFQ NSAQIAKALV

301 NAQVDFQAMW YTDQNHGIPG LSSKHLYTHM THFLKQCFSL S

Figure 3. Summary of previous hybridoma fusion that failed to produce anti-seprase monoclonal anitbodies.

As reported in the last progress report, mice were immunized with partially purified chicken embryo seprase. Sera from one mouse was shown to immunoprecipitate seprase activity and react with a 160 kDa protein in chiken embryo extracts. This mouse was sacrificed and used to produce hybridomas.

Summary of the fusion results:

583 clones grew up with HAT selection in 96 well plates. Each clone was transferred to a single well of a 24 well culture plates in 1 ml HAT growth medium. (5/29/1997-6/11/1997)

All clones were tested by immunoprecipitation and gelatin zymography to detect those supernatants producing antibodies capable of precipitating the proteolytic activity of seprase. (5/30/1997-6/30/1997)

21 hybridomas out of the original 583, were scored positive for immunoprecipitation of seprase activity. To avoid overgrowing and killing the clones, the positive wells were frozen until testing was completed.

Only 12 of the original 21 clones survived the freezing procedure.

The 12 remaining clones were subcloned by limiting dilution into 96 well plates.

Tested clones that grew up by immunopreciptiation. Subclone 8D2.E2 tested positive on two separate tests on 7/23/1997 and again on 7/25/97. Subclone 56.E6 tested weakly positive on 7/29/1997 and retested faintly positive on 7/30/1997.

Prepared ascites fluids of each of these hybridomas in an attempt to produce high-titer antibodies directed against seprase. Ascites fluid from the mice failed to immunoprecipitate seprase activity.

We were subsequently unable to successfully immunoprecipitate seprase activity with either 8D2.E2 or 56.E6 in any later tests.

We conclude that we lost the clones that were secreting antibodies against either seprase or seprase-associated proteins. The reason for this loss is unclear but could have been due to any of a number of well known problems associated with hybridomas and the cloning procedures.

[redacted]

Figures 4-9. Identification of antigenic regions in the C-terminal 341 amino acids of chicken embryo seprase.

The amino acid sequence determined for the C-terminal 341 amino acids was analyzed by for antigenic and exposed sites using the Mac Vector 3.5, version 6.0 software. Based on these data, peptides corresponding to amino acids 25-42 (peptide 1) and 123-136 (peptide 2) of this 341 amino acid polypetide were chosen for synthesis.

Figs. 4 & 5. C-terminal polypeptide The entire 341 amino acid polypeptide is analyzed for hydrophilicity, surface probability, flexibility, antigenic index, amphiphilic index, amphiphilic sheet, and secondary structure. Regions corresponding to peptides 1 and 2 are indicated by yellow high lighting on the antigenic index plot.

Figs. 6 & 7. Detailed analysis of peptide 1 (LRKERCQYYTARFSERSK) A 22 amino acid region of the C terminal 341 amino acid polypeptide including peptide 1 (yellow highlight on antigenic index plot) analyzed for hydrophilicity, surface probability, flexibility, antigenic index, amphiphilic index, amphiphilic sheet, and secondary structure.

Figs. 8 & 9. Detailed analysis of peptide 2 (YGGPCSQNVKHTFS) A 35 amino acid region of the C terminal 341 amino acid polypeptide including peptide 2 (yellow highlight on antigenic index plot) analyzed for hydrophilicity, surface probability, flexibility, antigenic index, amphiphilic index, amphiphilic sheet, and secondary structure.

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Untitled1 Protein Toolbox Plot
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Figure 4.

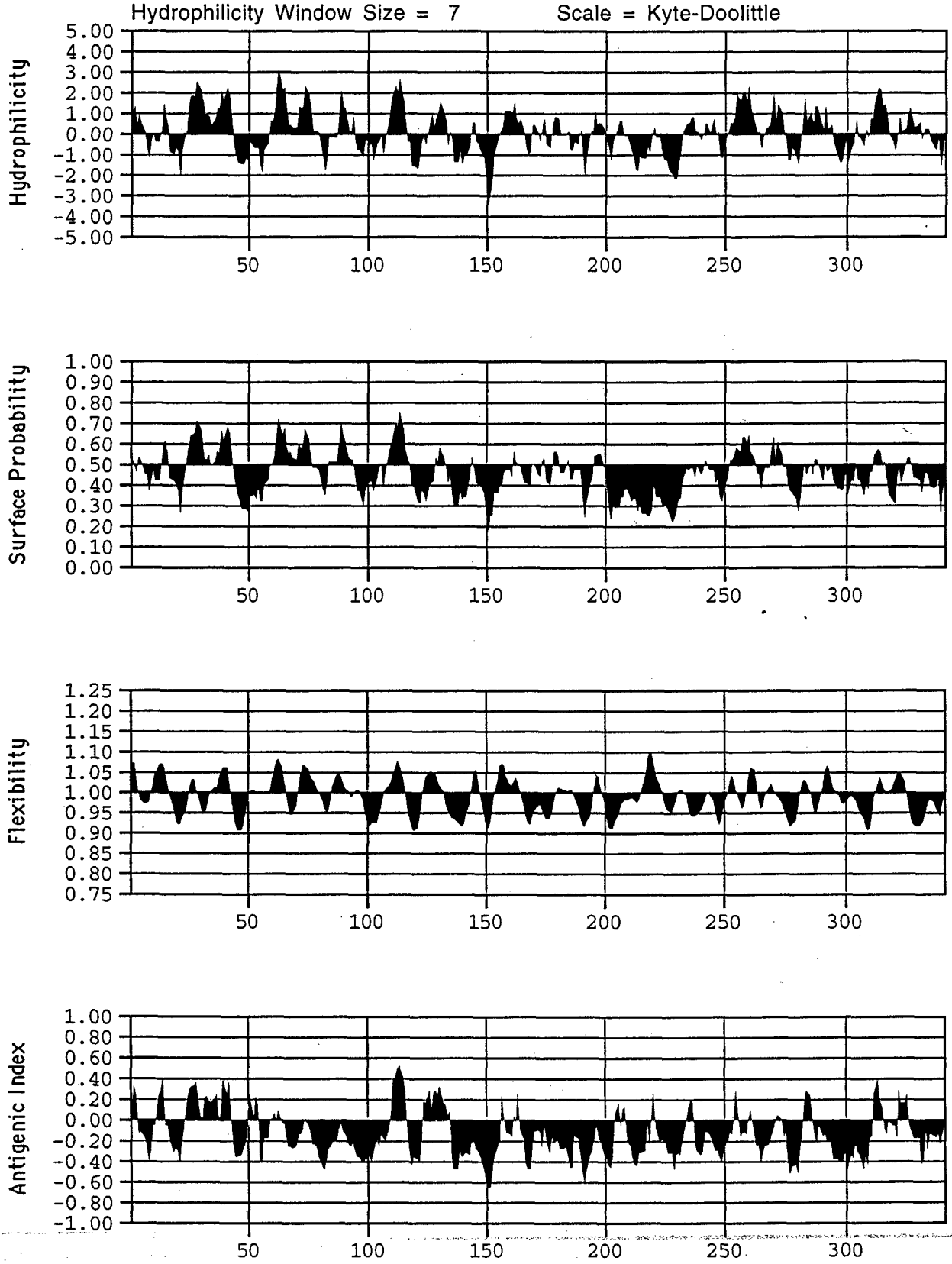

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Figure 5.

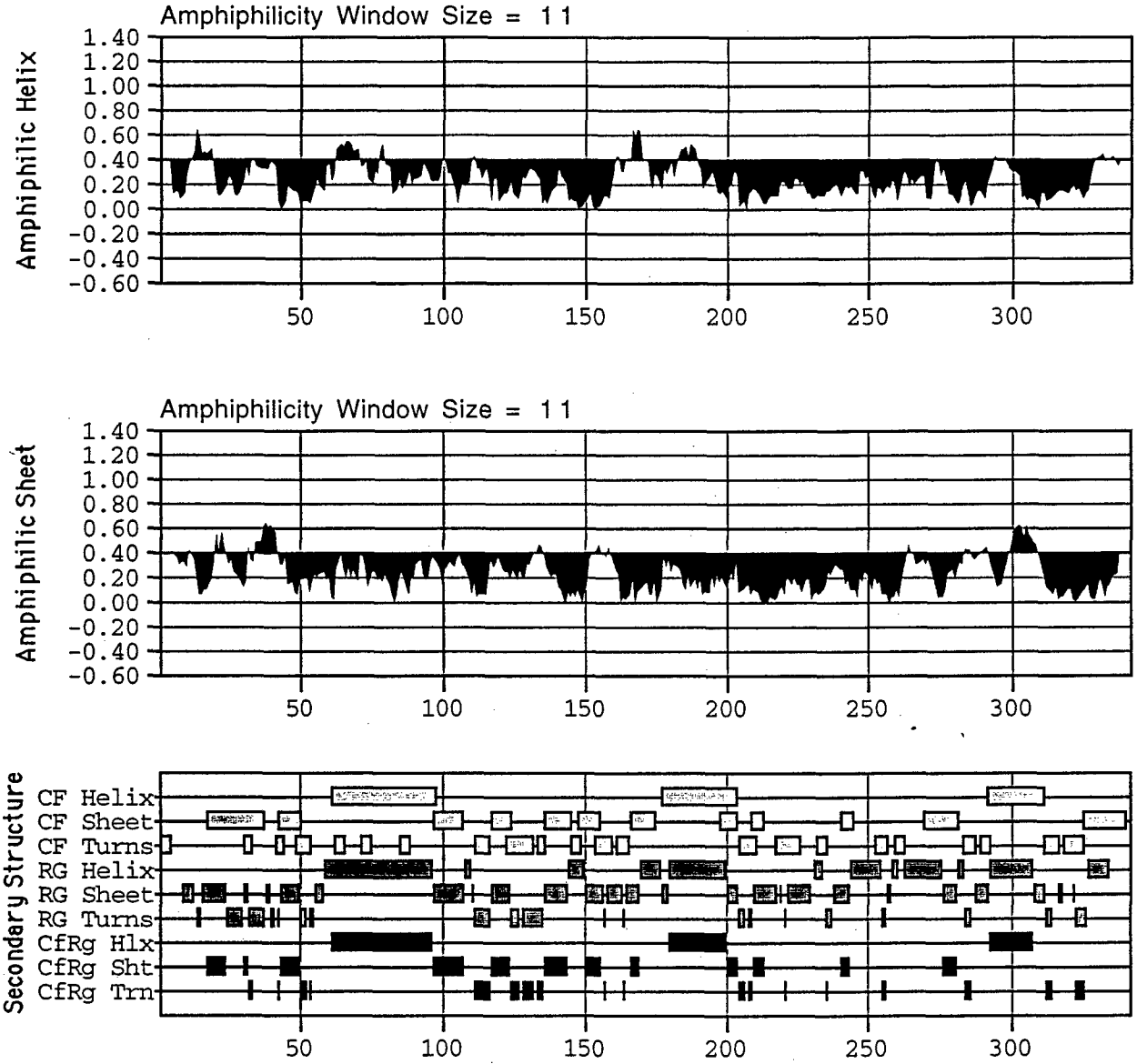

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Figure 6.

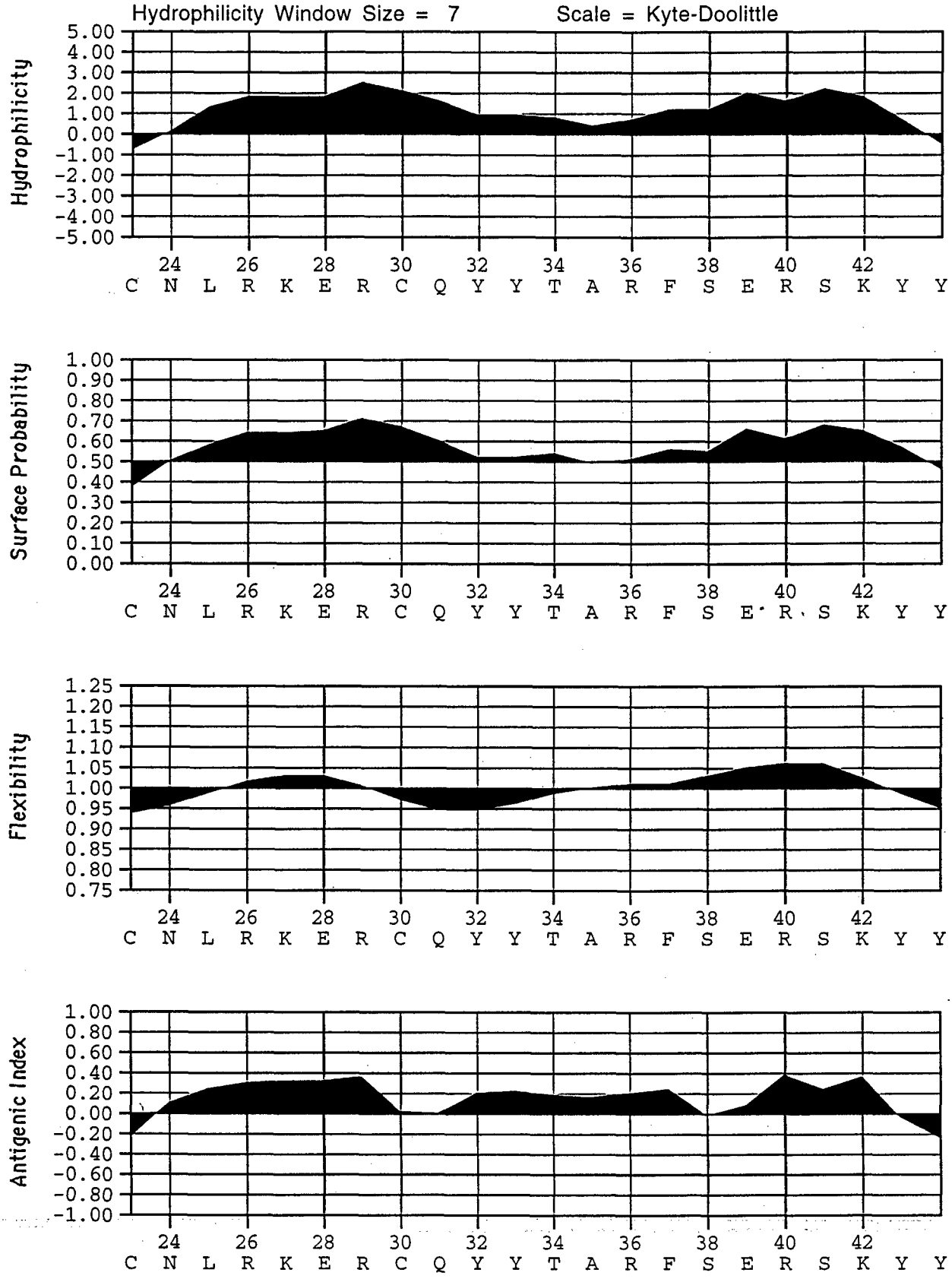

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Figure 7.

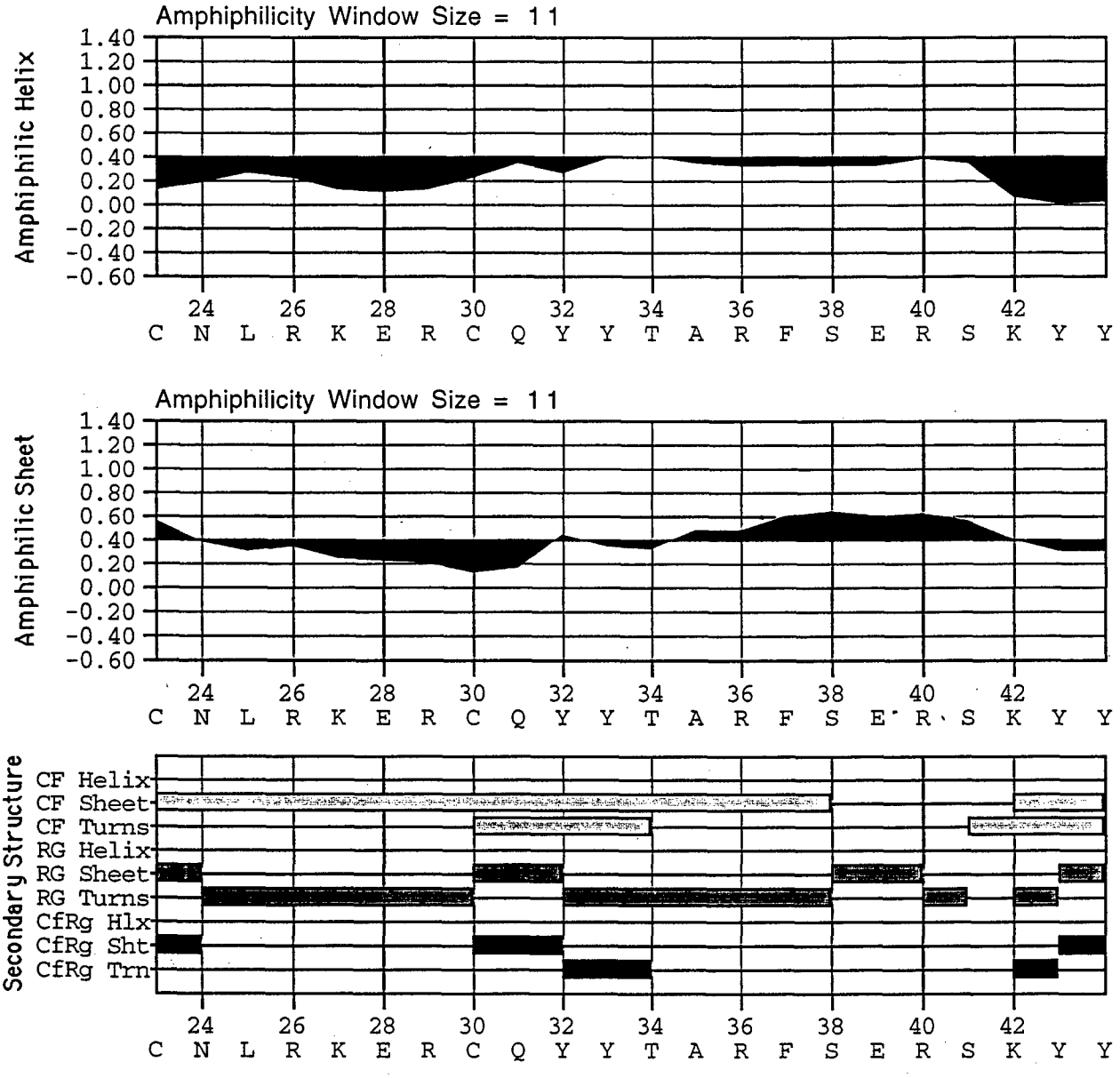

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Figure 8.

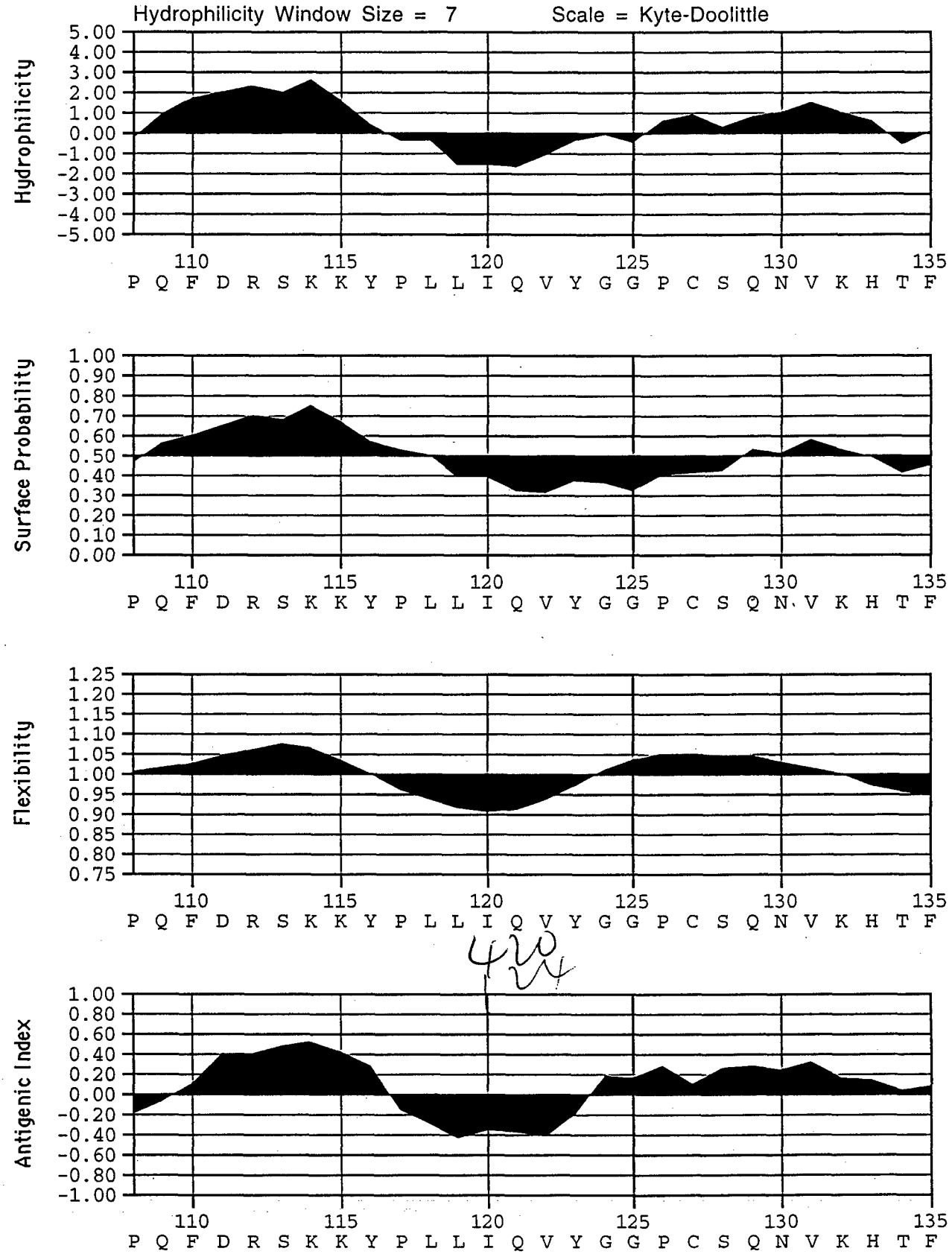

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Figure 9.

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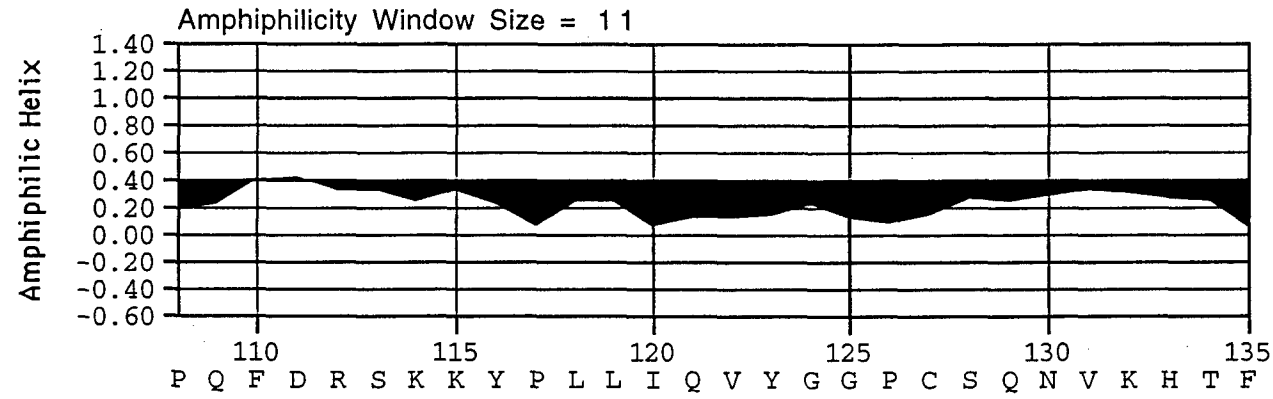
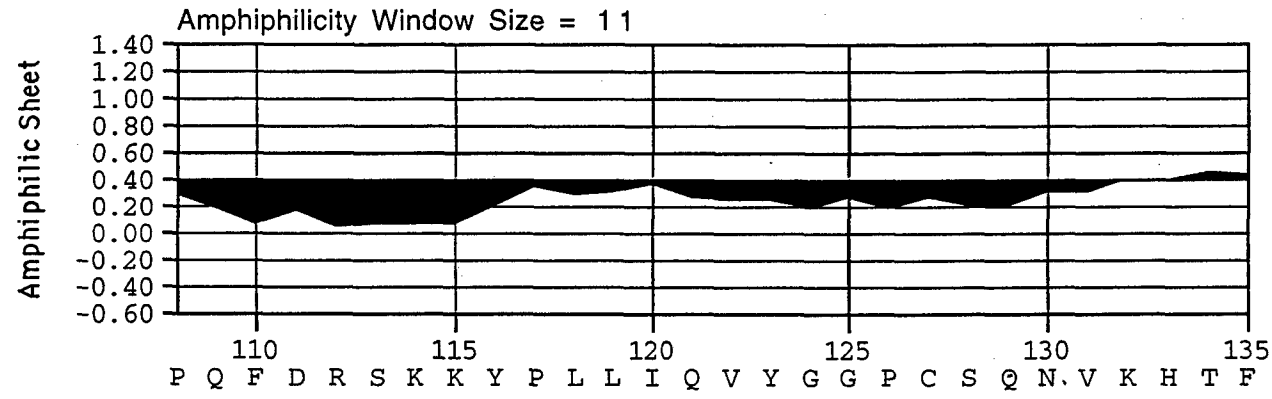
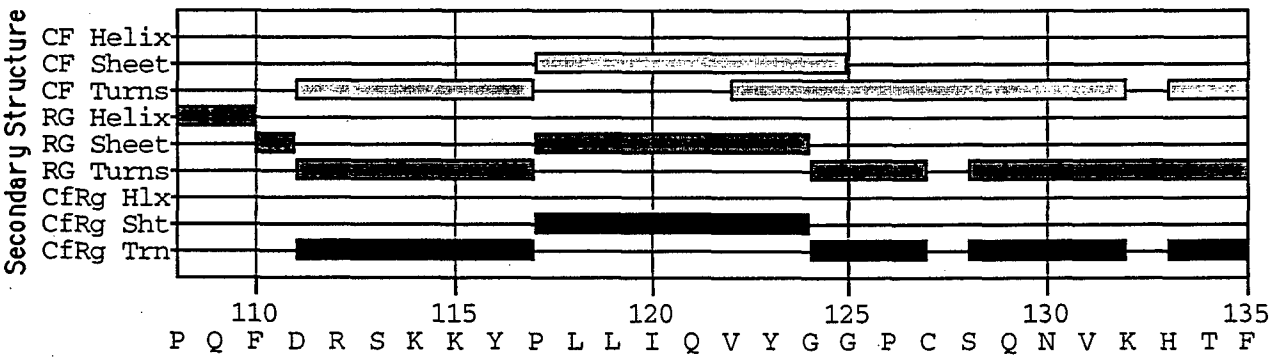






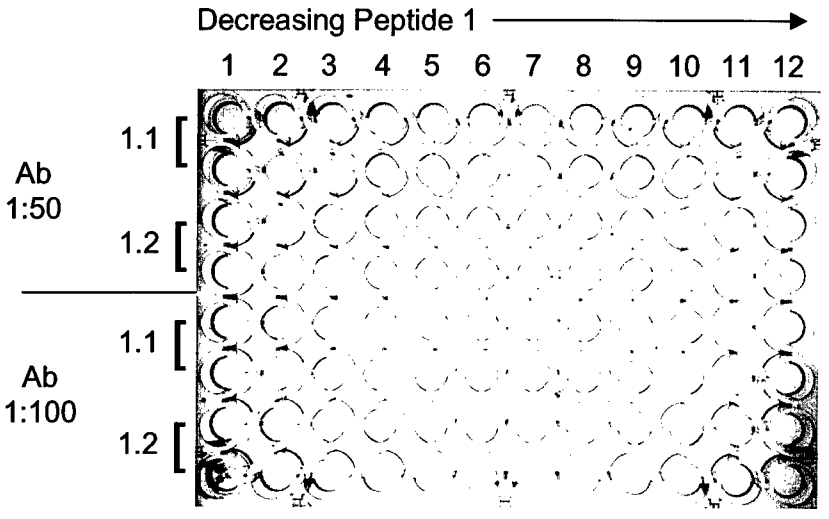


Figure. 10. ELISA analysis of sera from mice 1.1 and 1.2 immunized with peptide 1.

Photograph of a 96 well plate at the end of an ELISA. Sera obtained after the first boost from mouse 1.1 (rows A, B, E, & F) and mouse 1.2 (rows C, D, G, H) were used at 1:50 dilution (A-D) and 1:100 dilution (E-H). The wells were coated with serial dilutions of peptide 1 in each column (1-12) such that in column 1 the peptide was diluted 1:5, column 2 peptide was diluted 1:10, and so fourth through column 12 where peptide 1 was diluted 1:10,240. Serum from mouse 1.1 had a relatively high titer to peptide 1 and produced a yellow reaction color that decreased in intensity with dilution of the antigen (Rows A & B; E & F). In contrast serum obtained after the first boost from mouse 1.2 was of relative low titer and did not detect immobilized peptide 1 (Rows C & D; G & H).

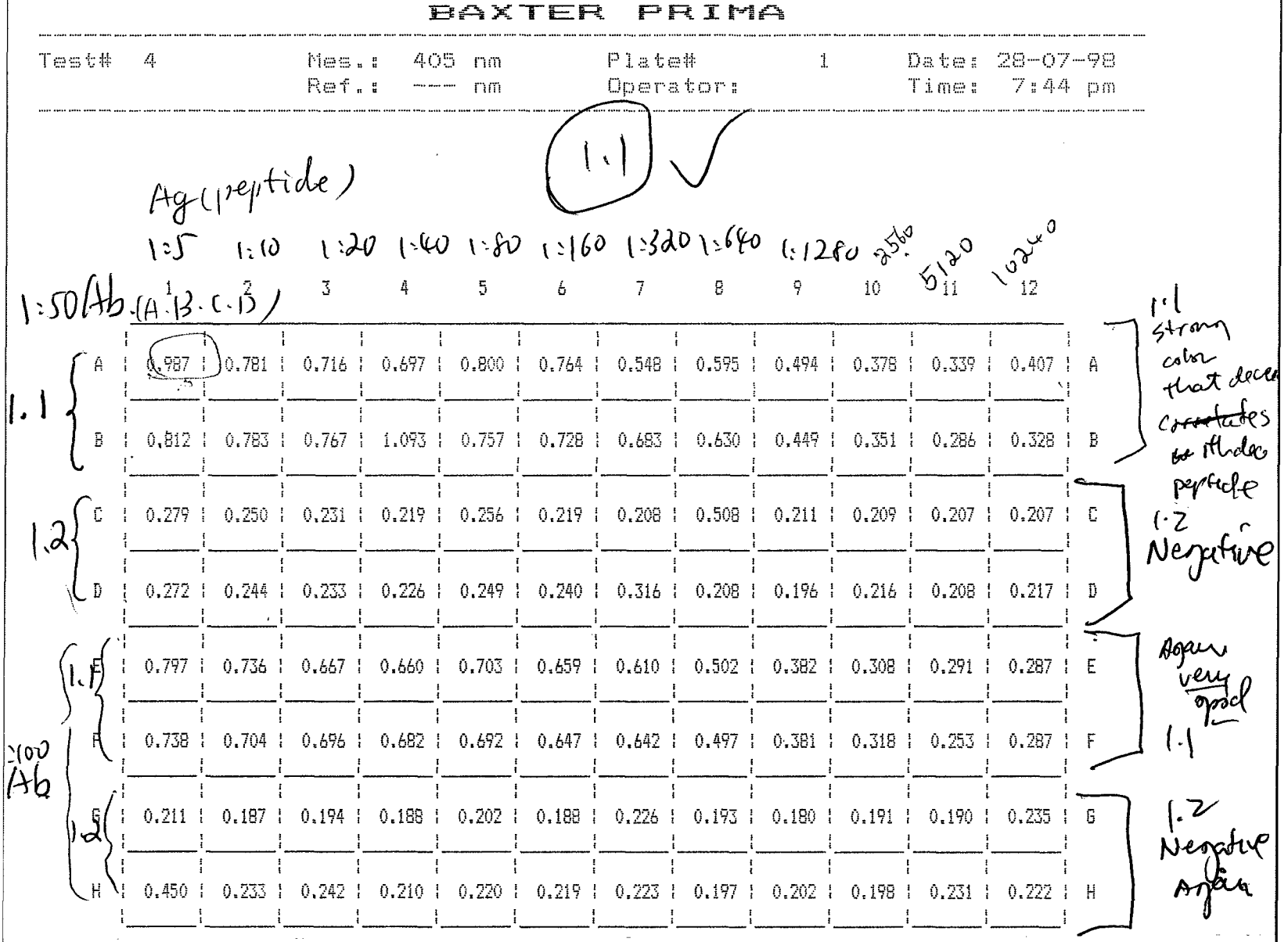
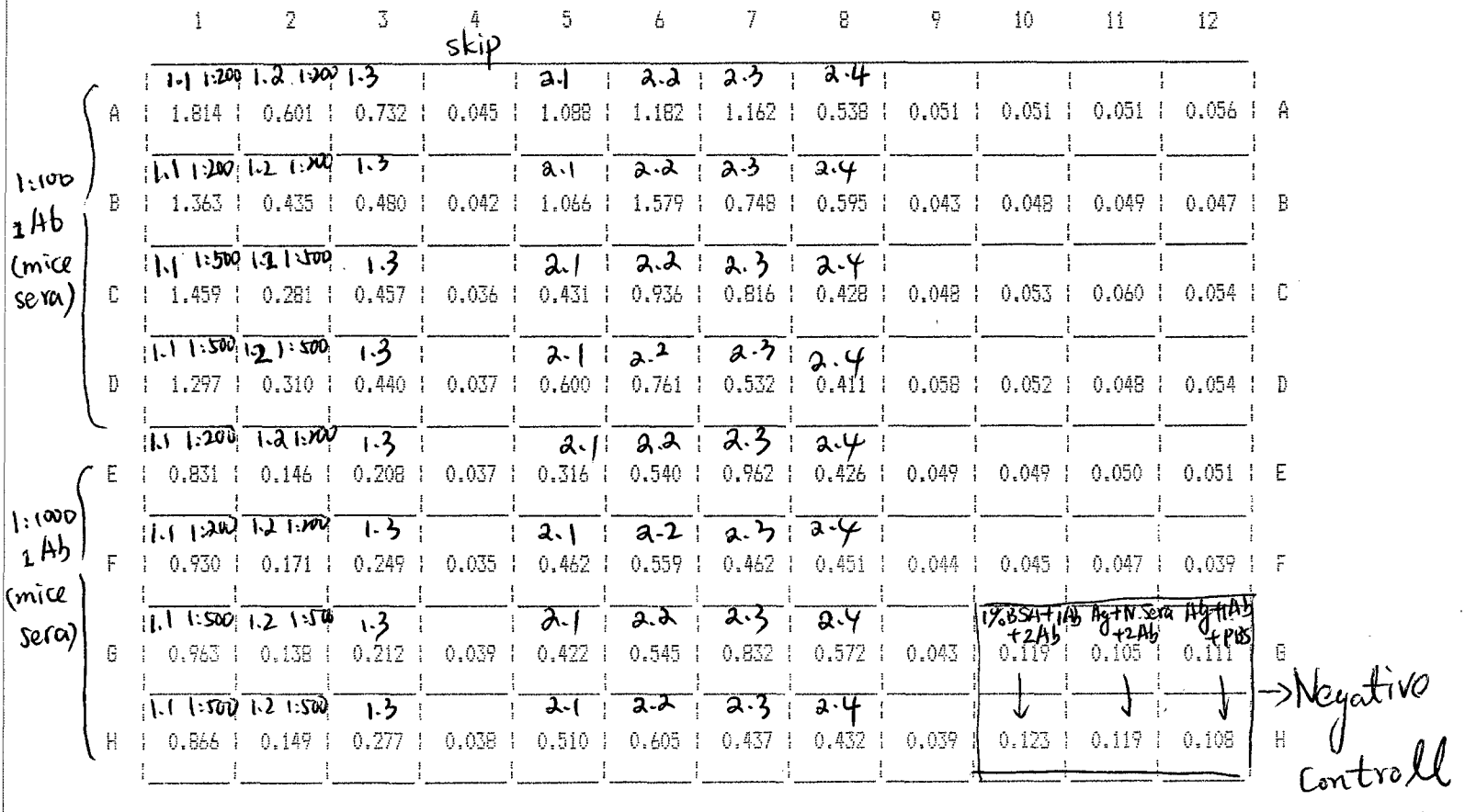


Figure 11. Determinations of the OD 405 for ELISA testing sera for titer to peptide 1.

Print out from a microtiter plate reader of the 96 well plate shown in figure 10 at the end of the ELISA. As described in figure 10, sera obtained after the first boost from mouse 1.1 (rows A, B, E, & F) and mouse 1.2 (rows C, D, G, H) were used at 1:50 dilution (A-D) and 1:100 dilution (E-H). The wells were coated with serial dilutions of peptide 1 in each column (1-12) such that in column 1 the peptide was diluted 1:5, column 2 peptide was diluted 1:10, and so fourth through column 12 where peptide 1 was diluted 1:10,240. Serum from mouse 1.1 had a relatively high titer to peptide 1 and produced a absorbance at 405 nm that decreased in intensity with dilution of the antigen (Rows A & B; E & F). In contrast serum obtained after the first boost from mouse 1.2 was of relative low titer and did not detect immobilized peptide 1 (Rows C & D; G & H).

BAXTER PRIMA

Test# 4 Mes.: 405 nm Plate# 1 Date: 14-08-98
Ref.: --- nm Operator: Time: 2:00 pm



	1	2	3	4	5	6	7	8	9	10	11	12	
	1.1 1:200	1.2 1:200	1.3		2.1	2.2	2.3	2.4					
A	1.814	0.601	0.732	0.045	1.088	1.182	1.162	0.538	0.051	0.051	0.051	0.056	A
	1.1 1:200	1.2 1:200	1.3		2.1	2.2	2.3	2.4					
B	1.363	0.435	0.480	0.042	1.066	1.579	0.748	0.595	0.043	0.048	0.049	0.047	B
	1.1 1:500	1.2 1:500	1.3		2.1	2.2	2.3	2.4					
C	1.459	0.281	0.457	0.036	0.431	0.936	0.816	0.428	0.048	0.053	0.060	0.054	C
	1.1 1:500	1.2 1:500	1.3		2.1	2.2	2.3	2.4					
D	1.297	0.310	0.440	0.037	0.600	0.761	0.532	0.411	0.058	0.052	0.048	0.054	D
	1.1 1:200	1.2 1:200	1.3		2.1	2.2	2.3	2.4					
E	0.831	0.146	0.208	0.037	0.316	0.540	0.962	0.426	0.049	0.049	0.050	0.051	E
	1.1 1:200	1.2 1:200	1.3		2.1	2.2	2.3	2.4					
F	0.930	0.171	0.249	0.035	0.462	0.559	0.462	0.451	0.044	0.045	0.047	0.039	F
	1.1 1:500	1.2 1:500	1.3		2.1	2.2	2.3	2.4					
G	0.963	0.138	0.212	0.039	0.422	0.545	0.832	0.572	0.043	0.119	0.105	0.111	G
	1.1 1:500	1.2 1:500	1.3		2.1	2.2	2.3	2.4					
H	0.866	0.149	0.277	0.038	0.510	0.605	0.437	0.432	0.039	0.123	0.119	0.108	H

Figure 12. ELISA of sera from mice immunized with peptides 1 and 2. Print out from a microtiter plate reader of the 96 well plate at the end of an ELISA testing sera obtained after the second boost from all mice injected with peptides 1 and 2. The sera were diluted to 1:100 (A-D) and 1:1000 (E-H). The wells were coated with 1:200 dilutions of the appropriate peptide (A & B, E & F) or 1:500 dilutions of the appropriate peptide (C & D; G & H). All sera had good reactivity with the peptides used as immunogen. In contrast, none of the negative control samples developed any reaction product (Rows G & H, Columns 10, 11 & 12). Negative controls included coating the plate with BSA instead of peptide then incubating with sera from mouse 1.1 and treating as described (Rows G & H; Column 10). Another control was substituting normal mouse serum (non-immune) for the primary antibody in wells coated with peptide 1 and treating as described (Rows G & H; Column 11). Finally, wells coated with peptide 1 were exposed to mouse 1.1 sera but the alkaline phosphatase conjugated second antibody was omitted (Rows G & H; Column 12). All 3 negative controls had acceptably low absorbances at 405 nm.

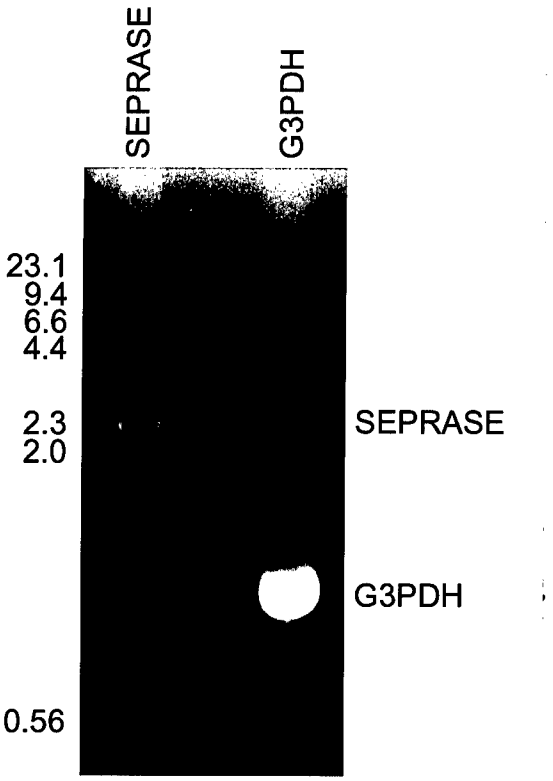


Figure 13. RT-PCR amplification of an apparently full length seprase cDNA from MDA-MB-436 human breast cancer cells.

Agarose gel electrophoresis showing the 2.4 kb and 1 kb amplification products produced with seprase-specific primers by RT-PCR from MDA-MB-436 human breast cancer cells (Sep). Glyceraldehyde 3 phophate dehydrogenase (G3PDH) was efficiently amplified from the same RNA preparation using G3PDH specific primers. Molecular sizes of the λ Hind III digest fragments (M) are given on the left x 10^{-3}.

Seprase, a Membrane-Bound Protease, Is Overexpressed by Invasive Ductal Carcinoma Cells of Human Breast Cancers

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The increased cell surface expression of the serine integral membrane protease, seprase, has been associated with the invasive behavior of human melanoma cell lines *in vitro*. The present study investigates the expression of seprase in malignant, premalignant, benign, and normal human breast tissues. The 170-kDa gelatinase activity of seprase was identified in extracts of infiltrating ductal carcinomas (IDC). Protein bands corresponding to the proteolytically active 170-kDa seprase dimer and its 97-kDa seprase subunit protein were identified by immunoblot analysis of IDC extracts using an antiserum elicited against immunoaffinity-purified seprase. Immunohistochemical analysis of seprase expression in 41 formalin-fixed and paraffin-embedded specimens of human breast tissue revealed preferential immunoreactivity with the malignant cells of IDC (27 cases). Within individual IDC specimens, the stromal cells or morphologically normal epithelium revealed low labeling that was always significantly less than the labeling of neoplastic cells. Lymph node metastases of IDC cells were also strongly positive, but the lymphoid tissue in affected nodes was not stained. Neoplastic cells in DC *in situ* (5 cases) exhibited variable levels of staining. Epithelial cells of benign fibroadenoma specimens (2 cases) and benign proliferative breast disease (5 cases) exhibited little or no immunoreactivity. Epithelial cells of normal breast tissue (1 case) were not stained. The overexpression of seprase by DC cells is consistent with seprase having a role in facilitating invasion and metastasis of IDC of the breast. The cell surface localization of seprase could be used to target therapeutic agents to malignant breast cells.

KEY WORDS: Extracellular matrix, Invadopodia, Invasion, Metastasis, Seprase, Serine integral membrane protease.

Mod Pathol 1998;11(9):855–863

Invasion with or without metastatic spread of breast cancer to distant sites has already occurred in a significant number of patients by the time their disease is detected (1). Significant improvement in patient survival might be achieved by halting the invasion process and containing the metastatic spread and growth of the disease. Extracellular matrix–degrading proteases are important in invasion and metastasis because their activities enable breast cancer cells to cross basement membrane and stromal extracellular matrix barriers and thus escape the breast (2, 3).

Invading breast cancer cells, like a variety of other invasive tumor cells, extend specialized plasma membrane protrusions called *invadopodia* that contact and degrade extracellular matrix (4, 5). Invadopodia can degrade many different purified extracellular matrix proteins as well as fully assembled natural extracellular matrices, suggesting that proteases capable of degrading most, if not all, extracellular matrix structural proteins are located on the surface of invadopodial membranes (6, 7). Several invadopodial proteases were identified (8); and one, seprase, was proposed as a marker of tumor cell invasiveness (7). Seprase is a serine integral membrane protease that was initially identified as a 170-kDa gelatinase activity in extracts of the invasive LOX human melanoma cell line (9). This activ-

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ity was not detected in noninvasive human melanoma cells lines. A similar, seprase-like protease was later identified as a 160-kDa gelatinase in transformed chicken embryo fibroblasts (6). In cell extracts, seprase exists as a 97-kDa monomer that is enzymatically inactive and as a noncovalently bound dimer of the 97-kDa protein that form the 170-kDa proteolytically active enzyme (10). The active dimer (170 kDa) can be entirely converted to the 97-kDa monomer protein by histidine modifying agents, heat, or exposure to acidic pH (10).

This study was performed to investigate seprase expression in malignant and benign human breast tissues using an antiserum raised against affinity-purified seprase. This antiserum reacts specifically with both the 97-kDa seprase protein and the 170-kDa active seprase dimer in extracts of human breast carcinomas. Immunohistochemical analysis reveals that infiltrating ductal carcinoma (IDC) cells are labeled intensely by the antiserum but surrounding stromal cells, lymphoid infiltrate, adipose tissue, and normal epithelial cells are not labeled or are labeled to a low level. DC *in situ* (DCIS) cells exhibited variable staining with antiserum to seprase, but hyperplastic cells in benign fibroadenomas and proliferative breast disease were only weakly labeled. Thus, seprase overexpression by malignant breast cells might be exploited for clinical prognosis and treatment.

MATERIALS AND METHODS

Generation of an Antiserum Against Seprase

White Leghorn chicken embryos, 9 days old, were purchased from Truslow Farms (Chestertown, MD). Seprase was purified from 220 (396 g) embryos. Various peripheral membrane proteins were removed by sequential extractions. First, the embryos were homogenized in 10 volumes of 10 mM Tris and 5 mM EDTA (pH 7.6). The insoluble material was collected by centrifugation and extracted with the same, low ionic strength buffer for 30 minutes at 37° C. The insoluble material was extracted with 10 mM Tris/hydrochloric acid (HCl), 0.6 M sodium chloride (NaCl), and 5 mM EDTA (pH 7.6) for 12 hours at 4° C. After each extraction, the insoluble material was collected by centrifugation, and the supernatant was discarded. The pellet was then extracted with 2.6% Triton X-114, 10 mM Tris/HCl, and 5 mM EDTA (pH 7.6). The supernatant (detergent extract) was recovered after centrifugation and used to continue purifying the protease.

The detergent extract was phase partitioned (11), and the detergent phase was separated from the aqueous phase by centrifugation. The detergent phase was diluted with cold deionized water (to 1L) to achieve a final concentration of less than 20 mM NaCl. The detergent phase was loaded at 70 mL/hr onto an 80-mL diethylaminoethyl-cellulose column (Whatman DE52; Whatman International, Hillsboro, OR) equilibrated in 0.5% Triton X-100, 5 mM EDTA, and 10 mM Tris/HCl (pH 7.5). The column was washed with 560 mL equilibration buffer, then eluted at 20 mL/hr with a 560-mL continuous gradient, ranging from 0 to 400 mM NaCl. Fractions (7 mL) were collected, and those with seprase activity were identified by zymographic analysis and pooled for purification on a monoclonal antibody (mAb) D43 column. The D43 mAb is a rat immunoglobulin (Ig) G2A that precipitates seprase activity and reacts with the 170-kDa active seprase dimer but not the 97-kDa monomer on immunoblots (7, 10).

Immunoaffinity columns were prepared by binding rabbit antirat IgG to protein A agarose and then binding mAb D43 to the rabbit antirat IgG. The resulting protein A agarose, rabbit antirat IgG, and mAb D43 complex was covalently linked together using dimethyl suberimidate-2HCl (Affinica Kit; Schleicher & Schuell, Keene, NH). Approximately 37 mg of diethylaminoethyl-purified seprase was loaded onto a 1-mL protein A agarose column linked in series to a second 1-mL D43 affinity column. Unbound proteins were washed from the columns with 10 ml of 0.5% Triton X-100, Tris-buffered saline (TBS; 20 mM Tris/HCl [pH 7.6], 150 mM NaCl) and 5 mM EDTA. The columns were then separated and eluted individually with 0.1% Triton X-100 and 0.1 M glycine/HCl (pH 2.4). Fractions (1.5 mL) were collected in tubes containing 70 μL of 2 M Tris base to neutralize the pH. For production of antisera, immunoaffinity purified seprase was injected into a 3-month-old female Harlan Sprague Daly rat. For injections, seprase was mixed 1:1 with Freund's Complete Adjuvant (GIBCO-BRL, Gaithersburg, MD), followed 4 weeks later with a boost (1:1), with Freund's Incomplete Adjuvant (GIBCO-BRL).

Preparation of Human Breast Carcinoma Tissue Extracts

The breast tumors that were used for biochemical and immunohistochemical analysis in this study did not receive radiation or chemotherapy before surgical excision of the tumor. Fresh human breast cancer tissue, identified by pathologists as IDC, was washed three times with cold phosphate-buffered saline (pH 7.4) to remove extracellular proteins and homogenized in 1% (v/v) Triton X-114 in TBS and 5 mM EDTA at 4° C. The insoluble material was eliminated by centrifugation and the extract used immediately or stored at −80° C. A phase separation step was performed after removal of the detergent insoluble material. The detergent phase was then purified by wheat germ agglutinin Sepharose chro-

matography (Vector Laboratories, Burlingame, CA), as previously described for melanoma seprase (9). Extracts of breast cancer tissue prepared in this way were used for zymographic and Western blot analysis.

Immunoblots

For Western blotting, samples were solubilized in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and subjected to electrophoresis through a 7.5% acrylamide gel. The proteins were transferred to nitrocellulose filters (0.45 μm) by diffusion in water overnight at 22° C. A representative transfer was stained with Ponceau-S (Sigma, St. Louis, MO) to evaluate the success of the transfer and to locate the molecular weight markers. Free protein binding sites on the nitrocellulose were blocked with 5% (w/v) nonfat dry milk, 0.1% (v/v) Tween-20, and TBS for 1 hour at 22° C. The rat antiserum directed against seprase was diluted 1:1000 in blocking buffer and applied to the nitrocellulose protein replicas for 1 to 2 hours at 22° C in a humidified chamber. Excess antibody was removed by washing the transfers five times with 0.1% (v/v) Tween-20 in TBS. The antibody-antigen complexes were prepared for detection by incubating the samples with a horseradish peroxidase conjugated antibody directed against rat IgG (Amersham, Arlington Heights, IL) diluted 1:3000 in blocking buffer for 1 hour at 22° C. After five washes in 0.1% Tween-20, the reactive bands were detected using a chemiluminescence detection system (ECL Kit, Amersham).

Gelatin Zymographic Analysis

Gelatin zymographic examination was routinely used for identifying seprase activity in extracts of IDCs and performed as described earlier (6). Zymograms used 3 mg/mL gelatin and 10% (w/v) acrylamide.

Immunohistochemical Analysis

Formaldehyde-fixed, paraffin-embedded archival tissue was cut into section 3 to 4 μm thick and mounted on polylysine-coated slides. The tissue was deparaffinized by two xylene washes and rehydrated by four washes of decreasing ethanol concentrations, followed by a single wash in distilled water. Trypsin was used to unmask hidden epitopes by breaking protein crosslinks resulting from the formalin fixation (12). Endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide. The slides were washed in TBS for 20 minutes and then incubated with 10% nonimmune normal goat serum at room temperature for 10 minutes. The excess blocking solution was removed, and the sections were incubated in a 1:2000 dilution of the primary rat antiserum at room temperature overnight. Antibody not bound to antigen was removed by washing with TBS for 10 minutes. The sections were then treated with biotin-labeled goat antirat IgG, diluted 1:2000. Free antibody was washed away using TBS, and the sections were incubated with avidin-biotin-peroxidase for 30 minutes. The sections were washed in TBS for 10 minutes, followed by incubation in the 0.1% hydrogen peroxide and the chromogen 3,3′-diaminobenzidine. The sections were washed in tap water for 5 minutes, counterstained with aqueous hematoxylin, and mounted on slides (13). The procedure used for immunohistochemical examination of the lymph node metastasis and normal human breast tissue, as well as for staining the specimens used to compile Table 1, was performed as described above except that the antibody against seprase was localized using a kit from BioGenex (San Ramon, CA) that relied on alkaline phosphatase conjugated to the IgG directed against rat IgG and fast red chromogen.

Seprase Expression in Malignant, Benign, and Normal Breast Epithelia

Forty-one surgical specimens from patients receiving initial treatment for breast conditions are arranged in Table 1 according to diagnoses and immunoreactivity for seprase. The cases are grouped from malignant, premalignant, benign, and normal diagnoses as IDC, DCIS, lobular carcinoma *in situ* (LCIS), fibroadenoma, proliferative breast disease, and normal breast tissue. Four of the DCIS cases (Cases 29–32) were diagnosed as comedocarcinoma. The lobular carcinoma *in situ* sections used in our study did not have evidence of invasion, but some sections had invading cells. Sections were stained by a standardized, conventional immunohistochemical technique. A score of 1+ is the lowest possible staining intensity. We also scored the intensity of seprase immunoreactivity for stromal cells and morphologically normal epithelial cells within malignant and benign neoplasms. The other clinical characteristics that we collected included the age of the patient, the presence of estrogen and/or progesterone receptor on tumor cells, the percentage of tumor cells in the S phase of the cell cycle, chromosomal ploidy, and histologic grading (I, II, or III) for increasing anaplasia of the tumor cells (14).

RESULTS

Immunoaffinity-purified seprase was used to produce an antiserum capable of recognizing seprase in formaldehyde-fixed and paraffin-embedded tissues. D43 is a rat monoclonal IgG2A

TABLE 1. Seprase Expression in Human Breast Tissues

Case	Diagnosis	Age (yr)	Size (cm)	Node status	ER/PR	% S phase	Ploidy	Grade	Neo	St	Ep
1	IDC	33	1.5	10/16	−/−		Aneu	III	1+	+/−	+/−
2	IDC	39	8	10/18	−/−	9	Aneu	III	1+	+/−	1+
3	IDC	72	0.7		+/−			I	2+	+/−	−
4	IDC	45	1	0/18	+/−	7	Dipl	I	2+	1+	1+
5	IDC	36	1	2/30	−/−	9	Aneu	III	2+	+/−	1+
6	IDC	45	1.7	0/12	+/+	10	Aneu	III	2+	+/−	1+
7	IDC	54	1.8	6/13		6	Dipl	II	2+		
8	IDC	31	2	0/14	+/+	8	Aneu	II	2+	+/−	
9	IDC	62	2.5	0/14	−/−	14	Aneu	III	2+	1+	−
10	IDC	50	2.6	3/8	+/+	4	Aneu	II	2+	1+	1+
11	IDC	28	4	1/16	+/+	21	Aneu	III	2+	+/−	1+
12	IDC	48	1.3	1/18	−/−	23	Aneu	III	2+	+/−	1+
13	IDC	48	6.5	4/4	+/−	12	Aneu	III	2+	−	+/−
14	IDC	44	8	0/3	−/−			III	2+	1+	1+
15	IDC	78	0.7		+/+		Aneu	I	3+	1+	+/−
16	IDC	72	1.2	1/2	+/+		Aneu	II	3+	+/−	
17	IDC	27	1.5	6/7		10	Aneu	III	3+	1+	
18	IDC	48	1.8	0/13	+/+	4	Dipl	II	3+	1+	−
19	IDC	54	2	1/18	+/−	6	Aneu	II	3+	1+	
20	IDC	64	2.5	0/10	+/−	9	Aneu	II	3+	1+	1+
21	IDC	81	4	5/17	+/+		Aneu	III	3+	1+	
22	IDC	61	1.3	0/12	+/−	3	Dipl	II	4+	1+	2+
23	IDC	40	1.8	0/20		8	Aneu	III	4+	1+	1+
24	IDC	83	2		+/+	8		II	4+	2+	
25	IDC	51	4	1/14		11	Aneu	II	4+	2+	
26	IDC	73	7	1/17	+/−	14	Aneu	III	4+	+/−	
27	IDC	60	0.9	0/18	+/+			II	4+	−	
28	DCIS	66		0/3	−/−		Aneu	II	+/−	+/−	
29	DCIS	39	0.7	0/16	+	2	Dipl	I	2+	+/−	+/−
30	DCIS	60	2.5	0/14	+/−	9	Aneu		2+	1+	1+
31	DCIS	47			−/−				2+	+/−	−
32	DCIS	50	4	0/15		11	Aneu		4+	2+	
33	LCIS	48		0/14	+/+	1	Dipl		1+	+/−	−
34	FIB	36							1+	−	
35	FIB	47		0/15					−	−	
36	PBD	45								1+	1+
37	PBD	36								1+	+/−
38	PBD	58								−	+/−
39	PBD	45								1+	1+
40	PBD	47								−	−
41	Normal tissue									−	−

1+, lowest positive staining intensity; aneu, aneuploid; dipl, diploid; DCIS, ductal carcinoma *in situ*; Ep, epithelial cells; St, stromal cells; ER, estrogen receptor; PR, progesterone receptor; FIB, fibroadenoma; IDC, infiltrating ductal carcinoma; LCIS, lobular carcinoma *in situ*; Neo, neoplastic cells; Node status, number of nodes positive for tumor cells/number of nodes examined; PBD, proliferative breast disease.

produced by Monsky *et al.* (7) and Pineiro-Sanchez *et al.* (10) that immunoprecipitates 170-kDa active seprase dimer and the 97-kDa seprase protein from extracts of human melanoma cells. Immunoblot analysis revealed that D43 reacted only with the 170-kDa active dimer and that immunoreactivity was lost after boiling or exposure to acid pH, so the epitope it recognizes was lost with dissociation or denaturation (7, 10). This limited recognition of seprase by the D43 mAb indicated that although it would be useful for purifying seprase, it might not recognize seprase in archival tissue specimens. Therefore, D43 was used to purify seprase for the purpose of generating a polyclonal antiserum that recognized many different epitopes on the seprase molecule.

Seprase was purified from detergent extracts of 9-day-old chicken embryos by sequential extractions to remove peripheral proteins and by Triton X-114 detergent extraction to solubilize seprase. Purification of seprase to near homogeneity was achieved by phase partitioning the detergent extract and subsequent chromatography of the detergent phase by anion exchange and mAb D43 immunoaffinity columns. Analysis of the proteins released from the D43 immunoaffinity column by SDS-PAGE and silver staining revealed that a 97-kDa protein corresponding to seprase was specifically bound by the column (Fig. 1A). Seprase was not retained by protein A agarose beads that did not have IgG attached, but a protein migrating at 66 kDa was retained by both columns (Fig. 1A). The affinity-purified seprase was used as immunogen to produce a polyclonal antibody. The resultant antiserum specifically identifies the 97-kDa seprase protein in chicken embryo extracts (Fig. 1B). The antiserum also identifies both the 97-kDa seprase monomer and the 170-kDa seprase dimer in extracts of human IDCs of the breast that were not boiled (Fig. 2). These human IDC extracts also re-

tain the 170-kDa gelatinase activity of seprase (Fig. 2). The molecular weights of the proteins identified by the antiserum in extracts of human breast carcinoma extracts were consistent with the established molecular weights of 170 kDa for the proteolytically active seprase dimer and 97 kDa for inactive monomeric seprase (7, 10).

Surgical specimens of human breast tissue (41 cases) were stained with antiserum directed against seprase (27 IDCs, 5 DCISs, 1 lobular carcinoma *in situ* (LCIS), 5 benign proliferative breast disease, 2 fibroadenomas, and 1 sample of normal breast tissue) (Table 1). The antiserum directed against seprase intensely labels invasive cells in IDC of the breast tumors, but labeling is much lower or absent from stroma, inflammatory tissue, or normal epithelium (Table 1; Fig. 3). The tumor cells in all of the 27 IDCs were positively stained for seprase, and the neoplastic cells were always more intensely labeled than stromal cells and morphologically normal epithelium in the same specimen. In no instance were IDC cells scored as weak or negative for immunoreactivity with antibodies to seprase. Moreover, the

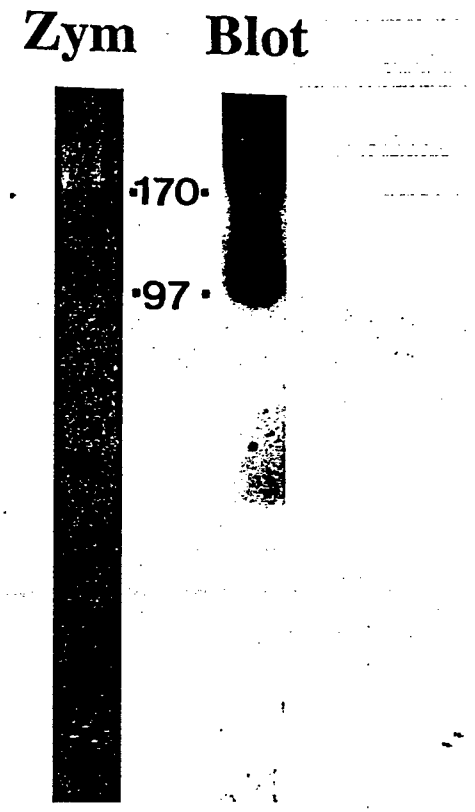

FIGURE 2. Identification of seprase in extracts of IDC of the breast. Seprase was present in extracts of tumors that were identified by pathologists as IDCs of the breast. These tumors were extracted with Triton X-114, and the proteins binding to a wheat germ agglutinin agarose column were subjected to SDS-PAGE and blotted onto nitrocellulose. Seprase activity at 170 kDa is apparent in this fraction, as seen by gelatin zymographic analysis *(Zym)*. The antiserum detects 170-kDa and 97-kDa proteins that correspond to the active seprase dimer and its inactive subunit *(Blot)*. Molecular weight markers are $\times 10^{-3}$ and are indicated between panels.

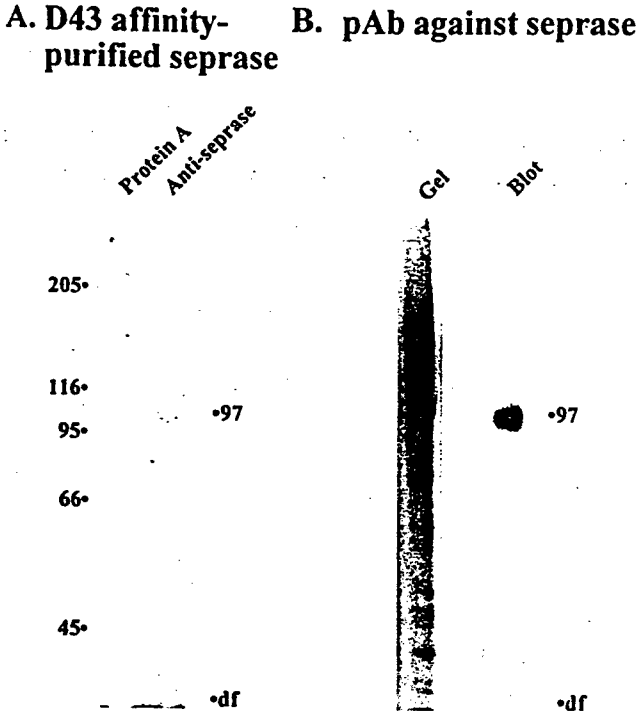

FIGURE 1. The antiserum raised against seprase shares specificity with the D43 mAb to seprase. A, D43 affinity-purified seprase. Silver-stained gel shows the chicken embryo 97-kDa protein that was eluted with acid from a mAb D43 column *(Anti-Seprase)*. No 97-kDa protein was detected eluting from the control column (Protein A). A 66-kDa protein is present in both samples and is thus bound nonspecifically. B, polyclonal antibody against seprase immunoblot. Western blot analysis shows proteins recognized by the polyclonal antibody generated against mAb D43 immunoaffinity-purified seprase. Proteins in a chicken embryo seprase extract were separated by SDS-PAGE and detected by Coomassie Blue staining *(Gel)*. The polyclonal antibody reacts only with a 97-kDa protein in samples in which seprase was boiled *(Blot)*. Molecular weights of the standard *(far left)* and the 97-kDa proteins are $\times 10^{-3}$, and *df* indicates the dye front.

majority of these neoplastic cells had moderate (2+), high (3+), or the highest (4+) levels of immunoreactivity. IDC cells in lymph node metastases were also strongly seprase positive, but lymphoid tissue is not labeled (Fig. 4). Neoplastic cells are labeled throughout the cytoplasm (Fig. 4), so seprase is not strictly localized to the cell surface of the neoplastic cells of primary IDC tumors and IDC lymph node metastases. Except for diagnosis as IDC, the relative intensity of seprase staining did not seem to correlate with other clinical variables including the patient age, the levels of estrogen or progesterone receptors, the percentage of cells in the S phase, or DNA ploidy (Table 1). In addition, there was no obvious correlation between levels of seprase expression at the primary site and prognostic indicators such as tumor size, nodal involvement, and histologic grade (Table 1).

We examined five DCISs, and all except one were positive for seprase expression (Table 1). Histologic evaluation of the seprase-negative DCIS showed no evidence of microinvasion, but foci of microinva-

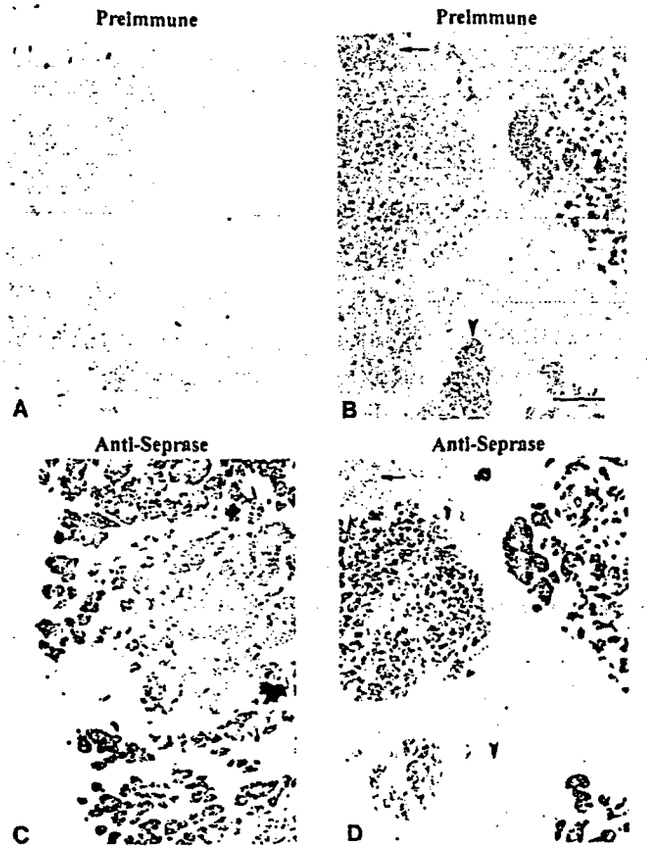

FIGURE 3. Malignant DC of the breast cells express high levels of seprase. A low power view of IDC of the breast shows clumps of neoplastic cells filling the ducts and invading the stroma. C, D: IDC cells are stained intensely by the antiserum directed against seprase. The tumor cells exhibit heterogeneity in seprase expression, with some cells more intensely labeled than others. D, normal stroma, noninvasive carcinoma *in situ (arrowheads)*, and inflammatory tissue *(arrows)* are not stained. A, B: no brown 3,3'-diaminobenzidine reaction product is seen in adjacent sections stained with the preimmune rat serum. The sections are counterstained with hematoxylin. Original magnifications, 28× (bar = 500 μm).

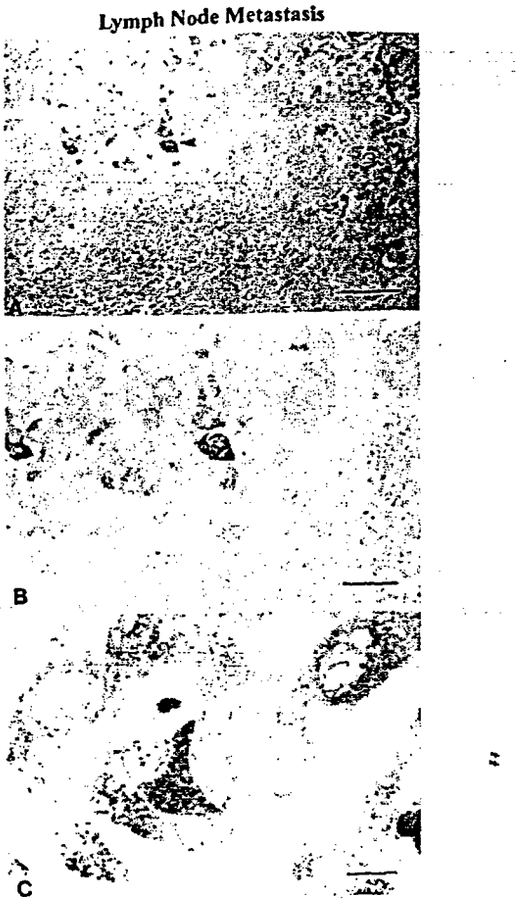

FIGURE 4. Seprase is expressed by breast cancer cells in lymph node metastases. In (A), (B), and (C), seprase is expressed by breast cancer cells that metastasized to lymph nodes. A, the large breast cancer cells *(arrowhead)* are easily distinguished from the lymphocytes in this node. The metastatic breast cancer cells vary in level of seprase expression, and some cancer cells stain much more intensely with the fast red reaction product than others. Original magnifications: A, 160× (bar = 100 μm); B, 800× (bar = 20 μm); C, 3500× (bar = 4 μm).

sion were noted in the four DCISs that were positive for seprase expression. These seprase-positive cases were also classified as comedocarcinomas. The significance of the variable seprase expression by DCIS is difficult to assess from this small number of cases that also has an unusually high percentage of DCISs with microinvasion. In addition to DCISs, we examined one LCIS specimen, and it had a low level of staining for seprase. We also studied two fibroadenomas and five cases of benign proliferative breast disease, all of which had low levels of immunoreactivity (1+) or were not labeled. The one normal breast specimen was not stained by the antiserum against seprase (Fig. 5). Seprase expression by epithelial cells in these benign breast conditions was always low, and these cells never expressed seprase at the moderate, high, or the highest levels (2+ , 3+, 4+) that were recorded in the majority of the malignant and premalignant cases. These immunohistochemical findings indicated that seprase was overexpressed by neoplastic cells in invasive ductal carcinoma. These observations were consistent with overexpression of seprase acting to facilitate invasion and metastasis by degrading extracellular matrix.

DISCUSSION

This immunohistochemical study used a seprase-specific antiserum to reveal overexpression of seprase by the neoplastic cells of IDC relative to its expression by the stromal and normal epithelial cells in these tumors. Although seprase is known to be present on the surface of invasive cells (7), it is not restricted to the plasma membrane. IDC cells also had a large intracellular pool of seprase that was apparently contained within small vesicles and might reflect the increased synthesis of this membrane protein. The overexpression of seprase by neoplastic IDC cells was also corroborated by the low levels of seprase expression observed in benign

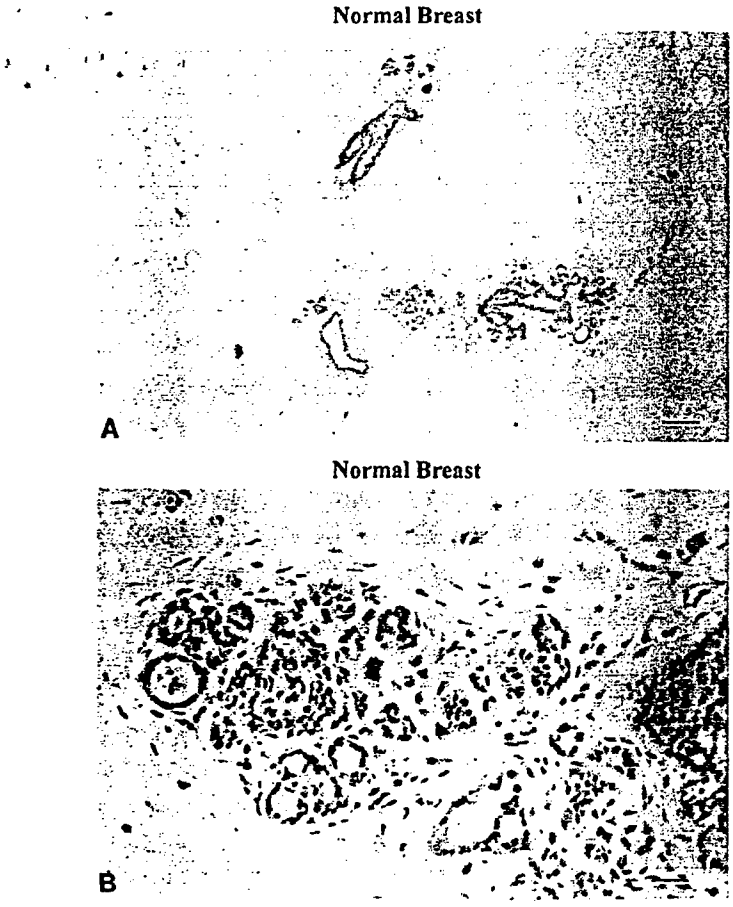

FIGURE 5. Normal breast tissue is not labeled by the antibody directed against seprase. No staining is detected in these normal breast epithelial cells stained with antibody against seprase. Original magnifications: A, 80× (bar = 100 μm); B, 800× (bar = 10 μm).

proliferative or normal breast cells. In contrast to malignant breast conditions, cells of fibroadenomas and epithelial cells of benign proliferative breast disease had little or no staining for seprase. Normal breast epithelium was not stained. Stromal cells in the benign lesions and in normal breast tissue did not express seprase. These findings supported the idea that seprase overexpression was a marker for invasiveness and extended this possibility from cells grown *in vitro* to clinical specimens of malignant human breast tissues.

Previous studies suggested that seprase overexpression might be a marker of an early event in the process of progression from the noninvasive premalignant to the invasive malignant phenotype (7, 9). Initially, this notion was based on examination of seprase activity in many different human melanoma cell lines with known invasive potentials (9). It was supported by the observed concentration of seprase on invadopodia, a location that places it at the sites of aberrantly high proteolysis of extracellular matrix caused by invading tumor cells (7, 15). Moreover, human melanoma cells that were sorted for the highest level of cell-surface seprase expression also revealed the highest degree of proteolysis of extracellular matrix (7). The proteolysis of extracellular matrix by invadopodia facilitates invasion by human breast cancer cell lines, and the level of proteolytic activity is positively correlated to the invasion potential of the cell lines (16). Thus, this evidence combined with the findings from this study suggests that seprase might have a role in conferring the invasive phenotype on human breast cancer cells.

The role of seprase in the malignant progression of breast cancer is not known, but it might facilitate invasion and metastasis of breast carcinoma cells by increasing their proteolysis of extracellular matrix. Seprase is an integral membrane serine protease that is embedded in the plasma membrane, with its catalytic domain exposed to the extracellular environment (7, 10). Seprase can degrade gelatin or denatured Type I collagen at the neutral pH of the extracellular fluid (6, 9); thus, it might facilitate invasion by degrading extracellular matrix directly. Seprase might also indirectly increase the extracellular matrix degrading–activity of tumor cells by acting in concert with other matrix-degrading proteases. It is known that human breast cancers express a variety of extracellular matrix–degrading proteases, including matrix metalloproteinase (MMP)-2 and MMP-9 (3, 17–20), stromelysin-3 (21), plasminogen activators (22–24), cathepsins B and L (25–28), cathepsin D (29) and MT1-MMP (30). The activity of these proteases is tightly regulated by transcriptional control, activation of latent proenzymes and by the levels of endogenous inhibitors for the different matrix-degrading proteases (3). Seprase and MT1-MMP are integral membrane proteases known to be overexpressed by human breast cancer cells. MT1-MMP apparently functions as an activator of latent MMP-2 by invasive breast carcinoma cells (31, 32). Seprase might also function to stimulate proteolysis of extracellular matrix indirectly by promoting the activity of other matrix-degrading proteases. There is no evidence that seprase can activate latent forms of secreted extracellular matrix–degrading proteases, but it could facilitate proteolysis of extracellular matrix by exposing hidden protease cleavage sites on extracellular matrix molecules.

Sequence analysis of human melanoma seprase revealed that it is a nonclassical serine integral membrane protease that is very closely related to fibroblast activation protein (FAP)-α and shares considerable sequence homology to dipeptidyl peptidase IV (10). The nucleotide sequence of the cDNA encoding seprase differs from that of FAP-α by changes or insertions of only 8 bp, resulting in a difference of 48 amino acids or 94% identity (10). Elevated expression of FAP-α was reported in reactive stromal fibroblasts of human epithelial cancers, including breast cancer, but the neoplastic cells had

lower expression of FAP-α (33). Those results were obtained using the F19 mAb directed against FAP-α and were in contrast to the findings presented here using the polyclonal antibody against seprase (33). Thus, despite their close sequence homology, seprase and FAP-α might have distinct roles in the invasion process, with seprase being more confined to tumor cells and FAP-α restricted to reactive stromal fibroblasts.

Seprase might be a good candidate for providing target specificity to therapeutic agents. Inhibition of the proteases that cancer cells use to degrade extracellular matrix is a promising area of translational research designed to control tumor spread and growth (34). Much of the progress in this arena was in the development of inhibitors of MMPs (35, 36), but inhibition of seprase might also impede the invasion process. Alternatively, the overexpression, cell surface localization and physical attachment of seprase to the surface of breast cancer cells might be exploited to target cytotoxins to malignant cells.

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Book Review

Gilstrap LC III, Faro S: Infections in Pregnancy, 2nd ed, 345 pp, New York, Wiley-Liss, 1997 ($79.95)

A publisher friend of mine told me once that, among the various medical specialists, gynecologists are ranked last on the list of most avid readers. I would submit that they most likely do not have time for lengthy books; if one were to find a format to deliver a message that can be read between two deliveries, more gynecologists could be reached. The case in point is the present book, which illustrates this principle well. Written by two experienced gynecologists specializing in gynecologic and obstetric infections, the book is a brief but most informative compendium dealing with some two dozen common infectious diseases that affect pregnant women. The text is to the point, typically amplified with diagnostic algorithms or summary tables. It reads well and is up-to-date. In my opinion, it is ideally suited for a busy gynecologist, but it should be of interest to other specialists, including pathologists, as well. It is no wonder that this useful book is already in its second edition. I am sure that a third, written in the same format, will follow in due time.

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Proteolysis of extracellular matrix by invadopodia facilitates human breast cancer cell invasion and is mediated by matrix metalloproteinases

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Breast cancer cell lines vary in invasive behavior and one highly invasive cell line (MDA-MB-231) proteolytically degrades extracellular matrix with invadopodia (Thompson *et al.* 1992, *J Cell Physiol*, 150 534–44; Chen *et al.* 1994, *Breast Can Res Treat*, 31 217–26). Invadopodial proteolysis of extracellular matrix is thought to be necessary for invasion; however, this has not been demonstrated directly. To obtain such evidence, normal (HBL-100) and malignant (MCF-7, MDA-MB-231) breast cells were evaluated for invadopodial proteolysis of extracellular matrix and invasive behavior. We report that invadopodial proteolysis of immobilized fibronectin is positively correlated with invasion of cells into type I collagen gels. Moreover, reducing the proteolytic activity of invadopodia with the metalloproteinase inhibitor, batimastat (BB-94), also decreases invasion indicating that breast cancer cell invasion is dependent upon proteolytically active invadopodia. © **Lippincott-Raven Publishers**

Keywords: (BB-94) batimastat, fibronectin, invadopodia, invasion, metastasis, MMP

Introduction

Invasive tumor cells have increased levels of a variety of extracellular matrix-degrading proteases that allow them to traverse complex basement membrane and stromal matrices (reviewed in [1–3]. Generally, the matrix-degrading proteases are secreted by tumor and stromal cells as inactive zymogens that require association with the tumor cell surface to become activated and capable of degrading extracellular matrix [1,2]. The cellular sites of abnormally high extracellular matrix-degrading activity can be investigated by growing invasive cells on monolayers of fluorescently labeled extracellular matrix proteins. Fluorescence-negative regions underneath the invasive cells are observed that indicate the positions where matrix proteolysis has occurred [4,5]. The proteolytic activity is discretely focused and corresponds to areas where plasma membrane protrusions termed 'invadopodia' extend from cell surfaces and contact the matrix [4, 6]. Invadopodia can degrade multiple extracellular matrix proteins including intact fibronectin-rich matrices produced by fibroblasts, fibronectin, laminin, type IV collagen, and type I collagen [5]. A variety of invasive cells exhibit invadopodia including human melanoma cells [7], transformed chicken fibroblasts [4–6], and human breast cancer cells [8,9]. Invadopodial proteolysis of extracellular matrix is thought to facilitate tumor cell invasion into extracellular matrix [3,6,10].

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Matrix metalloproteinases (MMPs) are important mediators of invadopodial degradation of extracellular matrix because an inhibitor of MMPs (NP-20) decreases invadopodial degradation of type I collagen by Rous sarcoma virus-transformed chicken embryo fibroblasts [11]. MMPs are a family of structurally related enzymes that together can degrade all components of the extracellular matrix and are known to be important in tumor cell invasion [1,2]. MMPs are synthesized as inactive proenzymes that require cleavage of the pro-peptide for activation of their proteolytic activities. Proteolytically active MMP-2 (gelatinase A) and MT1-MMP (membrane type 1-matrix metalloproteinase or MMP-14) are concentrated on invadopodial membranes suggesting that these enzymes are important mediators of invadopodial extracellular matrix degradation [11,12]. MT1-MMP is activated by furin-like enzymes prior to its expression on the cell surface [13,14] where it serves as a potent activator of latent MMP-2 [15,16]. Given the wide range of extracellular matrix substrates degraded by invadopodia, other proteases including other MMPs are likely to have a role in proteolysis of extracellular matrix by invadopodia.

This study was performed to determine if invadopodial proteolysis of extracellular matrix plays an important role in facilitating human breast cell invasion. Previously, it was shown that the invasive MDA-MB-231 human breast cancer cells use invadopodia to degrade films of fluorescent extracellular matrix molecules covalently attached to glutaraldehyde crosslinked gelatin [8,9]. Independently, others have shown that human breast cancer cell lines vary in their ability to invade into Matrigel and that human breast cancer cell lines vary in local invasion through the peritoneum when injected into the mammary fat pads of nude mice [17]. MDA-MB-231 cells were identified as invasive in both assays [8,17]. In contrast, MCF-7 human breast cancer cells were moderately invasive through Matrigel in Boyden chamber assays and, although these cells formed tumors when injected into the mammary fat pads of nude mice, the tumor cells did not invade through the peritoneum [17]. Although invadopodial proteolysis of extracellular matrix and invasiveness have been assessed separately, there has not been a direct evaluation of the role of invadopodial proteolysis of extracellular matrix in invasion. Here we correlate the HBL-100, MCF-7, and MDA-MB-231 human breast cell lines for invadopodial function as determined by degradation of fluorescently labeled or radiolabeled fibronectin and for invasiveness using type I collagen gels. The MMP inhibitor batimastat was used to inhibit the function of invadopodia to thereby investigate the role of invadopodia in breast cancer cell invasion. We show that invadopodial proteolysis of extracellular matrix facilitates human breast cancer cell invasion and is mediated by MMPs.

Materials and methods

Cell culture:
Human breast adenocarcinoma cell lines MDA-MB-231, MCF-7, and the normal breast cell line HBL-100 were obtained from American Type Culture Collection (Rockville, MD). All cell lines were maintained in Eagle's minimal essential medium (Gibco-BRL, Gaithersburg, MD), supplemented with 10% heat-inactivated FBS (FBS; Atlanta Biologicals, Norcross, GA), 10 μg/ml bovine insulin (Sigma, St. Louis, MO), 10 μg/ml glutamine (Gibco-BRL, Gaithersburg, MD), 1% penicillin-streptomycin (Gibco-BRL) 2×10^{-6} mM β-estradiol (Sigma) and maintained at 37°C in 95% air and 5% CO_2. The FBS used for growing cells was either complete (Figure 4), depleted of MMPs and TIMPs (Figures 1 and 2A) or depleted of MMPs, TIMPs and plasmin/plasminogen (Table 1, Figures. 2, 3B and 5) as described below.

Fluorescent fibronectin substrates for determining invadopodial proteolysis of extracellular matrix
This assay relies on growing cells on fluorescein isothiocyanate-labeled fibronectin that is covalently bound to a glutaraldehyde-crosslinked gelatin film attached to a glass coverslip. Fluorescence-negative regions underneath cells represent areas where the extracellular matrix has been degraded [4,5]. Human plasma fibronectin (Becton Dickinson Labware, Medford, MA) was coupled to fluorescein isothiocynate (Research Organics, Cleveland, OH) according to the manufacturer's instructions. The assays reported here were allowed to proceed for 24 or 72 h at 37°C in 95% air and 5% CO_2 prior to fixation and preparation for fluorescence microscopy.

Fluorescence microscopy
The cells were fixed, stained with rhodamine phalloidin (Molecular Probes, Inc., Eugene, OR) as described earlier [5]. The cells were observed using ×40, ×60 and ×100 objectives of a Nikon LABOPHOT microscope equipped for epifluorescence photomicroscopy and images were recorded using Ilford HP5 400 ASA black and white film. To quantify invadopodial matrix proteolysis, 10 micro-

scopic fields on each coverslip were randomly selected and the fluorescence of the fibronectin film visualized with the × 40 objective and scored for the presence or absence of matrix proteolysis as judged by the presence of focal fluorescence negative spots in areas where the matrix was degraded [4,5,8]. The results are presented as the average number of fields positive for matrix degradation per 10 fields examined.

[^{125}I]-fibronectin substrates for determining invadopodial proteolysis of extracellular matrix

This second invadopodial proteolysis assay utilized [^{125}I] fibronectin as the substrate and protease-depleted FBS in the growth medium. The assay involves: iodination of the fibronectin, coupling the [^{125}I]-fibronectin to crosslinked gelatin films, determination of [^{125}I]-fibronectin bound to the substrate, extensive washing to remove free [^{125}I]-fibronectin, and determination of invadopodial proteolysis.

Removal of interfering extracellular matrix-degrading proteases from FBS

Gelatin Sepharose chromatography was used to remove MMPs and TIMPs from FBS [18,19]. A 10 ml gelatin-Sepharose (Pharmacia Biotech, Uppsala, Sweden) was equilibrated with the binding buffer 20 mM Tris, 0.5 M NaCl, 1.0 mM $CaCl_2$, 10% glycerol (v/v), 0.05% BRIJ-35 (v/v), 0.02% NaN_3 (v/v), pH 7.6. MMPs and TIMPs in FBS (heat inactivated) were bound to the column by loading the serum (50 ml) onto the column at 35 ml/h. The void volume was discarded, and the flow-through, depleted of MMPs and TIMPs but containing the other serum components, was collected and sterile filtered. Gelatin zymography of breast cancer cell growth medium made 10% (v/v) with respect to FBS revealed no MMP activity (not shown).

For the batimastat inhibitor studies (batimastat kindly provided by British Biotech Pharmaceuticals, Ltd, Oxford, UK), the FBS was depleted of MMPs, TIMPs, and the broad spectrum protease, plasmin/plasminogen, that is abundant in serum. MMPs and TIMPs were removed as described and then lysine Sepharose was used to remove the plasmin/plasminogen [20]. A 10 ml lysine Sepharose column (Pharmacia Biotech) was packed and equilibrated with the same binding buffer as described above. The MMP/TIMP-depleted FBS (50 ml) was loaded onto the column at 35 ml/h. The MMP/TIMP and plasminogen/plasmin-depleted serum in the flow-through was collected and sterile filtered. Immunoblot analysis of breast cancer cell growth medium made 10% (v/v) with respect to MMP/TIMP and plasminogen/plasmin-depleted FBS revealed no plasminogen or plasmin immunoreactivity with a goat IgG directed against bovine plasminogen (American Diagnostics, Greenwich, CT) (not shown).

Iodination of fibronectin, coupling to crosslinked gelatin and determination of cpm [^{125}I]-fibronectin bound to the substrate

Fibronectin (50 μg) was iodinated with 0.5 mCi [^{125}I] using chloramine T (2 mg/ml) as described [21]. [^{125}I]-fibronectin was separated from free [^{125}I] by gel filtration using an excellulose GS column (Pierce, Rockford, IL) that had been equilibrated in 1 mg/ml bovine serum albumin (BSA) and phosphate buffered serum (PBS). An equivalent amount of [^{125}I]-fibronectin in 200 μl was coupled to the glutaraldehyde crosslinked gelatin film coating 15 mm glass round coverslips as previously described for fluorescent fibronectin [4,5,22]. The level of [^{125}I]fibronectin used in independent experiments varied from 2×10^6 cpm to 10^{10} cpm. The ^{125}I-labeled fibronectin was allowed to bind to the coverslips for at least 12 h. At the end of coupling, 1 μl of the coupling fluid was counted and the volume of coupling fluid was measured to determine the amount of [^{125}I]fibronectin that did and did not bind to each coverslip.

The [^{125}I]fibronectin-coupled crosslinked gelatin films were subjected to a series of washes designed to remove any free or weakly bound [^{125}I]fibronectin and to block exposed aldehyde groups. The washes included 3 ml of the following solutions: 70% ethanol (1 × 5 min, 22°C), PBS (3 × 5 min, 22°C), growth medium containing 10% MMP/TIMP and plasminogen/plasmin-depleted FBS (1 × 3 h, 37°C), PBS (2 × 5 min, 22°C), and serum-free growth medium (1 × 24 h, 37°C). A sample (10 μl) was taken to determine the level of [^{125}I]fibronectin in each wash. The total ^{125}I-fibronectin that did not bind each coverslip was determined by adding the radioactivity (cpm) in the unbound fraction to the sum of the radioactivity (cpm) released by all of the washes. Then the amount of [^{125}I]fibronectin bound to each coverslip was determined by subtracting the total unbound radioactivity from the radioactivity originally added to the coverslip.

Determination of invadopodial matrix proteolysis using immobilized [^{125}I]fibronectin

Human breast cells were harvested using trypsin-EDTA, diluted with growth medium containing 10% protease-depleted FBS and washed three times with sterile PBS. Coverslips were placed in the wells of a 6-well culture plate. Cells (1×10^5) were placed onto each coverslip in 200 μl of medium and allowed to attach to the [^{125}I]fibronectin for 1 h at 37°C. Then

2.8 ml of growth medium containing 10% MMP/TIMP and plasminogen/plasmin-depleted FBS containing no additives, DMSO, or 10 μM batimastat in DMSO was added to achieve a final volume of 3 ml. The plates were placed back into the incubator and 50 μl aliquots were taken from each well under sterile conditions and counted at 3, 24, 48, 72 and 96 h. At each time point, 50 μl of growth media containing 10 % protease-depleted FBS were put back into each well to maintain the 3 ml volume of the assay. The radioactivity of the aliquots was determined with a Packard gamma counter.

The total amount of ^{125}I released into the media for each time point was calculated and added to the sum of the radioactivity in the 50 μl aliquots from prior time points. This value was divided by the total cpm [^{125}I]fibronectin bound to that coverslip and multiplied by 100 to give the percentage of total bound ^{125}I released into the media.

Cell viabilities

The cytotoxicity of 72 h exposure to batimastat and DMSO was investigated by performing trypan blue exclusion assays as described by others [23].

Gelatin zymography

Gelatin zymography was performed essentially as described by Heussen and Dowdle [24] with 1 mg/ml gelatin co-polymerized into SDS-PAGE that was 10% (w/v) with respect to acrylamide.

Conditioned growth medium

To determine the protease activities released into the medium by human breast cancer cells and to investigate the inhibitory effects of batimastat on these proteases, MDA-MB-231 cells were grown to high density (7×10^5 cells/ml) in growth medium containing 10% (v/v) FBS in 75 cm^2 flasks. Complete growth medium was removed and the cells washed three times with 10 ml sterile PBS. After removing the final wash, the cells were incubated in serum-free minimal essential media for 48 h at 37°C in 5 % CO_2. The conditioned medium was collected, cells and debris removed by centrifugation (5000 *g*, 5 min), and concentrated 10 times using centricon 30 filter concentrators (Amicon, Inc., Beverly, MA). The concentrated conditioned medium was tested for proteases in the presence or absence of 10 μM batimastat by zymography.

Invasion assay

Type I collagen gel invasion assays were performed in 24-well plates as described by Liebersbach and Sanderson [25] except that the assays were allowed to proceed for 72 h. Quantification of the percentage of invading cells was achieved by first determining the number of non-invading cells that could be removed from the top of the gel with trypsin-EDTA and then counting the invading cells that were released from within the type I collagen gel by extensive collagenase digestion as described by Liebersbach and Sanderson [25]. The leading front of invasion was defined as the point where the two most distantly migrating breast cancer cells were simultaneously in focus in one field under ×200 magnification. After 72 h, each gel was searched in a Z pattern and the mean distance ± standard error of the mean was determined using the calibrated micrometer of a Nikon inverted phase contrast microscope. Within experiments, duplicate assays were performed for each cell line and each experiment was repeated at least two times.

Results

Invadopodial extracellular matrix degrading activities of human breast cell lines

The MDA-MB-231 human breast cancer cells are proteolytically active and degrade extracellular matrix substrates by an invadopodial-dependent mechanism [8,9]. Other work has shown that human breast cancer cell lines differ in invasive behavior as determined *in vitro* using a Boyden chamber invasion assay with Matrigel as the barrier to invasion and the invasiveness observed *in vitro* correlates closely with *in vivo* invasive behavior observed using the nude mouse model [17]. These workers identified the MDA-MB-231 cell line as one of the most invasive. The purpose of this study was to determine the role of invadopodial proteolysis of extracellular matrix in the invasion process.

The extracellular matrix-degrading activity of the human breast cell lines was investigated by growing the cells on fluorescently labeled fibronectin that was covalently coupled to a glutaraldehyde-crosslinked gelatin film. HBL-100 normal human breast cells do not degrade the matrix and the fibronectin substrate remains intact and uniformly fluorescent underneath the cells (Figure 1A, B). MCF-7 human breast cancer cells reveal limited degradation of the fluorescent-fibronectin film, with one microscopic field in ten having the spots of decreased fluorescence that are indicative of matrix proteolysis (not shown). The MDA-MB-231 breast cancer cell line reveals extensive degradation of the fluorescent fibronectin films, with five microscopic fields in ten having evidence of matrix degradation (Figure 1C, D).

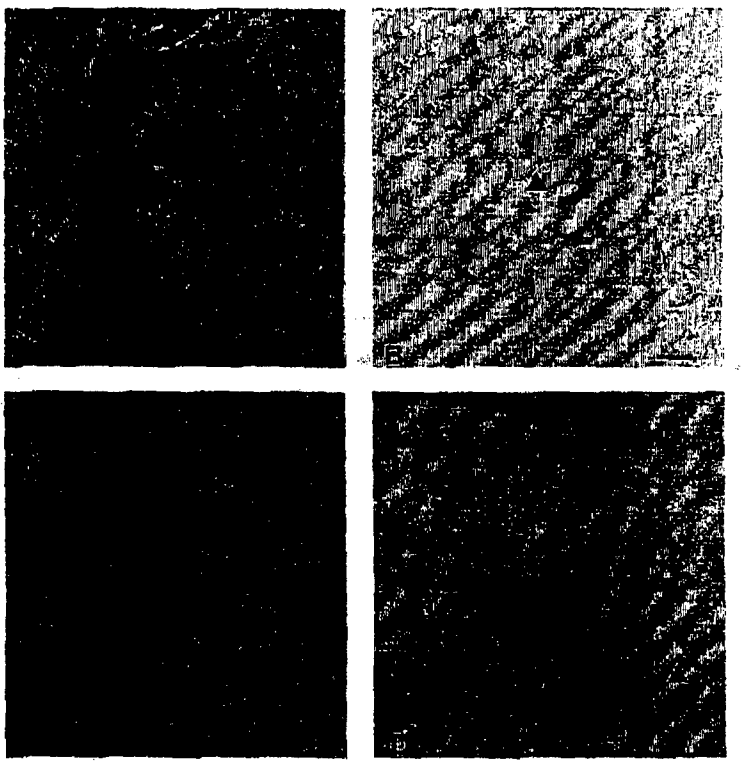

Figure 1. Normal HBL-100 human breast cells do not degrade fibronectin but malignant MDA-MB-231 human breast cancer cells degrade fibronectin. Normal HBL-100 cells are visualized by rhodamine-phalloidin fluorescence (A). The fibronectin substrate in the same microscopic field is visualized by fluorescein–fibronectin fluorescence respectively (B). Arrowheads point to identical locations in the microscopic fields. Malignant MDA-MB-231 human breast cells (C) degrade fibronectin that is covalently linked to the substrate (D). Arrowheads point to identical locations in the same microscopic field. The observed fluorescence-negative spots where the fibronectin has been removed are characteristic of invadopodia-mediated proteolysis (arrowheads: D). Cells were grown on the fibronectin films for 72 h. Bar = 10 μm.

The fact that MMP-2 and MT1-MMP are concentrated on invadopodia [11,12] led us to investigate the effect of MMP inhibition on proteolysis of extracellular matrix by MDA-MB-231 cells. Batimastat (Mr 477) is a broad spectrum MMP inhibitor that mimics one part of the principle MMP-cleavage site in collagen. Batimastat was used because it has nM IC50 values for inhibition of several MMPs including: MMP-1 (interstitial collagenase), MMP-3 (stromelysin), MMP-2 (gelatinase A), MMP-9 (gelatinase B) and MMP-7 (matrilysin) [26]. Batimastat also blocks the activation of MMP-2, presumably by inhibition of MMP-14 (MT1-MMP) [26]. Batimastat does not inhibit metalloproteinases such as angiotensin-converting enzyme and enkephalinase nor does it inhibit other proteases implicated in tumor cell invasion such as plasmin, urokinase-type plasminogen activator, and cathepsins (personal communication, Peter D. Brown, British Biotech Pharmaceuticals, Ltd. Oxford, UK). Other desirable properties of batimastat include low cytotoxicity and a minimal cytostatic effect [26,27]. Moreover, cells treated with batimastat exhibit normal migration in chemotactic assays, indicating that the adhesive and motile machinery of cells is not affected by batimastat [26].

Batimastat was used to investigate the effect of MMP inhibition on extracellular matrix proteolysis by invadopodia. Batimastat completely inhibited invadopodial degradation of fluorescent fibronectin films by MDA-MB-231 breast cancer cells (Figure 2C–F). Invadopodial degradation of extracellular matrix was apparent underneath the untreated MDA-MB-231 cells (Figure 2A, B). Neither the DMSO vehicle nor batimastat were toxic to the cells during incubation periods up to 72 h or at any of the batimastat concentrations used in this study as judged by trypan blue exclusion assays.

To quantify the extracellular matrix proteolysis, normal HBL-100 and malignant MDA-MB-231 breast cells were grown on immobilized [^{125}I] fibronectin and the radioactivity released from the substrate by the proteolytic action of the cells was determined. MDA-MB-231 cells release 11-fold more [^{125}I]fibronectin degradation products from the substrate than HBL-100 cells after 72 h (Figure 3A). The effect of the batimastat on the proteolysis of extracellular matrix by the MDA-MB-231 breast cancer cell line was investigated. Batimastat reduced the release of [^{125}I]fibronectin by MDA-MB-231 human breast cancer cells to below that released by the

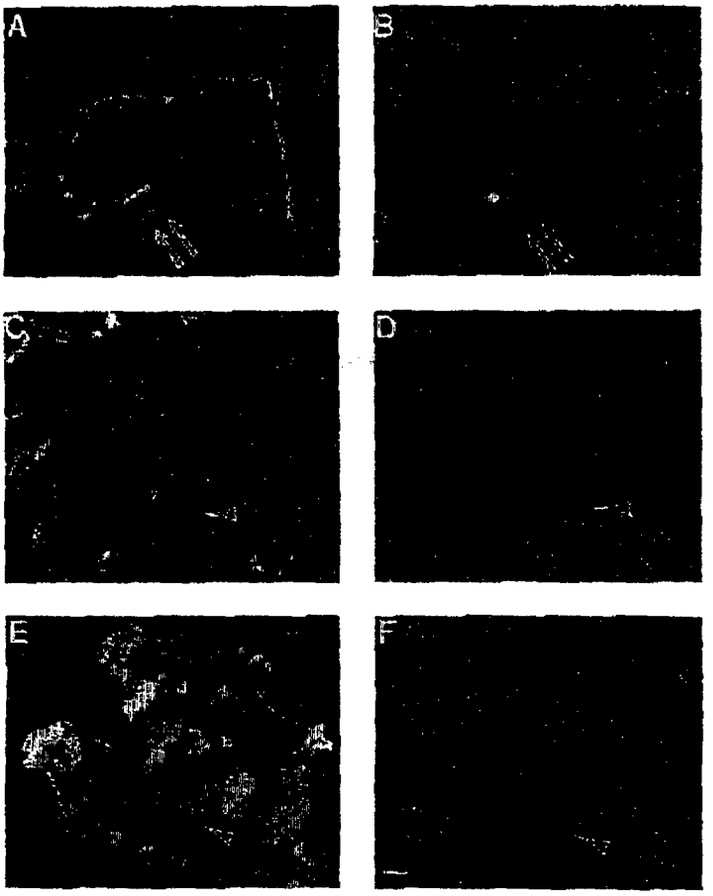

Figure 2. Inhibition of MDA-MB-231 invadopodial matrix degradation by batimastat. Cells (A, C, E) and underlying substrates (B, D, F) in the same microscopic fields (A-B, C-D, and E-F) are visualized for rhodamine-phalloidin (cells) and fluorescein-fibronectin (substrates) after growing for 24 h. Proteolytic degradation of fluorescent fibronectin substrates associated with invadopodia is detected in control cells (arrows: A and B) but not in cells treated with 0.5 μM (C and D) or 1.0 μM batimastat (E and F). These substrates had less fluorescent intensity than those used for Figure 1. Bar = 10 μm.

no-cell control (Figure 3B). The release of [^{125}I]fibronectin by MDA-MB-231 cells was only slightly reduced by the DMSO vehicle as compared to untreated cells (Figure 3B). Although it was not cytotoxic, batimastat was active as an inhibitor of MMPs. Batimastat inhibited the 92 and 72 kDa gelatinase activities released into serum-free medium by MDA-MB-231 cells (Figure 3C). The complete inhibition of MDA-MB-231 proteolysis of [^{125}I]fibronectin by batimastat suggests that virtually all of the observed invadopodial proteolysis of extracellular matrix by MDA-MB-231 cells is mediated by MMPs.

Invadopodial extracellular matrix degrading activity correlates with invasion potential of human breast cell lines

Invasion was evaluated by seeding the cells on top of a native type I collagen gel and allowing them to interact with the gel for 72 h (Figure 4). The percentage of cells that invaded into the type I collagen gels after 72 h was determined (Figure 4A). The HBL-100 normal human breast cell line is not invasive in this assay (Figure 4A, H100). The MCF-7 human breast cancer cell line is somewhat invasive (Figure 4A, MCF7) and the MDA-MB-231 human breast cancer cell line is the most invasive in this assay (Figure 4A, M231).

The degree of invasiveness was also reflected in measurements of the leading front of invasion. The leading front of invasion is defined as the deepest level in the gel where at least two cells are simultaneously in focus and is determined at the end of the experiment using the calibrated fine focus of an inverted microscope (Figure 4B). The HBL-100 normal human breast cell line did not invade and the leading front of cells was 100 μm or approximately one cell diameter into the gel (Figure 4B, H100). The human breast cancer cell lines invaded into type I collagen with the leading front of MCF-7 cells approximately six cell diameters into the gels and the leading front of MDA-MB-231 cells was 12 cell diameters into the gels (Figure 4B, MCF7 and M231).

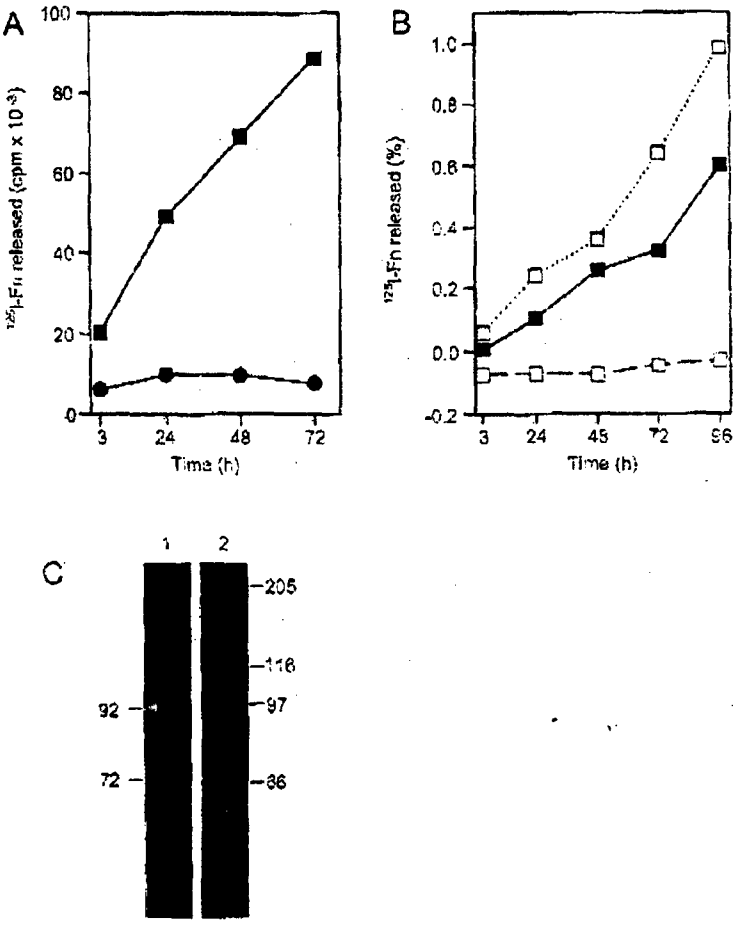

Figure 3. Quantification of matrix proteolysis by normal and malignant human breast cells. (A) Malignant MDA-MB-231 human breast cells (■) release up to 11-fold more fibronectin into the media than the normal HBL-100 human breast cells (●) over 72 h. Levels of radioactivity were determined in two separate experiments. The average of the two determinations after background subtraction is plotted at each time point. (B) Inhibition of MDA-MB-231 human breast cancer invadopodial proteolysis of matrix by the metalloproteinase inhibitor batimastat. Batimastat-treated (10 μM) MDA-MB-231 human breast cancer cells (-o-) release less [125I]fibronectin from the substrate than the no-cell control (background subtracted). MDA-MB-231 human breast cancer cells degrade [125I]fibronectin in the absence (... □ ...) or presence of the DMSO (■) vehicle. Results are expressed as percentage of substrate released and plotted to the average of determinations made in two separate experiments. The average of the two determinations after background subtraction is plotted for each time point. (C) Lane 1 is a zymogram showing gelatinase activities at 92 and 72 kDa secreted into serum-free growth medium conditioned by MDA-MB-231 cells. Lane 2 is a zymogram of the same sample incubated with 10 μM batimastat causing loss of the 92 and 72 kDa gelatinase activities.

Batimastat inhibits MDA-MB-231 human breast cancer cell invasion into type I collagen gels

The correlation between invadopodial matrix-degrading activity (Figures 1–3) and invasion into type I collagen gels (Figure 4) suggested that inhibition of invadopodial matrix proteolysis would decrease invasiveness of the malignant cells. To substantiate the observed correlation, the effect of inhibiting invadopodial proteolysis of matrix on invasiveness was investigated. Batimastat inhibits invasion of MDA-MB-231 cells into type I collagen gels (Figure 5A). Batimastat inhibited invasion from 2.7-fold to 10-fold relative to the percentage of invading cells observed in control cultures growing in the presence of DMSO (Figure 5A). Malignant MDA-MB-231 cells growing in growth medium or those growing in growth medium with added DMSO revealed large invading cell populations (Figure 5A). Normal HBL-100 cells had low numbers of cells invading the type I collagen gels (Figure 5).

Batimastat also reduced the leading front of invasion observed for MDA-MB-231 human breast cancer cells (Figure 5B). Batimastat caused the leading front of invasion to be indistinguishable from cells at the top of the gel or located less than half as deep as that observed for cells grown without batimastat (Figure 5B). MDA-MB-231 breast cancer cells growing in growth medium, or in growth medium with added DMSO revealed leading fronts of invasion ranging from 9 to 11 cell

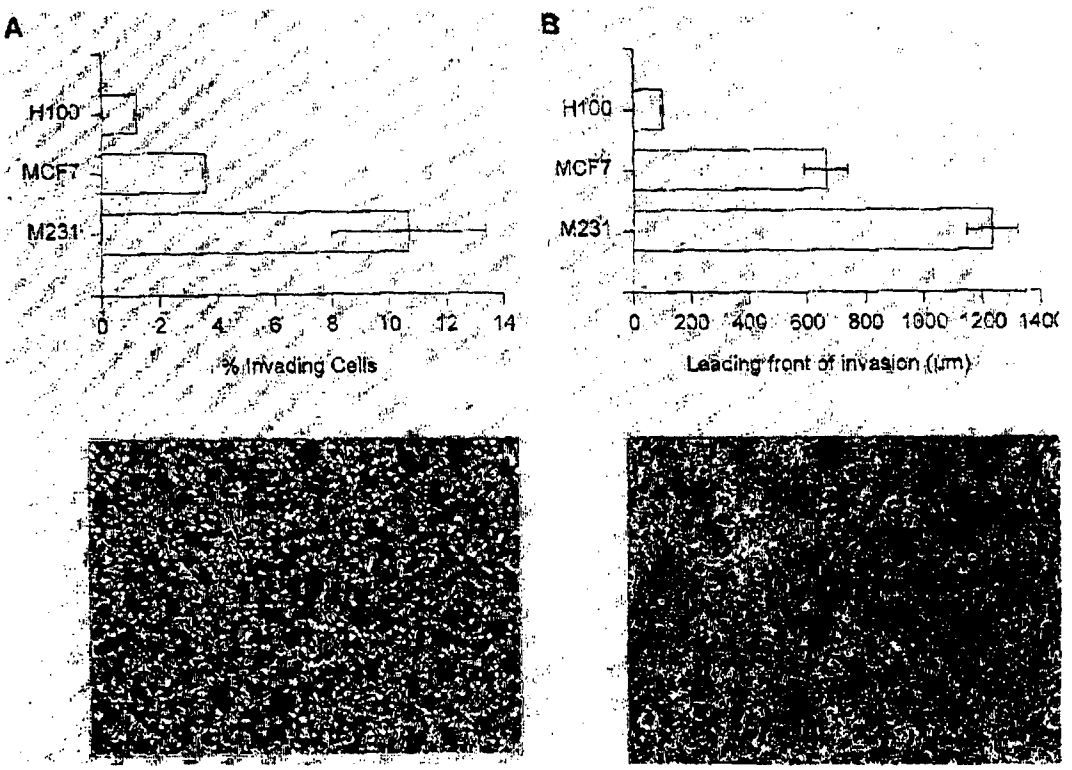

Figure 4. MDA-MB-231 human breast cancer cells invade into type I collagen gels. (A) Invasion of different human breast cell lines into type I collagen gels. The percentage of cells that invade into type I collagen after 72 h was determined for three different human breast cell lines. The HBL-100 normal human breast cell line (H100) is least invasive with only 1.2% ± 0.1 invading cells, the MCF-7 human breast cancer cell line (MCF7) is moderately invasive with 3.58% ± 0.4 invading cells, and the MDA-MB-231 human breast cancer cell line (M231) is most invasive with 10.7% ± 2.7 invading cells. In these experiments, cells were grown in medium containing 10% complete FBS. For each cell line, the bar length indicates the average value of 16 different determinations obtained in eight separate experiments. Error bars indicate ± the standard error of the mean. (B) Depth of the leading front of invasion into type I collagen gels of different human breast cell lines. The depth of the leading front of cells invading into type I collagen gels was determined after 72 h for three human breast cell lines. The HBL-100 cells (H100) invade least deeply into the type I collagen gels (100 μm, ± 5.8), MCF-7 cells (MCF7) invade to a moderate depth (667 μm, ± 72.7) and MDA-MB-231 cells (M231) invade furthest into the type I collagen gels (1233 μm, ± 88.2). For each cell line, the bars indicate the average value of 40 determinations obtained in the same eight experiments reported in (A). Error bars are drawn to ± the standard error of the mean. (C) MDA-MB-231 human breast cancer cells growing on top of a type I collagen gel. Cells (5×10^4) were seeded on top of the gel and allowed to invade for 72 h. In this panel, the plane of focus is the top of the gel. (D) Three MDA-MB-231 cells (arrows) are shown invading 600 μm into the type I collagen gel. They were located by focusing down from the top of the gel in the microscopic field shown in (C). The scale bar (100 μm) for (C) and (D) is shown in (D).

diameters into the gel (Figure 5B; M231 and M231 + DMSO). Normal HBL-100 cells invaded approximately 2.5 cell diameters into the type I collagen gels (Figure 5B, H100). Together, these results indicate that proteolysis of extracellular matrix by invadopodia facilitates human breast cancer cell invasion and is mediated by MMPs.

Discussion

Invadopodial proteolysis of extracellular matrix greatly facilitates invasion by human breast cancer cells. Two main lines of evidence support this conclusion. First, the invasion potential of breast cancer cells is directly correlated with the extracellular matrix degrading activity of their invadopodia.

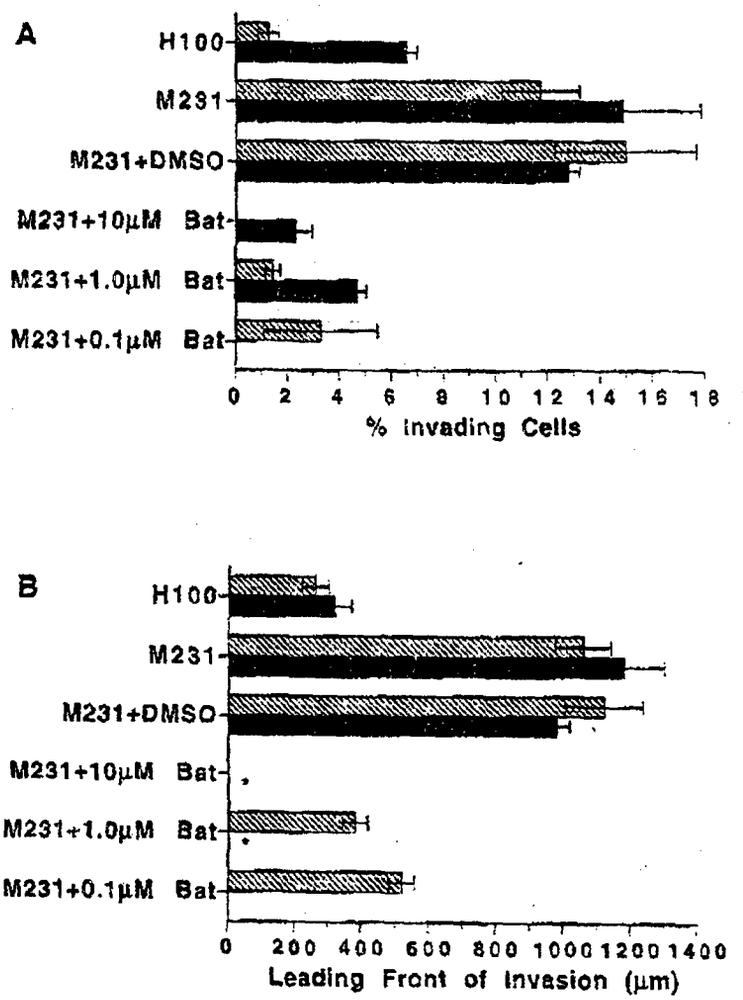

Figure 5. Batimastat inhibits the invasive behavior of MDA-MB-231 human breast cancer cells. The percentage of HBL-100 cells (H100) and MDA-MB-231 cells (M231) invading into type I collagen gels (A) and the leading front of invading cells (B) observed in two different experiments are shown (A, B: hatched bars = experiment 1, black bars = experiment 2). (A) Batimastat inhibits invasion of MDA-MB-231 breast cancer cells into type I collagen gels. Batimastat (1.0 μM) inhibited invasion up to 10-fold relative to DMSO control. Increased batimastat concentrations resulted in decreased invasion (experiment 1 (hatched bars): M231 + 0.1 μM Bat and M231 + 1.0 μM Bat; experiment 2 (black bars): M231 + 1 μM Bat and M231 + 10 μM Bat). The graphs are the average of two data points and the bars indicate the range of the determinations. (B) Batimastat reduces the distance traveled into the type I collagen gel by cells at the leading front of invasion. In experiment 1 (hatched bars), the leading front of invasion was reduced 2.2-fold by 1.0 μM batimastat (M231 + 1.0 μM Bat) and 2.9-fold by 0.1 μM batimastat. In experiment 2 (black bars), invading cells were not detected (*) below the top of the type I collagen gel in the batimastat-treated groups (M231 + 10 μM Bat* and M231 + 1.0 μM Bat*). The graphs are averages of measurements of the leading front of invasion at five locations within the gel and bars represent ± standard error of the mean.

For each cell line tested, the relative level of invadopodial proteolysis of extracellular matrix as measured using fluorescent or radiolabeled fibronectin substrates, positively correlated with each of two different measurements of invasiveness: (i) the percentage of cells invading into type I collagen gels and (ii) the distance traveled by cells at the leading front of invasion. Second, treatment of invasive MDA-MB-231 human breast cancer cells with the MMP inhibitor, batimastat, reduced invadopodial function as assessed by measuring cell-mediated proteolysis of immobilized fibronectin. Batimastat also reduced MDA-MB-231 cell invasion into type I collagen gels as reflected by the reduced percentages of invading cells and shorter distances invaded into the gels relative to untreated cells. Batimastat inhibition of invadopodial matrix proteolysis and invasion is apparently due to its inhibition of MMP proteolytic activities because batimastat effectively inhibited two gelatinase activities at 92 and 72 kDa that were secreted by the MDA-MB-231 cells. However, batimastat did not affect cell viability. The low cytotoxicity of batimastat and MMP inhibition by batimastat over the range of concentrations used in this study (0.1–10 μm) has also been observed for human MDA-MB-435 breast cancer cells [28]. The results presented here confirm separate observations regarding either invadopodial proteolysis or invasion of human breast cancer cells reported by others [8,17]. This study strengthens the linkage of

invadopodial proteolysis of extracellular matrix to the invasion process because both parameters were observed and manipulated in the same study.

MMPs play a critical role in mediating the invadopodial proteolysis of extracellular matrix that facilitates tumor cell invasion. This is evidenced by the effectiveness of the MMP inhibitor batimastat in simultaneously reducing invadopodial proteolytic activity and invasiveness of malignant breast cells. A number of MMPs have been implicated in having a role in proteolysis of extracellular matrix by human breast cancers including: MMP-2 (gelatinase A) [29, 30], MMP-9 (gelatinase B) [2, 31, 32], MMP-11 (stromelysin-3) [33], an 80 kDa MMP [34] and MMP-14 (MT1-MMP) [35]. Batimastat inhibition of MMPs has been effective in reducing the growth and spread of mammary tumors in animal models [28, 36]. Moreover, batimastat and a more bio-available derivative of batimastat called marimastat (BB-2516) are being evaluated in clinical trials for use as an anti-tumor therapeutics [37, 38]. The development of MMP inhibitors for use in anti-tumor therapies continues to be a promising area of research [26].

Although there is mounting evidence that MMPs have a critical role in tumor cell invasion into the complex basement membrane and stromal matrices within living organisms, the evidence also suggests that coordinated activity of other proteases and glycosidases in addition to MMPs is needed to efficiently degrade the matrix and enable invasion [1]. This study was primarily focused on the role of MMPs in breast cancer cell invasion because the gel substrate used, triple helical type I collagen, is resistant to proteolytic cleavage by other classes of proteases [2]. However, an *in vitro* study showed that in the presence of serum, batimastat only inhibited MDA-MB-231 proteolysis of human endothelial basement membranes by 30%. An additional 30–40 % inhibition of basement membrane degradation was achieved when batimastat was used in combination with inhibitors of urokinase-type plasminogen activators [39]. There is an apparent need for other classes of proteases for tumor cells to efficiently degrade complex extracellular matrices.

Elucidation of the mechanisms that concentrate MMPs and other extracellular matrix-degrading proteases to invadopodia may lead to new malignant cell diagnostics and strategies to inhibit their invasion. MMP-2 and other matrix-degrading proteases are not restricted to invadopodia. MMP-2 is secreted as a soluble enzyme that can be found within the cytoplasm of cells and embedded in the extracellular matrix as well as on invadopodia. The integral membrane MMP, MT1-MMP, has been identified as an activator of latent MMP-2 and proposed to serve as a cell surface receptor for MMP-2 [40]. In addition, the $\alpha_v\beta_3$ integrin has also been shown to serve as a cell surface receptor for MMP-2 [41]. MT-MMPs, integrins or both of these molecules may have a role in concentrating MMP-2 to invadopodial membranes of human breast cancer cells. It has been shown that MT1-MMP must be localized to invadopodia to stimulate the local degradation of extracellular matrix that is characteristic of invadopodia and that its cytoplasmic domain has a role in directing MT1-MMP to invadopodial membranes [12]. Another mechanism for recruitment of active proteases to invadopodial membranes could involve directed oligomerization of the subunits of integral membrane proteases such as seprase, fibroblast activation protein-α, dipeptidyl peptidase IV, and meprin [3]. This directed oligomerization might occur in response to signaling through integrins [42]. Perturbing the recruitment and assembly of proteases on the invadopodial surface might effectively reduce invadopodial proteolysis of extracellular matrix and limit invasion.

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Evaluation of seprase activity

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Abstract

Seprase is a serine protease that is integral to the plasma membrane and is overexpressed by invasive tumor cells (Piñeiro-Sánchez et al, 1997, J. Biol. Chem. 272:7595; Monsky et al, 1994, Cancer Res. 54:5702). Seprase activity is most often assessed by zymography, which is not a quantitative assay. This study establishes a relatively simple and quantitative method for determining seprase activity. The degradation of a ^{3}H-gelatin substrate is measured in the presence of 5 mM EDTA which inhibits matrix metalloproteinases but not seprase. The quantitative character of the assay was demonstrated using partially purified seprase from chicken embryos, a preparation that lacks detectable matrix metalloproteinase activity. In this assay, release of ^{3}H-gelatin fragments is linear over time for 1.5 µg/assay seprase concentration as well as for preparations concentrated or diluted by five fold (7.5 µg/assay and 0.3 µg/assay respectively). Additional experiments were performed to validate the quantification of seprase activity using the radiographic assay by comparing the results to zymography. Exposure to 22 or 37° C results in maximal seprase activity while exposure to 80 or 100° C completely abolishes seprase activity in both zymography and the radiographic assay. Exposure to 60° C abolished seprase activity as judged by zymography, but about 50 % gelatinase activity was observed using the ^{3}H-gelatin substrate. Immunopreciptiation with seprase-specific antibody specifically removed seprase and lowered the seprase activity remaining in the extracts as judged by both assays. Investigation of the seprase that was partially purified from human breast cancer tissue revealed that its specific activity (cpm gelatin fragments released / {mg protein x h}) is five times greater than that of seprase purified from chicken

embryos. This assay will be useful for determining the seprase activity in extracts of tumor tissues and cells as well as for identifying inhibitors of seprase.

Keywords: Breast cancer, gelatinase, matrix metalloproteinase, extracellular matrix, invasion

Introduction

Seprase was first identified as a 170 kDa gelatinase activity in detergent extracts of the human melanoma cell line LOX (1) and then as a 160 kDa gelatinase activity in detergent extracts of Rous sarcoma virus transformed, chicken embryo fibroblasts (2). Because seprase partitions to the detergent phase of Triton X-114 extracts, it was determined that seprase is an integral membrane protease (1, 2). The gelatinase activity of seprase was shown to be sensitive to heat, acid pH, phenyl methyl sulfonyl fluoride (PMSF) and N-methyl maleimide (NEM) but insensitive to β-mercaptoethanol, dithiothreitol, EDTA, 1,10 phenanthroline, pepstatin, and leupeptin; suggesting that seprase is a serine or cysteine protease (1, 2). The 170 kDa active enzyme is a complex of two identical 97 kDa subunits (3, 4). The molecular cloning and analysis of a cDNA encoding the human melanoma 97 kDa seprase subunit protein indicate that it is a type II transmembrane serine protease with the catalytic triad amino acid residues (S, D, H) arranged in the non-classical orientation (4, 5). Interestingly, the seprase subunit protein is proteolytically inactive despite the fact that it includes the entire catalytic domain. Seprase, apparently, requires oligomerization into dimers for proteolytic activity (4, 6). Inactivation of the seprase proteolytic activity is caused by agents that dissociate the subunit proteins such as acid pH, heat, and alkylating agents including diethyl pyrocarbonate (DEPC) and NEM, as well as by agents that modify the catalytic site, serine, such as PMSF and diisopropyl fluorophosphate (DFP) (4).

Seprase is thought to have a role in facilitating tumor cell invasion and metastasis (1, 6). Several observations support this hypothesis. First, seprase degrades extracellular matrix and is overexpressed by invasive tumor cells. For

example, high levels of seprase activity were detected in extracts of the highly invasive human melanoma cell line LOX , but seprase activity was not detected in extracts of 32 other tumor cell lines that were not invasive in *in vitro* assays (1). Seprase over-expression by invasive tumor cells has also been observed in pathologic specimens of human breast cancer tissue by immunohistochemistry (7). Second, seprase is localized on the plasma membrane where it could interact with extracellular matrix substrates, and it is concentrated on invadopodia, specialized protrusions of the plasma membrane that cause proteolysis of extracellular matrix (2, 3). Thirdly, a positive correlation between the level of seprase expression and invasive behavior of LOX human melanoma cells has been observed (3). Although overexpression of seprase is apparently an important feature of the invasive phenotype, methods for determining seprase activity are not well developed.

Seprase in extracts of cells and tissues has been analyzed by immunoblotting and gelatin zymography, techniques that are difficult to quantify and may not give accurate indications of the level of seprase activity. In addition, immunoblot analysis measures the level of 170 kDa and 97 kDa proteins independent of their proteolytic activities. Zymography is not generally used for quantification of proteolytic activity due to the saturation of the signal that occurs. Zymography and immunoblotting allow identification of seprase in crude extracts but require solubilization of the extract proteins in SDS followed by SDS-PAGE. These treatments can change the apparent activity of proteases (8). It is conceivable that zymography could reduce the apparent seprase activity by causing dissociation of some assembled dimers into inactive monomers.

This study was performed to establish a quantitative assay for the proteolytic activity of seprase in extract samples. A seprase activity assay was developed that is based on established procedures for measuring the gelatinase activities of MMPs (9). A ^{3}H-gelatin substrate was used in conjunction with buffers that included EDTA to promote seprase activity while inhibiting the activities of MMPs. The assay is shown to be linear over several concentrations of seprase. This assay will be useful for determinations of the net seprase activity in relatively crude extracts as well as for direct determinations of the activity of purified seprase.

Materials and Methods

Sources of seprase

The partial purification of seprase has been described (7) and is based on published methods (1, 4). Briefly, nine day old chicken embryos were rinsed 3 times with cold PBS, and used immediately or stored at -80° C. The embryos were homogenized and peripheral membrane proteins were removed by sequential extractions. The homogenate was extracted at 37° C with 10 volumes of 10 mM Tris, 5 mM EDTA, pH 7.6. The insoluble material was collected by centrifugation at 10,000 x *g* and extracted with 10 mM Tris-HCl, 0.6 M NaCl, 5 mM EDTA, pH 7.6 for 1 h at 4° C. The insoluble material was collected and extracted with 2.6 % Triton X-114, 10 mM Tris-HCl, 5 mM EDTA, pH 7.6. The soluble material was collected by centrifugation as described above and the detergent extract supernatant was recovered.

The detergent extract was phase partitioned (10), and the detergent phase was separated from the aqueous phase by centrifugation. The detergent phase was diluted and loaded at 70 ml/h onto an 80 ml DEAE-cellulose column (Whatman DE52, Whatman Labsales, Hillsboro, OR) equilibrated in 0.5 % Triton X-100, 5 mM EDTA, 10 mM Tris-HCl, pH 7.5. The column was washed and then eluted with a continuous 0 to 400 mM NaCl gradient in the equilibration buffer. Fractions with seprase activity were identified by zymography and pooled for purification by WGA agarose. Purification of seprase from 11.5 g of human breast tumor tissue was performed as described for chicken embryo seprase, except that the DEAE cellulose column was omitted.

Zymography: Zymography was performed essentially as described by Heussen

& Dowdle (11) with 1 mg/ml gelatin copolymerized into 10 % SDS polyacrylamide gel.

Gelatinase activity assay: Gelatin (100 mg, type A porcine skin, 300 bloom, Sigma, St. Louis, MO) in 10 ml borate buffer, pH 9.5 was labeled with 1 mCi ^{3}H-acetic anhydride (12). The ^{3}H-gelatin in the radiolabeled stock solution was separated from free isotope by gel filtration over an 80 ml Sephadex G-150 column at 22° C. A broad peak of labeled gelatin began eluting immediately after the void volume and was well separated from the sharp peak of unbound isotope. The ^{3}H-gelatin was mixed 1:2 with neutralized, acid soluble type I collagen (1 mg/ml, Sigma, St. Louis, MO) in 50 mM Tris-HCl, pH 7.6, 5 mM EDTA. The collagen was denatured by heating at 55° C for 30 min.

The gelatinase activity of seprase was determined using a modification of the assay of Stetler-Stevenson and co-workers (9). Reactions to determine the gelatinase activity of seprase were performed in triplicate in the wells of a 96 well microtiter plate with 13 µl seprase-containing fractions; 13 µl of ^{3}H-gelatin substrate, and 47 µl of the reaction buffer: 0.1 % Triton X-100, 50 mM Tris-HCl, 5 mM EDTA, pH 7.6. Samples to determine the background cpm were prepared by mixing 60 µl of reaction buffer and 13 µl substrate then precipitating the undigested gelatin with 20 µl TCA/TA on ice. Samples to determine the total counts were prepared by mixing 13 µl substrate, with 80 µl of reaction buffer but no TCA/TA was added to these samples. Experimental reactions were stopped at various time points and the undigested substrate precipitated by addition of 20 µl 10 % trichloro acetic acid, 5 % tannic acid (TCA/TA). Reactions were precipitated for 1 h on ice and then the precipitates were cleared by a 10 min, 1200 x g centrifugation at 4° C in a Beckman TJ-6 refrigerated table top centrifuge and a TH-4 rotor. Samples (60 µl) were mixed vigorously in 4.5 ml of Ecolume scintillant

(ICN Pharmaceuticals Inc., Costa Mesa, CA) and counted with a Packard 1600 TR liquid scintillation counter.

To determine if the assay was linear over several concentrations, seprase enriched fractions were concentrated 5 fold to 0.58 mg/ml using centricon 30 filter concentrators (Amicon, Inc, Beverly, MA) and then used as is, diluted to the original concentration, or diluted to 1/5 the original concentration with reaction buffer.

Determination of fold purification: The activity for each fraction was determined using the ^{3}H gelatin substrate and the gelatinase activity assay described above. The cpm of TCA/TA-soluble ^{3}H gelatin liberated per mg protein was determined in triplicate at 1, 5, 10, 24, and 30 h for each fraction, and the average of these determinations was plotted. A line was drawn to pass through the origin and use all available data by first order (linear) regression, as calculated by Jandel Sigma Plot for Windows (SPSS, Inc., Chicago, IL). For each fraction, the slope of the "cpm/mg" versus "time" line is equal to the specific activity of the seprase in the sample, and was used to determine the fold purification. Activity determinations have been performed on 3 other seprase preparations with similar results.

Immunoprecipitations: A rat polyclonal antiserum was elicited against affinity purified chicken embryo seprase as described earlier (7). Immunoprecipitations were performed using 15 µl rat serum directed against the 97 kDa protein or 15 µl of preimmune serum from the same rat, 200 µl protein A agarose (Pierce, Rockford, IL), and 200 µl of WGA-purified chicken embryo seprase. The subtraction of seprase was determined by gelatin zymography. Immunoprecipitations were also performed to

measure the effect of removing seprase activity on the gelatinase activity of partially purified seprase preprarations. These precipitations were performed by coating 100 µl of protein A beads with 30 µl or 60 µl of antiserum, washing away unbound proteins, and then exposing the antibody-coated protein A agarose to 200 µl WGA-purified chicken embryo 160 kDa protein. The gelatinase activity present in the fraction that was not bound to the antibody-coated beads was measured by soluble gelatinase activity assay described above. The gelatinase activities of the purified seprase preparation used for these experiments and of the fraction not bound by protein-A beads coated with nonimmune rat antiserum were also determined.

Protein determinations were performed using the bicinchoninic acid assay and bovine serum albumin dissolved in Tris-HCl, pH 6.8, 1 % (w/v) SDS as the standard protein (Pierce, Rockford, IL).

Results

A source of seprase was needed to enable development of a quantitative assay for seprase activity. Chicken embryo seprase was partially purified and used to establish a gelatinase activity assay with a ^{3}H-gelatin substrate. The partial purification procedure was developed from previously published methods for purifying human melanoma cell seprase (1, 4). A series of extractions removes many peripheral membrane proteins and then the seprase is solubilized with Triton X-114 and EDTA. Seprase activity partitions exclusively to the detergent phase and is further purified using DEAE-cellulose and WGA-agarose. Gelatin zymography reveals that the resulting preparation has high 160 kDa seprase activity but no detectable MMP activity (Fig. 1). Specifically, the 70 kDa chicken homolog of MMP-2 is not detected in the partially purified seprase preparations (Fig. 1). Proteolysis of a ^{3}H-gelatin substrate by this preparation was investigated by incubating the seprase and the ^{3}H-gelatin over time and then using TCA/TA to precipitate the undigested substrate at different time points. Measurement of the TCA/TA-soluble, radiolabeled gelatin degradation products at various time for several different levels of the same fraction of partially purified seprase protein (7.5 µg, 1.5 µg, and 0.3 µg) over 5 h reveals that the observed gelatinase activity increases with increasing concentrations of seprase (Fig. 2A). Plotting the slopes of this data (cpm/h) versus protein concentration reveals that these samples are within the linear range of the assay (not shown). Investigation of samples from different steps in the seprase enrichment procedure reveals a close correspondence between the increasing purification of seprase and increasing gelatinase activity (Fig. 2B). Moreover, these experiments were conducted over 30 hours and the assay remained

linear over this time frame (2B). The gelatinase activity assay reveals a 21.8 fold enrichment of the seprase activity by the purification procedure (Table 1).

Investigation of the temperature sensitivity of chicken embryo seprase reveals that seprase activity is not affected by 15 min preincubation at 22 or 37° C, because maximal activity is observed by both gelatinase activity assay (Fig. 2C) and zymography (Fig. 2D). However, a 15 min preincubation of the seprase at 80 or 100° C inactivates the seprase and no activity is observed in either assay (Fig. 2C & D). Preincubation of the seprase at 60° C partially inactivates the seprase as judged by gelatinase activity assay (Fig. 2C) but completely inactivates seprase as judged by zymography (Fig. 2D). Thus, the loss of seprase activity by heating is detected as a reduction in gelatinase activity by both assays.

An antiserum that recognizes both active 160 kDa chicken embryo seprase (Fig. 3A, Active) and the proteolytically inactive 97 kDa subunit of chicken embryo seprase (Fig. 3A, Inactive) was used to immunoprecipitate seprase. Immunoprecipitation with the seprase specific antibody removes much of the seprase and causes a corresponding drop in the seprase activity that remains in the extract. This specific reduction in seprase activity is detected by both zymography (Fig. 3B) and gelatinase activity assays (Fig. 3C). The antibody to seprase removes up to 50 % of the seprase activity from the unbound fraction (Fig. 3C, plot D) relative to the unbound fraction of control precipitates (Fig. 3C, plot B).

Seprase was also partially purified from human breast tumors (11.5 g) using the procedure described above for partial purification of chicken embryo seprase, except that the DEAE cellulose column was omitted. These preparations reveal a 170 kDa

gelatinase activity that is seprase because: i) the gelatinase is active in the presence of EDTA which inhibits MMPs, ii) the gelatinase activity requires detergent for solubilization and partitions to the detergent phase of Triton X-114 extracts (Fig. 4), and iii) the gelatinase binds to WGA. Seprase was the only gelatinase activity detected by zymography in these samples (Fig. 4). The gelatinase activity assay revealed that human breast cancer seprase is purified 21 fold by this procedure (Table 2). Moreover, breast cancer seprase is 5 times more active than chicken embryo seprase when the specific activities (cpm gelatin released/{mg protein x h}) of seprase from the two sources are compared (Tables 1 & 2).

Discussion

Seprase activity in extracts can be measured using a ^{3}H-gelatin substrate. This conclusion is supported by the close correlation of the relative seprase gelatinase activities in different samples as judged separately by the gelatinase activity assay and zymography. Specifically, loss or reduction of seprase activity by heating or immunoprecipitation resulted in corresponding losses or reductions in the observed seprase band of activity on zymograms and amount of soluble radiolabeled gelatin fragments. Concentrating or enriching seprase activity resulted in corresponding increases in seprase activity in both assays. One difference between zymography and the seprase activity assay was that after heating to 60° C seprase activity was observed in the gelatinase activity but not by zymography. This result likely reflects the re-naturation and re-association of a fraction of the seprase subunit proteins during the gelatinase activity assay. In zymography, such reassociation of seprase subunits probably would not occur due to SDS in the gel sample buffer binding to dissociated seprase subunits and the subsequent separation of the proteins by SDS-PAGE. The gelatinase activity assay allows the amount of apparent seprase activity to be determined relative to extract protein. In this way, the apparent specific activity of seprase was observed to be 5 times greater in extracts of human breast cancer tissue than in chicken embryo extracts. This finding is consistent with the overexpression of seprase in breast cancer tissues observed by immunohistochemistry (7) and suggests that breast cancer tissue has a high level of fully assembled, active seprase. It is also consistent with the differential expression of seprase in chicken embryonic tissues because the initial extract includes proteins from the entire embryo where some tissues

have detectable seprase activity and others do not (Kaushal, V.; Goldstein, L. A.; Chen, W.-T. and Kelly, T. manuscript in preparation). However, the possibility that the breast cancer extracts contain additional proteases that are not detected by zymography or reduced inhibitors of these proteases can not be completely ruled out.

Determination of seprase activity in extracts at physiologic pH and salt concentrations affords a means to investigate seprase activity under conditions that more closely resemble those found *in vivo*. Quantitative comparisons of activity can be made between different samples with the determinations focused on the active enzyme and not the level of inactive subunit proteins. The technique will be useful for measuring the level of seprase activity of cells that overexpress seprase as part of pathologic processes or because they have been genetically engineered for seprase overexpression. The seprase activity assay may be particularly useful for identification of molecules that inhibit seprase activity. Antibodies and other non-toxic inhibitors of seprase will be valuable for clarifying the role of seprase in tumor cell invasion and may ultimately have clinical utility.

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Figure Legends

Figure 1. Partial purification of seprase from chicken embryos.

A) The upper flow-chart outlines the purification procedures. The middle panels show the seprase activity in different fractions of chicken embryo seprase purification as detected by gelatin zymography. Seprase activity is extracted by 2.6 % Triton X-114 (Total detergent extract) and partitions strictly to the detergent phase (Detergent). The detergent phase was loaded onto a DEAE cellulose column. Seprase bound to the column and the majority of the activity eluted between 0.1 M NaCl and 0.25 M NaCl (DEAE column). This fraction was loaded onto a wheat germ agglutinin column. The seprase bound to the column, indicating that it is a glycoprotein, and was eluted with 10 % (w/v) n-acetyl glucosamine (WGA column upper and lower panels). Molecular weight markers are given on the right x 10^{-3}.

B) The panel on the left is an SDS-PAGE of the proteins in chicken embryo seprase that was partially purified as described above. The panel on the right is a gelatin zymogram of this same sample. Both gel and zymogram were stained with Coomassie Brilliant Blue R-250. Stained proteins are detected at 190 kDa and 160 kDa that commigrate with the gelatinase activity. Molecular weights are given on the right x 10^{-3}.

Figure 2. Quantification of the gelatinase activity of seprase

A) The seprase activity assay is linear over a 5 h time period using a ^{3}H-gelatin substrate and three concentrations of the same partially purified chicken embryo

seprase fraction: 7.5 µg (●), 1.5 µg (■) and 0.3 µg (▲). The TCA/TA-soluble ^{3}H-gelatin proteolyltic fragments were determined (CPM) over five hours (Time (h)). Gelatinase activity increases with increasing concentration of seprase. Assays were performed in triplicate in the presence of 5 mM EDTA, 0.1 % Triton X-100 and each point is the average of 3 assays after background subtraction. For each plot, the solid line represents the first order regression of the data.

B) Fractions from the purification of chicken embryo seprase were analyzed by gelatinase assay to determine the substrate hydrolyzed per mg of protein (CPM/mg) over time (h). The plots are the first order regressions for: the initial low ionic strength extraction (▼); total detergent extract (◆); detergent phase (■), DEAE pooled fractions (▲), and WGA pooled fractions (●). Observed gelatinase activities increase with increasing seprase purity. Assays were performed in triplicate in the presence of 5 mM EDTA, 0.1 % Triton X-100 and each point is the average of 3 assays after background subtraction.

C) Chicken embryo seprase (7.5 µg) was maintained at temperatures of 22, 37, 60, 80, or 100° C for 15 min and then used for gelatinase assay. Seprase was not affected by incubation at 22 (●) or 37° C (■), as the observed gelatinase activities are identical (first order regression for both sets of data results in superimposed lines). Gelatinase activity was significantly reduced by treatment at 60° C (▲), and destroyed by 80 (▼) or 100° C (◆). Assays were performed in triplicate in the presence of 5 mM EDTA, 0.1 % Triton X-100 and each point is the average of 3 assays after background subtraction.

D) By gelatin zymography, seprase activity is detected only in samples treated with 22 and 37° C. The activity is lost in samples exposed to 60, 80 and 100° C. Molecular weight is given x 10^{-3}.

Figure 3. The gelatinase activity assay determines specific reductions in seprase activity.

A) Immunoblot analysis of WGA-purified chicken embryo seprase. A polyclonal antibody to seprase recognizes the 160 kDa active seprase dimer together with a low level of a 97 kDa subunit protein (Active). Boiling this sample prior to electrophoresis results in loss of the 160 kDa protein and increased 97 kDa protein (Inactive).

B) Immunoprecipitation of the seprase-gelatinase activity as determined by zymography. Gelatin zymogram showing that chicken embryo seprase activity (white band at 160 kDa, both panels) in the original extract (Load lanes, both panels) is not significantly reduced by immunoprecipitation with preimmune serum (Preimmune/Not Bound). However much of the seprase activity is removed by immunoprecipitation with the antibody against seprase because much less activity is present in the unbound supernatant fraction (Not Bound/Anti-seprase) relative to that in the initial extract (Load/Anti-seprase). Molecular weights are given x 10^{-3}.

C) Immunoprecipitation of seprase-gelatinase activity as determined by an activity assay using a ^{3}H-gelatin substrate. Gelatinase activity in a seprase preparation used for immunoprecipitation experiments (A). Non-immune rat IgG did not

precipitate seprase activity and the activity remaining in the unbound fraction (B) is only slightly reduced relative to that in the initial extract. Seprase activity was specifically removed from the extract by immunoprecipitation with 30 µl (C) or 60 µl (D) rat polyclonal antibody against seprase, resulting in a reduction in the gelatinase activity remaining in the extract relative to the initial extract. Two separate experiments are shown (Black & Hatched bars) with bar height indicating the average of the three determinations. Error bars are drawn to the standard error of the mean.

Figure 4. Seprase activity is present in extracts of malignant human breast tumors. Seprase was enriched in the detergent phase of Triton X-114 extracts of human tumors that were evaluated by pathologists to be infiltrating ductal carcinomas of the breast. Seprase activity at 170 kDa and smearing to 205 kDa is apparent in this fraction as judged by zymography. Molecular weight markers are given x 10^{-3}.

Table 1. Enrichment of chicken embryo seprase

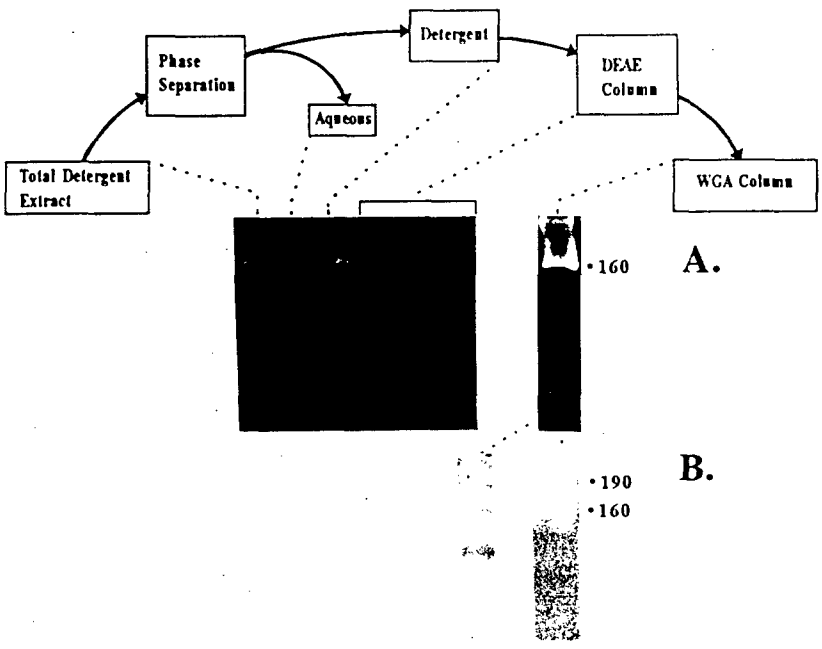
429.4 g chicken embryos

Fraction	Units activity (cpm/{mg x h})	Fold Purification	Protein (mg)
Initial Extract	263.9	-	7,238
TX-114 Extract	265.7	1.0	617.5
Detergent phase	328.1	1.2	328.9
DE-52	3,467.9	13.1	14.8
WGA	5,742.9	21.8	5.8

Table 2. Enrichment of human breast cancer seprase

11.5 g human breast cancer tumor

Fraction	**Units Activity (cpm/{mg x h})**	**Fold Purification**	**Protein (mg)**
Initial Extract	1,852	-	61.08
WGA	38,680	21	0.155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Aqueous
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WGA Column
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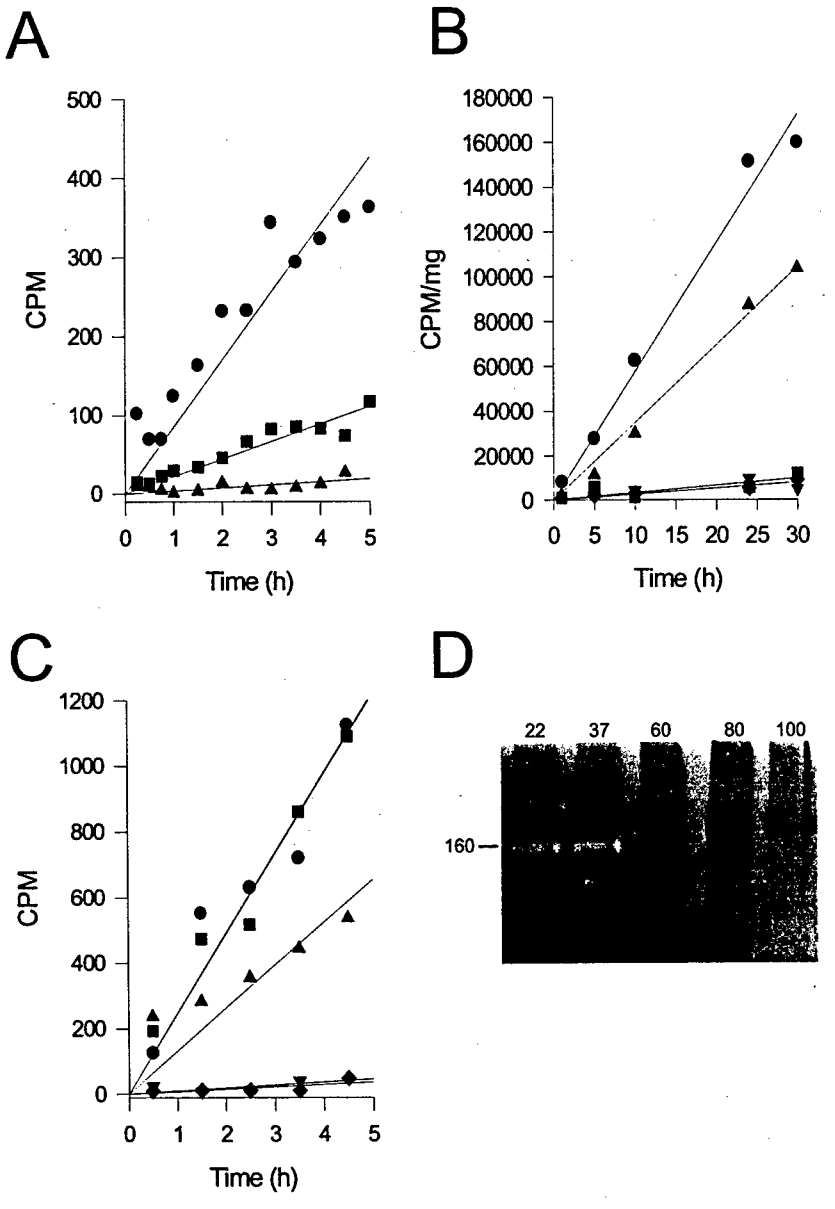
A.
•190
•160
B.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B
C
D
CPM
CPM/mg
Time (h)
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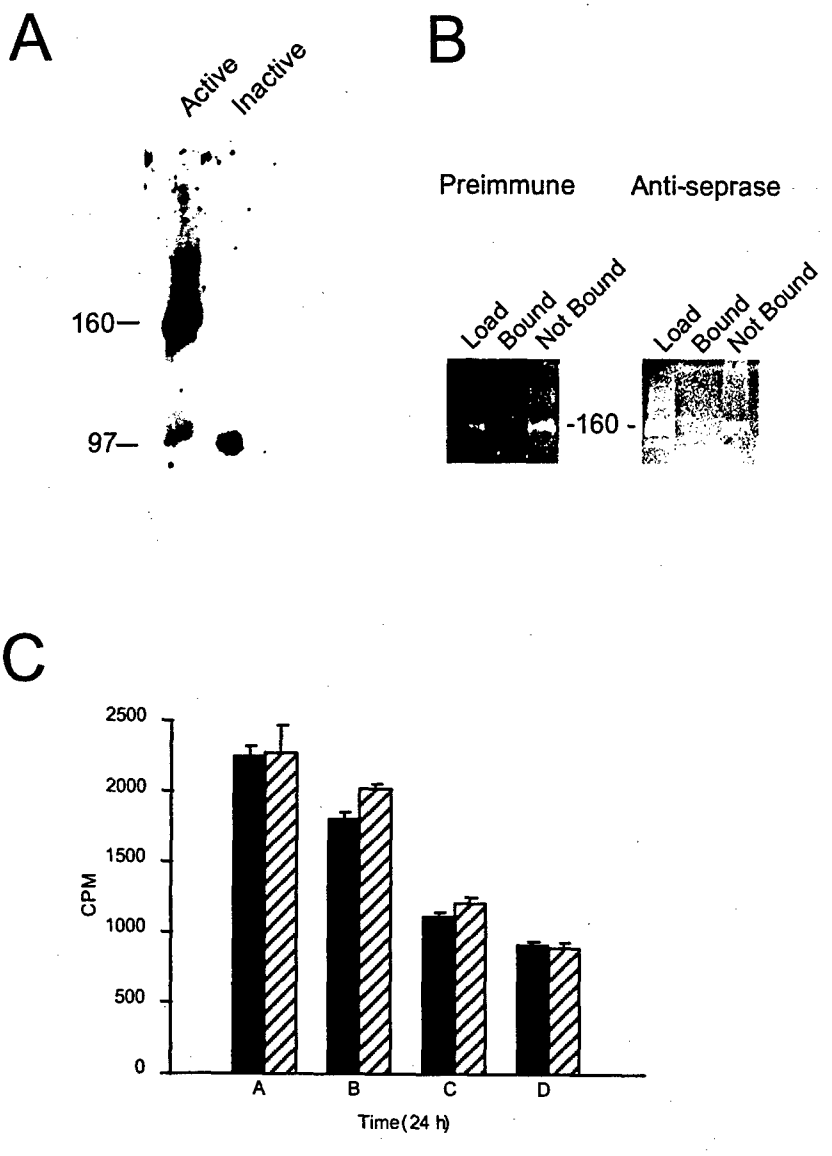
37
60
80
100
160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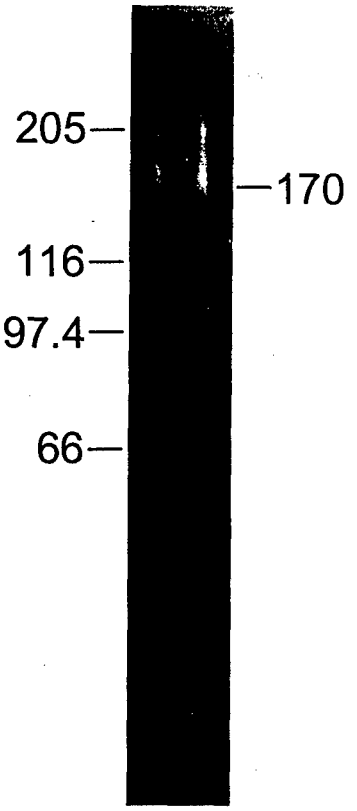
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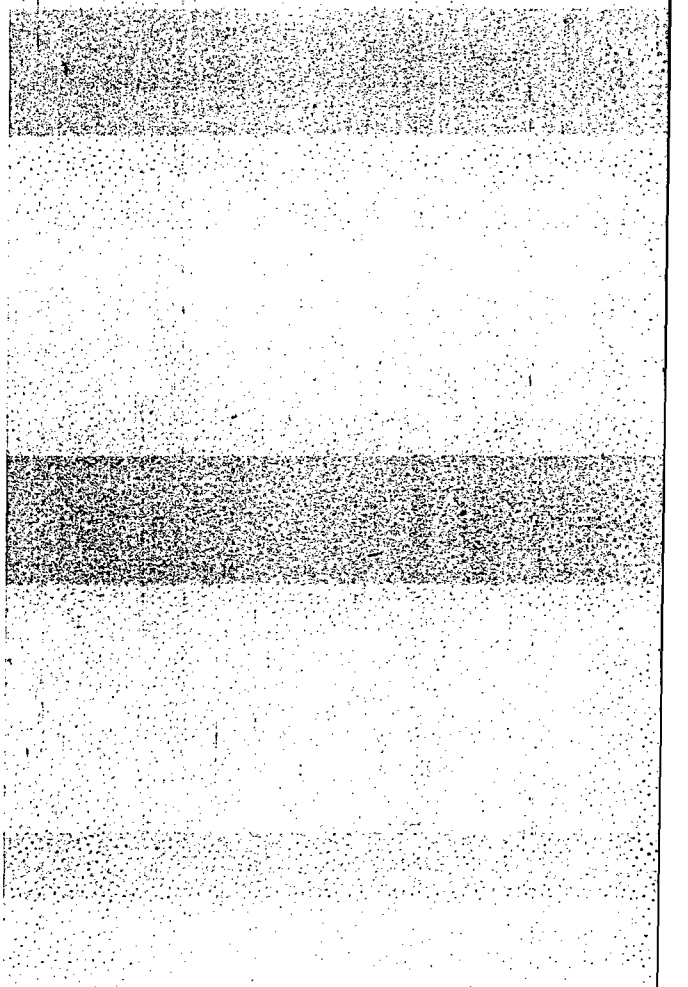

Recd 10/29/2001

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REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 17 Oct 01

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

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2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

PHYLIS M. RINEHART
Deputy Chief of Staff for
Information Management

Enclosure