

Award Number: DAMD17-01-1-0252

TITLE: An Epithelial-Derived, Integral Membrane, Kunitz-Type
Serine Protease Inhibitor in Breast Cancer

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REPORT DATE: August 2004

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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20050715 053

REPORT DOCUMENTATION PAGEForm Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY

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2. REPORT DATE

August 2004

3. REPORT TYPE AND DATES COVERED

Annual Summary (1 Aug 03 - 31 Jul 04)

4. TITLE AND SUBTITLEAn Epithelial-Derived, Integral Membrane, Kunitz-Type
Serine Protease Inhibitor in Breast Cancer**5. FUNDING NUMBERS**

DAMD17-01-1-0252

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**8. PERFORMING ORGANIZATION
REPORT NUMBER****9. SPONSORING / MONITORING****AGENCY NAME(S) AND ADDRESS(ES)**U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012**10. SPONSORING / MONITORING
AGENCY REPORT NUMBER****11. SUPPLEMENTARY NOTES**

Original contains color plates. All DTIC reproductions will be in black and white.

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE**13. ABSTRACT (Maximum 200 Words)**

In the current research plan, we proposed to study the anti-tumor and anti-protease activity of a membrane-bound Kunitz-type serine protease inhibitor (KSPI; also known as HAI-1). In order to investigate how HAI-1 regulates matriptase function, we investigated the cellular events associated with matriptase activation. During matriptase activation induced either by S1P or suramin, HAI-1 along with matriptase is translocated and accumulated at cell-cell junctions or in vesicle-like structures, which were named as matriptase activation foci. In activation foci, HAI-1 binds active matriptase to form a 120-kDa complex immediately following the activation of matriptase. The close temporal and spatial coupling of matriptase activation with its inhibition suggests that the proteolytic activity of this enzyme must be well controlled, and that the activation of matriptase substrates may be tightly regulated by this mechanism.

14. SUBJECT TERMSAnti-Tumor, Protease, Protease Inhibitor, Cancer Invasion
HGF Activation, Pro-uPA Activation, FGF**15. NUMBER OF PAGES**

10

16. PRICE CODE**17. SECURITY CLASSIFICATION
OF REPORT**

Unclassified

**18. SECURITY CLASSIFICATION
OF THIS PAGE**

Unclassified

**19. SECURITY CLASSIFICATION
OF ABSTRACT**

Unclassified

20. LIMITATION OF ABSTRACT

Unlimited

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Introduction:

The death of women with breast carcinoma mainly results from metastasis. Metastatic breast cancer cells must escape from a primary tumor and migrate through anatomical barriers in order to gain access to the blood or lymphatic system and establish at a new site in the body. Cellular motility and degradation of extracellular matrix (ECM) are two of the major events in breast cancer metastasis and can be promoted by stromal-derived, ECM-degrading protease systems, such as the urokinase type plasminogen activator (uPA) system and by motility factors, such as hepatocyte growth factor (HGF)/scatter factor (SF). In order to understand how breast cancer cells regulate both stromal-derived, ECM degradation and cellular motility for metastasis, we have discovered and characterized in human breast cancer a new epithelial-derived, type 2 integral membrane, serine protease, matriptase, and its cognate inhibitor, a Kunitz-type serine protease (KSPI), a type 1 integral membrane protein which was initially identified as an inhibitor of hepatocyte growth factor activator and named as HAI-1 (1-3). Both matriptase and HAI-1 have been implicated in the regulation of ECM-degradation of cellular motility (4). In the current research plan, we proposed to study the anti-tumor and anti-protease activity of this membrane-bound Kunitz inhibitor.

Body:

During the August 03-July 04 period we had addressed Aim 3 of the original proposal:

Year 3: In the third year, we will further characterize KSPI-transfected breast cancer cells regarding their *in vitro* and *in vivo* growth, invasion, and metastatic potential (Aim 2). In addition, we will begin our purification of KSPI complexes from T-47D and KSPI-transfected MDA MB-435 cells (Aim 3).

In the third year, we have finished the role of KSPI (now termed HAI-1) in matriptase activation and inhibition. The transfection of HAI-1 in breast cancer cells have been completed and reported in the annual report of first year, and the new HAI-1 complex turns out to be HGF activator complexed with HAI-1. In last report, we have finished structural and functional analysis of HAI-1, and the role of HAI-1 in matriptase expression. In the current report, we took cell biology approach to show how HAI-1 closely regulates matriptase.

In our previous studies (5-7), we showed that sphingosine 1-phosphate (S1P), a lysophospholipid with pleiotropic, growth factor-like activity, induces matriptase translocation to and activation at cell-cell contacts of 184 A1N4 immortal human mammary epithelial cells. S1P also simultaneously induces actin cytoskeletal rearrangement, cell-shape changes, formation of subcortical actin belts, and assembly of adherens junctions in 184 A1N4 cells. These S1P-induced cellular events are thought to be induced mainly via S1P receptors, members of endothelial differentiation gene (EDG) family (8;9). While we were investigating the role of S1P receptors in the activation of matriptase, we noticed that suramin, a sulfide-rich, anionic small molecule, could induce activation of matriptase to much higher levels than S1P, but did not change cell morphology. In the current study, we investigated how suramin induced matriptase in mammary epithelial cells, by comparing it with S1P, and set out to discern any common mechanisms for matriptase activation using each inducer.

Suramin-induced matriptase activation in immortal human mammary epithelial cells-To analyze the effect of suramin on activation of matriptase, we first determined the optimal concentration (Fig. 1A) and time course (Fig. 1B) of cellular treatments with this compound. Matriptase activation was determined by the levels of activated matriptase, using immunoblot analyses of cell lysates or immunofluorescent staining of fixed cells. For these studies we employed monoclonal antibody M69, which specifically recognizes the two-chain activated matriptase, but not the single-chain zymogen (5). Activation of matriptase, induced by suramin, occurred in a dose-response manner, with a minimal required concentration of 25 μ M (Fig. 1A). The activated matriptase was detected in complexes with its cognate inhibitor, HAI-1 at 120- and 85-kDa (Figs. 1 and 2). We also used another anti-matriptase monoclonal mAb M32, to determine the levels of matriptase. M32 mAb recognizes the third LDL receptor class A domain of matriptase (7), and thus can react with the single-chain, latent protease of 70-kDa, and with two-chain activated matriptase in the 120-kDa HAI-1 complex, but not with

the 85-kDa HAI-1 complex, which likely contains the serine protease domain of matriptase and the full length HAI-1 (Fig. 2). M32 reactivity provided data indicating the extent that latent matriptase is converted to its activated form, by comparing the levels of the 70-kDa form in controls to treated samples. For example, the majority of matriptase was converted to its activated form with 50 and 100 μ M of suramin. The appearance of matriptase-HAI-1 complexes induced by suramin was also observed in dose response manner by using anti-HAI-1 mAb M19. M19 recognizes the unbound, full-length HAI-1 at 55-kDa, and both 120- and 85-kDa matriptase-HAI-1 complexes (Fig. 1A). In contrast to matriptase, however, the majority of HAI-1 was detected in its unbound form, even at the treatment of 100 μ M of suramin. These data suggest that the complexed HAI-1 represents only a small portion of total HAI-1, even under conditions where the majority of matriptase is activated and bound to HAI-1. Therefore, 184 A1N4 cells express HAI-1 in much higher ratios than matriptase. Matriptase activation was detected within 5 min of suramin treatment, and reached to the maximal activation after 30-min treatment (Fig. 1B). This rapid response of matriptase activation is similar to that observed with S1P (6).

Inhibition of matriptase by HAI-1 simultaneously follows the activation of the protease-In order to further confirm that the 120- and 85-kDa bands, which were detected by these three mAbs, are matriptase-HAI-1 complexes, we first immunodepleted cell lysates of suramin-treated 184 A1N4 cells, using anti-HAI-1 mAb M19. Then we examined the depletion of both 120- and 85-kDa bands on immunoblots, using anti-HAI-1 mAb M19, anti-matriptase mAb M32, and anti-two chain matriptase mAb M69 (Fig. 2A). These combinations of immunodepletion and immunoblot confirmed that the protein bands with same size recognized by different mAbs on immunoblots are the same proteins. As expected, anti-HAI-1 mAb M19 completely depleted the 120-, 85-, and 55-kDa bands recognized by M19 on immunoblot. Both 120- and 85-kDa bands, but not the 70-kDa band, recognized by mAb M69 on immunoblot, were also depleted by anti-HAI-1 mAb M19. The complete depletion of the 120-, 85-, and 55-kDa, and the lack of immunodepletion of the 70-kDa matriptase bands of HAI-1 by anti-HAI-1 mAb M19 provided evidence that the immunodepletion was specific. These data also confirm that both 120- and 85-kDa bands are matriptase-HAI-1 complexes. The 85-kDa matriptase-HAI-1 complex was previously suggested to be the free form of activated matriptase (5;6;10). These data suggest that all activated matriptase is bound with HAI-1, immediately following its generation.

It is possible that the 85-kDa complex is derived from the 120-kDa complex. Indeed, the 120-kDa complex was converted to the 85-kDa complex by incubating the cell lysate at 37°C (Fig. 2 B). Because mAb M32 did not recognize the 85-kDa complex, the conversion of 120-kDa complex to 85-kDa complex could result from the loss of the noncatalytic domains of matriptase, which contain its third LDL receptor class A domain where the epitope recognized by M32 resides. Furthermore, addition of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) into the cell lyses buffer inhibited this conversion even for the samples incubated at 37°C. Because that the loss of noncatalytic domains of matriptase occurred only for activated matriptase, but not for latent matriptase, and because that DTNB can inhibit protein disulfide isomerase (11), it is very likely that, following cell lysis, protein disulfide isomerase cleaves the disulfide bond which connects the noncatalytic domains with the serine protease domain of activated matriptase. For single-chain matriptase, if cleavage of this disulfide linkage by protein disulfide isomerase occurs, the serine protease domain would not separate from the noncatalytic domain, as the protease would be still held as signal-chain by peptide bonds. In Figure 2C, we summarize the conversion of the 120-kDa to the 85-kDa matriptase complex and the position of epitopes recognized by our three mAbs.

S1P and suramin induce accumulation and activation of matriptase at activation foci-While both S1P and suramin induce matriptase activation in 184 A1N4 cells and share several common characteristics, such as in dose-response, rapid kinetics, and rapid inhibition of active matriptase by HAI-1, we have observed some differences between both. Specifically, suramin requires μ M concentration, relative to nM for S1P, to induce matriptase activation. Also, much higher proportions of latent matriptase were converted to activated matriptase in suramin-treated cells, compared to S1P-treated cells. In addition, S1P simultaneously induces changes in cell shape under the light microscope, but such changes were not observed for suramin. The S1P-

induced changes in cell shape are mainly associated with the S1P-induced actin cytoskeletal rearrangement, the formation of actin subcortical belts. Furthermore, destruction of actin cytoskeletal rearrangement inhibits S1P-induced matriptase activation (7). Therefore, it is of interest to investigate the role of the cellular events, particularly actin cytoskeletal rearrangement, in suramin-induced matriptase activation. In Figure 3, we compared suramin with S1P for their abilities to induce the redistribution of matriptase and actin cytoskeletal rearrangement. After growing cells in IMEM supplemented with 0.5% FBS for 2 days, matriptase was observed mainly in the cytoplasm with diffuse staining patterns. Little F-actin was observed in these cells. After thirty minutes of suramin treatment, while matriptase was still located in cytoplasm, the protease apparently concentrated at vesicle-like structures with various sizes (Fig. 3D), in stringing contrast to the cell-cell contact localization of matriptase induced by S1P (Fig. 3G). While filamentous actin structures were increased by suramin treatment, they were much less organized (Fig. 3E) in S1P-treated cells (Fig. 3H). Interestingly, pretreatment with suramin apparently did not affect the S1P-induced formation of subcortical actin belts and translocation of matriptase to cell-cell contacts (Figs. 3J and K).

We further examined the appearance and localization of activated matriptase (in red color) using Alexa-Fluor 647-labeled, mAb M69 in conjunction with total matriptase (in green color) using Alexa-Fluor 488-labeled, mAb M32 (Fig. 4). In spite of high levels of total matriptase in the cytoplasm, activated matriptase was not detected in 184 A1N4 cells after growth for 2 days (Fig. 4A), consistent with the results of immunoblot (Fig. 1). Thirty minutes after suramin treatment, activated matriptase was detected as spotty vesicle-like structures in cytoplasm (Fig. 4D), a pattern similar to total matriptase (Figs. 4E and 1D). When both images were merged, activated matriptase coincided with the spotty, total matriptase (Fig. 4F). The localization of activated matriptase, as well as the total matriptase in suramin-treated 184 A1N4 cells, was different from S1P-treated cells, where activated matriptase was at cell-cell contacts (Figs. 4G, H, and I). Despite these differences, activated matriptase appeared where total matriptase accumulates. In S1P-treated cells, activated matriptase was exclusively detected at cell-cell contacts particularly where more total matriptase accumulated. Similar to S1P-treated cells, suramin treatment causes accumulation of matriptase, but now in the cytoplasm, as spotty vesicle-like structures, where its activation may occur. These data suggest that accumulation of matriptase is a common theme for its activation, either at cell-cell contacts in S1P-treated cells, or in cytoplasm as spotty vesicle-like structures in suramin-treated cells. These observations are consistent with our hypothesis that matriptase activation occurs *via* autoproteolytic activation, in that dimerization or oligomerization of latent matriptase molecules occurs, leading to their cross-activational cleavage (10). Accumulation of matriptase at cell-cell junctions or in the cytoplasm as spotty, vesicle-like structures, which we term "activation foci", provides the platform for latent matriptase molecules to interact with each other.

HAI-1 is translocated to activation foci for matriptase activation in response to S1P or suramin treatment-HAI-1 was initially identified to be the cognate inhibitor of matriptase (12), and subsequently its role in matriptase activation was demonstrated (3). The extremely rapid, highly efficient inhibition of matriptase by HAI-1 coupled to the requirement of HAI-1 for matriptase activation, suggest that the inhibitor should be in physical proximity to matriptase during activation of the protease. Indeed, the inhibitor was co-translocated with matriptase to cell-cell contacts, in response to S1P treatment (Figs. 5A, B and C), and appeared with matriptase in the spotty, vesicle-like structures in the cytoplasm, in response to suramin treatment (Figs. 5E, F, and G). These results suggest that HAI-1, along with matriptase are translocated to activation foci for activation of matriptase. Therefore, for matriptase autoproteolytic activation, both S1P and suramin may translocate matriptase, HAI-1, and other unidentified components to the activation foci to form the activation complex.

Figure 1. Suramin induction of matriptase activation in a concentration- and time-dependent manner. (A) Dose-response of suramin-mediated matriptase activation. Serum-starved 184 A1N4 cells were treated with the indicated concentrations of suramin for 30 min. (B) Time course of suramin-induced matriptase activation. Serum-starved 184 A1N4 cells were stimulated with 50 μ M suramin for the indicated times. Equal amounts of total cell lysates were examined by western blotting with mAb M69, directed against activated matriptase, mAb M32, directed against matriptase, and mAb M19, directed against HAI-1.

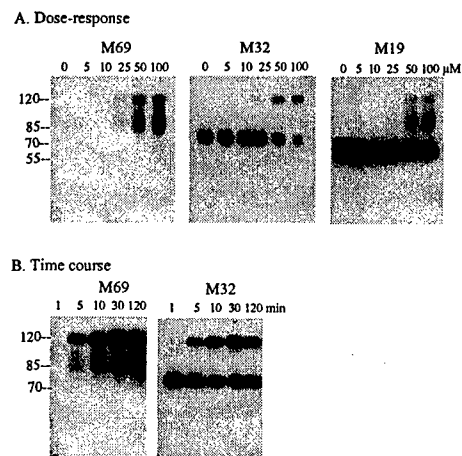


Figure 1

Figure 2. Detection of all activated matriptase in HAI-1 complexes. (A) Confirmation that 120- and 85-kDa bands are matriptase-HAI-1 complexes. In order to confirm that the 120- and 85-kDa bands are matriptase-HAI-1 complexes, the cell lysates of suramin-treated 184 A1N4 cells were immunodepleted using anti-HAI-1 mAb M19-Sepharose 4B. The total cell lysate (L) and the mAb M19-depleted cell lysate (D) were analyzed with western blotting with anti-HAI-1 mAb M19, anti-two-chain matriptase mAb M69, and anti-matriptase mAb M32. (B) The 85-kDa matriptase-HAI-1 complex is derived from the 120-kDa complex. Suramin-stimulated cells were lysed in lysis buffers with or without 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB). These cell lysates were incubated at 4°C or 37°C for 5 min, as indicated. The samples were examined by immunoblot analysis with mAbs M19, M32, and M69, respectively. (C) A proposed model for the conversion of the matriptase-HAI-1 complex from the 120 kDa to 85 kDa forms. The serine protease domain (SPD) of activated matriptase binds to the first Kunitz domain (KD1) of HAI-1 to form the 120-kDa complex. A proposed cleavage at the disulfide bond, which connects the serine protease domain (SPD) with noncatalytic domain of matriptase, occurs *via* the action of protein disulfide isomerase (PDI) to generate the 85-kDa complex, which contains only the serine protease domain (SPD) of matriptase and full length HAI-1. The epitope recognized by mAb M32 on the third LDL receptor class A domain is lost in the 85-kDa complex, along with the loss of noncatalytic domain of matriptase. Both epitopes, recognized by mAbs M69 and M19, are preserved in the 85-kDa complex.

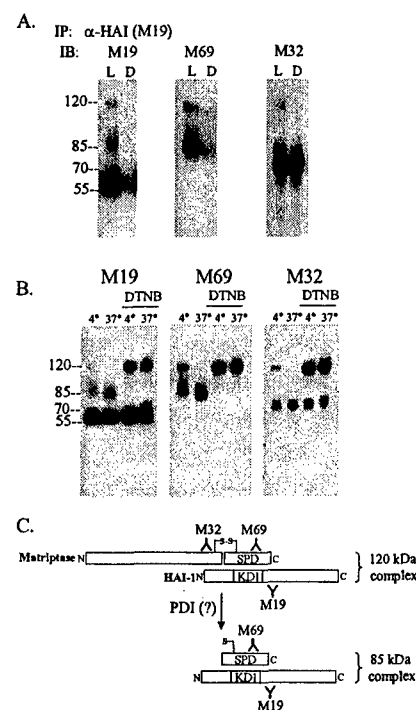


Figure 2

Figure 3. Effects of sphingosine-1-phosphate (S1P) and suramin on subcellular translocation of matriptase. Serum-starved 184 A1N4 cells were cultured in the presence or absence of S1P (50 ng/ml) or suramin (50 μ M) for 30 min. Cells were stained for F-actin with Texas red-conjugated phalloidin (red), for matriptase with Alexa Fluor 488-conjugated monoclonal Ab M32 (green), and for nuclei with 4',6-diamidino-2-phenylindole (DAPI, blue).

Figure 4. Induction of matriptase activation by S1P and suramin. Serum-starved 184 A1N4 cells were stimulated with S1P (50 ng/ml) or suramin (50 μ M) for 30 min. Cells were stained for total matriptase with Alexa Fluor 488-conjugated mAb M32 (green), for activated matriptase with Alexa Fluor 647-conjugated mAb M69 (red), and for nuclei with DAPI (blue).

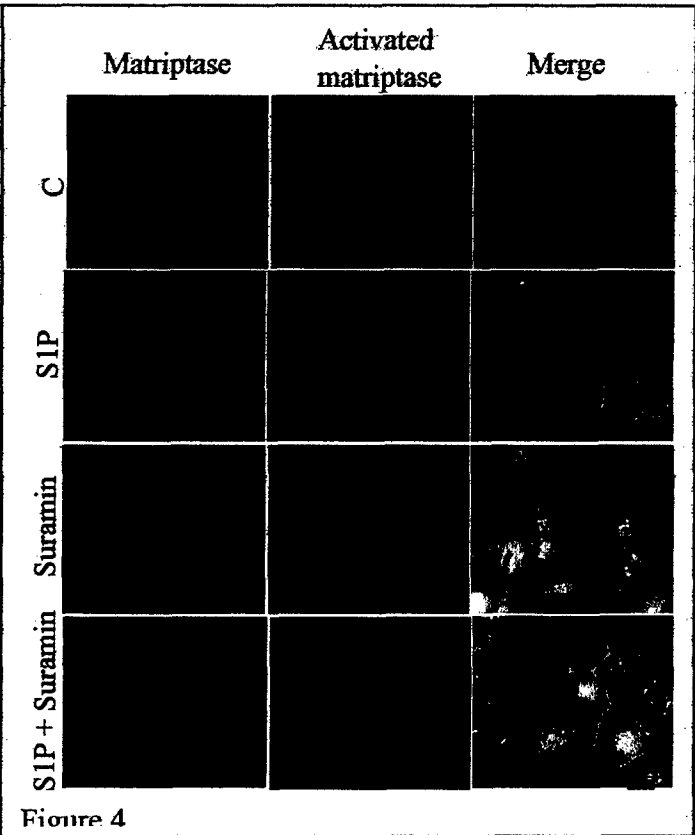
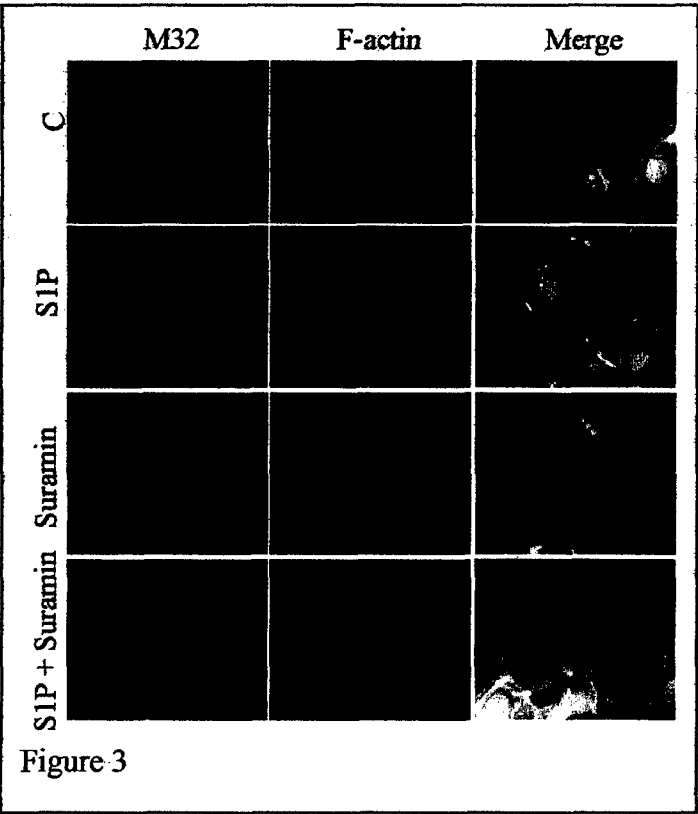
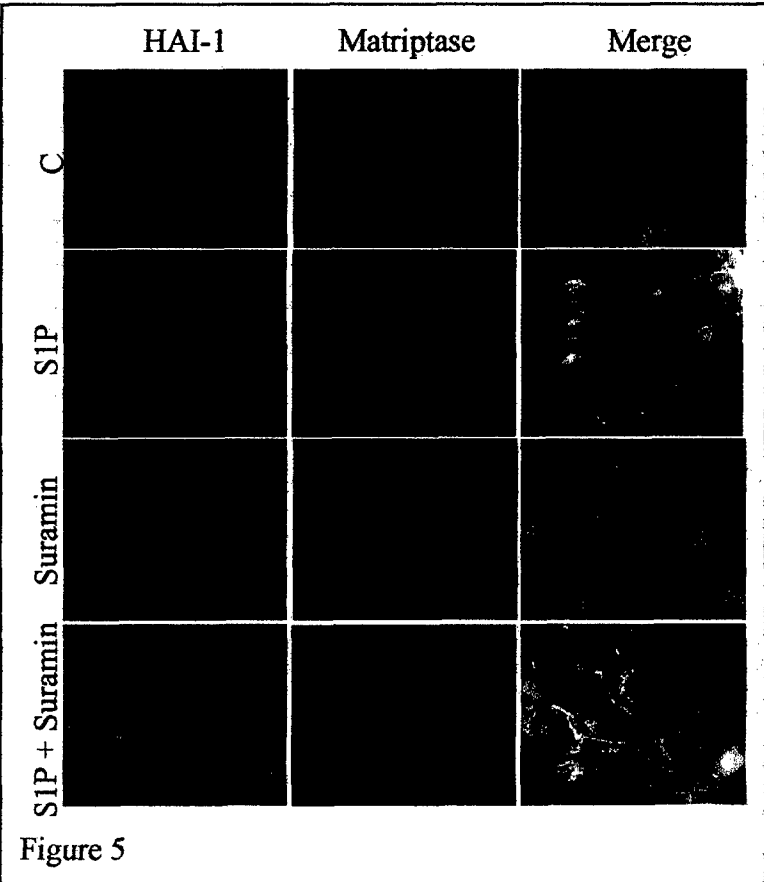


Figure 5. Translocation of HAI-1 to activation foci during matriptase activation
 Serum-starved 184 A1N4 cells were cultivated in the presence or absence of S1P (50 ng/ml) or suramin (50 μ M) for 30 min. Cells were stained for HAI-1 with Alexa Fluor 488-conjugated MAb M19 (green), for matriptase with Alexa Fluor 647-conjugated monoclonal Ab M32 (green), and for nuclei with (DAPI, blue).



Key research accomplishments:

- We have established two model systems to induce matriptase activations in mammary epithelial cells
- During matriptase activation, HAI-1 along with matriptase is translocated to specialized subcellular structures, named activation foci.
- In activation foci, HAI-1 binds active matriptase to form a 120-kDa complex immediately following the activation of matriptase.
- Therefore, all activated matriptase was detected in HAI-1 complex.
- The 120-kDa complex seems not stable and is readily converted to a 85-kDa complex probably via protein disulfide isomerase.

Reportable outcomes:

1. Oberst, M.D., Anders, J., Benaud, C., Lin, C.-Y., Dickson, R.D., and Johnson, M.D., (2001) Over-expression of the hepatocyte growth factor inhibitor (HAI-1) in human breast cancer cells: Effects on cell growth, morphology, and motility. 2nd General Meeting of International Protease Society, Muenchen, German.
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Conclusion:

In order to investigate how HAI-1 regulates matriptase function, we investigated the cellular events associated with matriptase activation. During matriptase activation induced either by S1P or suramin, HAI-1 along with matriptase is translocated and accumulated at cell-cell junctions or in vesicle-like structures, which were named as matriptase activation foci. In activation foci, HAI-1 binds active matriptase to form a 120-kDa complex immediately following the activation of matriptase. The close temporal and spatial coupling of matriptase activation with its inhibition suggests that the proteolytic activity of this enzyme must be well controlled, and that the activation of matriptase substrates may be tightly regulated by this mechanism.

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Appendices: None