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## ABSTRACT

In the current research plan, we proposed to study the mechanism for activation of matriptase, a membrane-bound serine protease. Previously, we showed matriptase is activated via transactivation, where the interactions among latent matriptase molecules, HAI-1, and other unidentified proteins are required for the proceeding of activation cleavage. In non-transformed mammary epithelial cells, matriptase activation can be induced by sphingosine 1-phosphate (S1P), a blood-borne bioactive phospholipid. We further showed that S1P induces matriptase translocation and accumulation at cell-cell contacts where activation occurs, a process depending on the assembly of adherens junctions and formation of subcortical actin belts in response to S1P exposure. In the final year, we showed that both matriptase and HAI-1 are accumulated at activation foci during matriptase activation. The dual roles of HAI-1 in matriptase activation and inhibition results in immediate inhibition of matriptase right after the activation of the protease. 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 proteolysis of matriptase substrates may be tightly regulated by this mechanism.

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## Introduction:

Invasion of cells from one tissue to a neighboring tissue has been observed both in physiological processes, such as organ development and wound healing, and pathological processes, such as breast cancer metastases. Physiological invasion is thought to be tightly controlled. The regulatory mechanism of physiological invasion may be lost in pathologic states, particularly in malignant progression, resulting in uncontrolled invasion. Degradation of extracellular matrix (ECM) and cell migration are two key events for cellular invasion. Proteases and protease inhibitors are implicated in cellular invasion due to their potential roles in localized degradation of ECM and in the activation of latent growth/motility/angiogenesis factors, such as hepatocyte growth factor (HGF)/scatter factor (SF). HGF/SF is a major regulatory molecule for epithelial cell migration. HGF/SF and most ECM-degrading protease systems, such as the uPA system and the matrix metalloproteases are, however, expressed *in vivo* by the stromal elements of human breast cancer. Therefore, breast cancer cell invasion has been proposed to be a collaboration between epithelial cancer cells and stromal cells. In order to understand the role of epithelial cells in cellular invasion, we have characterized an epithelial-derived, breast cancer-associated, integral membrane, trypsin-like serine protease, termed matriptase (1). Matriptase has been demonstrated to activate uPA and HGF/SF (2). These observations suggest that matriptase could act as a cell surface activator to recruit and activate stromal-derived ECM-degrading proteases and motility factors. Furthermore, activation of matriptase in nontumorigenic human mammary epithelial cells (HMEC) seems to be tightly regulated by bioactive lipids, mainly sphingosine 1-phosphate (S1P) (3;4). Surprisingly, this S1P-based, physiological regulatory mechanism may be lost during breast cancer progression (5). In the current research plan, we proposed to study the role of S1P receptors in matriptase activation and the structural requirements for the activation of matriptase.

## Body:

**Year 3:** We will finish all of the work proposed in Aim 3, including the determination of whether the CUB domains play the role of a "lock" for the autoactivation of matriptase, and whether matriptase may bind to itself.  
**Aim 3:** to investigate the role of the non-catalytic domains of matriptase in the activation of matriptase.

The work proposed for year 3 has been finished and published (6). Our conclusion is that the non-catalytic domains of matriptase, including LDL receptor class A domains and CUB domains, as well as posttranslational modifications, such as N-glycosylations, are required for matriptase activation. These studies were reported in year 1. During the June 04-May 05 period, we further investigated the cellular events associated with matriptase activation. We compared the physiological matriptase activation inducer, sphingosine 1-phosphate (S1P) and a newly discovered chemical inducer of matriptase activator, suramin, in terms of cellular events associated matriptase activation and the signaling pathways involved in both induced matriptase activation. These works have been published in American Journal Physiology-Cell Physiology (288:C932-C941, 2005). We report here with these works. Please refer to the attached reprint for the Figures.

In our previous studies (3;4;7), we showed that 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 activation 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 and 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 (3). Activation of matriptase, induced by suramin, occurred in a dose-responsive manner, with a minimal required concentration of 25  $\mu$ 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 those in treated samples. For example, the majority of matriptase was converted to its activated form with 50 and 100  $\mu$ M of suramin. The appearance of matriptase-HAI-1 complexes induced by suramin was also observed in dose-responsive 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 after treatment with 100  $\mu$ 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 SIP (4).

**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 almost completely depleted the 120-, 85-, and 55-kDa bands recognized by M19 on immunoblot. Both 120- and 85-kDa bands, recognized by mAb M69 on immunoblot, but not the 70-kDa band recognized by mAb M32, were also depleted by anti-HAI-1 mAb M19. The depletion of the 120-, 85-, and 55-kDa, and the lack of immunodepletion of the 70-kDa matriptase bands 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 (3;4;6). However, these current 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. 2B). 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 lysates 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 DTNB can inhibit protein disulfide isomerase (10), 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 single-chain by peptide bonds. In Figure 2C, we summarize our current theories

that the 85-kDa matriptase complex is converted from the 120-kDa complex, and show the positions 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  $\mu\text{M}$  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 cause 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 (Fig. 3A). Little F-actin was observed in these cells (Fig. 3B). 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 striking contrast to the cell-cell contact localization of matriptase induced by S1P (Fig. 3G). While filamentous actin structures were increased by S1P treatment (Fig. 3H), they were much less organized in suramin-treated cells (Fig. 3E). 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 by immunofluorescent staining (3;4;7). Thirty minutes after suramin treatment, activated matriptase was detected as spotty vesicle-like structures in cytoplasm (Fig. 4B), a pattern similar to total matriptase (Figs. 4A and 3D). When both images were merged, activated matriptase coincided with the spotty, total matriptase (Fig. 4C). 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 colocalized with total matriptase at cell-cell contacts (Figs. 4D, E and F). 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. Again, cellular pretreatment with suramin did not affect subsequent S1P-induced translocation of both latent matriptase and activated matriptase to cell-cell junctions (Figs. 4G, H, and I). 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 (6). 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 (11;12), and subsequently its role in matriptase activation was demonstrated (6). 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. After growth of 184 A1N4 cells for 2 days, the inhibitor and matriptase were localized in cytoplasm, surrounding the nuclei (Figs. 5A, B, and C). Both proteins were co-translocated to cell-cell contacts, in response to S1P treatment (Figs. 5D, E and F), and

appeared in the spotty, vesicle-like structures in the cytoplasm, in response to suramin treatment (Figs. 5G, H, and I). 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. F-actin polymerization is not required for suramin-induced matriptase activation--Previously we showed that S1P-induced matriptase translocation and activation at cell-cell contacts was dependent on actin cytoskeletal rearrangement (7). Therefore, it was of interest to investigate the role of actin cytoskeletal rearrangement in suramin-induced matriptase activation. As expected, activation of matriptase by S1P was significantly suppressed by pretreatment of 0.1  $\mu$ M cytochalasin D, which causes disruption of actin filaments and inhibition of actin polymerization (Fig. 6B). However, cytochalasin D, up to 1  $\mu$ M, failed to block matriptase activation induced by suramin (Fig. 6A). These data are parallel to the fact that suramin does not induce formation of subcortical actin belts, and suggest that actin filament polymerization is not required for suramin-induced accumulation and activation of matriptase.

Both S1P-induced and suramin-induced matriptase activation was inhibited by Ro 31-8220-- Because S1P induces various signal pathways, which may participate in matriptase activation, we used an array of chemical inhibitors, including the MEK kinase inhibitor PD98059 (50  $\mu$ M), PI3 kinase inhibitor LY 294002 (50  $\mu$ M), protein kinase C inhibitors Ro 31-8220, calmodulin inhibitor W-7 (30  $\mu$ M), and  $Ca^{2+}$ /calmodulin kinase II inhibitor KN-62 (10  $\mu$ M) to elucidate signaling pathways which may participate in matriptase activation. Only treatment of 184 A1N4 cells with Ro 31-8220 abolished S1P-induced activation of matriptase (Fig. 7B), suggesting the role of protein kinase C in matriptase activation. Other chemicals showed no obvious effect on the S1P-induced activation of matriptase (data not shown). Interestingly, despite differential response to cytochalasin D, both S1P-induced and suramin-induced matriptase activation was inhibited by Ro 31-8220 (Fig. 7). These data suggest that both S1P and suramin may share some common regulatory mechanisms for their ability to induce matriptase activation.

#### **Key research accomplishments:**

- (1) S1P-induced matriptase translocation to cell-cell contacts, where it is activated, is an F-actin polymerization-dependent process. Conversely, suramin-induced matriptase accumulation and activation at vesicle-like structures is an F-actin polymerization-independent process.
- (2) While matriptase activation can occur at different subcellular locations, both S1P- and suramin-induced matriptase accumulation forms unique subcellular structures, termed activation foci, where oligomerization of matriptase zymogens and HAI-1 may occur, promoting matriptase activation.
- (3) Furthermore, matriptase activation may be regulated by intracellular signaling, since Ro-31-8220, a bisindolylmaleimide protein kinase C inhibitor, inhibited both S1P- and suramin-induced activation.
- (4) The requirement of HAI-1 for matriptase activation, and the coincidence of HAI-1 and matriptase in activation foci apparently provide rapid access of HAI-1 for inhibition of matriptase, immediately following its activation.

#### **Reportable outcomes:**

1. Oberst, M.D., Singh, B., Ozdemirli, M., Dickson, R.B., Johnson, M.D., and Lin, C.-Y. (2003) Characterization of Matriptase Expression in Normal Human Tissues. *J. Histochem. and Cytochem.* 51:1017-1025
2. Oberst, M.D., Williams, C., Johnson, M.D., Dickson, R.B., and Lin, C.-Y. (2003) The activation of matriptase requires its noncatalytic domains, serine protease domain, and its cognate inhibitor. *J. Biol. Chem.* 278:26773-26779.
3. Hung RJ, Hsu I. W., Dreiling J. L., Lee M. J., Williams C. A., oberst M. D., Dickson R. B., and Lin C.-Y. (2004) Assembly of Adherens Junctions Is Required for Sphingosine 1-phosphate-induced Matriptase Accumulation And Activation at Mammary Epithelial Cell-cell Contacts. *Am J Physiol Cell Physiol.* 286:C1159-69.

4. Lee M.-S., Kiyomiya K., Benaud C., Dickson R.B., and Lin C.-Y. (2005) Simultaneous activation and HAI-1-mediated inhibition of matriptase induced at activation foci in human mammary epithelial cells. *Am J Physiol Cell Physiol.* 288:C932-C941.
5. Oberst MD, Chen LY, Kiyomiya KI, Williams CA, Lee MS, Johnson MD, Dickson RB, Lin CY. (2005) Hepatocyte growth factor activator inhibitor 1 (HAI-1) regulates activation and expression of matriptase, a membrane-bound serine protease. *Am J Physiol Cell Physiol.* (In press)

**Conclusion:**

We identified a chemical compound, suramin, to be a very potent inducer of matriptase activation. By comparing S1P and suramin, we conclude that matriptase activation is closely regulated and occur via translocation and accumulation of matriptase at activation foci, at which matriptase and other activation-required component accumulate. In addition, we discovered that matriptase activation and HAI-1-mediated inhibition simultaneously occur, suggesting the proteolytic activity of matriptase is closely controlled.

**Reference:**

1. Lin, C. Y., Anders, J., Johnson, M., Sang, Q. A., and Dickson, R. B. (1999) *J Biol.Chem.* **274**, 18231-18236
2. Lee, S. L., Dickson, R. B., and Lin, C. Y. (2000) *J Biol.Chem.* **275**, 36720-36725
3. Benaud, C., Dickson, R. B., and Lin, C. Y. (2001) *Eur.J Biochem.* **268**, 1439-1447
4. Benaud, C., Oberst, M., Hobson, J. P., Spiegel, S., Dickson, R. B., and Lin, C. Y. (2002) *J Biol.Chem.* **277**, 10539-10546
5. Benaud, C. M., Oberst, M., Dickson, R. B., and Lin, C. Y. (2002) *Clin Exp.Metastasis* **19**, 639-649
6. Oberst, M. D., Williams, C. A., Dickson, R. B., Johnson, M. D., and Lin, C. Y. (2003) *J Biol.Chem.* **278**, 26773-26779
7. Hung, R. J., Hsu, I., Dreiling, J. L., Lee, M. J., Williams, C. A., Oberst, M. D., Dickson, R. B., and Lin, C. Y. (2004) *Am J Physiol Cell Physiol* **286**, C1159-C1169
8. Lee, M. J., Van Brocklyn, J. R., Thangada, S., Liu, C. H., Hand, A. R., Menzeleev, R., Spiegel, S., and Hla, T. (1998) *Science* **279**, 1552-1555
9. Van Brocklyn, J. R., Tu, Z., Edsall, L. C., Schmidt, R. R., and Spiegel, S. (1999) *J Biol.Chem.* **274**, 4626-4632
10. Lahav, J., Wijnen, E. M., Hess, O., Hamaia, S. W., Griffiths, D., Makris, M., Knight, C. G., Essex, D. W., and Fardale, R. W. (2003) *Blood* **102**, 2085-2092
11. Shimomura, T., Denda, K., Kitamura, A., Kawaguchi, T., Kito, M., Kondo, J., Kagaya, S., Qin, L., Takata, H., Miyazawa, K., and Kitamura, N. (1997) *J Biol.Chem.* **272**, 6370-6376
12. Lin, C. Y., Anders, J., Johnson, M., and Dickson, R. B. (1999) *J Biol.Chem.* **274**, 18237-18242

**Appendices:**

A reprint of the following paper is provided:

Lee M.-S., Kiyomiya K., Benaud C., Dickson R.B., and Lin C.-Y. (2005) Simultaneous activation and HAI-1-mediated inhibition of matriptase induced at activation foci in human mammary epithelial cells. *Am J Physiol Cell Physiol.* 288:C932-C941.



## Simultaneous activation and hepatocyte growth factor activator inhibitor 1-mediated inhibition of matriptase induced at activation foci in human mammary epithelial cells

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**Lee, Ming-Shyue, Ken-ichi Kiyomiya, Christelle Benaud, Robert B. Dickson, and Chen-Yong Lin.** Simultaneous activation and hepatocyte growth factor activator inhibitor 1-mediated inhibition of matriptase induced at activation foci in human mammary epithelial cells. *Am J Physiol Cell Physiol* 288: C932–C941, 2005. First published December 8, 2004; doi:10.1152/ajpcell.00497.2004.—Activation of single-chain, latent matriptase, a type II transmembrane serine protease, depends on the weak proteolytic activity of its own zymogen as well as its cognate inhibitor, hepatocyte growth factor activator inhibitor 1 (HAI-1). Oligomerization of matriptase zymogens and HAI-1, and probably its interaction with other proteins, has been proposed to occur during matriptase activation. In the present study, we examined the cellular events associated with matriptase activation triggered either by the physiological inducer sphingosine 1-phosphate (S1P) or by a chemical inducer, the polyanionic compound suramin. S1P-induced matriptase translocation to cell-cell contacts, where it is activated, is an F-actin polymerization-dependent process. Conversely, suramin-induced matriptase accumulation and activation at vesicle-like structures is an F-actin polymerization-independent process. While matriptase activation can occur at different subcellular locations, both S1P- and suramin-induced matriptase accumulation form unique subcellular structures, termed activation foci, where oligomerization of matriptase zymogens and HAI-1 may occur, promoting matriptase activation. Furthermore, matriptase activation may be regulated by intracellular signaling, because Ro 31-8220, a bisindolylmaleimide protein kinase C inhibitor, inhibited both S1P- and suramin-induced activation. The requirement of HAI-1 for matriptase activation and the coincidence of HAI-1 and matriptase in activation foci apparently provide rapid access of HAI-1 for the inhibition of matriptase immediately after its activation. Indeed, all activated matriptase was detected in complexes with HAI-1 only 5 min after suramin stimulation. The close temporospatial coupling of matriptase activation with its inhibition suggests that the proteolytic activity of this enzyme must be well controlled and that the proteolysis of matriptase substrates may be tightly regulated by this mechanism.

sphingosine 1-phosphate; suramin

MATRIPTASE, a member of the recently described type II transmembrane serine protease family (6, 22, 28), has been implicated in cancer invasion and metastasis (10, 27). The protease could function as a membrane catalyst on breast cancer cell surfaces to recruit and activate urokinase-type plasminogen activator, an important extracellular matrix-degrading protease system, and hepatocyte growth factor, an important cell motility factor (14, 17, 27, 29). The protease consists of multiple

domains, including a short cytoplasmic domain at the NH<sub>2</sub> terminus followed by a putative transmembrane domain; a sperm protein, enterokinase, and agrin domain; two tandem C1r/C1s, urchin embryonic growth factor, and bone morphogenetic protein-1 (CUB) domains; four tandem low-density lipoprotein (LDL) receptor class A domains; and a trypsinlike serine protease domain at its COOH terminus (12, 19, 30, 31). In addition to its role in cancer, expression of matriptase is observed mainly in most epithelium-containing tissues (24). The distribution of matriptase suggests its role in epithelial biology. Indeed, matriptase is an essential component of the profilaggrin-processing pathway in keratinocytes and a key regulator of epidermal terminal differentiation (21). These activities are important for epidermal barrier formation and postnatal survival as shown in matriptase-knockout mice (20).

Besides its increased expression, such as in human breast cancer (10), matriptase could play a role in cancer malignancy, such as in ovarian cancer, because of an imbalance of the protease relative to its cognate inhibitor, hepatocyte growth factor activator inhibitor 1 (HAI-1), in favor of proteolysis (23). Furthermore, deregulated activation and aberrant subcellular localization of the protease may also contribute to its role in breast cancer malignancy (2). In immortal mammary epithelial cells, activation of matriptase occurs at cell-cell junctions upon cellular stimulation with serum or sphingosine 1-phosphate (S1P) (1, 3, 7). However, breast cancer cells constitutively activate matriptase and concentrate the activated protease at membrane ruffles (2), a relocalization that may convert matriptase from a well-regulated cell junctional protease in mammary epithelial cells to a deregulated invasion protease at the leading edges of breast cancer cells. Furthermore, oligosaccharide modifications of matriptase by *N*-acetylglucosaminyltransferase V (GnT-V) in cancer cells could enhance the stability of matriptase, contributing to the prometastatic effect of GnT-V (8, 9).

Activation of matriptase requires cleavage at its canonical activation motif to convert the single-chain zymogen to a two-chain active protease, a common theme for most of the serine proteases (1). Interestingly, the activation cleavage of matriptase may not be carried out by other active proteases, because matriptase activation depends on its own active site triad and requires its noncatalytic domains and even its cognate inhibitor HAI-1 (25). Therefore, we have proposed that the cleavage at the activation motif of one matriptase zymogen

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molecule may be preceded by the weak proteolytic activity of another matriptase zymogen molecule as a process of transactivation (25). Dimerization or oligomerization of matriptase molecules thus may serve as a key step during matriptase autoproteolytic activation, a mechanism observed with C1r protease (5) and caspase 8 (33). The noncatalytic domains and posttranslational modifications of matriptase, as well as LDL receptor class A domain of HAI-1, could provide the structural basis for the oligomerization of these molecules during matriptase activation (25). In the present study, we sought to further understand the mechanisms that govern the process and regulation of matriptase autoproteolytic activation and the role of HAI-1 in matriptase functionality by characterizing and comparing the effects of two different exogenous inducers of matriptase activation in terms of the cellular events and signaling pathways involved in the activation of matriptase. Our data reveal that upon the stimulation of these two inducers, both matriptase and HAI-1 were translocated and accumulated at cell-cell junctions or vesicle-like structures where matriptase was activated. The close spatial association between matriptase and HAI-1 also allowed HAI-1 to inhibit active matriptase immediately after its activation.

#### MATERIALS AND METHODS

**Chemicals and reagents.** Formaldehyde solution was purchased from EM Science/EM Industries (Bibbstown, NJ). Cytochalasin D, suramin, and Ro 31-8220 were obtained from Biomol (Plymouth Meeting, PA). All other chemical reagents were obtained from Sigma (St. Louis, MO) unless otherwise specified.

**Cell lines and culture conditions.** Immortalized 184 A1N4 human mammary epithelial cells, a gift from Dr. Martha Stampfer (Lawrence Berkeley National Laboratory, Berkeley, CA), were maintained routinely as previously described (1). For time-kinetic and dose-response experiments, 184 A1N4 cells were seeded at a density of  $1 \times 10^5$  cells per well in a six-well plate for 2 days. Cells were treated with different concentrations of suramin or for various durations with 50  $\mu$ M suramin as indicated in the figure legends. Control cells were treated with DMSO alone. For immunofluorescent staining, 184 A1N4 cells were seeded in a well with a coverglass on the bottom of 12-well plates in improved minimum essential medium (IMEM) containing 0.5% FBS. Two days after being plated, cells were treated with 50  $\mu$ M suramin or 50 ng/ml S1P in IMEM for 30 min. Control cells were treated with DMSO alone. After the treatment, cells were stained with fluorescent dye-labeled monoclonal antibodies (MAb) as described in the figure legends.

**Monoclonal antibodies.** Human matriptase protein was detected using MAb M32, which interacts with the third LDL receptor class A domain of matriptase (7, 18). The activated form of matriptase was detected using MAb M69, which recognizes an epitope present only in the activated (two-chain) form of the enzyme (1). Human HAI-1 was analyzed using MAb M19 (18).

**Conjugation of MAb with fluorescent dyes.** To conjugate MAb with fluorescent dyes, 1 mg of MAb, including M32, M69, and M19, were dissolved in 0.1 M NaHCO<sub>3</sub> and then labeled with Alexa Fluor 488, 594, or 647 (Molecular Probes, Eugene, OR) according to the manufacturer's instructions.

**Western blot analysis.** Cell lysates for Western blotting were prepared by lysing cells in 1% Triton X-100 in PBS after the cells were washed twice in PBS. The nuclei and cell debris were removed by centrifugation at 10,000 g for 10 min at 4°C. The protein concentration was determined using bicinchoninic acid protein assay reagents (Pierce, Rockford, IL) according to the manufacturer's protocol. For immunoblotting, an aliquot of the total lysate protein was electrophoresed in a 7.5% SDS gel under nonboiled and nonreduced

conditions and transferred onto Protran nitrocellulose membranes (Schleicher & Schuell, Keene, NH). The membranes were incubated with MAb as indicated, and the proteins were visualized using Western Lightning Chemiluminescence Reagent Plus (PerkinElmer, Boston, MA).

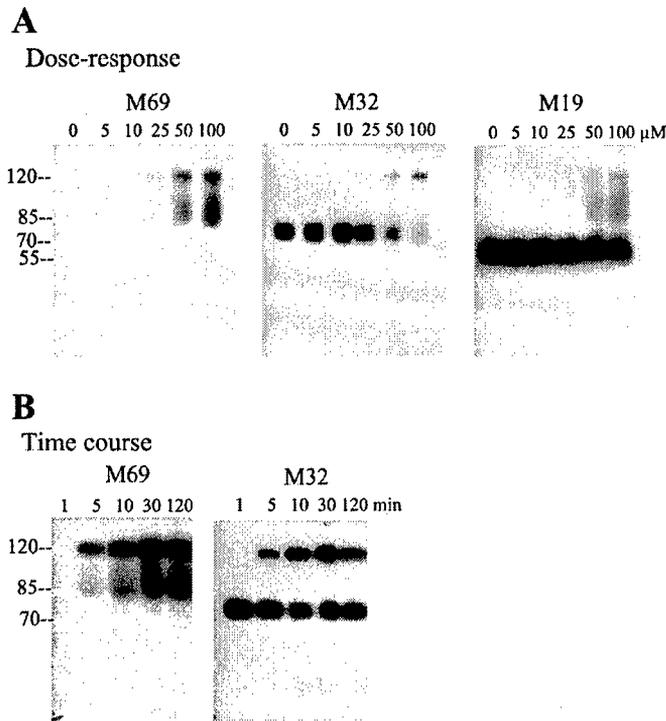
**Immunodepletion.** An aliquot of 400  $\mu$ l of total cell lysates from suramin-stimulated 184 A1N4 cells was incubated with 30  $\mu$ l of MAb M19-conjugated Sepharose 4B (5 mg of M19 per ml of Sepharose 4B beads) at 4°C for 2 h. The supernatant was separated from M19-Sepharose 4B by centrifugation.

**Immunofluorescence microscopy.** Cells were plated onto microcoverglasses and grown for 2 days. Subconfluent cells were subjected to the treatments indicated in each figure legend. Cells were then fixed in 3.7% formaldehyde and permeabilized within 0.05% Triton X-100 in PBS for 20 min at room temperature. Cells were washed with PBS three times. Matriptase, activated matriptase, and HAI-1 were detected with Alexa Fluor dye-conjugated M32, M69, and M19, respectively. F-actin was visualized using Texas red-labeled phalloidin (Molecular Probes), and nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). FITC-labeled anti-mouse IgG was used for the staining controls, which resulted in a weak, diffuse background (data not shown). After fluorescent staining, coverglasses were mounted with Prolong Antifade (Molecular Probes) and the fluorescent images were captured using the MetaVue software package (Universal Imaging, Downingtown, PA) in conjunction with a Nikon Eclipse E600 digital fluorescence microscope.

#### RESULTS

In previous studies (1, 3, 7), investigators at our laboratory showed that S1P, a lysophospholipid with pleiotropic, growth factor-like activity, induced 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 the endothelial differentiation gene family (16, 32). 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 could, but that did not change cell morphology. In the present study, we investigated how suramin induced matriptase activation in mammary epithelial cells by comparing it with S1P and set out to discern any common mechanisms for matriptase activation using each inducer.

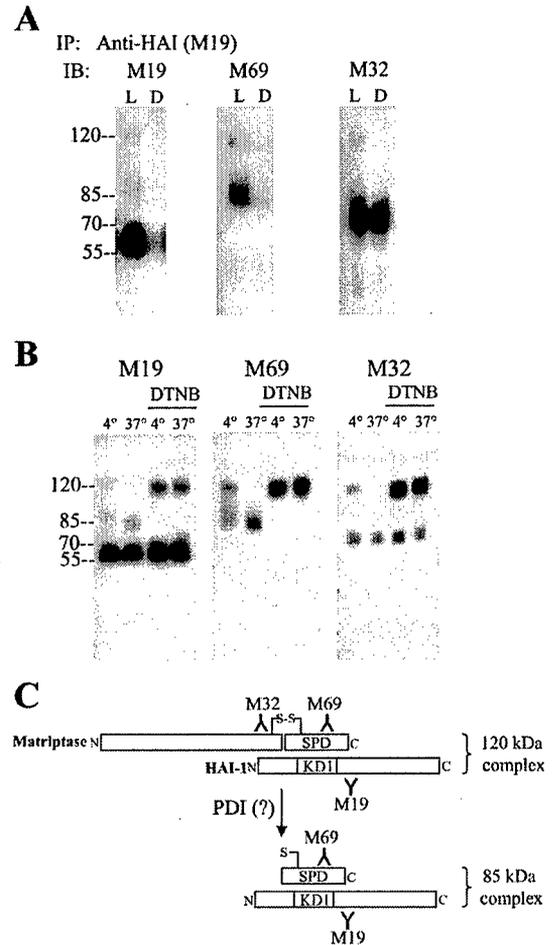
**Suramin-induced matriptase activation in immortal human mammary epithelial cells.** To analyze the effect of suramin on the activation of matriptase, we first determined the optimal concentration (Fig. 1A) and time course (Fig. 1B) of cellular treatments using this compound. Matriptase activation was determined by the levels of activated matriptase using immunoblot analysis of cell lysates and immunofluorescent staining of fixed cells. For these studies, we used MAb M69, which specifically recognizes the two-chain activated matriptase but not the single-chain zymogen (1). Activation of matriptase induced by suramin occurred in a dose-responsive manner, with a minimal required concentration of 25  $\mu$ 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 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



**Fig. 1.** Suramin induction of matriptase activation in a concentration- and time-dependent manner. *A*: dose-response relationship of suramin-mediated matriptase activation. Serum-starved 184 A1N4 cells were treated with the indicated concentrations of suramin (in  $\mu\text{M}$ ) for 30 min. *B*: time course of suramin-induced matriptase activation. Serum-starved 184 A1N4 cells were stimulated with 50  $\mu\text{M}$  suramin for the indicated times. All control cells were treated with improved minimum essential medium (IMEM) alone. Equal amounts of total cell lysates were examined using Western blotting with MAb M69 directly against activated matriptase, with MAb M32 directly against matriptase, and with MAb M19 directly against hepatocyte growth factor activator inhibitor 1 (HAI-1).

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 to which latent matriptase is converted to its activated form by comparing the levels of the 70-kDa form in controls with those in treated samples. For example, the majority of matriptase was converted to its activated form with 50 and 100  $\mu\text{M}$  suramin. Matriptase-HAI-1 complexes induced by suramin also were observed to appear in a dose-responsive manner in association with anti-HAI-1 MAb M19. M19 recognizes the unbound, full-length HAI-1 at 55 kDa and both the 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 after treatment with 100  $\mu\text{M}$  suramin. These data suggest that the complexed HAI-1 represents only a small portion of total HAI-1, even under conditions in which 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 (3).

*Inhibition of matriptase by HAI-1 simultaneously follows the activation of protease.* To further confirm that the 120- and 85-kDa bands detected using these three MAb were matriptase-HAI-1 complexes, we first immunodepleted cell lysates of suramin-treated 184 A1N4 cells using anti-HAI-1 MAb M19. Subsequently, we examined the depletion of both 120- and 85-kDa bands on immunoblots using anti-HAI-1 MAb M19,



**Fig. 2.** Detection of all activated matriptase in HAI-1 complexes. *A*: confirmation that 120- and 85-kDa bands are matriptase-HAI-1 complexes. 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 (IP) using anti-HAI-1 MAb M19-Sepharose 4B. The total cell lysate (L) and the MAb M19-depleted cell lysate (D) were analyzed using 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 using immunoblot analysis with MAb M19, M32, and M69, respectively. *C*: a proposed model for the conversion of the matriptase-HAI-1 complex from the 120- 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 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 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 MAb M69 and M19, are preserved in the 85-kDa complex.

anti-matriptase MAb M32, and anti-two-chain matriptase MAb M69 (Fig. 2A). These combinations of immunodepletion and immunoblot confirmed that the protein bands of the same size recognized by different MAb on immunoblots were the same proteins. As expected, anti-HAI-1 MAb M19 almost completely depleted the 120-, 85-, and 55-kDa bands recognized by M19 using immunoblotting. Both 120- and 85-kDa bands recognized by MAb M69 using immunoblotting, but not the 70-kDa band recognized by MAb M32, were also depleted by anti-HAI-1 MAb M19. The depletion of the 120-, 85-, and 55-kDa bands, and the lack of immunodepletion of the 70-kDa matriptase bands, by anti-HAI-1 MAb M19 provide evidence that the immunodepletion was specific. These data also confirm that both 120- and 85-kDa bands were matriptase-HAI-1 complexes. The 85-kDa matriptase-HAI-1 complex was previously suggested to be the free form of activated matriptase (1, 3, 25). However, the present data suggest that all activated matriptase is bound with HAI-1 immediately after 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. 2B). Because MAb M32 did not recognize the 85-kDa complex, the conversion of the 120-kDa complex to a 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, the addition of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) into the cell lysis buffer inhibited this conversion even for the samples incubated at 37°C. Because the loss of noncatalytic domains of matriptase occurred only for activated matriptase and not for latent matriptase, and because DTNB can inhibit protein disulfide isomerase (15), it is very likely that, after cell lysis, protein disulfide isomerase cleaves the disulfide bond that 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 will not separate from the noncatalytic domain, because the protease will still be held as single-chain matriptase by peptide bonds. Figure 2C summarizes our current theory that the 85-kDa matriptase complex is derived from the 120-kDa complex and shows the positions of epitopes recognized by the three MAb that we used.

*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 dose-response relationship, rapid kinetics, and rapid inhibition of active matriptase by HAI-1, we have observed some differences between them. Specifically, suramin requires micromolar concentration, compared with nanomolar concentration for S1P, to induce matriptase activation. Also, compared with S1P-treated cells, much higher proportions of latent matriptase were converted to activated matriptase in suramin-treated cells. In addition, S1P simultaneously induced changes in cell shape under the light microscope, but such changes were not observed with suramin. The S1P-induced changes in cell shape were associated mainly with the S1P-induced actin cytoskeletal rearrangement, that is, 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 Fig. 3, we compared suramin with S1P for their abilities to induce the redistribution of matriptase and cause 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 (Fig. 3A). Little F-actin was observed in these cells (Fig. 3B). After 30 min of suramin treatment, while matriptase was still located in cytoplasm, the protease apparently concentrated at vesicle-like structures with various sizes (Fig. 3D), in striking contrast to the cell-cell contact localization of matriptase induced by S1P (Fig. 3G). While filamentous actin structures were increased by S1P treatment (Fig. 3H), they were much less organized in suramin-treated cells (Fig. 3E). Interestingly, pretreatment with suramin apparently did not affect the S1P-induced formation of subcortical actin belts and translocation of matriptase to cell-cell contacts (Fig. 3, J and K).

We further examined the appearance and localization of activated matriptase (red) using Alexa Fluor 647-labeled MAb M69 in conjunction with total matriptase (green) using Alexa Fluor 488-labeled MAb M32 (Fig. 4). Despite high levels of total matriptase in the cytoplasm, activated matriptase was not detected using immunofluorescent staining in 184 A1N4 cells after growth for 2 days (1, 3, 7). Thirty minutes after suramin treatment, activated matriptase was detected as spotty vesicle-like structures in cytoplasm (Fig. 4B), a pattern similar to total matriptase (Figs. 4A and 3D). When both images were merged, activated matriptase coincided with the spotty total matriptase (Fig. 4C). The localization of activated matriptase, as well as the total matriptase, in suramin-treated 184 A1N4 cells was different from that in S1P-treated cells, where activated matriptase was colocalized with total matriptase at cell-cell contacts (Fig. 4, D–F). Despite these differences, activated matriptase appeared where total matriptase accumulated. In S1P-treated cells, activated matriptase was exclusively detected at cell-cell contacts, particularly where more total matriptase accumulated. Similarly to S1P-treated cells, suramin treatment caused accumulation of matriptase, but now in the cytoplasm, as spotty, vesicle-like structures, where its activation may occur. Again, cellular pretreatment with suramin did not affect subsequent S1P-induced translocation of both latent matriptase and activated matriptase to cell-cell junctions (Fig. 4, G–I). 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 (25). 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 (18, 26), and subsequently its role in matriptase activation was demonstrated (25). The extremely rapid, highly efficient inhi-

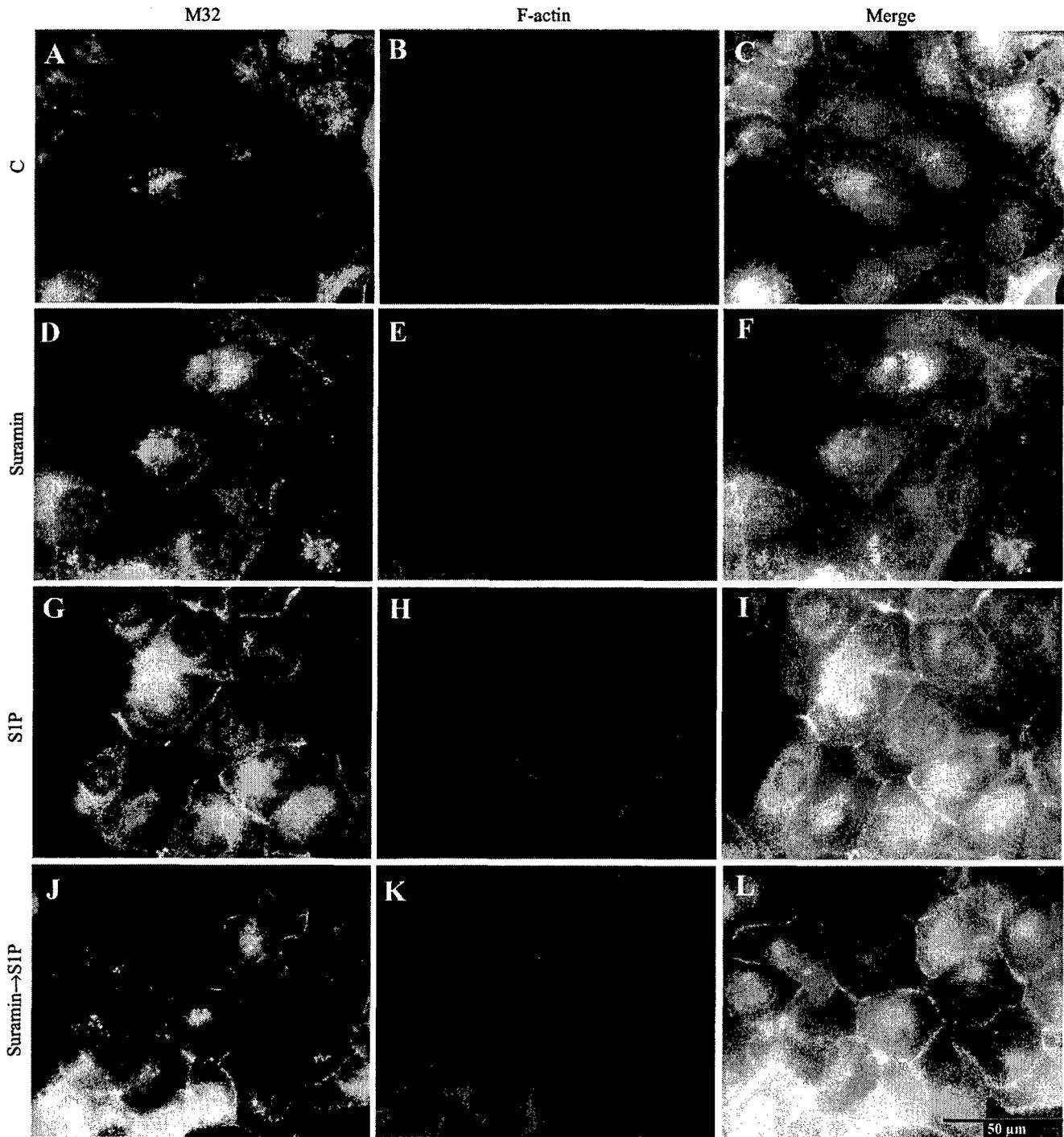


Fig. 3. Effects of sphingosine 1-phosphate (S1P) and suramin on subcellular translocation of matriptase (A–L). Serum-starved 184 A1N4 cells were cultured in the presence or absence of S1P (50 ng/ml) or suramin (50  $\mu$ M) for 30 min. Control cells (C) were cultured in IMEM alone. Cells were stained for F-actin with Texas red-conjugated phalloidin (red; B, E, H, and K), for matriptase with Alexa Fluor 488-conjugated MAb M32 (green; A, D, G, and J), and for nuclei with 4',6-diamidino-2-phenylindole (DAPI) (blue; C, F, I, and L).

hibition of matriptase by HAI-1, coupled with the requirement of HAI-1 for matriptase activation, suggest that the inhibitor should be in physical proximity to matriptase during activation of the protease. After growth of 184 A1N4 cells for 2 days, the inhibitor and matriptase were localized in the cytoplasm, surrounding the nuclei (Fig. 5, A–C). Both proteins were cotrans-

located to cell-cell contacts in response to S1P treatment (Fig. 5, D–F) and appeared in the spotty, vesicle-like structures in the cytoplasm in response to suramin treatment (Fig. 5, G–J). These results suggest that HAI-1 and matriptase are translocated to activation foci for the activation of matriptase. Therefore, for matriptase autoprolytic activation, both S1P and

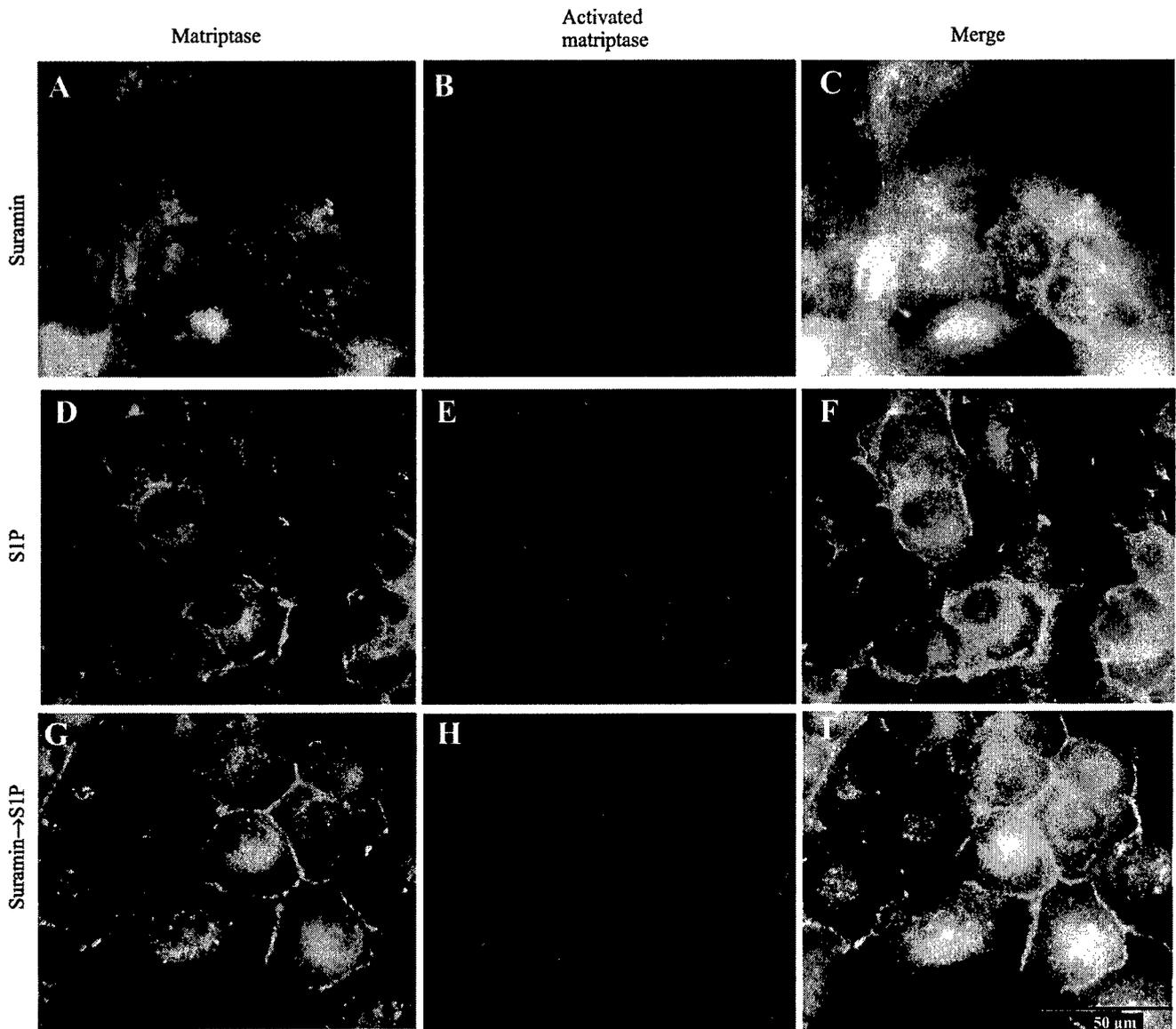


Fig. 4. Induction of matriptase activation by SIP and suramin (A–I). Serum-starved 184 A1N4 cells were stimulated for 30 min with SIP alone (50 ng/ml; D–F), suramin alone (50  $\mu$ M; A–C), or suramin followed by SIP (G–I). Cells were stained for total matriptase with Alexa Fluor 488-conjugated MAb M32 (green; A, D, and G), for activated matriptase with Alexa Fluor 647-conjugated MAb M69 (red; B, E, and H), and for nuclei with DAPI (blue; C, F, and I).

suramin may translocate matriptase, HAI-1, and other unidentified components to the activation foci to form the activation complex.

*F-actin polymerization is not required for suramin-induced matriptase activation.* Previously, we showed that SIP-induced matriptase translocation and activation at cell-cell contacts was dependent on actin cytoskeletal rearrangement (7). Therefore, it was of interest to investigate the role of actin cytoskeletal rearrangement in suramin-induced matriptase activation. As expected, activation of matriptase by SIP was significantly suppressed by pretreatment of 0.1  $\mu$ M cytochalasin D, which causes disruption of actin filaments and inhibition of actin polymerization (Fig. 6B). However, cytochalasin D up to 1  $\mu$ M concentration failed to block matriptase activation induced by suramin (Fig. 6A). These data are parallel to the fact that suramin does not induce formation of subcortical actin

belts and suggest that actin filament polymerization is not required for suramin-induced accumulation and activation of matriptase.

*Both SIP- and suramin-induced matriptase activation is inhibited by Ro 31-8220.* Because SIP induces various signal pathways, which may participate in matriptase activation, we used an array of chemical inhibitors, including the MEK kinase inhibitor PD-98059 (50  $\mu$ M), phosphatidylinositol 3-kinase inhibitor LY-294002 (50  $\mu$ M), protein kinase C (PKC) inhibitors Ro 31-8220, calmodulin inhibitor *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7; 30  $\mu$ M), and  $\text{Ca}^{2+}$ /calmodulin kinase II inhibitor 1-(*N,O*-bis[5-isoquinolinesulfonyl]-*N*-methyl-L-tyrosyl)-4-phenylpiperazine (KN-62; 10  $\mu$ M) to elucidate signaling pathways that may participate in matriptase activation. Only treatment of 184 A1N4 cells with Ro 31-8220 abolished SIP-induced activation of matriptase

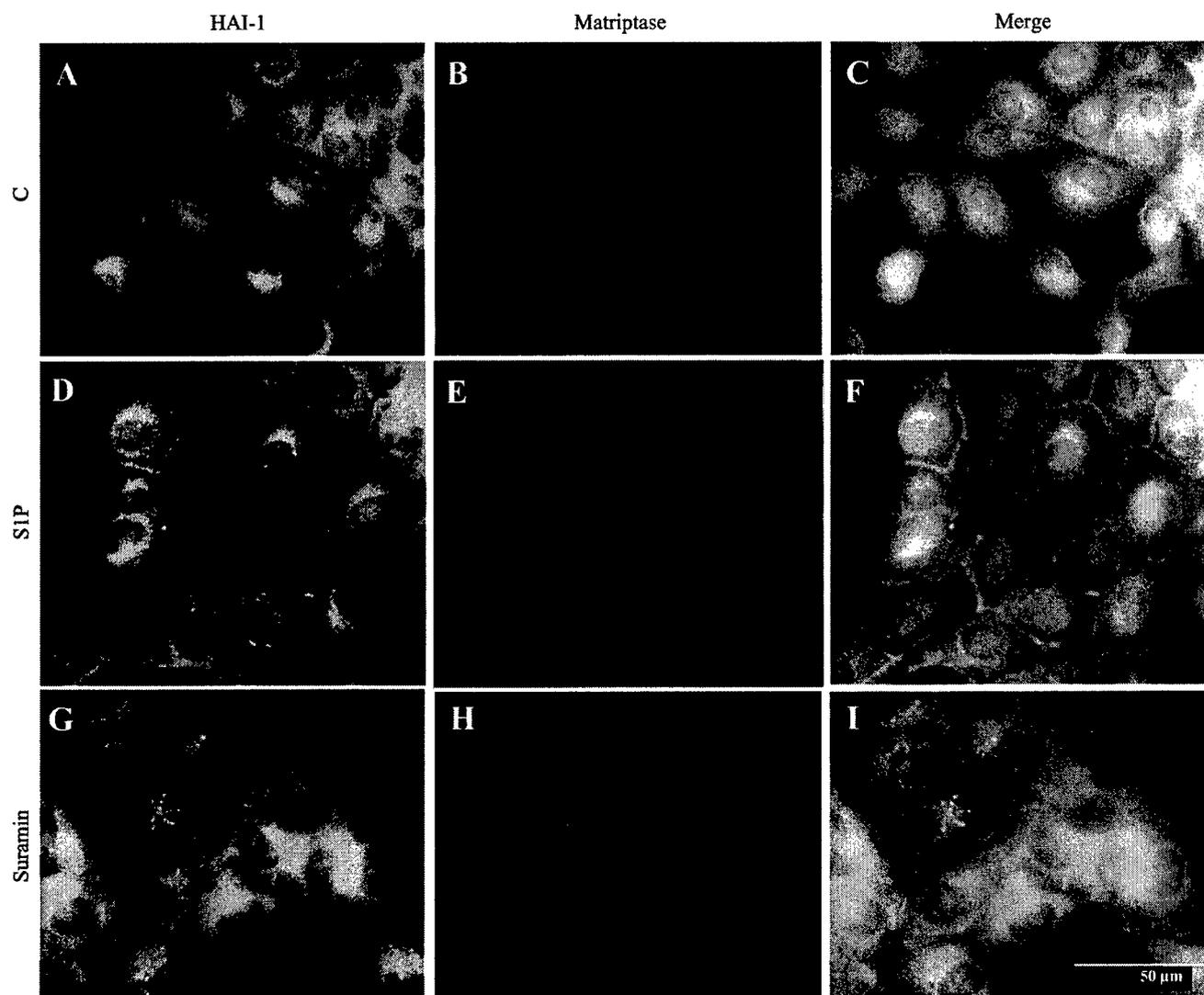


Fig. 5. Translocation of HAI-1 to activation foci during matriptase activation. Serum-starved 184 A1N4 cells were cultivated in the presence or absence of SIP (50 ng/ml; *D–F*) or suramin (50  $\mu$ M; *G–I*) for 30 min. Control cells were cultured in IMEM alone (*A–C*). Cells were stained for HAI-1 with Alexa Fluor 488-conjugated MAb M19 (green; *A*, *D*, and *G*), for matriptase with Alexa Fluor 647-conjugated MAb M32 (red; *B*, *E*, and *H*), and for nuclei with DAPI (blue; *C*, *F*, and *I*).

(Fig. 7*B*), suggesting the role of PKC in matriptase activation. Other chemicals showed no obvious effect on the SIP-induced activation of matriptase (data not shown). Interestingly, despite differential response to cytochalasin D, both SIP-induced and suramin-induced matriptase activation was inhibited by Ro 31-8220 (Fig. 7). These data suggest that both SIP and suramin may share some common regulatory mechanisms for their ability to induce matriptase activation.

#### DISCUSSION

From a biochemical point of view, the process of matriptase activation represents a proteolytic cleavage at its canonical serine protease activation motif, converting the single-chain zymogen to the two-chain, active protease. If this process is carried out by other active proteases, as is the case for most serine proteases, one could expect that matriptase activation will be a simple matter by the regulation of its protease activator. However, our data indicate that matriptase activation

is a complex process, because the current results show that the translocation of matriptase to activation foci, the formation of putative activation complex, and the involvement of protein kinases are required for it to occur and because previous studies conducted at our laboratory showed that matriptase activation requires its own intact active triads and noncatalytic domains, and even its cognate inhibitor HAI-1. Thus all of these data suggest that matriptase activation occurs through a tightly regulated process; importantly, its activation is dependent on its own protease activity but is not based on other active proteases. In immortal mammary epithelial cells, this activation event can be induced either by its physiological inducer SIP to occur at cell-cell junctions or by chemical inducer suramin to occur at vesicle-like structures. In activation foci, the cleavage of matriptase at the canonical activation motif of its zymogen may be carried out by another matriptase zymogen molecule as a process of transactivation. Activation by zymogen could serve as the major mechanism for subse-

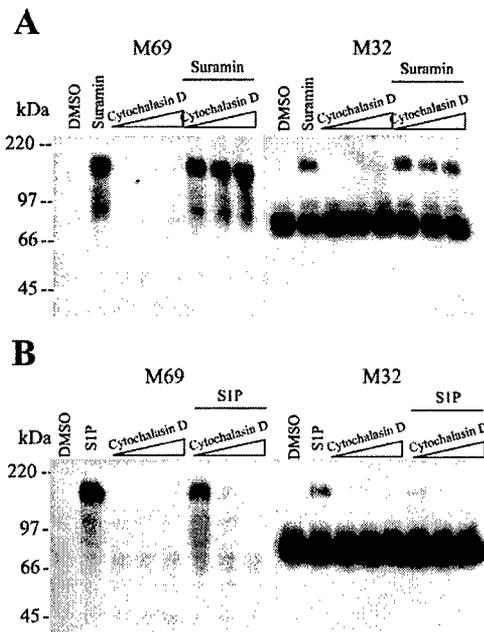


Fig. 6. Effect of cytochalasin D on suramin- or SIP-induced matriptase activation. Serum-starved 184 A1N4 cells were pretreated with 0.01, 0.1, or 1  $\mu$ M cytochalasin D for 30 min. Control cells were treated with DMSO alone. Cells were then cultivated for 30 min with 0.01, 0.1, or 1  $\mu$ M cytochalasin D in the presence or absence of 50  $\mu$ M suramin (A) or 50 ng/ml SIP (B). Equal amounts of total cell lysates were analyzed using Western blotting with MAb M69 or M32 to detect the activated or total matriptase, respectively.

quent matriptase activation along the cell-cell contacts (in the case of SIP stimulation) or at the vesicle-like structures (in the case of suramin stimulation). Because the cleavage preference of matriptase perfectly matches the flanking sequences of its activation motif, the newly produced, active matriptase may also activate latent matriptase. However, this possibility could depend on the competition for active matriptase among the latent matriptase, its physiological substrates (as yet unknown), and its competitive inhibitor HAI-1.

HAI-1 was initially identified as a cognate matriptase inhibitor (18) and was subsequently shown also to be required for matriptase activation (25). Its inhibitory role results from the binding of its first Kunitz domain with active matriptase (14). However, its participation in matriptase activation is mainly via its LDL receptor class A domain, which could provide the structural basis for protein-protein interactions in the formation of matriptase activation complexes. The translocation and concentration of both matriptase and HAI-1 in activation foci in response to cellular stimulation by suramin or SIP further support the close temporospatial relationship between these two molecules during matriptase activation. Therefore, HAI-1 may directly or indirectly contact latent matriptase in the activation complex. This close spatial proximity of HAI-1 to active matriptase must result in a very efficient and rapid inhibition of active matriptase immediately after matriptase activation. This inhibitor-dependent activation could be the explanation for all activated matriptase in HAI-1 complexes (Figs. 1 and 2). The coupling of matriptase activation with its inhibition apparently makes the half-life of active matriptase very short. Therefore, it is plausible that the substrates of matriptase may also be present in the activation foci or that the

matriptase substrates could even be required for full matriptase activation. This mechanism could allow matriptase activation, action on substrates, and inhibition to occur simultaneously.

The dual role of HAI-1 in matriptase activation and inhibition appears to be reminiscent of the role of tissue inhibitor of matrix metalloproteinase-2 (TIMP-2) in the regulation of matrix metalloproteinase-2 (MMP-2). It has been established that MMP-2 activation occurs via a mechanism to form a trimolecular complex of TIMP-2, MMP-2 zymogen, and MT1-MMP in 1:1:1 stoichiometry (11). When the concentration of TIMP-2 is low, TIMP-2 cannot bind to active MMP-2, leading to collagen degradation by these active MMP-2. At the high concentration of TIMP-2, TIMP-2 functions as a great inhibitor to block MMP-2 activity (13). Similarly, HAI-1 is necessary to form oligomeric complexes with matriptase zymogens and/or other yet identified proteins to facilitate matriptase activation. The coupling of protease activation with inhibition ensures a tight control in the protease function, avoiding harmful effects of proteolysis.

Because matriptase is likely to be activated via autoproteolytic activation and because only the first protease in a protease cascade needs this activation mechanism, we have hypothesized that matriptase may be at the pinnacle of an as yet poorly characterized protease cascade. The complicated regulatory mechanisms that govern matriptase activation, action on its substrates, inhibition, and subcellular localization seem parallel to its potential leading position in a protease cascade. By these regulatory mechanisms, activation of matriptase and the putative protease cascade would occur only at the right time, when the exogenous inducers were present, and only at the right place, such as at cell-cell junctions where its substrates might be colocalized or cotranslocated. Depletion of the exogenous inducers, as well as HAI-1 inhibition, provides the mechanisms for fast and timely switch-off of the protease cascade. Ectodomain shedding of matriptase-HAI-1 complexes could be the mechanism for their cellular clearance.

Although SIP and suramin induce matriptase activation at different subcellular locations, both activation inducers cause translocation and accumulation of matriptase, HAI-1, and probably other required components at activation foci. These common processes for matriptase activation indicate some

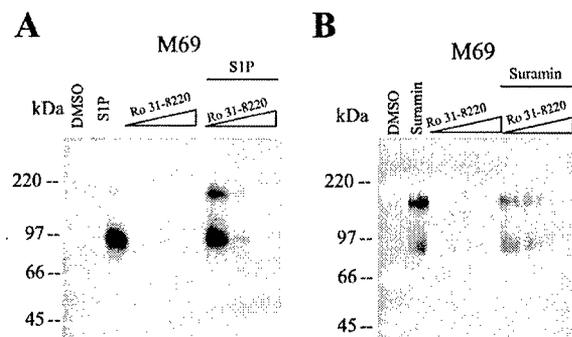


Fig. 7. Effect of Ro 31-8220 on SIP- or suramin-induced matriptase activation. Serum-starved 184 A1N4 cells were pretreated with 0.1, 1, or 5  $\mu$ M Ro 31-8220 for 30 min. Control cells were treated with DMSO alone. Cells were then cultivated for 30 min with 0.1, 1, or 5  $\mu$ M Ro 31-8220 in the presence or absence of 50 ng/ml SIP (A) or 50  $\mu$ M suramin (B). Equal amounts of total cell lysates were analyzed using Western blotting with MAb M69 to detect the activated form of matriptase.

basic mechanisms for matriptase activation: the involvement of the cell membrane and intracellular signaling. Both cell-cell junctions and vesicle-like structures contain lipid bilayers for the anchoring and trafficking of these proteins, consistent with the transmembrane protein characteristics of matriptase and HAI-1. The involvement of PKC-like protein kinases in both SIP- and suramin-induced matriptase activation suggests that intracellular signaling may contribute to the trafficking and oligomerization of these proteins at activation foci, consistent with the role of PKC in vesicle trafficking (4). Interestingly, this protein kinase-based intracellular signaling appears to depend on the assembly of F-actin and adherens junctions in response to SIP stimulation; suramin, however, could induce this intracellular signaling in the absence of F-actin.

In conclusion, in response to exogenous activation inducers, matriptase, its cognate inhibitor HAI-1, and possibly other unidentified proteins translocate and accumulate at activation foci, either at cell-cell junctions or at vesicle-like structures inside cells. Activation foci provide a good milieu in which matriptase can interact with those essential components and initiate matriptase activation. After its activation, active matriptase is rapidly bound to HAI-1. Subsequently, the matriptase-HAI-1 complex is shed into the extracellular milieu. Thus our data indicate that activation and HAI-1-mediated inhibition of matriptase are well organized and controlled at activation foci in human mammary epithelial cells.

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#### REFERENCES

- Benaud C, Dickson RB, and Lin CY. Regulation of the activity of matriptase on epithelial cell surfaces by a blood-derived factor. *Eur J Biochem* 268: 1439–1447, 2001.
- Benaud CM, Oberst M, Dickson RB, and Lin CY. Deregulated activation of matriptase in breast cancer cells. *Clin Exp Metastasis* 19: 639–649, 2002.
- Benaud C, Oberst M, Hobson JP, Spiegel S, Dickson RB, and Lin CY. Sphingosine 1-phosphate, present in serum-derived lipoproteins, activates matriptase. *J Biol Chem* 277: 10539–10546, 2002.
- Chen YW, Lang ML, and Wade WF. Protein kinase C- $\alpha$  and - $\delta$  are required for Fc $\alpha$ R (CD89) trafficking to MHC class II compartments and Fc $\alpha$ R-mediated antigen presentation. *Traffic* 5: 577–594, 2004.
- Gaboriaud C, Thielens NM, Gregory LA, Rossi V, Fontecilla-Camps JC, and Arlaud GJ. Structure and activation of the C1 complex of complement: unraveling the puzzle. *Trends Immunol* 25: 368–373, 2004.
- Hooper JD, Clements JA, Quigley JP, and Antalis TM. Type II transmembrane serine proteases: insights into an emerging class of cell surface proteolytic enzymes. *J Biol Chem* 276: 857–860, 2001.
- Hung RJ, Hsu I, Dreiling JL, Lee MJ, Williams CA, Oberst MD, Dickson RB, and Lin CY. Assembly of adherens junctions is required for sphingosine 1-phosphate-induced matriptase accumulation and activation at mammary epithelial cell-cell contacts. *Am J Physiol Cell Physiol* 286: C1159–C1169, 2004.
- Ihara S, Miyoshi E, Ko JH, Murata K, Nakahara S, Honke K, Dickson RB, Lin CY, and Taniguchi N. Prometastatic effect of N-acetylglucosaminyltransferase V is due to modification and stabilization of active matriptase by adding  $\beta$ 1–6 GlcNAc branching. *J Biol Chem* 277: 16960–16967, 2002.
- Ihara S, Miyoshi E, Nakahara S, Sakiyama H, Ihara H, Akinaga A, Honke K, Dickson RB, Lin CY, and Taniguchi N. Addition of  $\beta$ 1–6 GlcNAc branching to the oligosaccharide attached to Asn 772 in the serine protease domain of matriptase plays a pivotal role in its stability and resistance against trypsin. *Glycobiology* 14: 139–146, 2004.
- Kang JY, Dolled-Filhart M, Ocal IT, Singh B, Lin CY, Dickson RB, Rimm DL, and Camp RL. Tissue microarray analysis of hepatocyte growth factor/Met pathway components reveals a role for Met, matriptase, and hepatocyte growth factor activator inhibitor 1 in the progression of node-negative breast cancer. *Cancer Res* 63: 1101–1105, 2003.
- Karagiannis ED and Popel AS. A theoretical model of type I collagen proteolysis by matrix metalloproteinase (MMP) 2 and membrane type 1 MMP in the presence of tissue inhibitor of metalloproteinase 2. *J Biol Chem* 279: 39105–39114, 2004.
- Kim MG, Chen C, Lyu MS, Cho EG, Park D, Kozak C, and Schwartz RH. Cloning and chromosomal mapping of a gene isolated from thymic stromal cells encoding a new mouse type II membrane serine protease, epithin, containing four LDL receptor modules and two CUB domains. *Immunogenetics* 49: 420–428, 1999.
- Kinoshita T, Sato H, Okada A, Ohuchi E, Imai K, Okada Y, and Seiki M. TIMP-2 promotes activation of progelatinase A by membrane-type 1 matrix metalloproteinase immobilized on agarose beads. *J Biol Chem* 273: 16098–16103, 1998.
- Kirchhofer D, Peek M, Li W, Stamos J, Eigenbrot C, Kadkhodayan S, Elliott JM, Corpuz RT, Lazarus RA, and Moran P. Tissue expression, protease specificity, and Kunitz domain functions of hepatocyte growth factor activator inhibitor-1B (HAI-1B), a new splice variant of HAI-1. *J Biol Chem* 278: 36341–36349, 2003.
- Lahav J, Wijnen EM, Hess O, Hamaia SW, Griffiths D, Makris M, Knight CG, Essex DW, and Farndale RW. Enzymatically catalyzed disulfide exchange is required for platelet adhesion to collagen via integrin  $\alpha$ 2 $\beta$ 1. *Blood* 102: 2085–2092, 2003.
- Lee MJ, Van Brocklyn JR, Thangada S, Liu CH, Hand AR, Menzelev R, Spiegel S, and Hla T. Sphingosine-1-phosphate as a ligand for the G protein-coupled receptor EDG-1. *Science* 279: 1552–1555, 1998.
- Lee SL, Dickson RB, and Lin CY. Activation of hepatocyte growth factor and urokinase/plasminogen activator by matriptase, an epithelial membrane serine protease. *J Biol Chem* 275: 36720–36725, 2000.
- Lin CY, Anders J, Johnson M, and Dickson RB. Purification and characterization of a complex containing matriptase and a Kunitz-type serine protease inhibitor from human milk. *J Biol Chem* 274: 18237–18242, 1999.
- Lin CY, Anders J, Johnson M, Sang QA, and Dickson RB. Molecular cloning of cDNA for matriptase, a matrix-degrading serine protease with trypsin-like activity. *J Biol Chem* 274: 18231–18236, 1999.
- List K, Haudenschild CC, Szabo R, Chen W, Wahl SM, Swaim W, Engelholm LH, Behrendt N, and Bugge TH. Matriptase/MT-SP1 is required for postnatal survival, epidermal barrier function, hair follicle development, and thymic homeostasis. *Oncogene* 21: 3765–3779, 2002.
- List K, Szabo R, Wertz PW, Segre J, Haudenschild CC, Kim SY, and Bugge TH. Loss of proteolytically processed filaggrin caused by epidermal deletion of matriptase/MT-SP1. *J Cell Biol* 163: 901–910, 2003.
- Netzel-Arnett S, Hooper JD, Szabo R, Madison EL, Quigley JP, Bugge TH, and Antalis TM. Membrane anchored serine proteases: a rapidly expanding group of cell surface proteolytic enzymes with potential roles in cancer. *Cancer Metastasis Rev* 22: 237–258, 2003.
- Oberst MD, Johnson MD, Dickson RB, Lin CY, Singh B, Stewart M, Williams A, al-Nafussi A, Smyth JF, Gabra H, and Sellar GC. Expression of the serine protease matriptase and its inhibitor HAI-1 in epithelial ovarian cancer: correlation with clinical outcome and tumor clinicopathological parameters. *Clin Cancer Res* 8: 1101–1107, 2002.
- Oberst MD, Singh B, Ossandon M, Dickson RB, Johnson MD, and Lin CY. Characterization of matriptase expression in normal human tissues. *J Histochem Cytochem* 51: 1017–1025, 2003.
- Oberst MD, Williams CA, Dickson RB, Johnson MD, and Lin CY. The activation of matriptase requires its noncatalytic domains, serine protease domain, and its cognate inhibitor. *J Biol Chem* 278: 26773–26779, 2003.

26. Shimomura T, Denda K, Kitamura A, Kawaguchi T, Kito M, Kondo J, Kagaya S, Qin L, Takata H, Miyazawa K, and Kitamura N. Hepatocyte growth factor activator inhibitor, a novel Kunitz-type serine protease inhibitor. *J Biol Chem* 272: 6370–6376, 1997.
27. Suzuki M, Kobayashi H, Kanayama N, Saga Y, Suzuki M, Lin CY, Dickson RB, and Terao T. Inhibition of tumor invasion by genomic down-regulation of matriptase through suppression of activation of receptor-bound pro-urokinase. *J Biol Chem* 279: 14899–14908, 2004.
28. Szabo R, Wu Q, Dickson RB, Netzel-Arnett S, Antalis TM, and Bugge TH. Type II transmembrane serine proteases. *Thromb Haemost* 90: 185–193, 2003.
29. Takeuchi T, Harris JL, Huang W, Yan KW, Coughlin SR, and Craik CS. Cellular localization of membrane-type serine protease 1 and identification of protease-activated receptor-2 and single-chain urokinase-type plasminogen activator as substrates. *J Biol Chem* 275: 26333–26342, 2000.
30. Takeuchi T, Shuman MA, and Craik CS. Reverse biochemistry: use of macromolecular protease inhibitors to dissect complex biological processes and identify a membrane-type serine protease in epithelial cancer and normal tissue. *Proc Natl Acad Sci USA* 96: 11054–11061, 1999.
31. Tanimoto H, Underwood LJ, Wang Y, Shigemasa K, Parmley TH, and O'Brien TJ. Ovarian tumor cells express a transmembrane serine protease: a potential candidate for early diagnosis and therapeutic intervention. *Tumour Biol* 22: 104–114, 2001.
32. Van Brocklyn JR, Tu Z, Edsall LC, Schmidt RR, and Spiegel S. Sphingosine 1-phosphate-induced cell rounding and neurite retraction are mediated by the G protein-coupled receptor H218. *J Biol Chem* 274: 4626–4632, 1999.
33. Yang X, Chang HY, and Baltimore D. Autoproteolytic activation of pro-caspases by oligomerization. *Mol Cell* 1: 319–325, 1998.

