The Surface of Prostate Carcinoma DU145 Cells Mediates the Inhibition of Urokinase-type Plasminogen Activator by Maspin

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ABSTRACT
Maspin is a novel serine protease inhibitor (serpin) with tumor suppressive potential in breast and prostate cancer, acting at the level of tumor invasion and metastasis. It was subsequently demonstrated that maspin inhibits tumor invasion, at least in part, by inhibiting cell motility. Interestingly, in cell-free solutions, maspin does not inhibit several serine proteases including tissue-type plasminogen activator and urokinase-type plasminogen activator (uPA). Despite the recent biochemical evidence that maspin specifically inhibits tissue-type plasminogen activator that is associated with fibrinogen or poly-L-lysine, the molecular mechanism underlying the tumor-suppressive effect of maspin remains elusive. The goal of this study was to investigate the effect of maspin on cell surface-associated uPA. In our experimental system, we chose prostate carcinoma DU145 cells because these cells mediate plasminogen activation primarily by uPA, as shown by two different colorimetric enzyme activity assays. Purified recombinant maspin produced in baculovirus-infected Spodoptera frugiperda Sf9 insect cells [rMaspin(i)] binds specifically to the surface of DU145 cells, inhibits the DU145 cell surface-bound uPA, and forms a stable complex with the uPA in DU145 cell lysate. The inhibitory effect of rMaspin(i) on cell surface-bound uPA was similar to that of an uPA-neutralizing antibody and was reversed by a polyclonal antibody against the reactive site loop sequence of maspin. The Kᵢ value for rMaspin(i) in cell surface-mediated plasminogen activation was 20 nM, which is comparable to the Kᵢ values for plasminogen activator inhibitor 1 and plasminogen activator inhibitor 2, respectively. Furthermore, the proteolytic inhibitory effect of rMaspin(i) was quantitatively consistent with its inhibitory effect on the motility of DU145 cells in vitro. Our data demonstrate an important role for the prostate carcinoma cell surface in mediating the inhibitory interaction between rMaspin(i) and uPA. Thus, future maspin-based therapeutic strategies may prove useful in blocking the invasion and metastasis of uPA-positive prostate carcinoma.

INTRODUCTION
Maspin is a Mr 42,000 protein having an overall sequence homology with members of the serine protease inhibitor serpin superfamily (1). The maspin gene has been mapped to chromosome 18q21.3–q23 (2), within a cluster of serpins including PAI-2 and squamous cell carcinoma antigen (3). Accumulated evidence shows an inverse correlation between maspin expression and the progression of breast cancer (1, 4). In addition, maspin expression is down-regulated in prostate carcinoma cells compared with that in immortalized normal prostate epithelial cells (5, 6).

Biological studies demonstrate a tumor-suppressive role of maspin, acting at the levels of tumor invasion and metastases. Mammary carcinoma MDA-MB-435 cells transfected with maspin cDNA are significantly inhibited in invasion and motility assays in vitro and are inhibited in tumor growth and metastasis in nude mice (1, 5, 7). It has been shown independently that induction of maspin expression in four different breast tumor cell lines by γ-linolenic acid dramatically reduces cell motility in vitro (8). Furthermore, purified recombinant maspin proteins produced in three different expression systems inhibit the invasion and motility of an array of breast cancer cell lines as well as three prostatic carcinoma cell lines in culture (5, 9). A study by Seftor et al. (10) shows that purified recombinant maspin provokes changes of the integrin profiles on the MDA-MB-435 cell surface in favor of a more benign epithelial phenotype. Recently, Zhang et al. (11) have shown that recombinant mouse maspin inhibits prostate tumor mitogenesis in vitro and inhibits human prostate tumor-induced neovascularization in a xenograft mouse model. Hence, maspin protein, either reexpressed in carcinoma cells or as an exogenously added purified reagent, may find novel therapeutic applications in the intervention of both breast and prostate cancers.

The enthusiasm about the potential therapeutic value of maspin in treating human malignancy is hampered by the lack of understanding of the molecular mechanism of maspin. Central to this issue, maspin does not act as a classical serpin in cell-free conditions, i.e., it does not inhibit a series of serine proteases including tPA and uPA (12–14), although the RSL sequence of maspin is critical for its biological activities (1, 5, 11). After intensive research, tPA preactivated by fibrinogen or poly-L-lysine has been identified as a target of maspin (12). Interestingly, detailed kinetic data suggest that recombinant maspin may use its two segregated domains to interact with the catalytic and regulatory domains of tPA, respectively (12). The complex interaction between recombinant maspin and tPA suggests that the novel proteolytic inhibitory property of maspin depends not only on its intrinsic structural features but also on the microenvironment of the target enzyme.

We report here that rMaspin(i) competitively inhibited plasminogen activation mediated by the surface of prostate carcinoma DU145 cells. In contrast, rMaspin(i) did not inhibit purified uPA, tPA, and plasmin in cell-free conditions. Under our culture conditions, the DU145 cell surface used primarily uPA, but not tPA, to convert plasminogen to plasmin. We show that rMaspin(i) forms a stable complex with uPA in DU145 cell lysate. Furthermore, the maspin proteolytic inhibitory effect correlated quantitatively with the inhibition of cell motility in vitro. To our knowledge, this is the first evidence that maspin inhibits uPA. Moreover, our data demonstrate an important role of the prostate carcinoma cell surface in mediating the inhibitory interaction between maspin and uPA. Thus, future maspin-based therapeutic strategies may prove useful in blocking the invasion and metastasis of uPA-positive prostate carcinomas.
MATERIALS AND METHODS

Cell Culture. Human prostate DU145 carcinoma cells (American Type Culture Collection) were maintained in RPMI 1640 (Life Technologies, Inc.) medium supplemented with 5% fetal bovine serum (Hyclone) in a cell culture incubator (constantly set at 37°C with 6.5% CO2). The CM was produced by incubating the cells (approximately 70% confluent) for 24 h in a SF keratinocyte growth medium (KGM; Life Technologies, Inc.) supplemented with epidermal growth factor (5 ng/ml). All CM thus obtained was concentrated by approximately 10-fold in Centricon-10 units (Amicon).

Chemicals and Reagents. Antibodies against the RSL sequence of maspin protein Abs4A were produced and purified as described previously (1). Purified recombinant PAI-1 was a generous gift from Dr. Thomas Reilly (Dupont Merck, Wilmington, DE). Reagents of molecular biology grade for protein gel electrophoresis, protein staining, and protein concentration analysis were purchased from Bio-Rad. Trypan blue solution was purchased from Flow Laboratories (McLean, VA). Proteolytic enzymes, protease substrates, and specific protease inhibitors purchased from American Diagnostica include recombinant single-chain tPA, high molecular weight recombinant uPA, synthetic chromogenic peptide substrate of uPA (Spectrozyme UK), synthetic chromogenic peptide substrate of tPA (Spectrozyme tPA), Glu-type plasminogen, purified plasmin, chromogenic plasmin substrate (Spectrozyme PL), and 0.1% Triton X-100.

RESULTS

Purification of Monomeric rMaspin(i). The purified recombinant innate human PAI-2. Unless otherwise specified, all other chemicals and reagents were of the highest purity and obtained from Sigma. Purification of Monomeric rMaspin(i). rMaspin(i) was first purified by a two-step chromatographic procedure as described previously (9). This rMaspin(i) preparation was further purified by a heparin column procedure using the medium-pressure BioLogic automated chromatographic system (Bio-Rad). Briefly, 10 mg of the initially purified rMaspin(i) were loaded on to a heparin column. The unbound molecules were removed from the column by an extensive washing (20 column volumes) with 20 mM Tris-HCl (pH 7.6). The bound proteins were eluted by a NaCl gradient (0–0.5 M) in 20 mM Tris-HCl (pH 7.6). The fractions eluted at 0.1 mM NaCl contained the purified monomeric rMaspin(i). The purified rMaspin(i) was sterilized by filtration through a 0.2 µm membrane for subsequent biochemical and biological analyses.

Coupled Colorimetric Assay for Plasminogen Activation in Conditioned Culture Medium. Aliquots of 80 µl of reaction buffer (50 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, 0.2 µg/ml leupeptin, 0.32 mM Glu-plasminogen, 0.2 mM Spectrozyme PL, and 0.1% Triton X-100) were added to the wells of a 96-well microplate. Glu-plasminogen was omitted in the blank reactions. The enzyme reaction was initiated by the addition of 20 µl of the concentrated CM of DU145 cells. The photometric absorbance of the reaction mixtures at 405 nm was monitored at 37°C over the next 20 min using a Bio-Rad Benchmark microplate reader.

Detection of Cell Surface-associated Plasminogen Activators by CAAs. CCA were seeded at 10,000 cells/well in 96-well plates in the maintenance medium and allowed to reach approximately 90% confluence. The cells were washed once with PBS and analyzed in a PBS-based reaction buffer (100 µl/well) containing 0.8 mM MgCl2 and 0.2 µg/ml leupeptin. The reactions were initiated by the addition of the chromogenic amidolytic substrate specific for either uPA (Spectrozyme UK) or tPA (Spectrozyme tPA) to a final concentration of 0.2 mM. No amidolytic substrate was added in the blank reactions. The photometric absorbance of the reaction mixtures at 405 nm was monitored at 37°C over the next 30 min.

CCA-CSPA. Cells were prepared as described for the CAA. A total of 100 µl of the reaction mixture (PBS, 0.2 mM Spectrozyme PL, 0.32 mM Glu-plasminogen, 0.8 mM MgCl2, 0.2 µg/ml leupeptin, and various protein factors at the indicated final concentrations) was added to the cells in each well. The reaction was initiated by the addition of Glu-plasminogen, which was omitted in the blank reactions. The photometric absorbance of the reaction mixtures at 405 nm was monitored at 37°C over the next 20 min.

Chemical Cross-linking Assay. One hundred µg of the total lysate of DU145 cells were incubated in the presence or absence of 5 µg of rMaspin(i) at 37°C for 30 min. In parallel, 1 µg of purified uPA protein was incubated with 5 µg of rMaspin(i) (or BSA as a negative control) at 37°C for 30 min. The above-mentioned reaction mixtures were incubated with 0.1% glutaraldehyde on ice for 30 min and then quenched by glycine (pH 8.0) at a final concentration of 100 mM. The resulting mixtures were analyzed by Western blotting with either the polyclonal antibody against the NH2-terminal variable sequence of maspin [Abs3A (1)] or the monoclonal antibody against the A-chain of uPA (American Diagnostica).

Immunofluorescent Staining of Maspin. Cells cultured in 8-well chamber slides (Nunc) in the maintenance medium were washed with PBS and incubated with PBS ± 80 nM rMaspin(i) for 30 min. The cells were gently washed twice with PBS, fixed with 4% freshly prepared paraformaldehyde/ PBS for 10 min, blocked with Abs4A (5 µg/ml), and subsequently blocked with R-phycocyanin-antirabbit IgG (H+L) (Zymed), as described previously (5). The nuclear DNA was briefly counterstained with Hoecht 33528 at a final concentration of 10 µg/ml. The stained cells were viewed and photographed under a Leica fluorescence microscope.

ELISA Detection of rMaspin(i) Bound to DU145 Cells. Cells seeded at 105 cells/well in a 96-well microplate were incubated in the maintenance medium for 12 h. The cells were washed with PBS, incubated overnight at 4°C in 100 µl/well SF keratinocyte growth medium containing rMaspin(i) at various concentrations. The cells were washed gently, blocked with 1% BSA/ PBS for 1 h, and incubated with 15 µg/ml Abs4A for 1 h, and detected by the color reaction of horseradish peroxidase-conjugated antirabbit IgG as described by Harlow and Lane (15).

In Vitro Motility Assays Using MICS. Briefly, the serum-starved cells (24 h) were seeded onto the 8 µm polycarbonate membrane insert (precoated with 50 µg/ml Matrigel) of the MICS chamber. rMaspin(i), Abs4A, or a preincubated mixture of rMaspin(i) and Abs4A (37°C, 30 min) was added directly to the cell cultures at the indicated final concentrations. The MICS chamber was incubated for 6 h at 37°C with 6.5% CO2. Cells attached to the bottom side of the polycarbonate membrane insert were fixed, stained, and counted under the microscope as described previously (5).

Miscellaneous Procedures. Cell staining with SRB was performed as described by Garbin et al. (16). The trypan blue cell staining procedure was performed as described by Broman et al. (17). Plasminogen-dependent gelatinolytic zymogram was performed as described previously (18). SDS-PAGE gel electrophoresis was performed as described by Laemmli (19). Nondenaturing protein gel electrophoresis was performed as described by Lomas et al. (20). Western blotting of maspin was performed as described previously (1). Silver nitrate staining of protein electrophoresis gels and protein concentration analyses using the Bio-Rad reagents were performed according to the manufacturer’s instructions.

Fig. 1. Purification of intact monomeric rMaspin(i) by heparin affinity chromatography using the BioLogic System (Fast Protein Liquid Chromatography). A, silver-stained SDS-PAGE gel. Lane 1, molecular weight standards; Lane 2, 0.75 µg of the starting rMaspin(i) preparation; Lane 3, 0.75 µg of rMaspin(i) eluted with 0.1 mM NaCl. B, silver-stained, nondenaturing protein gel electrophoresis gel. Lane 1, molecular weight standards (phosphorylase b, Mr 94,000; BSA, Mr 66,000; carboxy anhydridase, Mr 31,000; β-lactoglobulin, M 21,000). Lanes 2 and 3 each contain 0.75 µg of protein from two different aliquots of the same elution fraction shown in Lane 3 in A. The aliquot of rMaspin(i) in Lane 2 had been stored at −70°C, whereas the aliquot in Lane 3 had undergone a series of freezing and thawing over a 1-month period.
mogenous (Lane 3), whereas the starting material (Lane 2) had impurities of higher and lower molecular weights. The yield of this highly purified rMaspin(i) was estimated to be at least 10% (w/w) of the total extractable proteins of the host insect cells.

To test whether the purified rMaspin(i) was in a uniform conformation, an aliquot of rMaspin(i) shown in Lane 3 of Fig. 1A was analyzed by nondenaturing protein electrophoresis followed by silver staining. As shown in Fig. 1B, only one protein band was detected (Lane 2). Furthermore, the purified rMaspin(i) remained homogeneous after being frozen and thawed several times over the next month (Lane 3). The identity of rMaspin(i) was further confirmed by Western blotting using the polypeptide-derived polyclonal antibody against the RSL sequence of maspin, Abs4A (Ref. 1; data not shown).

**Cell Surface-associate uPA as a Target of rMaspin(i).** To address the possibility that maspin may inhibit cell surface-associated uPA, it was critical to use a homogenous cell population that produces uPA as the predominant plasminogen activator. For this reason, human prostate carcinoma cell line DU145 was chosen for our study. Fig. 2A shows the zymographic profile of the plasminogen-dependent gelatinolytic activities of both the total cell lysate and the SF-CM of DU145 cells. As compared with the purified tPA and uPA standards, both the lysate and the SF-CM of DU145 cells contained primarily uPA, although a trace amount of tPA was also detected.

To further identify the plasminogen activator(s) produced by DU145 cells, the SF-CM of DU145 cells was first tested by the coupled assay for plasminogen activation. As shown in Fig. 2B, the SF-CM of DU145 cells was active in converting Glu-plasminogen to plasmin, which in turn led to the cleavage of Spectrozyme PL, as shown by an increased photometric absorbance at 405 nm (12). The uPA-neutralizing antibody blocked this plasminogen activation activity in a dose-dependent manner. In contrast, the IPA-neutralizing antibody had no significant effect. CAA was also performed. When the chromogenic amidolytic substrate specific for uPA or tPA was added to the monolayer of DU145 cells, only the uPA substrate, Spectrozyme UK, was cleaved (Fig. 2C). The uPA-neutralizing antibody, but not a IPA-neutralizing antibody, blocked the cleavage of Spectrozyme UK. In parallel experiments, the IPA-specific substrate, Spectrozyme tPA, was not cleaved.

There appeared to be a specific interaction between rMaspin(i) and the DU145 cell surface. DU145 cells do not produce endogenous maspin (Refs. 5 and 6; Fig. 3A). However, after the cells were treated with rMaspin(i) and washed thoroughly, cell-bound rMaspin(i) was detected by immunofluorescent staining under a nonpermeablizing condition (Fig. 3A). In addition, when added to the SF culture medium, rMaspin(i) at low concentrations (<100 nm) bound to DU145 cells in a dose-dependent manner (Fig. 3B). As the concentration of rMaspin(i) exceeded 100 nm, the amount of cell surface-bound rMaspin(i) was not further increased, suggesting that the binding of rMaspin(i) had reached a level of saturation.

To examine the effect of rMaspin(i) on DU145 cell surface-mediated plasminogen activation, the CCA-CSPA was used. As shown in Fig. 4A, rMaspin(i) at a final concentration of 90 nm exhibited a significant inhibition of DU145 cell surface-mediated plasminogen activation. In addition, purified Abs4A blocked the inhibitory activity of rMaspin(i) in a dose-dependent manner, whereas the preimmune rabbit IgG had no significant effect. It is worth noting that throughout this biochemical assay, cells remained intact, showing little or no plasma membrane permeability by trypan blue and a full recovery of proliferation in the subsequent cell growth assay (data not shown).

The number of remaining cells in each reaction, as quantified by the SRB staining procedure (16), was used to normalize the initial velocities of plasminogen activation.

To further test whether rMaspin(i) inhibits a specific plasminogen activator on the surface of DU145 cells, cells were preincubated with specific neutralizing antibody of uPA or tPA and analyzed by CCA-CSPA. As shown in Fig. 4B, the uPA-neutralizing antibody inhibited DU145 cell-mediated plasminogen activation to a similar extent as rMaspin(i). Cells preincubated with the uPA-neutralizing antibody were not further inhibited by rMaspin(i) in this assay. In contrast, the tPA-neutralizing antibody neither affected the basal level cell-mediated plasminogen activation nor altered the proteolytic inhibitory effect of rMaspin(i). These data further support a specific inhibitory activity of rMaspin(i) toward the cell surface-associated uPA.

rMaspin(i) did not seem to act as a protease substrate. Fig. 4C shows the Western blotting of rMaspin(i) before (Lane 2) and after (Lane 3) the CCA-CSPA, respectively. rMaspin(i) was not degraded during CCA-CSPA. Under our assay conditions, the cleavage of the plasmin substrate Spectrozyme PL was dependent on the exogenous supply of either Glu-plasminogen (data not shown) or plasmin.
weights of approximately 80,000, 120,000, and 125,000, respectively (Lane 3). This polymerization pattern resembles that seen with the trypsin-cleaved maspin, a Mr 38,000 NH₂-terminal fragment of maspin (13). These data suggest that rMaspin(i) did not form a complex with soluble uPA but rather was significantly degraded.

When rMaspin(i) was incubated with DU145 lysate, however, a new protein band of approximately Mr 100,000 was detected by Western blotting of maspin (Fig. 6, Lane 5). When the same membrane containing Lanes 4 and 5 was rebotted with the uPA antibody, a uPA-containing protein of approximately Mr 100,000 was detected in the mixture of DU145 cell lysate and rMaspin(i) (Fig. 6, Lane 7). In the absence of rMaspin(i), only the monomeric uPA of Mr 55,000 was detected in DU145 cell lysate (Fig. 6, Lane 6). Given that the combined molecular weight of rMaspin(i) and uPA is 97,000, it is likely that the new band of approximately Mr 100,000 detected by both Abs3A and the monoclonal antibody against uPA was the complex between rMaspin(i) and uPA in DU145 cells.

The Biologically Active rMaspin(i) Acts as a Competitive Inhibitor of the Cell Surface-mediated Plasminogen Activation. Kinetic analyses were performed to further investigate the mode of increased cleavage of Spectrozyme PL by exogenously added pure plasmin, however, was not affected by rMaspin(i) (Fig. 4B). Thus, it seemed unlikely that rMaspin(i) directly inhibited plasmin.

rMaspin(i) Exerts Differential Effects on Cell Surface-associated and Free Plasminogen Activators. Because DU145 cells secreted both tPA and uPA into the culture media (Fig. 2A), it was important to investigate whether rMaspin(i) inhibited the unbound plasminogen activators. As shown in Fig. 5A, rMaspin(i) added at a final concentration of 90 nM did not inhibit the basal level activity of plasminogen activation in the SF-CM of DU145. The exogenously added tPA and uPA both significantly stimulated the plasminogen activation in the CM. However, in each case, rMaspin(i) failed to inhibit the accelerated plasminogen activation.

Interestingly, the preincubation of DU145 cells with purified uPA did not lead to an elevated plasminogen activation on the cell surface, nor did this treatment change the inhibitory activity of rMaspin(i) (Fig. 5B). It is possible that the surface of DU145 cells was saturated with the endogenous uPA and thus could not accommodate additional plasminogen activators added exogenously. It has been reported that, under an acidic condition, the cell surface-associated uPA may be stripped and subsequently replaced by purified uPA (21). However, treatment of DU145 cells by this method caused significant damage to the cell membrane, as indicated by an increased trypan blue permeability (data not shown), thus preventing an accurate assessment of the binding as well as the activity of uPA on DU145 cell surface.

To investigate whether rMaspin(i) interacts directly with uPA, chemical cross-linking experiments were performed. As shown in Fig. 6, the polymerization of rMaspin(i) in cell-free solution resulted in a high molecular weight rMaspin(i) oligomer (Mr >125,000; Fig. 6, Lane 1). When rMaspin(i) was incubated with purified uPA, three major polymer forms of rMaspin(i) were detected with molecular weights of approximately 80,000, 120,000, and >125,000, respectively (Lane 3). This polymerization pattern resembles that seen with the trypsin-cleaved maspin, a Mr 38,000 NH₂-terminal fragment of maspin (13). These data suggest that rMaspin(i) did not form a complex with soluble uPA but rather was significantly degraded.

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The Biologically Active rMaspin(i) Acts as a Competitive Inhibitor of the Cell Surface-mediated Plasminogen Activation. Kinetic analyses were performed to further investigate the mode of
The apparent kinetic parameters were obtained from the Lineweaver-Burk plots. The initial velocities of plasminogen activation were measured by a coupled plasminogen activation assay, and the rate of the plasminogen activation mediated by DU145 cell surface-associated uPA was measured by a CCA-CSPA assay. The initial velocities of plasminogen activation were normalized by the amount of DU145 cells, and the error bars represent the SDs.

**DISCUSSION**

We show here that purified rMaspin(i) acts as a competitive inhibitor of DU145 cell surface-associated uPA, having an apparent $K_i$ value of 20 nM. The proteolytic inhibitory activity of rMaspin(i) correlates with its inhibition of the motility of DU145 cells in vitro. These data support the hypothesis that maspin blocks cell motility and invasion, at least in part, by inhibiting the tumor cell-mediated plasminogen activation.

The biological activity of rMaspin(i) in inhibiting cell motility and invasion has been initially localized on the cell membrane (5). Data from the current study demonstrated an important role for the epithelial cell surface in regulating the inhibitory interaction between rMaspin(i) and the localized plasminogen activation system. First, rMaspin(i) bound to intact DU145 cells in a saturable fashion (Fig. 3), suggesting that it may interact with a specific cell surface-associated molecule. Second, rMaspin(i) exhibited a strong inhibitory effect on DU145 cell surface-mediated plasminogen activation, comparable with that of PAI-1 and PAI-2 in parallel experiments (Fig. 7). In contrast, rMaspin(i) did not inhibit free uPA (12–14) in solution, nor did it inhibit the plasminogen activators secreted by DU145 cells to the CM (Fig. 5). Moreover, we showed that rMaspin(i) forms a complex with the DU145 cell-associated uPA but acted as a substrate of purified uPA in solution (Fig. 6).

The DU145 cell surface may conceivably enhance the inhibitory interaction between rMaspin(i) and its target plasminogen activators via two mechanisms that are not mutually exclusive. On one hand, specific interactions between uPA and its associated proteins on the cell surface may render uPA prone to inhibition by rMaspin(i). It is proposed that the activation of uPA on the cell surface is mediated by a specific receptor, uPAR, which not only controls the localized plasminogen activation (22–24) but also regulates cell-matrix interaction with Abs4A, but not with the preimmune rabbit IgG, abolished the inhibitory activity of rMaspin(i). Thus, the RSL of rMaspin(i) was critical for both its proteolytic inhibitory activity (Fig. 4A) and its biological activity in inhibiting tumor cell motility.

**Fig. 6.** The molecular interaction between rMaspin(i) and uPA. Mixtures of rMaspin(i), purified single-chain uPA, DU145 cell lysate, and BSA in the indicated combinations were cross-linked using a glutaraldehyde procedure and immunoblotted with Abs3A against maspin (Lanes 1–5) or with the monoclonal antibody against uPA (Lanes 6 and 7; see “Materials and Methods”).

**Fig. 5.** The differential effects of rMaspin(i) toward cell surface-associated and free plasminogen activators. DU145 cells cultured in a 96-well microplate were incubated for 30 min at 37°C with 50 µl of SF medium (1 and 2), or SF medium plus 8 NIH units/ml tPA (3 and 4), or SF medium plus 8 NIH units/ml uPA (5 and 6). The resulting CM were collected and analyzed by the coupled plasminogen activation assay in the presence (2, 4, and 6) or absence (1, 3, and 5) of rMaspin(i) at a final concentration of 90 nM (shown in A). In parallel, the remaining cells were analyzed by CCA-CSPA in the presence (2, 4, and 6) or absence (1, 3, and 5) of rMaspin(i) at a final concentration of 90 nM (shown in B). The changes of photometric absorbance at 405 nm for each reaction were normalized by the number of remaining cells ($A_{550\text{nm}}$). In both A and B, the data represent an average of quadruplicate repeats, and the error bars represent the SDs.

The changes of photometric absorbance at 405 nm for each reaction were normalized by the number of remaining cells ($A_{550\text{nm}}$). In both A and B, the data represent an average of quadruplicate repeats, and the error bars represent the SDs.

**Fig. 7.** The biphasic dose-response curves of the Lineweaver-Burk plots with PAI-1 and PAI-2 were obtained (data not shown). The slopes of these plots were replotted against the concentration of the corresponding inhibitor as described previously (5). As shown in Fig. 7B, the replot of the slopes derived from Fig. 7A versus the concentrations of rMaspin(i) was linear. Based on Fig. 7B, an apparent $K_i$ value of approximately 20 nM was deduced. Interestingly, rMaspin(i) became less inhibitory as its concentration was further increased to 240 nM (Fig. 7A). In parallel, both recombinant PAI-1 and PAI-2 exhibited competitive inhibition, to slightly different extents, on DU145 cell-mediated plasminogen activation. The Lineweaver-Burk plots with PAI-1 and PAI-2 were obtained (data not shown). The slope of the Lineweaver-Burk plot was initially increased as the concentration of rMaspin(i) increased from 0 to 90 nM, demonstrating a dose-dependent inhibition by rMaspin(i). Furthermore, these plots intersect close to the 1/v axis, suggesting a constant maximum velocity ($V_{\text{max}}$). Thus, rMaspin(i) appeared to inhibit the cell surface-mediated plasminogen activation as a competitive inhibitor. As shown in Fig. 7B, the replot of the slopes derived from Fig. 7A versus the concentrations of rMaspin(i) was linear. Based on Fig. 7B, an apparent $K_i$ value of approximately 20 nM was deduced. Interestingly, rMaspin(i) became less inhibitory as its concentration was further increased to 240 nM (Fig. 7A).

In parallel, both recombinant PAI-1 and PAI-2 exhibited competitive inhibition, to slightly different extents, on DU145 cell-mediated plasminogen activation. The Lineweaver-Burk plots with PAI-1 and PAI-2 were obtained (data not shown). The slopes of these plots were replotted against the concentration of the corresponding inhibitor as described previously (5). The apparent $K_i$ values for PAI-1 and PAI-2 deduced from these replots were approximately 10 and 25 nM, respectively. We have shown previously that rMaspin(i) inhibits the invasion and motility of three prostate carcinoma cell lines including DU145 (5). To test whether repurified monomeric rMaspin(i) retained biological activity, we performed the MICS motility assay as described previously (5). As shown in Fig. 8, the purified monomeric rMaspin(i) exhibited a biphasic dose-dependent inhibition on the motility of DU145 cells with a nadir at 125 nM. This biphasic dose-response curve coincides with the biphasic effects of rMaspin(i) on DU145 cell-mediated plasminogen activation (Fig. 7A). In addition, precautionary measures were taken to ensure that the purified rMaspin(i) was free of any contaminating uPA.
action through its close association with integrins, the transmembrane receptors of extracellular matrix proteins (25). The elegant study of Schwartz et al. (26) demonstrates that uPAR-bound uPA and free uPA are inhibited by serpin PAI-3 via different mechanisms.

On the other hand, the cell surface microenvironment may play a critical role in mediating the transition of rMaspin(i) from a latent conformation to an active conformation. Based on a current paradigm, the initial docking of the RSL of an inhibitory serpin into the catalytic site of the target enzyme induces a massive β sheet rearrangement of the serpin molecule, leading to a stabilized enzyme/inhibitor complex (27, 28). Accordingly, based on protein sequence alignments, serpins such as chick ovalbumin and maspin, whose RSL may not undergo such conformational rearrangement without substantial energy compensation, were predicted to be noninhibitory (29). However, a study by Mellet et al. (30) showed that chick ovalbumin could be converted to a potent competitive serine protease inhibitor by heat denaturation. In fact, the activity of many inhibitory serpins such as PAI-1 can be greatly enhanced by serpin cofactors (31, 32). Central to the molecular mechanism of maspin, we show here that the RSL of rMaspin(i) was required for its inhibitory effect on the motility of DU145 cells (Fig. 8) and further supports the role of maspin as an inhibitory serpin.

Furthermore, given the earlier in vitro evidence that the M, 38,000 NH₂-terminal fragment of rMaspin(i) specifically interacts with the regulatory domain of tPA (12), it is likely that the functional determinants of maspin are not restricted to its RSL. It is worth noting that PAI-1 uses its NH₂-terminal domain to interact with heparin and vitronectin (31–33). The latter is an extracellular matrix component and regulates cellular functions via its integrin receptor and uPAR (25). An X-ray crystallographic study of cleaved PAI-1 revealed two additional potential regulatory sites on β strand 3A and 5A, respectively, that may render PAI-1 inactive on additional intermolecular interaction (34). Future study is needed to address what other functional domains of rMaspin(i) are involved in its specific interaction with the cell surface-bound plasminogen activators. In addition, because rMaspin(i) stimulates the adhesion of mammary carcinoma MDA-MB-435 cells to fibronectin and alters the integrin profiles on the cell surface (10), it is of particular importance to investigate whether fibronectin binds directly to maspin and regulates its proteolytic inhibitory activity.

Regarding the biphasic dose effect of rMaspin(i) on DU145 cell surface-mediated plasminogen activation (Fig. 7A) and on cell motility (Fig. 8), our chemical cross-linking evidence (Fig. 6) supports an earlier notion that purified recombinant maspin may undergo a concentration-dependent polymerization (35). Although the intact monomeric rMaspin(i) used in this study did not undergo fast spontaneous polymerization and was not degraded during storage (Fig. 1), at higher local concentrations, rMaspin(i) may polymerize and lose its proteolytic inhibitory potency. Meanwhile, current evidence does not exclude the possibility that the reduced biochemical and biological activities of rMaspin(i) at higher concentrations are caused by an increased nonspecific interaction between rMaspin(i) and the plasminogen activation system because rMaspin(i) has been shown to use its NH₂-terminal domain to interact with the regulatory domain of tPA (12). Furthermore, because the specific receptors and other molecules associated with the plasminogen activation system on the cell surface are of fundamental importance in cell biology (22–26, 31–33, 36–39), an inhibitor of plasminogen activators such as rMaspin(i), when added...
at high doses, may not only shift the balance against the enzymatic activity of its target but may also trigger further compensating cellular responses.

It was advantageous to use DU145 cells to investigate the effect of maspin on cell-associated uPA because these cells do not produce endogenous maspin but express abundant uPA and uPAR (40). However, this choice may also limit our conclusion. Future studies are needed to clarify whether maspin inhibits the uPA associated with other cell lines and whether maspin competes with other known plasminogen activators such as PAI-1 and PAI-2 to inhibit the cell surface-bound uPA. On the other hand, the experimental design of this study may not appropriately address the potential inhibitory interaction between maspin and pericellular tPA. tPA is thought to act as a secreted enzyme, which does not depend on the cell surface for its activity (41–44). To date, the role of tPA on the cell surface-mediated plasminogen activation remains unclear. It is important to point out that DU145 cells produce a trace amount of tPA (Fig. 2A). However, the tPA activity was not detected by either CCA-CSPA or the cell-based CAA (Fig. 2 and B and C).

In conclusion, we provide the first evidence that rMaspin(i) acts as a potent inhibitor of cell surface-bound uPA, comparable with PAI-1 and PAI-2 in parallel experiments. We demonstrated that the DU145 cell surface played a critical role in mediating the inhibitory interaction between rMaspin(i) and uPA. Moreover, the proteolytic inhibitory activity of rMaspin(i) correlates quantitatively with its inhibition of the motility of DU145 cells in vitro. These data suggest a molecular mechanism underlying the tumor-suppressive role of maspin. It is reasonable to hypothesize that novel maspin-based therapeutic strategies may prove useful in blocking uPA-mediated tumor invasion and metastasis. To this end, it is of particular importance to note that different inhibitors of plasminogen activators may play distinct roles in tumor progression. For example, PAI-1, along with uPA and uPAR, is causatively involved in the progression of breast cancer and prostate cancer (45–48). In contrast, maspin, which is down-regulated in breast cancer (45–48) and PAI-1 and PAI-2 in parallel experiments. We demonstrated that the DU145 cells are a target of the tumor suppressor gene maspin. Proc. Natl. Acad. Sci. USA, 93: 499–504, 1996.


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