Sufficiency of the Reactive Site Loop of Maspin for Induction of Cell-Matrix Adhesion and Inhibition of Cell Invasion: Conversion of Ovalbumin to a Maspin-like Molecule

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Running Title: The role of the RSL of maspin in cell matrix adhesion and cell invasion
SUMMARY

Maspin, an ov-serpin, inhibits tumor invasion and induces cell adhesion to extracellular matrix molecules. Here, we use maspin/ovalbumin chimeric proteins and the maspin reactive site loop (RSL) peptide to characterize the role of the RSL in maspin-mediated functions. Replacement of the RSL plus the C-terminal region or the RSL alone of maspin with that of ovalbumin resulted in the loss of the stimulatory effect on adhesion of corneal stromal cells to type I collagen, fibronectin and laminin and of mammary carcinoma MDA-MB-231 cells to fibronectin. Maspin with ovalbumin as the C-terminal region retained activity suggesting the maspin C-terminal polypeptide is not required. An R340Q mutant retained full maspin activity, however, an R340A mutant lost activity. This indicates the arginine side chain at the putative P1 site forms a hydrogen bond and not an ionic bond. The RSL peptide (P10-P5’, aa 330-345) alone induced cell-matrix adhesion of mammary carcinoma cells and corneal stromal cells and inhibited invasion of the carcinoma cells. Substitution of the RSL of ovalbumin with that of maspin converted inactive ovalalbumin into a fully active molecule. Maspin bound specifically to the surface of the mammary carcinoma cells with a $k_d$ of 367 ± 67 nM and 32.0 ± 2.2 x 10⁶ binding sites/cell. The maspin RSL peptide inhibited binding suggesting the RSL is involved in maspin binding to cells. Sufficiency of the maspin RSL for activity suggests the mechanism by which maspin regulates cell-matrix adhesion and tumor cell invasion does not involve the serpin mechanism of protease inhibition.
INTRODUCTION

Maspin is a 42 kDa protein synthesized by normal epithelial cells of a variety of mammalian organs such as mammary gland, prostate, skin, and cornea (1),(2). Synthesis of maspin has also been identified in the nonepithelioid cells of the corneal stroma, both in situ and in cell culture (2). The expression of maspin however, is lost after the second passage of cultured stromal cells. These later passage cells mimic the wound activated stromal fibroblasts which are much more mobile than stromal cells in the normal corneas. Expression of maspin is also lost or down regulated in many invasive carcinoma cells (3,4). Down regulation of maspin in carcinoma tissues correlates with progression and metastasis of tumors (5-7). Both the later passage corneal stromal cells and the invasive carcinoma cells respond to exogenously added maspin (2,3).

Several biological activities of maspin have been characterized, which suggest a role for maspin as a tumor suppressor and an inhibitor of angiogenesis. Addition of recombinant maspin or transfection of the maspin gene into carcinoma cells inhibits cell migration and invasion in vitro and reduces tumor growth and metastasis in vivo (3,8). Maspin also inhibits the in vitro migration and proliferation of endothelial cells, and blocks bFGF-induced neovascularization in the rat corneal pocket model (9). To date, the underlying mechanisms of maspin action on inhibition of tumor invasion and angiogenesis are not well established. Nonetheless, maspin can regulate adhesion of cultured corneal stromal cells and carcinoma cells to extracellular matrix (ECM\(^1\)) molecules (2,10). Pretreatment with recombinant maspin increases adhesion of cultured corneal stromal cells to several ECM molecules, including type I and type IV collagen, laminin and fibronectin (2), whereas it induces adhesion of carcinoma cells only to fibronectin and not to gelatin, laminin, type I and type IV collagens or fibrinogen (10). Stimulation of cell-ECM adhesion by maspin likely involves a mechanism by which maspin up-regulates expression of integrins since the level of \(\alpha 5\) integrin (the \(\alpha\) component of the fibronectin receptor) on the cell surface is induced in carcinoma cells pretreated with recombinant maspin (10).
Maspin shares sequence homology with the serpins (serine protease inhibitors) of the ovalbumin-type subfamily, which includes ovalbumin, plasminogen activator inhibitor-2 (PAI-2), squamous cell carcinoma antigen (SCCA) and bomapin (PI10) (11). Most serpins are inhibitors of specific proteases that react with an exposed reactive site loop (RSL) at the top of the molecule (Fig. 1). Whether maspin is a protease inhibitor is still controversial. Early in vitro studies showed that maspin does not inhibit a number of representative proteases (12). One recent study demonstrated maspin can inhibit prostate carcinoma cell surface-associated plasminogen activation by urokinase type plasminogen activator (uPA) (13), however, another study, using uPA bound to its receptor on tumor cells and tPA bound to vascular smooth muscle cells, fibrin or the prion protein, reported maspin does not inhibit either of these two plasminogen activators (14).

Although an intact RSL of maspin is required for inhibition of migration and invasion of mammary gland and prostate carcinoma cells (8), nothing is known about which region of the maspin molecule is required for induction of increased cell-ECM adhesion. Preincubation with blocking antibodies to the RSL of maspin (8), deletion from the P7’ residue of the RSL to the C-terminal end (15), and cleavage of maspin at the putative P1 site Arg within the RSL (16) destroyed the ability of maspin to regulate the migration and invasion of carcinoma cells. We hypothesized that stimulation of cell-ECM adhesion by maspin also utilizes the RSL.

We investigated the structure-function relationship of maspin using maspin/ovalbumin chimeras rather than using maspin deletion mutants as previously studied, due to a concern about proper folding of the mutant molecules. A tertiary structure model of maspin based on the crystal structure of ovalbumin (PDB: 1OVA) suggests maspin adopts a common serpin structure in which the C-terminal region (Strands B4 and B5) lies inside the molecule (Fig. 1). Deletion of this region could disrupt the overall serpin conformation resulting in an inactive molecule whereas swapping equivalent regions between maspin and a homologous serpin would potentially preserve the overall structure. Expression of yeast
recombinant maspin/ovalbumin chimeras as secreted proteins increases the probability the proteins are properly folded. Ovalbumin was chosen for swapping domains with maspin because the physical properties of both molecules are similar. Maspin and ovalbumin are similar in size and share about 30% amino acid sequence identity. Neither molecule undergoes a urea-induced unfolding transition from a stressed (S) form to a heat-stable relaxed (R) form (12,17). Furthermore, the hinge region (P8 to P12) of ovalbumin and maspin differs from that of inhibitory serpins in which a multiple alanine stretch is conserved (18). Most importantly, unlike maspin, ovalbumin does not induce cell-ECM adhesion (2).

In this study, we explored the function of the RSL domain of maspin on cell-ECM adhesion and tumor invasion using a region swapping approach between maspin and ovalbumin to preserve the serpin structure. The studies reported here document that 1) the maspin RSL domain, but not the C-terminal region, is required, 2) the RSL peptide is sufficient for induction of increased cell-ECM adhesion of corneal stromal cells and carcinoma cells and inhibition of carcinoma cell invasion, 3) replacement of the RSL of ovalbumin with that of maspin can convert ovalbumin to a maspin-like molecule and 4) the RSL can compete for specific binding of maspin to carcinoma cells.

EXPERIMENTAL PROCEDURES

Materials

The yeast expression system including YEpFLAG-1 vector, *Saccharomyces cerevisiae* protease-deficient strain BJ3505 (pep4::HIS3 prb-Δ1.6 HIS3 lys2-208 trp1-Δ101 ura3-52 gal2 can1) was obtained from Sigma (St. Louis, MO). *Escherichia coli* strain JM 109 was purchased from Promega (Madison, WI). ProSTAR™ Ultra HF RT-PCR System and PfuTurbo® Hotstart DNA polymerase were from Stratagene (La Jolla, CA). All oligonucleotide primers were from GIBCO BRL® Custom Primers (Life Technologies, Rockville, MD). The Ni-NTA™ agarose was obtained from QIAGEN (Valencia,
CA). All restriction endonucleases were from New England BioLabs® Inc. (Beverly, MA). The CytoMatrix™ adhesion assay strips coated with human type I collagen, fibronectin or laminin were purchased from Chemicon International, Inc (Temecula, CA). Calcein acetoxymethyl ester (calcein AM), CyQuant®GR dye and BODIPY®-TR-X succinimidyl ester, was obtained from Molecular Probes (Eugene, OR). Clostripain (Endoproteinase-Arg-C) was from Worthington Biochemical Corporation (Lakewood, NJ). Cell-Essentials, Inc (Boston, MA) synthesized a 15-amino acid RSL polypeptide (P10-P5'). The purity was greater than 95% and the composition confirmed by mass spectroscopy. Human mammary gland carcinoma MDA-MB-231 cells and mouse T-lymphoma cells transfected with pAc-neo-OVA, E.G7-OVA were obtained from American Type Culture Collection (Manassas, VA). An immortalized normal human corneal stromal cell line was a generous gift from Dr. MA Watsky (University of Tennessee College of Medicine, Memphis, TN). Leibovitz’s L-15 medium, Dulbecco’s modified Eagle’s medium (D-MEM) with high glucose and L-glutamine and without pyruvate, RPMI-1640 medium with L-glutamine, and neomycin (G418) were obtained from Invitrogen (Carlsbad, CA). Defined fetal bovine serum (FBS) was from Hyclone (Logan, UT). MITO+™ serum extender and Matrigel™ were from BD Biosciences (Bedford, MA). Polycarbonate membrane Transwell® chamber inserts were purchased from Corning Costar Corporation (Cambridge, MA). Cyprofloxacin was obtained from Bayer Corporation (Kankakee, IL). Unless specified, all other reagents and medium components for the yeast and bacterial cultures were purchased from Sigma.

Cell Cultures

The MDA-MB-231 cells were maintained in Leibovitz’s L-15 medium with 10% FBS and 10 μg/ml ciprofloxacin at 37°C without CO₂. The mouse T-lymphocyte E.G7-OVA cells were grown at 37°C
with 5% CO$_2$ in RPMI 1640 medium with 2 mM L-glutamine, 18 mM sodium bicarbonate, 25 mM glucose, 10 mM HEPES, 1.0 mM sodium pyruvate, 0.05 mM 2-mercaptoethanol, 0.4 mg/ml G418 and 10% FBS. The immortalized normal human corneal stromal cells (19), which mimic activated migratory wound corneal stromal cells and have the same properties as passaged human corneal stromal cells, were routinely grown in D-MEM medium containing 5% FBS, 1x MITO$^+$ and 10 g/ml ciprofloxacin in a humidified incubator with 5% CO$_2$ at 34°C.

The MDA-MB-231 human mammary carcinoma cells and immortalized corneal stromal cells do not synthesize maspin in contrast to normal mammary epithelial and corneal epithelial cells as determined by RT-PCR and Western blots using previously reported conditions for these assays (2).

**Construction of Maspin/Ovalbumin-swap Mutants**

The human maspin gene was previously obtained from human corneal epithelium by RT-PCR as described by Ngamkitidechakul et al.(2). Total RNA from the T lymphocyte cell line containing the ovalbumin gene was extracted using TRI-Reagent. The full-length ovalbumin gene was specifically amplified by RT-PCR using the ProSTAR™ Ultra HF RT-PCR System according to the manufacturer’s procedure (Stratagene) in the Mastercycler® personal thermocycler (Eppendorf, Westbury, NY). In the RT step, a specific ovalbumin antisense primer (Table I) was used to generate ovalbumin cDNA. PCR amplification of the cDNA was performed using two primers in which the *EcoR* I site was incorporated into the 5’ end of the sense primer and *Bgl* II into the 5’ end of the antisense primer. The annealing temperature for the PCR was 65°C. The gel-purified 1.2 kb PCR product was digested with *EcoR* I and *Bgl* II, and ligated into the YEplF expression vector (20). DNA sequencing at the MCW Protein and Nucleic Acid Facility confirmed the nucleotide sequences of both human maspin and chicken ovalbumin cDNA. Three ovalbumin (OV)/maspin-RSL swap mutants and one chimeric maspin/OV-RSL swap mutant were
constructed by the overlap extension PCR method of Ho et al. (21) using the primers listed in Table I. For each mutant construction, two sets of primers were utilized to amplify two separate overlapping PCR products that contain the mutation (or insertion) together with either upstream or downstream sequences. These PCR products were amplified using PfuTurbo® Hotstart DNA polymerase according to the manufacturer’s instructions using the optimal annealing temperature at 60°C. Two PCR fragments were joined together through overlapping sequences and PCR using primers from the 5’ and 3’ ends of the appropriate gene. An annealing temperature of 55°C was used. The full-length PCR products were cut with EcoRI and BglII and cloned directly into the yeast expression vector YEpHF. The MCW Protein and Nucleic Acid Facility confirmed the reading frame and sequences of all mutants.

To construct a site-specific P1 R to Q and R to A maspin mutants, the YEpHF-Maspin template vector was directly amplified by the method of Weiner et al (22) using two primers containing the mutation sequences (Table I) and PfuTurbo® Hotstart DNA polymerase. After 18 cycles of PCR amplification (55°C annealing), the PCR reaction was treated with DpnI (1 U) at 37°C for 1 hr to remove the parental template. The purified PCR products were transformed into JM 109 E. coli cells. A colony containing the plasmid with the desired mutation was chosen and the mutated sequences confirmed by sequencing.

**Expression and Purification of Yeast Recombinant HIS/FLAG-tagged Proteins**

All recombinant proteins were produced as N-terminal HIS/FLAG tag fusion proteins in a yeast system. The yeast expression vector YEpHF was modified from the original vector YEpFLAG-1 by adding a six-histidine tag upstream of the FLAG peptide tag (20). Due to the presence of an α-factor leader peptide sequence, the recombinant proteins were expressed and secreted outside the *S. cerevisiae* yeast BJ3505 into the buffered expression medium (2% peptone, 1% yeast extract, 3% glycerol, 1% glucose and 100 mM K2HPO4, pH 6.4). After growing at 30°C for 72
hrs, 1 liter of the culture medium was collected and concentrated to 200 ml by ultrafiltration (Amicon) under nitrogen gas at 60 psi. The concentrate was pH-adjusted to 8.0.

The yeast rHIS/FLAG–tagged protein was purified by mixing the concentrated yeast conditioned medium with 10 ml of Ni-NTA™ agarose resin overnight at 4°C. The resin was washed with 50 mM phosphate buffer, pH 8.0 containing 300 mM NaCl and 10 mM imidazole. Subsequently, the recombinant protein was eluted with 50 mM phosphate buffer, pH 7.0 containing 300 mM NaCl and 250 mM imidazole. The eluted protein was concentrated using an Ultrafree spin concentrator (Millipore), and dialyzed in phosphate buffered saline (PBS) pH 7.4 to remove the imidazole. Identical results were obtained in the presence and absence of the N-terminal His tag and the FLAG sequence for the maspin functions measured here.

Arginine-specific Proteolysis of Recombinant Maspin and the R340Q and R340A mutants

Endoproteinase-Arg-C (clostripain) was incubated with the yeast recombinant wild-type maspin, the R340Q and R340A mutants to confirm the mutagenesis. The enzyme was preactivated at room temperature for 2 hrs in 1 mM calcium acetate containing 2.5 mM dithiothreitol. The recombinant proteins (50 µg) in 50 mM ammonium bicarbonate, 0.2 mM calcium acetate and 2.5 mM dithiothreitol were incubated with the enzyme (50:1 molar ratio) at 37°C. At various time points, aliquots were removed and E-64 (10 µM) was added to stop the enzymatic reaction. For zero time points, 5 µg of the proteins were mixed with E-64 at 10 µM final concentration and then the enzyme was added. SDS sample buffer was added to the reaction samples and then boiled. The samples were subjected to SDS-PAGE and the proteins visualized by Coomassie brilliant blue staining. After 2 min of incubation with the protease, the wild-type maspin was cleaved with the appearance of a band corresponding to the size expected for a cleavage at R340, however, the maspin R340Q and R340A mutants were not cleaved over the first 60 min (data not shown). However, with time additional identical smaller products were noted in
Cell-ECM Adhesion Assay

MDA-MB-231 cells or corneal stromal cells were incubated with serum-free medium containing either 0.5 µM (MDA-MB-231 cells) or 1.0 µM (corneal stromal cells) of maspin, the maspin/ovalbumin chimeras or the RSL peptide for 18 hr prior to cell adhesion assay performed according to the protocol previously used by us and others (2,10). The maspin RSL peptide was used at 1 µM final concentration in both cell types. The adhesion assay was performed using CytoMatrix adhesion strips as previously described (2). Briefly, after preincubation with maspin, the cells were harvested using 2 mM EDTA in PBS. Both cell types were resuspended in RPMI 1640 containing 1x MITO+. The MDA-MB-231 cells and the corneal stromal cells were plated at a density of 2x10^4 cells/well and allowed to attach for one hour at 37°C on fibronectin-coated wells for the carcinoma cells and on type I collagen-, fibronectin-, or laminin-coated wells for the corneal stromal cells. Fibronectin was used for the MDA-MB-231 cells because maspin stimulates adhesion only to this ECM molecule (10). Collagen type I was used for the corneal stromal cell studies because it is the major extracellular molecule present in the corneal stroma (2). Fibronectin and laminin, ECM molecules found at lower amounts also were used for selected experiments. Following washing with PBS, the adherent cells were stained with crystal violet and quantified using a microplate reader (Bio-Tek Instruments, Winooski, VT). The experiments were repeated 4-5 times with 3-5 replicates per experiment.

In Vitro Tumor Cell Invasion Assay

The invasion assay was performed using the standard Matrigel method of Sternlicht et al. (23) with several modifications. Briefly, polycarbonate (8-µm) Transwell inserts were precoated with 2
mg/ml Matrigel. MDA-MB-231 cells (5 x 10^4) were incubated in the absence or presence of 0.5 µM rHIS/FLAG maspin, ovalbumin, the maspin/ovalbumin chimeras, the R340Q and R340A maspin mutants or the RSL peptide in Leibovitzs L-15 medium containing 1% FBS, 1x MITO+ and 10 µg/ml ciprofloxacin. After 48-hr of incubation at 37°C in a humidified incubator without CO₂, the cells in the bottom well were labeled with the fluorogenic vital dye calcein AM (5 µM final concentration). Fluorescence was measured using a CytoFluor™ fluorescence microplate reader (Millipore) with Ex: 480 nm and Em: 530 nm filters. Cells in duplicate wells without transwell inserts served as controls for cell proliferation and/or death during the incubation period. Invasion was calculated by dividing the relative fluorescence value of invading cells by that of total cells plated in duplicate wells without transwell inserts. Invasion of the control was set at 100%. The experiments were repeated 4-5 times with 3-5 replicates per experiment.

**Tumor Cell-Maspin Binding and Competition Assay**

Yeast recombinant maspin was conjugated with the fluorescent dye BODIPY®-TR-X, succinimidyl ester, purified and the degree of labeling, 5 BODIPY molecules per maspin molecule, determined according to the manufacturer’s procedure (Molecular Probes). To perform the binding assay, increasing amounts of BODIPY-TR-X-maspin (0.2 - 8.0 µM) were incubated in the dark with MDA-MB-231 cells at 4°C for 90 min in serum-free medium containing 0.1% bovine serum albumin (BSA). Next, the cells were washed three times with ice-cold PBS with 0.1% BSA. The binding was determined by measuring fluorescence using the CytoFluor microplate reader (Excitation: 580 nm, Emission 640 nm). The fluorescence values were converted to maspin concentration using a linear BODIPY maspin reference standard curve. To determine nonspecific binding, the assay was performed in the presence of a fifty-fold excess of non-labeled maspin. Specific binding was calculated by subtraction of the non-
specific binding from the total binding. Determination of the number of cells was carried out using CyQuant® GR as described in the manufacturer’s protocol (Molecular Probes) with an excitation of 485 nm and an emission of 530 nm and a linear standard cell curve. A nonlinear regression curve for specific binding was calculated using five sets of data and plotted using SigmaPlot with the Enzyme Kinetics/Pharmacology Module (SPSS, Chicago, IL). K_d and B_max values and curve statistics were determined from nonlinear regression analysis using the same program.

Competition assays of cell-maspin binding were conducted using 4 µM fluorescent-labeled maspin. The RSL peptide at 2, 10, 20- or 100-fold excess was added to cells along with BODIPY-TX-R-maspin. Non-specific binding was determined by adding a 50x excess of unlabeled maspin. Bound labeled maspin was quantified in the presence and absence of each competitor as described above. The experiments were repeated 3 times with 3-5 replicates per experiment.

**Statistical Analysis**

Overall differences among the treatment groups were determined using a one-way analysis-of-variance and differences between individual treatments were determined using the Student-Newman-Keuls test by SigmaStat software (SPSS Inc., Chicago, IL).
RESULTS

To elucidate the significance of the RSL in the induction of increased cell-matrix adhesion by maspin, we constructed chimeric mutants between maspin and the homologous non-inhibitory serpin, ovalbumin, a molecule that does not alter adhesion of corneal stromal cells to ECM molecules (2). The RSL to the C-terminal end (aa 331-375), the C-terminal region downstream of the RSL (aa 346-375) and the RSL of maspin (aa 331-345) were individually replaced using the equivalent portions of ovalbumin (Fig. 1).

Effect of OV/Maspin-RSL Chimeras on Cell-ECM Adhesion

In contrast to maspin, the Maspin/OV-RSL/OV-C swap mutant containing the RSL to the C-terminal end of ovalbumin did not stimulate adhesion of either the corneal stromal cells to type I collagen (Fig. 2A) or the carcinoma cells to fibronectin (Fig. 2B). However, when only the C-terminal domain of ovalbumin (Maspin/Masp-RSL/OV-C-Term) was swapped into the maspin molecule, adhesion was stimulated in both cell systems with all ECMs, suggesting the C-terminal portion of maspin is not directly associated with this activity. In contrast, replacement of the RSL of maspin alone with that of ovalbumin (Maspin/OV-RSL/Maspin-C-Term) abolished the effect on adhesion in both the corneal stromal cells to type I collagen (Fig. 2A) and the carcinoma cells to fibronectin. Therefore, the RSL of maspin is required for enhancement of cell-ECM adhesion.

Effect of Mutation of the Putative P1 Residue, R340 on Cell Adhesion

To determine whether arginine at the putative P1 site within the RSL of maspin is required for maspin activity on cell-ECM adhesion, we mutated the R340 at the P1 site to a Q in one mutant and to an A in a second mutant. The R340Q mutant and wild type maspin, increased adhesion of corneal stromal cells to type I collagen (Fig. 3A) and of mammary carcinoma cells to fibronectin (Fig. 3B). The R340Q
mutant was significantly more active than maspin for the carcinoma cells. In contrast, the R340A mutant lost activity. This mutant failed to induce adhesion of the corneal stromal cells (Fig. 3A) but showed intermediate activity between maspin and the control for the carcinoma cells (Fig. 3B). This loss of activity is not due to a major change in conformation because limited proteolysis using endoproteinase-Arg-C over 6 hr showed the same degradation pattern for this mutant as that for the active R340Q mutant. Thus, Gln can replace Arg at the putative P1 site but Ala cannot fully substitute for Arg at this site.

**Effect of the Maspin RSL Polypeptide on Cell-ECM Adhesion**

Since the RSL of maspin is required for the increase in adhesion of cells to ECM, we next examined whether the RSL is sufficient to induce this activity. A synthetic polypeptide corresponding to the 15 amino acid residues (P10-P5', aa 331-345) within the RSL of maspin was assayed to determine its effects on adhesion of cells to ECMs. The RSL peptide induced adhesion similar to maspin for both the corneal stromal cells and the carcinoma cells (Fig 4A and B).

**Conversion of Ovalbumin to a Maspin-like Molecule**

To further characterize the role of the RSL of maspin, we generated and tested a chimeric ovalbumin mutant in which the RSL of maspin was exchanged for the RSL of ovalbumin. As shown in Fig. 5, the Ovalbumin/Masp-RSL/OV-C-Term was also able to stimulate adhesion of stromal cells to type I collagen and carcinoma cells to fibronectin. Thus, ovalbumin can be converted by the replacement of only the RSL into a maspin-like molecule that induces cell-ECM adhesion.
Requirement of the Presence of the RSL of Maspin for Induction of Adhesion of Corneal Stromal Cells to Laminin and Fibronectin

Maspin not only stimulates adhesion of human corneal stromal cells to type I collagen but also to laminin and fibronectin (2). To determine whether the RSL of maspin is critical for the stimulation of maspin adhesion to multiple extracellular matrix molecules, two mutants, the Maspin/OV-RSL/Masp-C and the Ovalbumin/Masp-RSL/OV-C were chosen for use in the adhesion assays. The ability of maspin to stimulate adhesion of the corneal stromal cells to fibronectin and laminin in addition to type I collagen was lost upon removal of the maspin RSL and its replacement with the RSL of ovalbumin (Fig. 6). In addition, the RSL of maspin was sufficient to convert ovalbumin into a molecule that can stimulate adhesion of the corneal stromal cells not only to type I collagen but also to fibronectin and laminin. Thus, the RSL of maspin is critical for the stimulation of adhesion of human corneal cells to multiple extracellular matrix molecules.

Effect of the Chimeric Maspin/OV Mutants, the Putative P1 mutants, the Maspin RSL Polypeptide on Carcinoma Cell Invasion

The RSL of maspin was previously shown to be important for inhibition of carcinoma cell invasion (8,15) (16). Deletion of the P7 residue through the C-terminal end of maspin (aa 347-375) and trypsin cleavage of maspin at the P1 site Arg within the RSL resulted in loss of the activity. However, these modifications can potentially alter the overall structure of maspin leading to an inactive molecule. To better demonstrate the requirement of the RSL of maspin for inhibition of carcinoma cell invasion, we tested the maspin/ovalbumin chimeras using an in vitro tumor cell invasion assay. As predicted, the RSL of maspin is required because substitution of the RSL of maspin with that of ovalbumin (Masp/OV-
RSL/Masp C) abolished the activity (Fig. 7). Substitution of Arg at the putative P1 site with Gln (RQ) retained inhibitory activity while this activity was lost in the Ala mutant (RA). The ovalbumin maspin/containing the RSL of maspin (OV/Masp-RSL/OV C) inhibited invasion of the MDA-MB-231 mammary gland carcinoma cells further establishing the role of the RSL. These results suggest inhibition of carcinoma cell invasion by maspin utilizes the P10-P5’ residues within the RSL. The RSL peptide was tested and the results confirmed a functional role of this region. Similar to the effect on increased cell-ECM adhesion, this peptide was sufficient to inhibit invasion of the carcinoma cells through Matrigel.
Determination of the Kinetics of Maspin Binding to Mammary Gland Carcinoma Cells and the Ability of the RSL Peptide to Inhibit Maspin Binding

The ability of the RSL peptide to mimic maspin activity led us to hypothesize that the effects induced by maspin are initiated by maspin binding to the cells through the RSL. Maspin binding to mammary gland carcinoma cells is specific and saturable (Figure 8). The data fits best for a one binding site model with an $r^2 = 0.98$. The $k_d$ determined by non-linear regression was $367 \pm 67$ nM and the $B_{max}$ was $5.44 \pm 0.24$ pmol/10^5 cells. The calculated number of maspin binding sites per cell was $32.0 \pm 2.2 \times 10^6$. These values indicate that maspin binds to the carcinoma cells through a high number of low affinity sites. Using near saturating amounts of labeled maspin (4µM), the RSL peptide even at 2x (8µM) competed for specific binding of maspin to the carcinoma cells (Fig. 9). The degree of inhibition of specific maspin binding did not significantly change from 2 and 100x of the RSL peptide. These results suggest that maspin can bind to the cell surface through the RSL.
DISCUSSION

In the present study, we show that the RSL of maspin is required and sufficient to induce cell-ECM adhesion in two different cell types and for the inhibition of invasion of carcinoma cells. Stimulation of carcinoma cell adhesion to fibronectin and normal corneal stromal cell adhesion to type I collagen, fibronectin and laminin and inhibition of invasion of carcinoma cells requires the presence of the 15-residue reactive loop (P10-P5’, aa 331-345) of maspin because replacement of the RSL in maspin with the ovalbumin RSL abolishes the activity. However, Gln can replace Arg 340 in the P1 site within the RSL of maspin and retain the ability to stimulate cell-ECM adhesion and inhibit cell invasion. In contrast, mutation of the Arg 340 to an Ala residue results in the loss of these activities. This would indicate that the positive charge of Arg does not form a critical ionic bond but the side chain of this residue probably is involved in the formation of a critical hydrogen bond. Our putative maspin model predicts the side chain of Arg 340 is exposed suggesting an intermolecular interaction of this residue. Inhibitory activity toward target proteases is lost or decreased upon mutation of a charged P1 site residue of inhibitory serpins to non-charged residues (24-26). If Arg is in fact the P1 site of maspin, the lack of an effect of the R340Q mutant on increased cell-ECM adhesion suggests inhibition of proteases such as uPA and tPA by maspin does not contribute to the induction of adhesion by maspin. A similar conclusion was drawn in a recent study, which showed that although maspin inhibits migration of vascular smooth muscle cells, it does not inhibit tPA bound to these cells nor uPA bound to tumor cells (14).

In contrast to the effect of substitution of Arg 340 with Gln on cell-ECM adhesion and invasion, the putative P1 Arg of maspin cannot be substituted with this residue for inhibition of carcinoma cell migration (9). Although both this study and ours used multiple cell types and ECMs, the results within each set of experiments are consistent. The migration of the mammary carcinoma cells through Matrigel coated membranes was inhibited by maspin but not by an R340Q mutant (9). The same results were shown for endothelial cell migration through membranes coated with gelatin and skin fibroblast and keratinocyte
migration though non-coated membranes. The R340Q mutant retained the ability to stimulate adhesion of the carcinoma cells to fibronectin and the corneal stromal cells with type I collagen, fibronectin and laminin (data not shown). This mutant also inhibited invasion of mammary carcinoma cells through Matrigel, the same matrix used for the migration assay. Thus, the differences probably are due to the mode of interaction of maspin with the cells rather than the extracellular matrix molecule present. In the adhesion assay, the cells were preincubated with maspin for 18 hrs, the medium removed, the cells released with EDTA and replated in serum- and maspin-free medium on ECM coated wells for one hr (10). In the invasion assay, the cells were mixed with maspin, placed into the Transwell inserts and allowed to migrate for 48 hrs. In the migration assay, maspin was placed in the chamber on the opposite side of the membrane from the cells (9), which results in the establishment of a concentration gradient across the membrane. Thus, the observed differences for the R340Q mutant may be due to the specific experimental conditions of the different assays or to distinct mechanisms of action of maspin.

The C-terminal region of maspin is not required for adhesion-promoting activity since the maspin/ovalbumin chimera containing the C-terminal end (aa 346-375) of ovalbumin remains fully active. Previous studies showed that deletion of the P7’ residue through the C-terminal results in the loss of maspin activity (9,15). In this deletion mutant, the internal β-strands B4 and B5 and the exposed β-strand C1 (Fig. 1) are missing. These results suggest the C-terminal region, which is a highly conserved region of serpins (27,28), is required for proper conformation of the molecule including the RSL.

Not only is the RSL of maspin required, it is sufficient to induce cell-ECM adhesion in both cell systems and to inhibit carcinoma cell invasion. Both the RSL peptide and the ovalbumin mutant containing the RSL of maspin can efficiently inhibit carcinoma cell invasion and induce increased adhesion of carcinoma cells to fibronectin and corneal stromal cells to several ECMs. The sufficiency of the RSL peptide is unusual because the serpin inhibition mechanism requires additional regions of the
protein. This mechanism involves proteolytic cleavage at the P1 site, insertion of the RSL into β-sheet A and translocation of the attached protease to the opposite side of the molecule (29). Thus, the stimulation of cell-matrix adhesion and the inhibition of invasion probably do not involve the serpin mechanism of protease inhibition. However, a role of the RSL as a canonical protease inhibitor cannot be ruled out.

Conversion of inactive ovalbumin to a maspin-like molecule by swapping the RSL is atypical for the serpin system. A number of studies have explored the possibility of exchanging activities and/or specificities among serpins by swapping the RSL regions between inhibitory serpins such as α1-antitrypsin and α1-antichymotrypsin (30). Except for the highly identical (90%) serpins SCCA1 and SCCA2 (31), replacement of the RSL is not sufficient to transfer the specificity of one serpin to another (30). However, the specificity of the inhibitory activity of serpins has been changed by site-specific mutagenesis within the RSL (32,33).

The use of the RSL in a role that does not involve the serpin mechanism of inhibition is also unusual. Although the RSL of non-inhibitory serpins such as pigment epithelial-derived factor (PEDF) and thyroxine-binding globulin (TBG) acts as a substrate rather than an inhibitor for proteases, the RSL is not a functional domain responsible for their activities. Cleavage of the exposed RSL does not affect the neurite-promoting function of PEDF (34). Since the hormone-binding site in TBG resides in β-sheets B and C, cleaved TBG still can bind to thyroxine (35). The RSL of cortisol binding globulin (CBG) is also not a hormone-binding domain, yet cleavage of the RSL by elastase reduces the binding affinity for cortisol (36). In contrast, maspin uses the RSL for induction of increased cell-matrix adhesion of corneal stromal cells and carcinoma cells and inhibition of invasion of carcinoma cells. Although the RSL of maspin is sufficient for these activities of maspin, other domains are essential for other functions of maspin. The anti-angiogenic effect involves the N-terminal domain (aa 1-139) of maspin (9) and the collagen-binding domain of maspin maps to amino acid residues at positions 84-112 (37). Therefore, several different parts of
the maspin molecule may play distinctive and/or cooperative roles supporting many functions of maspin.

Mammary carcinoma cells bound maspin specifically and saturably through low affinity binding sites. The $k_d$ for maspin binding to the cells is similar to the concentration required for maximal biological activity. The maspin concentration for maximal inhibition of invasion of mammary carcinoma cells is 170-770 nM (16), which is in the same range of the $k_d$ determined here for the binding of maspin to the carcinoma cells. The relationship of the $k_d$ to the concentration required to exert maximal biological activity has also been observed for another serpin, PEDF. The $k_d$ for binding of PEDF to Y 79 retinoblastoma cells and to cerebellar granule neurons is similar to the concentration required for neurotrophic activity (38).

Not only is the 15 amino acid RSL peptide required and sufficient to mimic activity of maspin, it also competes for maspin binding to cells. The RSL does not completely inhibit 100% of maspin binding to the mammary carcinoma cells, yet the maspin binding experiments indicate only one binding site on the cells. If the binding site on maspin is larger than just the RSL, there is the possibility that when the RSL is bound to the cell surface that complete steric hindrance of maspin binding is not achieved; therefore, residual maspin binding activity may still be observed. Nevertheless, the inhibition of binding of maspin to the carcinoma cells by the RSL suggests the RSL of maspin is involved in binding of the protein to the carcinoma cell surface.

In summary, the RSL of maspin, but not the C-terminal domain (aa 346-375), is required for stimulation of corneal stromal and carcinoma cell adhesion to ECM and inhibition of carcinoma cell invasion in vitro. The putative P1 Arg can be substituted with Gln but not Ala and retain its full ability to increase adhesion suggesting this residue is involved in a hydrogen bond. Ovalbumin can be converted to a fully active maspin-like molecule by substitution of the RSL of ovalbumin with that of maspin. Maspin binds to the carcinoma cell surface with a $k_d$ in the range needed for biological activity. The RSL of
maspin can compete with the full maspin molecule for this binding. The sufficiency of the RSL for stimulation of adhesion and inhibition of invasion suggests the serpin mechanism of inhibition is not involved.
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FOOTNOTES

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1The abbreviations used are: aa, amino acids; BSA, bovine serum albumin; CBG, cortisol binding globulin; ECM, extracellular matrix; Em, emission, Ex, excitation, FBS, fetal bovine serum; Masp/Masp-RSL/OV-C, maspin mutant containing the C-terminal domain of ovalbumin; Masp/OV-RSL/OV-C, maspin mutant containing the RSL and the C-terminal domain of ovalbumin; Masp/OV-RSL/Masp-C, maspin mutant containing only the RSL of ovalbumin; OV/Masp-RSL/OV-C, ovalbumin mutant containing the RSL of maspin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PAI, plasminogen activator inhibitor; PBS, phosphate buffered saline; PEDF, pigment epithelial-derived factor; rHIS/FLAG, recombinant histidine/FLAG tag; R340Q, maspin Arg to Glu P1 mutant; RSL, reactive site loop; RT-PCR, reverse transcriptase-polymerase chain reaction; serpin, serine protease inhibitor; SCCA, squamous cell carcinoma antigen; TBG, thyroxine binding globulin; uPA, urokinase-type plasminogen activator.
FIGURE LEGENDS

Fig. 1. Model of maspin and amino acid sequence alignment of the reactive site loop (RSL) region to the C-terminal end of maspin and ovalbumin. A) A tertiary structure of maspin was constructed based on the structure of native ovalbumin (1OVA) using SWISS-MODEL, an automated comparative protein-modeling server (http://www.expasy.ch/swissmod/SWISS-MODEL.html). This model exhibits a general serpin folding with an exposed RSL (strand). The C-terminal domain (aa 346-375) is located in the central portion of the molecule (spacefill). B). Amino acid sequence alignment of regions including the RSL (underline) and C-terminal domain of human maspin (aa 325-375). Ovalbumin (aa 334-386) shows the most heterogeneity in the RSL area. Identical sequences are shown in bold letters.

Fig. 2. The RSL of maspin is required for cell-ECM adhesion. A) Normal corneal stromal cells were pretreated for 18 hrs with 1 µM yeast rHIS/FLAG maspin, ovalbumin or maspin/ovalbumin chimeras. The cells were washed, harvested and replated on type I collagen-coated wells. After 1 hr incubation, attached cells were stained with crystal violet and the dye measured at 550 nm. B) After an 18 hr pretreatment with 0.5 µM yeast rHIS/FLAG proteins, mammary gland carcinoma MDA-MB-231 cells were tested as given in (A) for their adhesion to fibronectin for 1 hr. (Error Bars = Standard Deviation, * p< 0.05 relative to the control)

Fig. 3. The P1 arginine within the RSL of maspin can be substituted by glutamine but not with alanine for full cell-ECM adhesion stimulation. rHIS/FLAG maspin, the R340Q or R340A maspin mutant protein was added to cultures of corneal stromal cells (1 µM) or MDA-MB-231 cells (0.5 µM). After 18 hr of incubation at 37°C, the stromal cells were tested for their adhesion properties to type I collagen (A) and MDA-MB-231 to fibronectin (B) (Error Bars = Standard Deviation, * p< 0.05 relative to the control, +
p< 0.05 relative to maspin)

**Fig. 4. The RSL of maspin is sufficient to induce cell-ECM adhesion.** Corneal stromal cells and MDA-MB-231 cells were pretreated for 18 hrs with rHIS/FLAG maspin (1 µM, stromal cells; 0.5 µM, carcinoma cells) or maspin RSL peptide (1 µM). Adhesion of the stromal cells to type I collagen (A) and MDA-MB-231 cells to fibronectin (B) was determined as described in Fig 2. (Error Bars = Standard Deviation, * p< 0.05 relative to the control)

**Fig. 5. The RSL of maspin converts ovalbumin to a maspin-like molecule.** After pretreatment for 18 hrs with rHIS/FLAG maspin or maspin/ovalbumin containing the RSL, the stromal cells (1 µM) and carcinoma MDA-MB-231 cells (0.5 µM) were plated in wells coated with type I collagen and fibronectin, respectively. Adhesion of the stromal cells to type I collagen (A) and MDA-MB-231 cells to fibronectin (B) was determined as described in Fig 2. (Error Bars = Standard Deviation, * p< 0.05 relative to the control)

**Fig. 6. The RSL of maspin is critical for induction of adhesion to multiple extracellular matrix molecules.**

After pretreatment for 18 hrs with rHIS/FLAG maspin or maspin/ovalbumin chimeras, the stromal cells (1 µM) were plated in wells coated with fibronectin [___], laminin [///], or type I collagen [xxxxx] respectively. Adhesion was assayed as given in Fig 2. (Error Bars = Standard Deviation, * p< 0.05 relative to the fibronectin control, ** p< 0.05 relative to the laminin control, *** p< 0.05 relative to the type I collagen control)

**Fig 7. The RSL of maspin inhibits carcinoma cell invasion.**

MDA-MB-231 cells were incubated with 0.5 µM rHIS/FLAG maspin, ovalbumin, the maspin/ovalbumin or the RSL in Transwell insert chambers precoated with Matrigel. The cells were
allowed to invade through the Matrigel layer and the membrane at 37°C for 48 hrs. The cells in the bottom wells and in duplicate wells without transwell inserts were labeled with calcein AM and fluorescence measured with Ex: 480 nm and Em: 530 nm filters. (Error Bars = Standard Deviation. * p< 0.05 relative to the control)

Fig. 8. Binding kinetics of maspin to mammary carcinoma cells.

BODIPY-TR-X-maspin (0.2 to 8 µM) was incubated with MDA-MB-231 cells at 4°C for 90 min. Bound fluorescent labeled maspin was quantified (Ex: 580 nm, Em: 640 nm) as described in the Methods Section. Specific binding was determined by subtracting the nonspecific binding determined in the presence of a fifty-fold excess of non-labeled maspin. Non-linear regression analysis using the Pharmacology/Enzyme Kinetic Module of SigmaPlot was used to determine the $k_d$ and $B_{max}$ values. (Error Bars = Standard Deviation of 5 replicates)

Fig. 9. Inhibition of maspin binding to mammary carcinoma cells by the RSL peptide of maspin.

The RSL peptide at 2-, 10-, 20- or 100-fold excess was incubated with MDA-MB-231 cells at 4°C for 90 min along with BODIPY-TR-X-maspin at 4 µM. Bound fluorescent labeled maspin was quantified in the presence and absence of the RSL peptide as described in the Methods Section. Specific binding was determined by subtracting nonspecific binding determined in the presence of a fifty-fold excess of non-labeled maspin. (Error Bars = Standard Deviation, * p< 0.05 relative to the control)
Table 1

| Type               | Name                              | Sequence                                      |
|--------------------|-----------------------------------|-----------------------------------------------|
| *Wild-type         | EcoRI -Maspin-N (sense)           | 5’-GTCGAATTCATGGATGCCCTGCAACTAG-3’            |
|                    | Bgl II -Maspin (antisense)        | 5’-GGCAGATCTTTAAGGAAGACAGAATTTTG-3’           |
|                    | EcoR I -OV (sense)                | 5’-TGAATTCATGGGCTCCATCGGTGCA-3’               |
| *                 | Bgl II -OV (antisense)            | 5’-GCAGATCTTTAAGGGAAAACACATCT-3’              |
| ---                 | OV-RSL/Masp-C (sense)             | 5’-GCAAGCGTCTCTGATGAATGCTGCC-3’               |
| ---                 | OV-RSL/Masp-C (antisense)         | 5’-GGTCAGCAATTCAATTCATCAGAGCGCTTG-3’          |
| Masp/OV-RSL (sense) | 5’-GAAATAAATCTGAAGATGGTAGAGGTTGAGTGGTGA-3’ |
| Masp/OV-RSL (antisense) | 5’-GACCTACCACCTCTCTCTCAATCTTCAGTTATTC-3’ |
| OV/Masp-RSL (sense) | 5’-AATGAAGCAGGGGGATTCCATAGAGGTG-3’ |
| OV/Masp-RSL (antisense) | 5’-TATGGAAATCCCCGCTGGCTTCTGATTTGC-3’ |
| Masp-RSL/OV-C (sense) | 5’-CTGCAGCAACAAGGAAGATTTAGGGCTGAC-3’ |
| Masp-RSL/OV-C (antisense) | 5’-CCTAAATCTTCCTCGTGTCGAGATCCGTG-3’ |
| &Maspin RQ mutant | Maspin RQ (sense)                 | 5’-GTGCCAGGAGCAAGATCTCCGACGAC-3’              |
| &Maspin RQ mutant | Maspin RQ (antisense)             | 5’-GTGCCAGGAGCAAGATCCGACGAC-3’                |
| &Maspin RA mutant  | Maspin RA (sense)                 | 5’-GTGCCAGGAGCAAGATCCGACGAC-3’                |
| &Maspin RA mutant  | Maspin RA (antisense)             | 5’-GTGCCAGGAGCAAGATCCGACGAC-3’                |

* Restriction sites EcoR I or Bgl II (italic letters) at 5’ overhang of sense or antisense, respectively of both ovalbumin and maspin (bold letters).

--- Sequences in bold letters complementary to ovalbumin or maspin (bold letters) with 5’ overhang containing ovalbumin or maspin sequences (nonbold) for overlap PCR extension.

‡Site-directed mutation (bold and underlined letters).
Figure 3

A

% Cell Adhesion

Control  Maspin  RQ  RA

B

% Cell Adhesion

Control  Maspin  RQ  RA
Figure 6

Graph showing % Cell Adhesion for different conditions: Control, Maspin, Masp/OV-RSL/Masp-C, OV/Masp-RSL/OV-C. The graph includes error bars indicating variability. Significant differences are marked with stars: * (p < 0.05), ** (p < 0.01), *** (p < 0.001).
Figure 7

% Cell Invasion

Control, Maspin, Masp/OV-RSL/Masp-C, RQ, RA, OV/Masp-RSL/OV-C, RSL, Ovalbumin
Figure 8

$k_d = 367 \pm 67 \text{ nM}$

$B_{max} = 5.44 \pm 0.24 \text{ pmol/10}^5 \text{ cells}$

$r^2 = 0.980$
Figure 9

pmol maspin bound/10^5 Cells

RSL Added

0  2x  10x  20x  100x
Sufficiency of the reactive site loop of maspin for Induction of cell-matrix adhesion and inhibition of cell Invasion: Conversion of ovalbumin to a maspin-like molecule
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