Maspin, the Molecular Bridge between the Plasminogen Activator System and β1 Integrin That Facilitates Cell Adhesion*

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Maspin is a non-inhibitory serine protease inhibitor (serpin) that influences many cellular functions including adhesion, migration, and invasion. The underlying molecular mechanisms that facilitate these actions are still being elucidated. In this study we determined the mechanism by which maspin mediates increased MCF10A cell adhesion. Utilizing competition peptides and mutation analyses, we discovered two unique regions (amino acid residues 190–202 and 260–275) involved in facilitating the increased adhesion function of maspin. In addition, we demonstrate that the urokinase-type plasminogen activator (uPA)/uPA receptor (uPAR) complex is required for the localization and adhesion function of maspin. Finally, we showed that maspin, uPAR, and β1 integrin co-immunoprecipitate, suggesting a novel maspin-uPA-uPAR-β1 integrin mega-complex that regulates mammary epithelial cell adhesion.

Maspin is a non-inhibitory serine protease inhibitor (serpin)4 that was originally identified as a type II tumor suppressor protein in mammary epithelial cells (1). One major tumor suppressor function of maspin is suppression of tumor cell motility, as it inhibits tumor cell migration/invasion in vitro and suppresses metastasis in mouse models (1–7). Several studies show that pericellular maspin inhibits cell motility by enhancing cell adhesion (2, 3, 8, 9). In addition to its tumor suppressing functions, our laboratory showed that maspin is also essential for normal fetal development as maspin knock-out mice are embryonic lethal during the peri-implantation stage partially due to disrupted visceral endodermal cell adhesion (10). The underlying molecular mechanism by which maspin regulates cell adhesion is currently unknown and under intense investigation. To date, there are two proposed pathways utilized by maspin to increase cell-extracellular matrix (ECM) adhesion; that is, the plasminogen activation system and β1 integrin signaling (9, 11–13).

The plasminogen activation system is believed to be a central player in several different processes important for tumor progression and metastasis (14–16). In this system urokinase-type plasminogen activator (uPA), a serine protease, binds to its glycosylphosphatidylinositol-anchored receptor (uPAR) and readily activates plasminogen to initiate a protease cascade resulting in localized ECM degradation for the purpose of cell migration (17, 18). It has been suggested that maspin integrates into the plasminogen activation system. Maspin inhibits prostate carcinoma cell migration and invasion by strengthening mature focal adhesion contacts, reducing uPA activity by internalizing the maspin-uPA-uPAR complex and by binding to pro-uPA, thus blocking its activation (12). Although maspin is classified as a serpin and decreases pericellular uPA activity, maspin does not directly inhibit uPA proteolytic activity (19–21). Together, these studies demonstrated that maspin can reduce prostate carcinoma cell migration and invasion by internalization of cell surface maspin-uPA-uPAR complexes.

The second proposed cell adhesion pathway involves maspin associating with β1 integrin, thus, altering integrin-mediated signaling. Initial studies investigating the anti-invasive function of maspin showed that MDA-MB-435 breast carcinoma cells treated with exogenous maspin had increased expression of α5- and α3-integrins. In addition to altered integrin expression profile, maspin stimulated focal adhesion and stress fiber formation in MDA-MB-231 breast carcinoma cells to a fibronectin matrix acting through the α5β1 integrin receptor (3, 22). We have suggested that cell surface maspin co-localizes with β1 integrin to increase MCF10A cell adhesion. This increased adhesion was facilitated by amino acids residues 139–225 in the maspin molecule (9). Another study demonstrated that maspin inactivation of β1 reduces vascular smooth muscle cell migration on laminin or fibronectin matrices (13). A peptide mimicking the G α-helix (G-helix, amino acids 237–251) region of maspin was both essential and sufficient for inhibiting cell migration, but it had no effect on cell adhesion (13, 23). These discoveries showed that the G-helix of maspin regulates cell migration, but another region (amino acids 139–225) is involved in regulating cell adhesion.

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4 The abbreviations used are: serpin, serine protease inhibitor; ECM, extracellular matrix; MEF, mouse embryo fibroblasts; RCL, reactive center loop; uPA, urokinase-type plasminogen activator; uPAR, urokinase-type plasminogen activator receptor.
Initially thought to simply localize uPA for ECM degradation, recent evidence indicates that uPAR also initiates intracellular signaling cascades that regulate cell adhesion, migration, and proliferation independent of protease activity (24). In fact, it is now becoming clear that uPAR can specifically modify integrin functions to regulate ECM binding, cell adhesion, and migration (24–26).

We speculate that maspin acts as an integrator of these two systems, ultimately leading to decreased cell migration and increased cell adhesion. Therefore, the objective of this study was to determine the intramolecular region(s) of maspin necessary for its pro-adhesive function and decipher its mechanism of action. We demonstrate two different regions proximal to the reactive center loop (RCL) of maspin that are responsible for maspin-mediated MCF10A cell adhesion. Importantly, this enhanced adhesion is dependent on the presence of both uPA and uPAR and is present in a complex with uPA-uPAR-β1 integrin on the cell surface. Together, we suggest that maspin coordinates both uPA-uPAR and β1 integrin receptors to regulate both mammary epithelial cell-ECM adhesion and migration.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—For immunoprecipitation and functional blocking experiments, we used the rabbit anti-human uPA and anti-human uPA (American Diagnostics). We used mouse monoclonal anti-maspin (BD Pharmingen) and rabbit polyclonal anti-β1 integrin (Chemicon) for both immunoprecipitation and immunoblot probing. An affinity-purified rabbit polyclonal antibody raised against maspin RCL peptide (AbS4A) was used from previous studies (1). Both the horseradish peroxidase-conjugated secondary antibodies and maspin (AbS4A) was used from previous studies (1). Both the horseradish peroxidase-conjugated secondary antibodies and maspin peptides were obtained from Sigma. Human uPA was purchased from Chemicon.

Cell Culture—MCF10A, immortalized human mammary luminal epithelial cells (CRL-10317; American Type Culture Collection), were cultured in Dulbecco’s modified Eagle’s medium (DMEM/F-12 (Invitrogen) containing 5% donor horse serum, 20 μg/ml epidermal growth factor, 100 μg/ml cholera toxin, 10 μg/ml insulin, 500 μg/ml hydrocortisone, 50 units/ml penicillin, and 50 μg/ml streptomycin at 37 °C and 5% CO2. All growth factors and hormones were purchased from Sigma.

For maspin secretion studies, MCF10A cells either directly ordered from ATCC (low passage) or carried in our laboratory for 2 years (high passage) were maintained in defined keratinoocyte serum-free medium (Invitrogen) supplemented with ciprofloxacim and MITO+ (BD Biosciences). Cells were passaged weekly and fed three times per week. Conditioned medium was collected on the day of passage, centrifuged to remove cell debris, and concentrated 24× using a 30-kDa spin filter (Millipore). Harvested protein was then used for Western blot analysis, as described later. Control (uPAR+/+) and uPAR-deficient (uPAR−/−) murine embryonic fibroblasts (MEFs) were isolated from embryos as previously described (27).

Design of Maspin Peptides—We recently demonstrated that amino acid residues 139–225 within maspin are important for increased MCF10A cell adhesion to its self-deposited matrix (9). To determine the surface-exposed amino acid sequences that may regulate cell adhesion, we analyzed the three-dimensional structure of maspin by Molsoft ICM-pro software. Subsequently, we purchased peptides (Sigma) composed of 16–23 amino acids (depending on the hydrophilicity and molecular weight) to test in adhesion assays.

Construction, Expression, and Purification of Glutathione S-Transferase (GST)-Maspin and Mutants—GST-tagged maspin (GST-maspin) was produced as previously described (28). Point mutants were constructed with a QuikChange multisite-directed mutagenesis kit (Stratagene) according to the manufacturer’s instruction. The primers are as follows: control mutant D177A, S178L, T180P (forward, 5′-GGATGAAGAAATTCCTCAGATAGGCTTTCAATGTCGGGAAAATTTCTTCATCC); single (E201K) mutant (forward, 5′-GATGAATCCTTAAAGGCCACTTTCTGTGGG; reverse, 5′-CCTCAAGCCAGAGCTTAAGTTCATCA); double (K268E, K270E) mutant (forward, 5′-GGCCAATGCGAAGTCCGACATTTCCCTCCC; reverse, 5′-GGAGGGAAAGTTCGAATTCGGCATTGGCC). To develop the triple mutant (E201K,K268E,K270E), primers of mutant K268E, K270E, and E201K were used as the template. To verify their fidelity, the constructs were sequenced. The constructs were transformed into Escherichia coli BL21 cells and expressed and purified according to the manufacturer’s instructions (GE Healthcare).

Adhesion Assay—Assays utilizing endogenous ECM proteins generated by MCF10A cells were performed as previously described (29). MCF10A cells were plated in 96-well dishes and allowed to reach confluence. Cells were washed with PBS and treated for 5 min with fresh sterile 20 mM NH4OH followed by extensive water washes. Wells were blocked with heat-denatured BSA (10 mg/ml) for 1 h at room temperature. Subconfluent cultures were trypsinized, washed with 37 °C serum-free DMEM/F-12 medium, and incubated with either antibodies or recombinant proteins (500 nm) for 30 min at 37 °C (when assaying endogenous maspin, enzyme-free cell dissociation solution was used instead of trypsin). In all assays 2.0 × 104 cells were plated in triplicate and allowed to adhere for 30 min at 37 °C. The GST protein was used as a control. Wells were washed with 37 °C serum-free DMEM/F-12, and adhered cells were fixed with 5% glutaraldehyde and stained with crystal violet dye. Cell adhesion was determined by the reading at 590 nm subtracted by the blank value (determined by BSA-coated wells, 5% of maximal cell adhesion). Cell adhesion was plotted as the percentage of the corresponding control value.

Cell surface stripping of uPA from uPAR was conducted as previously described (30). In brief, subconfluent cultures were washed twice with DMEM/F-12 supplemented with 20 mM HEPES and 1 mg/ml BSA. Then cells were incubated with 50 mM glycine-HCl (pH 3.0) with 100 mM NaCl at 25 °C for 2 min, and the reaction was halted by neutralizing with 500 mM HEPES (pH 7.4). In the function-blocking studies, cells were first incubated with the function blocking anti-uPAR or control rabbit IgG for 10 min; uPA and recombinant GST-maspin was added for another 20 min.

Maspin Binding Assay—Bacterial recombinant GST-maspin was labeled with 125I as described previously (31). Wild type...
(WT) and uPAR−/− MEFs (5.0 × 10^4 cells) were cultured in DMEM with 10% FBS on 96-well plates overnight. Cells were chilled on ice for 30 min, washed 3 times with ice-cold DMEM, then blocked on ice for 60 min using blocking buffer (DMEM containing 5% heat-inactivated BSA). MEFs were incubated with increasing concentrations of 125I-GST-maspin in blocking buffer at 4 °C for 90 min. After incubation, unbound 125I-GST-maspin protein was removed by washing 3 times with blocking buffer. Specific 125I-GST-maspin binding was determined by subtracting the detected radioactivity by the nonspecific binding (determined in the presence of a 50-fold excess of nonlabeled GST-maspin).

In the studies evaluating the uPA-uPAR interactions, 125I-GST-maspin was treated with ABS4A and S-20 (Santa Cruz) antibodies, which block the RCL and N-terminal domains, before adding to WT MEF cells. In one set, WT MEF cells were treated with an anti-mouse uPAR antibody (R&D), which blocks uPA binding to uPAR. In the other set, WT MEF cells were stripped of uPA, then treated with the anti-mouse uPAR antibody and then administered uPA (800 nM) and 125I-GST-maspin. Results were reported as 125I-GST-maspin binding to WT MEF cell surface as a percentage of control (IgG) antibody treatment.

**Immunoprecipitation and Western Blot**—Because MCF10A cells express uPAR at low levels, we overexpressed uPAR in MCF10A cells. Lysates from MCF10A cells overexpressing uPAR were prepared in lysis buffer: 50 mM Tris (pH 7.4), 1% Triton X-100, 1% sodium deoxycholate, 150 mM NaCl, 5 mM EDTA (pH 8.0), 5 mM PMSF, and protease inhibitor mixture (Thermo Scientific). Cellular debris was cleared from lysates by centrifugation, and protein concentration was determined by the BCA Protein Assay (Pierce). Whole cell extracts (500 μg of protein) were incubated overnight (constant rocking) with 5 μg of specific antisera or control rabbit irrelevant antisera (K67) at 4 °C. Protein A-Sepharose-coupled beads (Amersham Biosciences) were added and incubated for 2 h at 4 °C under constant agitation. Beads were centrifuged, washed 3 times with ice-cold lysis buffer, and boiled for 5 min in sample buffer containing 5% β-mercaptoethanol. Samples were separated on SDS-PAGE gels, transferred to a PVDF membrane (GE Healthcare), and probed for β1 integrin, uPAR, and maspin. Appropriate secondary antibodies were added, and proteins were visualized with enhanced chemiluminescence substrate (Pierce).

**Data Analysis**—Statistical differences between two individual groups were determined using an unpaired t test. Statistical significance was considered when the p value was less than 0.05.

**RESULTS**

**Maspin Is Exported from MCF10A Cells**—One key function of maspin is the regulation of cell motility and adhesion. After its discovery, researchers determined that maspin was ubiquitous in the cellular cytoplasm but was also localized to secretory vesicles and the cell surface (32). Additional studies by our laboratory and others further confirmed that maspin can be secreted or localized to the cell surface (8, 9, 33). Despite these findings, a recent study has suggested that maspin is an obligate intracellular protein that is not associated with the cytoskeleton or present on the cell surface (34). Therefore, we tested whether MCF10A cells from our laboratory can export maspin into the culturing medium.

In these experiments, we utilized human corneal epithelial cells as a positive control for maspin secretion. Studies have shown that maspin is expressed and secreted from corneal epithelial cells, which seems to regulate stromal wound healing and maintaining avascularity (8). Additionally, human corneal epithelial cells provide further evidence that maspin can be secreted from cells. Because the existence of extracellular maspin is crucial for the findings presented in this manuscript, we sought to verify that the MCF10A cells in our laboratory can export maspin.

The culturing media was obtained from two different MCF10A populations; (i) cells obtained directly from ATCC or (ii) cells that have been cultured in our laboratory for 2 years and analyzed by Western immunoblot for maspin protein expression. Maspin was detected in all media and cells tested (Fig. 1A).

Interestingly, using densitometry analysis, we discovered that the detection of extracellular maspin is significantly reduced in high passage MCF10A cells compared with the low passage cells (Fig. 1B). Additionally, intracellular maspin expression was elevated in the high passage MCF10A cells compared with the low passage cells. These results suggest that maspin is exported from MCF10A cells, and maspin secretion may be reduced by extended culturing conditions.

**Two Proximal Sites to the Reactive Center Loop Are Responsible for MCF10A Cell Adhesion**—MCF10A cells deposit ECM proteins and can nucleate adhesive complexes typical of epithe-

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**FIGURE 1. Maspin is exported from MCF10A cells.** A, human corneal epithelial cells (HCEC), used as a control, or low and high passage MCF10A cells were maintained in defined keratinocyte serum-free medium. Conditioned medium was collected, centrifuged to remove cell debris, and concentrated. Cells (2 × 10^6) were solubilized with radioimmune precipitation assay buffer containing protease inhibitors. Protein levels were measured by Pierce Coomassie reagent, and protein (20 μg) was run on 10% SDS-PAGE. Proteins in 50 μl of the 24× conditioned medium were separated on SDS-PAGE and visualized by Western blots (WB) using mouse anti-maspin (BD Biosciences) or rabbit anti-GAPDH (Chemicon). B, shown is a densitometry analysis of extracellular (media) and intracellular (cells) maspin expression from low passage (black bars) and high passage (gray bars) MCF10A cells. Error bars, S.E. of four replicates. Statistical analysis was done by t test where p < 0.05 (*).
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lia (35, 36). In our cell adhesion model, we previously demonstrated that the region between amino acids 139 and 225 in maspin appears to mediate cell adhesion (9). However, the exact region(s) involved has not been elucidated.

To determine the region(s) of maspin that is involved in adhesion, we developed a variety of competitive peptides. First, we developed peptides that dissect the aforementioned region (amino acid residues 139–225) into peptides corresponding to amino acid residues 137–158, 169–189, 181–202, 190–211, and 203–225. Then, using Molsoft ICM software to analyze the tertiary structure of maspin, we developed peptides to other regions of maspin that are exposed such as the RCL (329–343), 260–275, and the control peptide (97–112). Using these peptides, we determined which region of maspin is involved in mediating MCF10A cell adhesion.

The only peptides (derived from amino acid residues 139–225) that did compete with GST-maspin were the 181–202 and 190–211 peptides (Fig. 2A). These results have identified the 190–202 region of maspin is partially responsible for mediating cell adhesion. In addition, we discovered a novel region between amino acids 260 and 275 that is also important in regulating maspin-mediated MCF10A cell adhesion (Fig. 2A). Although the 190–202 (blue) and 260–275 (green) peptides are separated by 58 amino acids in the primary structure of maspin, they are adjacent to one another in the tertiary structure (Fig. 2B).

To further demonstrate that these regions are involved in maspin-mediated adhesion, we developed mutants of GST-maspin; single mutant (E201K), double mutant (K268E,K270E), triple mutant (E201K,K268E,K270E), and a control mutant (D177A, S178L, T180P). The single (E201K) mutant corresponds to peptide 181–202, whereas the double (K268E,K270E) mutant corresponds to peptide 260–275. The control (D177A, S178L, T180P) mutant failed to have any reduced cell adhesion. However, both the single and double GST-maspin mutants had significantly reduced MCF10A cell adhesion (by 16 and 17%, respectively) as compared with WT GST-maspin (Fig. 2C). The triple (E201K,K268E,K270E) mutant had an additive effect whereby MCF10A cell adhesion was decreased by ~28% when compared with WT GST-maspin (Fig. 2C). These findings support the notion that proximal amino acid residues from 190–202 and 260–275 in maspin mediate its effect on MCF10A cell adhesion.

Maspin Localization to the Cell Surface Requires the uPA-uPAR Complex—Recent accumulating evidence suggests an important role of the uPA-uPAR complex in regulating cell adhesion (24, 37). Because maspin is localized with the uPA-uPAR complex at the cell surface (12), we investigated whether uPAR is involved in maspin binding to the cell surface. Using WT and uPAR<sup>−/−</sup> MEFs, we found that WT MEFs displayed specific <sup>125</sup>I-GST-maspin binding, whereas uPAR<sup>−/−</sup> MEFs were absent of any specific <sup>125</sup>I-GST-maspin binding using concentrations within the normal physiological range (<500 nM) (Fig. 3A). These results suggest that uPAR expression is necessary for maspin to bind or localize to the cell surface.

In this study and others (11), we have implied that a region proximal to the RCL of maspin is involved in mediating cell adhesion. Therefore, we determined the binding of <sup>125</sup>I-GST-maspin to WT MEF cells that were incubated with either control (IgG), RCL (AB54A), or N-terminal (S-20) blocking antibodies. We found that only the RCL (and/or surrounding area) and not the N terminus of maspin is necessary for maspin binding to the surface of WT MEFs (Fig. 3B).

To determine whether the uPA-uPAR complex is required for <sup>125</sup>I-GST-maspin binding to MEF cell surface, we used an antibody that disrupts uPA binding to uPAR. In the first set of experiments, treatment with this antibody did not change the binding of <sup>125</sup>I-GST-maspin to WT MEF cells (Fig. 3C, first set). However, <sup>125</sup>I-GST-maspin binding to the WT MEF cell surface is reduced when cells were stripped of uPA and incubated with the uPAR blocking antibody before reintroducing exogenous uPA and <sup>125</sup>I-GST-maspin (Fig. 3C). These results demonstrated that cell surface localization of maspin requires not only uPAR but the uPA-uPAR complex.

Removal of Pericellular uPA Mitigates Maspin-mediated Cell Adhesion—As stated earlier, maspin binds to both uPA and pro-uPA, causing reduced cell attachment by strengthening mature focal adhesion contacts (12). A current model proposes that an unidentified region of maspin, in close proximity to the RCL, is responsible for maspin binding to uPA (and pro-uPA) (11). In this study, we have demonstrated that the amino acid residues from 190–202 and 260–275 in maspin mediate its effect on cell adhesion, and these regions are proximal to the RCL (Fig. 2). Using this information, we hypothesized that the association of maspin and uPA may mediate cell adhesion.

To test this hypothesis, we acid-stripped endogenous uPA before the addition of GST-maspin and evaluated the effect on MCF10A cell adhesion. Stripping uPA substantially blocks maspin-induced MCF10A cell adhesion (Fig. 4A). To verify that this ablation of maspin-induced adhesion was a direct effect of uPA removal, we resupplied uPA-stripped MCF10A cells with exogenous uPA (800 nM). In fact, the addition of exogenous uPA rescues the maspin-mediated adhesion of uPA-stripped MCF10A cells (154.06% of GST control, Fig. 4A). These experiments demonstrate that uPA is needed for maspin-mediated adhesion.

The uPA-uPAR Complex Is Necessary for Maspin-mediated Cell Adhesion—These combined studies revealed that uPA or uPAR removal from the surface of MCF10A cells ablates maspin localization to the cell surface (Fig. 3) and its ability to enhance cell adhesion (Fig. 4A). However, whether uPA and uPAR function independently or if the localization and adhesion functions of maspin require the uPA-uPAR complex is still not clear. Therefore, we utilized uPAR blocking antibody (anti-uPAR, blocks free uPA from binding to uPAR) to elucidate the role(s) of the uPA-uPAR complex in maspin-mediated adhesion.

For these experiments, we first stripped endogenous uPA and then incubated the cells with control rabbit IgG or anti-uPAR for 10 min, and finally, after the addition of exogenous uPA (800 nM) and GST-maspin (500 nM), the cells were allowed to adhere. At a lower concentration of the anti-uPAR (0.25 μg/ml), there was a slight decrease in cell adhesion, albeit not significant. However, at higher concentrations of anti-uPAR (0.5 and 1.0 μg/ml), maspin-mediated MCF10A cell adhesion was significantly inhibited compared...
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FIGURE 2. Peptides or mutation of maspin (E201, K268, and/or K270) inhibit MCF10A cell adhesion to self-deposited matrix. A, MCF10A cells were harvested with enzyme-free dissociation buffer and preincubated with the indicated peptides. Cells (2.0 × 10^4 cells) were seeded, and adhesion was measured after 30 min using colorimetric reaction as described under “Experimental Procedures.” Peptides 181–202, 190–211, and 260–275 inhibited cell adhesion, whereas no significant effect was detected with other peptides. Each sample was measured in triplicate. Results are representative of three independent assays. Statistical analysis was done by Student’s t test where p < 0.05 (*) and p < 0.01 (**).

with control antibody treatment (Fig. 4B). These findings demonstrate that disrupting uPA binding to uPAR impairs maspin-mediated adhesion.

FIGURE 3. uPAR and uPA-uPAR complex are required for maspin localization to MEF cell surface. A, wild type (WT, black circles) and uPAR−/− (white circles) MEFs were incubated with 125I-GST-maspin in DMEM containing 5% BSA or at 4 °C for 90 min. Cell surface binding was determined by measuring gamma radiation. B, pretreatment of 125I-GST-maspin with two different maspin antibodies shows that the RCL region is necessary for maspin binding to WT MEF cell surface. The ABS4A antibody (black bars) blocks the RCL region, whereas the S-20 antibody (white bars, Santa Cruz Biotechnology) blocks the N-terminal region. C, shown is inhibition of maspin binding to WT MEF cell surface by the anti-mouse uPAR antibody (R&D), which blocks uPA binding to uPAR. WT MEF cells were treated with the uPAR antibody either without uPA stripping or with uPA stripping and the addition of exogenous uPA. Results are reported as GST-maspin binding as a percentage of control (IgG) antibody treatment. Error bars, S.E. of six replicates. Statistical analysis was done by Student’s t test where p < 0.05 (*) and p < 0.01 (**).
Alternatively, if MCF10A cells are not stripped of uPA, the addition of the blocking uPAR antibody has no effect on maspin-mediated cell adhesion (Fig. 4C). The inability of the uPAR blocking antibody to reduce MCF10A cell adhesion implicates the uPA-uPAR complex as an effector of maspin-mediated cell adhesion.

Maspin Forms a Complex with uPAR and β1 Integrin—Earlier investigations suggest that maspin associates with β1 integrin, thus inhibiting cell migration and enhancing cell adhesion (9, 13, 23). In addition, numerous recent studies have discovered that uPAR and integrin receptors co-localize with one another and observe that a bidirectional communication exists between them (15, 24). Together, we postulated that maspin physically associates with both uPAR and β1 integrin, thus producing a complex that regulates both cell motility and adhesion.

We determined the biophysical interaction between maspin, uPAR, and β1 integrin by immunoprecipitating cell lysates from MCF10A cells overexpressing uPAR (Fig. 5). We found that maspin co-precipitated with both uPAR and β1 integrin. Although these results corroborate previous studies showing association between maspin-uPAR (12), maspin-β1 integrin (9, 13), and uPAR-β1 integrin (38, 39), this will be the first time showing the association of all three proteins, the maspin-uPAR-β1 integrin complex.

DISCUSSION

Tumor metastasis involves recognition, degradation, and migration through the surrounding ECM, a process intrinsically dependent on cell-ECM adhesion. Many different proteins (and/or protein complexes) are involved in promoting or inhibiting tumor metastasis. Maspin is a tumor-suppressing protein that is abundantly produced in normal mammary luminal epithelial and myoepithelial cells (1). Upon tumor progression, maspin expression is significantly reduced or lost in breast and prostate carcinoma cell lines and tissues (1, 3, 6, 40). One of the major tumor-suppressing functions of maspin is its ability to inhibit tumor cell motility and invasiveness, which is partially mediated by enhancing cell adhesion. We found previously, that the region between amino acids residues 139–225 in maspin facilitates increased mammary luminal epithelial (MCF10A) cell adhesion (9). However, the underlying region and molecular mechanism by which maspin regulates cell adhesion is currently unknown and under intense investigation. In this study we uncovered a unique region proximal to the

FIGURE 5. Co-immunoprecipitation of maspin with uPAR and β1 integrin. Protein extracts (500 µg) from MCF10A cells overexpressing uPAR were immunoprecipitated with antibodies to uPAR (American Diagnostics), maspin (BD Pharmingen), and β1 integrin (Chemicon). The Ki67 antibody (Chemicon) was used as a negative control. Eluted immunoprecipitates were separated by SDS-PAGE and analyzed by Western immunoblots (WB) as indicated.
RCL of maspin that mediates its adhesive function and discovered a novel maspin-uPA-uPAR-β1 integrin complex that facilitates maspin-mediated cell adhesion. These findings are the first to implicate maspin as an integrator of the urokinase activation system and integrin receptors by which cell adhesion and migration are regulated.

Many studies have suggested or implied that maspin is secreted or present/associated at the cell surface (3, 8, 9, 13, 32, 33, 40, 41). We have previously shown that maspin is present on the extracellular surface of MCF10A cells, and this regulates cell adhesion (9). Because a recent study refutes that maspin is not expressed extracellularly (34), we sought to rectify the differences between these opposing findings. In this study, we demonstrate that MCF10A cells gradually lose maspin expression through continued cell culturing. This finding is similar to that observed with isolated human coronary stromal cells (8) and could explain the differences seen from different laboratory reports (34).

To more precisely determine the region involved in maspin-mediated cell adhesion, we developed several smaller peptides from the 139–225 region of maspin. The crystal structure of maspin reveals several structural motifs within this region including the third strand of the A β-sheet (s3A), third strand of the C β-sheet (s3C), and first strand of the B β-sheet (s1B) (41, 42). Due to the surface-exposed amino acid side chains, we hypothesized that s3C and s1B (amino acids 180–210) are important for adhesion. However, we found that s1B (peptides 181–202 and 190–211), not s3C (peptide 169–189), was important in mediating cell adhesion. Moreover, a point mutation (E201K) within this region significantly reduced MCF10A cell adhesion. We can extrapolate using the overlapping amino acids of these peptides that amino acids 190–202 (TDTKPVQMMNMEA) in maspin are integral for maspin-mediated cell adhesion. Interestingly, these amino acids are highly conserved between human, mouse, rat, and chicken species (84% sequence identity and 100% similarity), suggesting their importance throughout evolution (41).

In this study we found that the s1B and s2C regions of maspin are important in mediating MCF10A cell adhesion. These regions are proximal to the RCL region of maspin, which is the putative uPA binding site on maspin. Recent studies found that maspin binds with uPA (and pro-uPA) and can then be localized on the cell surface by uPAR (12, 19, 20). The co-precipitated complex of maspin-uPA-uPAR inhibits prostate carcinoma cell migration and invasion due at least in part to the strengthening of mature focal adhesion contacts (12). Therefore, we proposed that the s1B and s2C regions of maspin facilitate cell adhesion and addressed whether maspin-mediated cell adhesion relies on the plasminogen activation pathway.

We showed here that removal of endogenous uPA from the surface of MCF10A cells abrogated maspin-mediated adhesion. However, administration of exogenous uPA restored maspin-mediated cell adhesion in a concentration-dependent manner. These results imply that exogenous (or cell surface-localized) uPA expression is necessary for the pro-adhesion function of maspin.

Localization of uPA to cell-cell contacts and focal adhesions is mediated by its receptor, uPAR. Previous reports demonstrate that maspin co-localizes with the uPA-uPAR complex, and the cell surface localization of the maspin-uPA-uPAR complex is lost when the glycosylphosphatidylinositol anchor is cleaved by phospholipase C and rendering uPAR soluble (12). Using these earlier findings, we found maspin localized on the surface of WT MEFs but was not detected on the surface of uPAR−/− MEFs. Therefore, we conclude that maspin binds to uPA (and potentially pro-uPA), and the uPA-uPAR complex localizes maspin to the cell surface.

An important implication to these findings is that altered cell surface expression of uPA or uPAR will result in altered maspin localization and cell adhesion. For instance, cells under siege from increased phospholipase and protease activity liberate uPAR from its glycosylphosphatidylinositol tether (43, 44). Additionally, uPAR expression on the outer leaflet of the cell membrane is regulated by endocytosis, and maspin can initiate endocytosis of the maspin-uPA-uPAR complex (12, 45, 46). These factors will determine whether maspin is detected or functions on the cell surface.

Because uPAR is a glycosylphosphatidylinositol-anchored receptor, it cannot transduce an intracellular signal by itself. However, numerous studies have shown that in addition to focusing extracellular uPA proteolysis, uPAR also acts as a genuine signaling receptor (15, 37). Many cellular responses modulated by the uPA-uPAR complex, including cell migration and adhesion, typically utilize transmembrane signaling to produce these effects. In a breakthrough study, Wei et al. (38) demonstrated that uPAR interacts with and modifies the function of β1 integrin, thus influencing cell adhesion and directional migration. The interaction between uPAR and β1 integrin seems to be dependent on uPA (or pro-uPA) binding to uPAR (47, 48). Although uPAR and β1 integrin co-localize and co-immunoprecipitate, there is still debate whether they interact directly or through a “linker” protein (49).

The interactions between maspin and β1 integrin have been suggested for many years. In the initial study, MDA-MB-435 breast carcinoma cells overexpressing maspin exhibited increased selective adhesion to a fibronectin matrix, mediated by the α5β1 integrin receptor (3). Our laboratory followed up by demonstrating that maspin regulates the early steps of cell adhesion by associating with and signaling through β1 integrin (9, 50). These studies showed that cell adhesion can be regulated by the association of maspin and β1 integrin. More recently, the maspin-β1 integrin complex has been shown to inhibit vascular smooth muscle cell migration. The innovative study by Ravenhill et al. (23) implicated the G β-helix (G-helix) region (amino acids 237–251) of maspin was sufficient to block vascular smooth muscle cell and carcinoma cell migration. Interestingly, this G-helix peptide itself was unable to affect maspin-mediated cell adhesion, but a mutation in the G-helix region of full-length maspin reduced maspin-mediated cell adhesion (23). In this manuscript we have already shown that maspin localization to the cell surface requires uPAR. However, other studies (including studies from our laboratory) suggest that maspin binds and signals through β1 integrin. Therefore, we investigated if maspin co-immunoprecipitated with uPAR, β1 integrin, or both uPAR and β1 integrin. We found that maspin is associated with a large multiprotein signaling complex,
Maspin Promotes Cell Adhesion via uPA-uPAR-β1 Integrin

FIGURE 6. Proposed model of the maspin complex that regulates cell adhesion. In the absence of maspin, uPA bound to its receptor (uPAR) can associate (either directly or indirectly, dotted lines) with β1 integrin. This association in turn activates a variety of signaling cascades that facilitate increased cell migration, increased matrix metalloproteinase (MMP) expression, and cell proliferation. We propose that the introduction of exogenous or increased endogenous expression of maspin facilitates an altered conformation of the mega-protein complex, thus promoting cell adhesion and reduced cell migration.

which includes uPAR and β1 integrin, and this complex association seems to be localized on the cell surface (9). Therefore, we show here a novel complex whereby maspin integrates (or associates with) the plasminogen activation system and β1 integrin, thus regulating cell adhesion and migration (Fig. 6).

There are two different possibilities for the sequence of events that regulate maspin-mediated adhesion and migration. The first possibility implies that uPAR and β1 integrin are not in a complex. Maspin binds to uPA (via s1B and s2C residues 190–202 and 260–275, respectively) and is localized on the cell surface by maspin-uPA binding to uPAR. Then, the maspin-uPA-uPAR complex associates with and inactivates β1 integrin (via the G-helix of maspin binding to β1 integrin), thus reducing cell migration potentially by increasing cell adhesion. The second possibility, which is probably more prevalent in cancer cells, involves uPAR and β1 integrin already in complex, thus facilitating enhanced migration capabilities. Then, the maspin-uPA complex binds to (or associates with) the uPAR-β1 integrin complex, thereby inducing a conformational or lateral mobility change in uPAR, which causes an altered physical association (and/or activation state) with β1 integrin, eventually resulting in decreased cell migration and/or increased cell adhesion. This novel maspin-uPA-uPAR-β1 integrin mega-complex integrates several potential signaling pathways, which provides a potential explanation for the dual functionality of maspin, i.e. inhibit cell migration and promote cell adhesion.

In addition to these four proteins, we cannot rule out the possibility of other proteins associating in this mega-complex. In particular, caveolin is required for the formation of uPAR-β1 integrin complexes and uPAR-dependant adhesion (38, 51, 52). Caveolin may act as a regulator of structure and organize the vast number of signaling kinases such as focal adhesion kinase and p130Cas-tyrosine kinase (53). Additionally, we have not addressed the identity of which α integrins would be associated in this mega-complex. In fact, we have purposefully excluded the α-integrins identities because the variation of integrin subtypes may facilitate which signal transduction pathway is necessary for a specific cell type. For instance, MCF10A cells deposit a matrix composed mainly of laminin-332 (35), in which α5β1 integrin is the primary adhesion receptor (54, 55). Alternatively, recent studies discovered that maspin inhibited vascular smooth muscle cell migration by specifically inhibiting α5β1 activation (13, 23). These cell-specific nuances are important and need to be investigated in future studies.

In conclusion, we show here that maspin produces a novel coordination of the plasminogen activation system and integrin receptors, which is necessary to facilitate its anti-migratory ability. We have identified that the s1B and s2C regions (amino acid residues 190–202 and 260–275, respectively) of maspin are responsible for its effects on cell adhesion and the necessity of the uPA-uPAR complex for this effect. Additionally, we provide a link between the two separate models proposed to mediate the anti-migratory effects of maspin (namely the plasminogen activator system and β1 integrin adhesion receptor). The ability of maspin to integrate into this signaling complex seems to be retained even in tumor cells, as recombinant maspin still facilitates anti-migratory signaling even after its expression is lost (1, 3, 40, 56, 57). The uPA-uPAR complex has been implicated as a facilitator of malignant tumor progression for more than 30 years (16). However, despite this plethora of knowledge, the therapeutic targeting of the uPA-uPAR system has remained elusive. This study furthers our understanding of the uPA-uPAR system and may potentially lead to novel maspin-based therapeutics for a variety of malignancies.

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