

Maspin is physically associated with $\beta 1$ integrin regulating cell adhesion in mammary epithelial cells

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ABSTRACT Maspin is a tumor-suppressor serpin (serine protease inhibitor), which inhibits cell invasion and migration. Here, we analyzed maspin function in cell adhesion in nontransformed mammary epithelial cells and investigated the underlying mechanism involved in this process. We report that maspin acts in the early steps in the cell adhesion process. Addition of recombinant maspin rapidly increased MCF-10A cell adhesion to the endogenously deposited matrix, and conversely both an antimaspin antibody (Ab) and maspin knockdown by RNA interference resulted in decreased cell adhesion. Mutation analyses revealed that a region of 86 amino acids located between aa 139 and aa 225 was responsible for maspin effect on adhesion. In addition, we show that maspin is associated with detergent-insoluble cortical cytoskeleton elements. Collectively, these results suggest that maspin is part of the supramolecular structure of the adhesion plaque and it modulates cell adhesion via a $\beta 1$ integrin-dependent mechanism.—Cella, N., Contreras, A., Latha, K., Rosen, J. M., and Zhang, M. Maspin is physically and functionally associated with $\beta 1$ integrin regulating cell adhesion in mammary epithelial cells. *FASEB J.* 20, E000–E000 (2006)

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TUMOR CELLS NEED to recognize and invade the surrounding extracellular matrix (ECM) in order to undergo metastatic growth in distant sites, a process intrinsically dependent on cell adhesion to the ECM. Maspin (SERPINB5) is a tumor-suppressor gene belonging to the family of serpins (serine protease inhibitors). Maspin is expressed in many tissues such as the mammary gland, prostate, skin, stomach, and uterus (1) and is down-regulated in carcinoma cells (2). Maspin has distinct biological effects, including inhibition of tumor growth, cell migration, and invasion (3) and inhibition of angiogenesis (4, 5). Maspin's tumor-suppressing effect appears to depend in part on its ability to increase cell adhesion to ECM (6, 7). The molecular mechanisms underlying maspin's diverse activities are under intense investigation. While the inhibition of the pericellular urokinase-type plasminogen activator (uPA) system by maspin has been reported in prostate tumor cells (8, 9), biochemical

analyses under several different biological contexts have not detected any inhibitory activity on several different proteases (10, 11). These differences may reflect different cell models and/or experimental approaches. Alternatively, it could indicate that maspin has a different mechanism of action other than protease inhibition. We have shown that transgenic animals expressing maspin in the mammary gland have smaller and fewer alveolar structures, lack functional differentiation, and could not nurse their young (12). No increase in protease inhibitory activity was detected in the mammary gland extracts of transgenic mice, indicating that another mechanism underlies maspin's function in this system. Since the majority of the studies on maspin have been performed on tumor models that do not express maspin, the mechanism of action and cellular distribution of endogenously expressed maspin have not been investigated. In this study, we take advantage of the MCF-10A human mammary epithelial cell model, which naturally expresses high levels of maspin, to investigate its role in cell adhesion and the mechanism underlying this process.

MATERIALS AND METHODS

Antibodies and reagents

Monoclonal antibody (mAb) anti-human maspin (Pharmin-gen) was used for Western blot and immunofluorescence analyses on MCF-10A cells. An affinity-purified rabbit polyclonal antibody (pAb) raised against maspin reactive site loop (RSL) peptide (AbS4A) (2) and a mouse polyclonal anti-maspin (whole molecule) Ab were both used in cell adhesion assays. For immunoprecipitation assay, we used a purified rabbit polyclonal anti-maspin Ab. Both mouse and rabbit antisera have been produced in our laboratory. For the studies of $\beta 1$ integrin, the following antibodies were used: A rabbit pAb (Chemicon) was used for immunofluorescence analyses and immunoprecipitation. Purified function-blocking anti- $\beta 1$ integrin (AIIB2) (a gift from Dr. Karl S. Matlin) was used in cell adhesion assays. Anti-tubulin Ab was purchased from Sigma, Inc., and anti-human MHC I Ab (W6/32,

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Sigma) was a gift from Dr. Michael Z. Gilcrease. A polyclonal anti-bax was from Upstate Cell Signaling (Charlottesville, VA). The following secondary antibodies were used: Alexa Fluor® 594-conjugated goat antimouse IgG (Molecular Probes, Eugene, OR), and FITC-conjugated goat anti-rabbit (Zymed) were used for MCF-10A staining. For Western blot analyses, horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit IgGs were employed (Sigma, St. Louis, MO). Reconstituted basement membrane (Matrigel) was from BD Biosciences, and soybean trypsin inhibitor was from Roche Biochemicals.

Cell culture

MCF-10A human mammary cells (CRL-10317; American Type Culture Collection) were cultivated as monolayers in Dulbecco's modified Eagle medium (DMEM)/F12 (Invitrogen) containing 5% donor horse serum, 20 µg/ml epidermal growth factor (EGF), 100 ng/ml cholera toxin, 10 µg/ml insulin, 500 µg/ml hydrocortisone, 50 U/ml penicillin, and 50 µg/ml streptomycin at 37°C and 5% CO₂. Growth factors and hormones were purchased from Sigma. For three-dimensional (3D) cultures, cells were trypsinized, transferred to trypsin inhibitor solution, washed with serum-free medium, and embedded in Matrigel (5×10⁵ cells/ml). After Matrigel solidification cells were overlaid with the medium described above and allowed to grow for at least 15 d, a time required for cells to form spheroids with hollow lumens. Medium was changed every two days.

RNA interference

Sequences of small interfering RNA (siRNA) for the maspin mRNA have been previously described (13). Selected siRNAs (229 and 455) were cloned into pSUPER.retro.puro vector (Oligo Engine) and transfected into PT67 packing cell line (Clontech, Palo Alto, CA). Virus-containing supernatants were added to MCF-10A cells for 2 d in the presence of 2 µg/ml of polybrene. A commercially available siRNA negative-control (sequence 1; Ambion) and an empty pSUPER.retro were used as controls. Two days after infection cells were selected with 1 µg/ml of puromycin. Maspin down-regulation by RNAi was confirmed by Western blot analysis, quantified by Image J analysis, and cell pools stably expressing maspin siRNAs were used in adhesion assays.

Immunoprecipitation

MCF-10A cells were cross-linked with 2 mM DTSSP (3,3'-Dithiobis [sulfosuccinimidylpropionate]) plus 2 mM DSS (Disuccinimidyl suberate) in PBS for 30 min at room temperature. Reaction was stopped with 50 mM Tris, pH 7.4. Cell lysates were prepared in modified radio-immuno-precipitation assay (RIPA) buffer: 50 mM Tris pH 7.4, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycolate, 150 mM NaCl, 1 mM Na₃VO₄, 10 mM NaF, 10 mM β-glycerophosphate, 1 mM EDTA pH 8.0, 1 mM EGTA, 100 mM lactose, 1 mM PMSF, 5 µg/ml aprotinin, and 5 µg/ml leupeptin. Extracts were cleared by centrifugation and protein concentration was determined by the Bradford method. Whole cell extract (500 µg) was incubated with 2 µl of specific antisera or control rabbit irrelevant antiserum at 4°C for 2 h. Protein A-Sepharose-coupled beads (Amersham Pharmacia Biotech) were added and incubated for 30 min to 1 h at 4°C under constant agitation. Beads were centrifuged, washed three times with ice-cold extraction buffer containing 25 mM lactose, and boiled for 5 min in sample buffer containing 5% β-mercaptoethanol. Samples were split into two and loaded separately

into 9 and 12% SDS-PAGE gels, respectively. Both gels were transferred to PVDF membrane (Bio-Rad) and analyzed by Western blot with anti-β1 integrin (9% gel) and antimaspin (12% gel). Appropriate secondary antibodies were added and proteins were visualized with enhanced chemiluminescence (ECL) chemiluminescent substrate (Pierce).

Preparation of Triton X-100 soluble and insoluble fractions

Fractionation was as described (14). Briefly, MCF-10A cells were washed twice with microtubule stabilization buffer (0.1 M pipes, pH 6.9; 2 M glycerol; 1 mM EGTA; 1 mM magnesium acetate) and extracted on ice for 5 min in buffer containing 0.2% Triton X-100, 1 mM sodium orthovanadate, 1 mM PMSF, 5 µg/ml aprotinin, and 5 µg/ml leupeptin. The insoluble fraction was solubilized at 4°C for 20 min in 1× RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycolate supplemented with protease inhibitors). Both fractions were cleared by centrifugation, and supernatants were analyzed by Western blot as described in previous section.

Construction, expression and purification of his/glutathione S-transferase (GST)-recombinant maspin

Glutathione S-transferase (GST) and his fusion proteins (Amersham Pharmacia Biotech and BD Bioscience Clontech, respectively) were transformed in *E. coli* BL21 cells, expressed, and purified as instructed by the manufacturers. GST-maspin containing a Arg→Gln point mutation (Mp*), C-terminal deletion (Mp-ΔRSL) and N-terminal deletion (Mp-ΔN) have been previously described (5), as well as fragment containing aa 1–139 (Mp-N) (13). To generate Mp(1–225) and Mp(225–346) mutants, we introduced a *Bgl*II site in maspin cDNA by site directed mutagenesis (Stratagene kit) using the following primers: sense: 5'-CCTTTCCA-GAATACAGATCTGAGTATGCTCATTG-3'; antisense: 5'-CAA-TGAGCATACTCAGATCTGTATTCTGGAAAGG-3', comprising nucleotides 847 to 880. This vector was digested with *Bgl*II and *Eco*RI, generating a fragment encoding a peptide from amino acids 225 to 346, which was subcloned to the *Bam*HI and *Eco*RI sites of pGEX-4T1 bacteria expression vector Mp(225–346). The generation of Mp(1–225) was performed by the digestion of mutant pGEX-Mp construct with *Bgl*II and *Eco*RI (an additional *Eco*RI site is located in the vector sequence flanking the stop codon of maspin cDNA), blunt-ending the restriction sites and self-ligation of the blunt-ends.

Adhesion assay

For the generation of the endogenous MCF-10A-deposited matrix, we followed the method described previously (15). MCF-10A 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, and wells were extensively washed with water. Wells were blocked with 10 mg/ml of heat-denatured BSA in PBS (80°C 15 min) for 1 h at room temperature. Subconfluent cultures were trypsinized, washed with warm serum-free DMEM/F12 medium, and incubated with antibodies or recombinant proteins (0.5 µM) for 30 min at 37°C (when assaying endogenous maspin enzyme-free cell dissociation solution (Cell and Mol. Technologies, Inc.) was used instead of trypsin). In all the assays, 2 × 10⁴ cells/well were plated in triplicates and allowed to adhere for 30 min at 37°C. When his-maspin was used, an irrelevant his-protein was used as a control. Wells were washed with warm serum free DMEM/F-12 and adhered cells were

fixed with 5% glutaraldehyde and stained with crystal violet. A blank value corresponding to BSA-coated wells ($\leq 5\%$ of maximal cell adhesion) was automatically subtracted. Dye was solubilized in 10% acetic acid, and absorbance was determined at 590 nm. For the function blocking study, cells were first incubated with the anti- $\beta 1$ function blocking Ab for 10 min and recombinant maspin was added for another 25 min. Alternatively, cells were incubated first with maspin for 10 min, followed by the Ab for 25 min. Adhesion was plotted as percentage of the control value. Statistical analysis was done by the Student's *t* test with $P < 0.05$ considered statistically significant or by the ANOVA analysis followed by post hoc test.

Fluorescence microscopy

For all the assays cells were plated on glass coverslips at 50–70% density. To determine the extracellular accessibility of maspin, incubation with the first Ab was done without fixation or permeabilization on ice, to avoid its internalization (16). To block nonspecific sites, cells were first incubated in D-MEM/F12 containing 2% BSA for 1 h at 4°C. Maspin AbS4A Ab (anti-RSL) was then added in the same medium for 2 h at 4°C. Preimmune serum was used as a negative control. Cells were then washed with ice-cold PBS, fixed with 4% paraformaldehyde, and treated with appropriate secondary antibodies for 30 min at room temperature. Cells were washed again and incubated with 10–20 mg/ml of Hoechst 33342 for nuclei staining (Molecular Probes). To investigate maspin association with cytoskeleton, cells were first treated with cytoskeleton (CSK) buffer for 3 min (10 mM pipes, pH 6.8; 300 mM sucrose; 100 mM sucrose; 100 mM NaCl; 3 mM $MgCl_2$; 1 mM EGTA; 0.5% Triton X-100; protease inhibitors), washed twice with PBS, and fixed with 4% paraformaldehyde for 15 min at room temperature. For the other staining reactions, cells were fixed in 4% paraformaldehyde and reaction proceeded as described above. Single optical section was analyzed by a laser-scanning confocal microscope (model 510; Carl Zeiss MicroImaging, Inc.) using a Plan-Apochromat 63X/1.4 numerical aperture (NA) objective lens for Fig. 1, a Plan-Apochromat 100X/1.4 NA for Figs. 6B and 7 or a Plan-Neofluar 40X/1.30 NA for Fig. 6C. The acquisition software used was LSM image browser (Carl Zeiss MicroImaging, Inc.).

Images analysis

Colocalization was done using the public domain ImageJ program, developed at the U.S. National Institutes of Health (<http://rsb.info.nih.gov/ij/>) using the Colocalization Test plugin developed by Wayne Rasband (Research Services Branch, National Institute of Mental Health, Bethesda, MD) and Tony Collins (Wright Cell Imaging Facility, University Health Network, Toronto, Canada), available at <http://www.uhnresearch.ca/facilities/wcif>. The Colocalization Test calculates the Pearson's correlation coefficient (Robs) between the two selected channels (red and green) (17) and compares it with coefficients that are calculated if there was only random overlap (Rrand) (18). The observed correlation (Robs) between the red and the green channel was considered significant if it was greater than 95% of the Rrand values. Pearson's correlation ranges from -1 to 1 , and is equal to 1 for 100% colocalization and 0 for random overlap of proteins. The test has been applied to background-subtracted images.

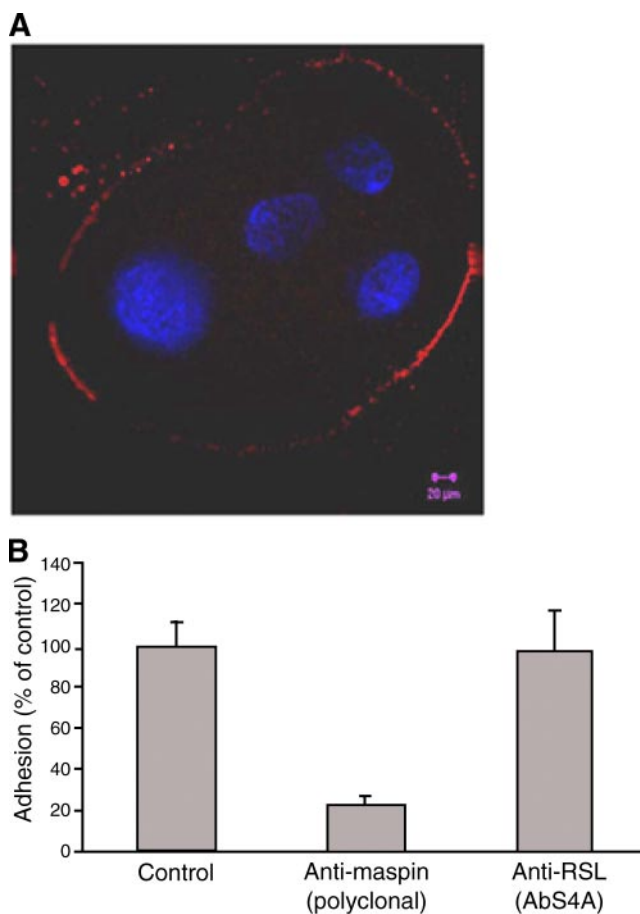


Figure 1. Polyclonal anti-maspin Ab, but not an anti-RSL Ab, generated against the RSL domain of maspin, inhibits MCF-10A cell adhesion to the self-deposited matrix. *A*) MCF-10A cells were plated on coverslips and stained with the rabbit anti-RSL (AbS4A) Ab on ice, to prevent the internalization of the Ab. Preimmune serum revealed no staining (data not shown). This image is representative of two independent assays. Confocal microscope analysis revealed maspin on the cell surface. Bar, 20 μ m. *B*) MCF-10A cells were harvested with enzyme-free dissociation buffer, and preincubated with the indicated antibodies. 2×10^4 cells/well were seeded and cell adhesion was measured after 30 min using colorimetric reaction as described in Materials and Methods. Polyclonal anti-maspin inhibited cell adhesion by 76%, whereas no effect was detected with the anti-RSL Ab. Control, preimmune serum. Each sample was measured in triplicate. Result is representative of three independent assays. Statistical analysis was done by a *t* test ($P < 0.05\%$).

RESULTS

Maspin rapidly modulates cell adhesion

MCF-10A is a nontransformed immortalized human mammary epithelial cell line, which has been extensively used as a model to explore different aspects of cell-extracellular matrix (ECM) interactions. These cells deposit endogenous ECMs and can nucleate adhesive complexes typical of epithelia (19, 20). In addition, MCF-10A cells can recapitulate other epithelial features when grown in a 3D environment, undergoing growth arrest, forming uniform spherical colonies that

present polarized cells with a hollow lumen and basal secretion of basement membrane components (21). These cells express high endogenous levels of maspin, providing an excellent model for investigating its function in cell adhesion. Since adhesion primarily involves the interaction between the cell membrane and the substrate, we first investigated if maspin is present on the cell surface by immunofluorescence. To address this issue, cells grown on coverslips were incubated with anti-maspin without any permeabilization or fixation on ice, in order to prevent Ab internalization (see details in Material and Methods). Figure 1A showed maspin labeling along the perimeter of the cell, confirming that it is present on the external side of the plasma membrane. Given that maspin is expressed on the cell surface, we asked if incubation of cells with anti-maspin could prevent cell adhesion to its endogenously assembled matrix. To test this possibility, subconfluent MCF-10A cells were harvested with enzyme-free dissociation buffer, preincubated with polyclonal anti-maspin Ab or preimmune Ab (control) in serum-free medium, and allowed to adhere to the self-deposited matrix for 30 min. We observed a 76% decrease in cell adhesion in the presence of a polyclonal anti-maspin antiserum compared to the control serum (Fig. 1B).

To gain further support for this finding, we employed RNA interference to specifically down-regulate maspin expression in these cells. Maspin siRNA 229 and 455 have been previously described (13). MCF-10A cells were transfected with these siRNA-expressing vectors and selected, and maspin protein down-regulation was confirmed by Western blot analysis (Fig. 2A, upper panel). Protein loading was controlled by reprobing the membrane with an anti-tubulin Ab (Fig. 2A, lower panel). Normalization of maspin bands with that of tubulin indicated that maspin down-regulation by RNAi was ~82% in both 229 and 455 siRNAs cells (Fig. 2B). siRNA-expressing cells exhibited significantly less adhesion compared with the control cells (Fig. 2C), further supporting maspin's role in adhesion. Previously, Seftor et al. observed an increase in integrin gene expression after 18–24 h of incubation with recombinant maspin (22), suggesting that maspin acts indirectly in the adhesion process. In our experiments, however, maspin effect was observed within 30 min. To re-ensure that, in our model, maspin acts in a different kinetics, we applied a similar approach, incubating MCF-10A cells with recombinant GST-maspin for a short period of time. For this purpose cells were trypsinized, which renders the endogenous maspin inactive (10, 23, 24). After harvesting, cells were washed and preincubated either with GST or increasing concentrations of GST-maspin and adhesion assay was carried out as described in Material and Methods. We observed a dose-dependent increase in cell adhesion, which was up to 3-fold higher than control cells incubated with GST alone (Fig. 2D). We conclude that exogenously added recombinant maspin can rapidly increase MCF-10A cell adhesion to its endogenous

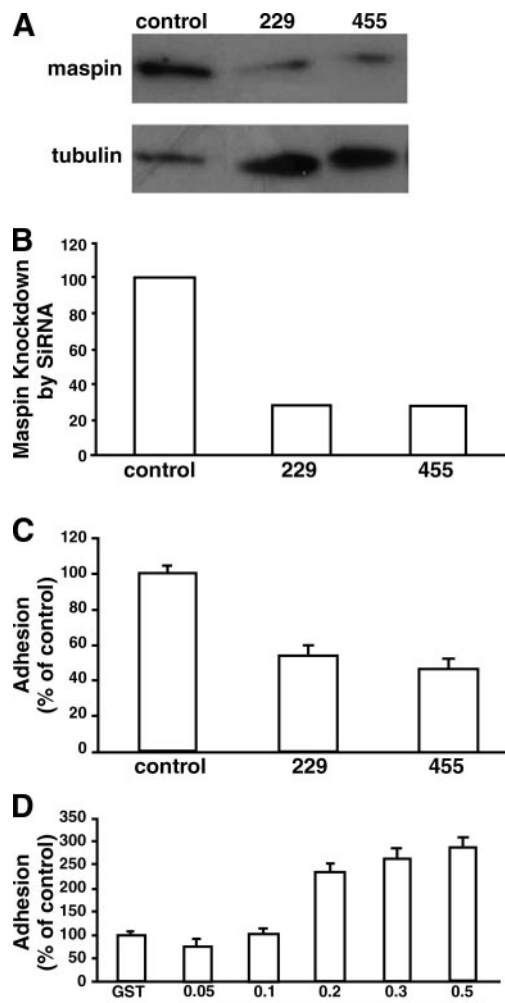


Figure 2. Maspin suppression by siRNA decreases cell adhesion and addition of recombinant maspin increases cell adhesion. A) MCF-10A cells were stably infected with control vector (control) or with maspin siRNA 229 and 455 vectors and maspin protein levels were analyzed by Western blot (upper panel, 15 μ g/lane). The membrane was subsequently reprobed with an anti-tubulin Ab for loading control (lower panel). B) Bands were quantified by Image J analysis and specific maspin down-regulation was plotted in arbitrary units. C) MCF-10A cells stably expressing empty vector (control) or maspin siRNA 229 and 455 vectors were plated on endogenous matrix (2×10^4 cells/well), and cell adhesion was quantified as described in Materials and Methods. siRNA expressing cells adhered 46.7% (229) and 53.6% (455) less than control cells. D) MCF-10A cells were preincubated with GST (0.5 μ M) or GST-maspin at the indicated concentrations for 30 min. 2×10^4 cells/well were plated on endogenous matrix, and cell adhesion was quantified as described in Materials and Methods. We observed a dose-dependent increase in cell adhesion in the presence of maspin. This result is representative of three independent assays. Statistical analysis was done by a *t* test ($P < 0.05$).

matrix. Altogether, these results indicate that in the MCF-10A model maspin interferes with early steps in the adhesion process and therefore appears to act differently from previous studies done in tumor and stromal cells.

Maspin promotes cell adhesion independently of its RSL domain

The RSL is a conserved serpin domain, which is essential for its antiprotease activity (25). The maspin RSL domain possesses unique sequence and structure, which makes it unlikely to be involved in protease inhibition (11). However, it has been shown to be essential for maspin-mediated inhibition of cell invasion and migration (7, 23). To test whether maspin RSL domain is also involved in its rapid action on cell adhesion in normal mammary epithelial cells, MCF-10A cells were incubated with a purified pAb raised against the RSL domain (AbS4A) of maspin (2) and allowed to adhere to its endogenous matrix as described above. Surprisingly, the treatment with anti-RSL Ab had no detectable effect on MCF-10A adhesion to the self-deposited matrix (Fig. 1B). This lack of effect was not due to inaccessibility of the maspin RSL domain, since this Ab could detect maspin on the cell surface by immunofluorescence (Fig. 1A). To confirm this observation we used two independent approaches: 1) we compared the effect of wild-type (WT) recombinant maspin with that of mutant proteins bearing a point mutation in the RSL loop (Mp*) and a C-terminal deletion of the RSL domain (Mp-ΔRSL) (Fig. 3; and 2) WT maspin was preincubated with an anti-RSL Ab before incubation with cells (23, 26). Under both conditions, the ability of maspin to increase cell adhesion was assayed as described above. We observed that recombinant maspin carrying either a C-terminal deletion or point mutation in the RSL resulted in an increased adhesion, comparable to WT maspin (Fig. 3B). In addition, no difference in cell adhesion could be detected when maspin was preincubated with an anti-RSL Ab (Fig. 3C).

We next attempted to determine which portion of maspin is important for this rapid induction of adhesion. For this purpose we generated several maspin constructs for the production of mutant maspin proteins as described in Materials and Methods. Initially, we used proteins containing only the first 139 amino acids (Mp-N) and another fragment where this N-terminal portion had been deleted (Mp-ΔN) (5) (Fig. 4). We observed that Mp-N failed to increase cell adhesion, while the carboxyl half of the molecule retained most of the effect observed with WT maspin (Fig. 4B). To further identify the functional domain, we constructed two additional mutants, which contain amino acids 1–225 and amino acids 225–346, respectively (Fig. 4A), and tested these proteins in cell adhesion assays. Mp(225–346) had no effect on MCF-10A adhesion to the endogenous matrix (Fig. 4C). Mp(1–225), in contrast, retained a decreased, but significant ability to sustain cell adhesion (Fig. 4C). All together, these experiments indicate that maspin RSL domain is not involved in maspin-dependent increase in cell adhesion. However, a portion of the 86 residues between amino acid 139–225 appears to contain the domain responsible for the rapid effect of maspin on

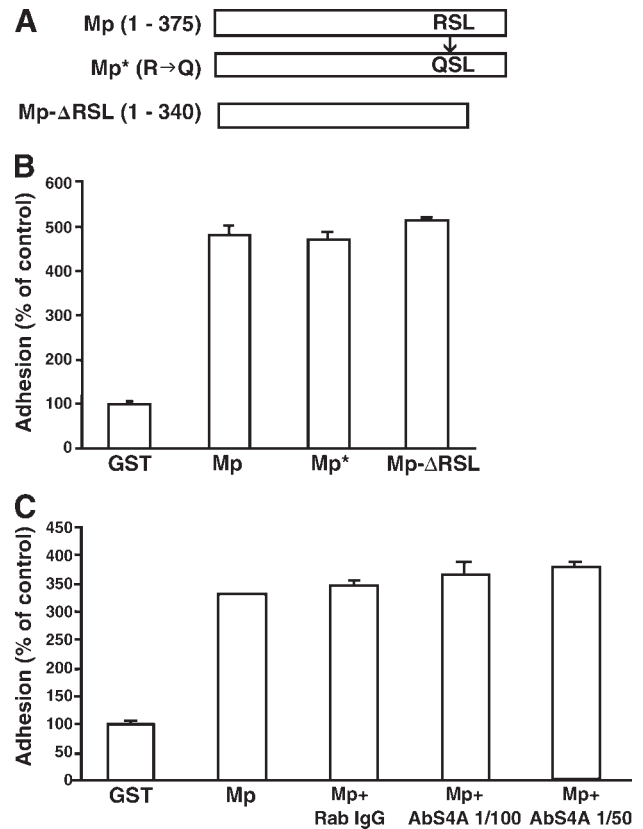


Figure 3. Maspin RSL domain is not involved in maspin-induced rapid adhesion to MCF-10A endogenous matrix. *A*) Recombinant GST-maspin constructs used in adhesion assays: Mp(1–375 aa) – WT GST-maspin; Mp*(R→Q) – point mutation on the RSL domain (arginine to glutamine); Mp-ΔRSL(1–340 aa) – C-terminal deletion. *B*) MCF-10A cells were preincubated with the indicated recombinant proteins (0.5 μM) for 30 min. 2×10^4 cells/well were plated on endogenous matrix and adhesion was quantified as described in Materials and Methods. *C*) Antibodies were preincubated with GST-maspin at the indicated dilutions for 30 min and sequentially incubated with cells for additional 30 min. Adhesion assays were performed as described. We observed a 2- to 3-fold increase in cell adhesion in the presence of maspin, which did not change significantly when cells were incubated with mutants of the RSL region or with anti-RSL Ab. Result represents three independent assays. Statistical analysis was done by ANOVA ($P < 0.05\%$).

MCF-10A adhesion to the self-deposited matrix. These findings further support the idea that maspin acts via a different mechanism in the MCF-10A model.

Maspin can override the inhibiting effect of anti-β1 integrin

Cells interact with the ECM proteins via transmembrane proteins, of which the integrin superfamily of cell surface receptors has been the most studied. MCF-10A matrix is mainly composed of laminin-5 (19), to which these cells adhere predominantly via α3β1 integrin (27, 28). Therefore, in order to investigate the involvement of β1 integrin in maspin-induced increase in adhesion, we examined the effect of maspin in adhesion in

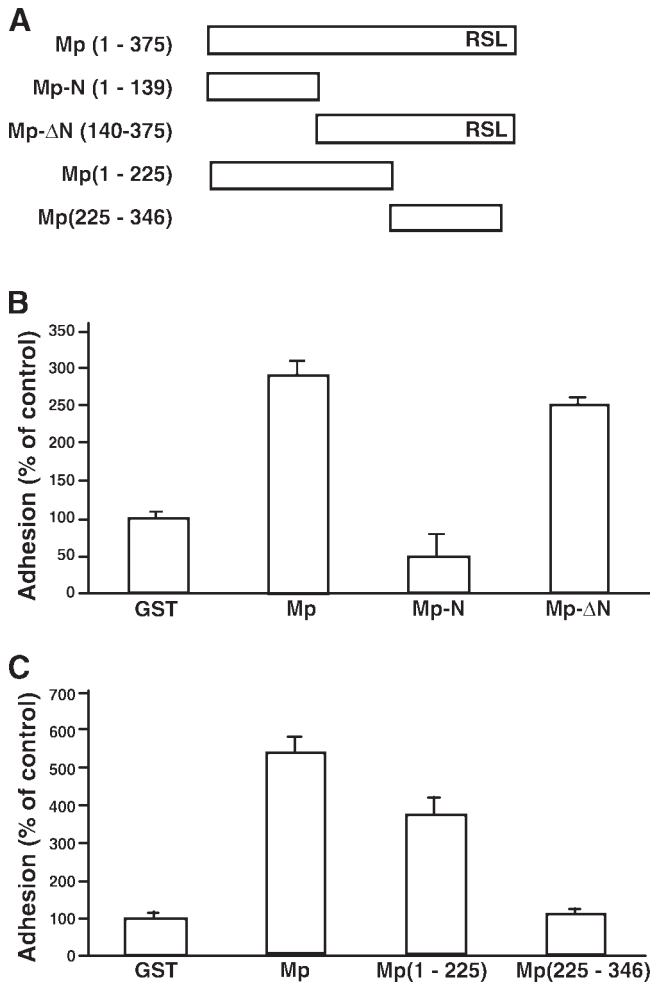


Figure 4. Rapid effect of maspin on MCF-10A adhesion depends on its N-terminal domain. *A*) Recombinant GST-maspin constructs used in adhesion assays. *B* and *C*) MCF-10A cells were preincubated with the indicated recombinant proteins (0.5 μ M) for 30 min and allowed to adhere for another 30 min. Adhesion was measured as described in Materials and Methods. Note that the domain between amino acids 139–225 is involved in maspin-induced increase in cell adhesion. This result is representative of three independent assays. Statistical analysis was done by ANOVA ($P < 0.05\%$).

conditions where this integrin subunit was occupied by the function-blocking Ab AIIB2 (Fig. 5). As shown in previous results, cells exhibited a 5-fold increase in cell adhesion in the presence of maspin (Fig. 5, bar number 3). Surprisingly, cells that were preincubated with AIIB2 followed by incubation with his-maspin still exhibited 5-fold increase in adhesion (Fig. 5, compare bar number 5 with inset), albeit 50% less cells adhered (compare bars 3 and 5). Since adhesion of MCF-10A cells to its self-deposited matrix is mediated by β 1 integrin, the concentration of AIIB2 used (1 μ g/ml) was probably not enough to completely block all the β 1 integrin sites, since we observed only a 50% adhesion inhibition with this concentration (inset). Therefore, the unblocked β 1 integrin sites most likely account for the same fold increase in adhesion in the presence of maspin. No effect was observed when an anti-human

MHC I Ab was used (bar number 4), indicating that this effect was specific for β 1 integrin. Interestingly, when maspin was added before the anti- β 1 integrin, the inhibiting effect given by this integrin Ab was no longer observed (Fig. 5, bar number 6). This result indicates that even though the total adhesion is inhibited, preincubation of cells with maspin could override the inhibition.

Maspin colocalizes and is physically associated with β 1 integrin

Given that maspin effect appears to be membrane-associated and can interfere with the adhesion inhibition given by anti- β 1 integrin, we postulated that maspin could also be physically associated with this integrin subunit. We tested this hypothesis by coimmunoprecipitation using antimaspin and anti- β 1 integrin antibodies. MCF-10A cells were chemically cross-linked, lysed in RIPA buffer, and immunoprecipitated with antimaspin or anti- β 1 integrin antibodies under stringent conditions to disrupt the noncovalent association between the integrin and maspin. An irrelevant rabbit antiserum was used as a negative control (Fig. 6A). Following immunoprecipitation, protein complexes were dissociated under reducing condition to break covalent cross-linked bonds. Western blot analysis of the precipitated material revealed that cell lysates contained substantial amounts of maspin (Fig. 6A, left lower panel, lane 2) and β 1 integrin (Fig. 6A, right upper panel, lane 5). In addition, we observed that β 1 integrin was detected in the maspin immunoprecipitate (Fig. 6, left upper panel, lane 2). Conversely, maspin was also detected in the β 1 integrin immunoprecipitate (Fig. 6A,

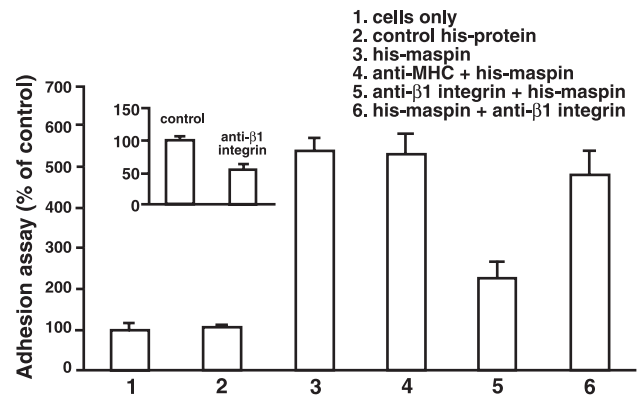


Figure 5. Maspin can override the inhibiting effect of anti- β 1 integrin. MCF-10A cells were harvested and preincubated with indicated recombinant proteins. Anti-MHC I (4) or anti- β 1 integrin (5) were incubated with cells for 10 min (1 μ g/ml), and recombinant his-maspin was added subsequently (0.5 μ M) for another 25 min. In bar number 6, his-maspin was added to cells first (10 min), and anti- β 1 integrin was added subsequently. Adhesion was measured as described previously. This result is representative of four independent experiments. Inset: anti- β 1 integrin down-regulates MCF-10A adhesion by itself. Statistical analysis was done by ANOVA ($P < 0.05\%$).

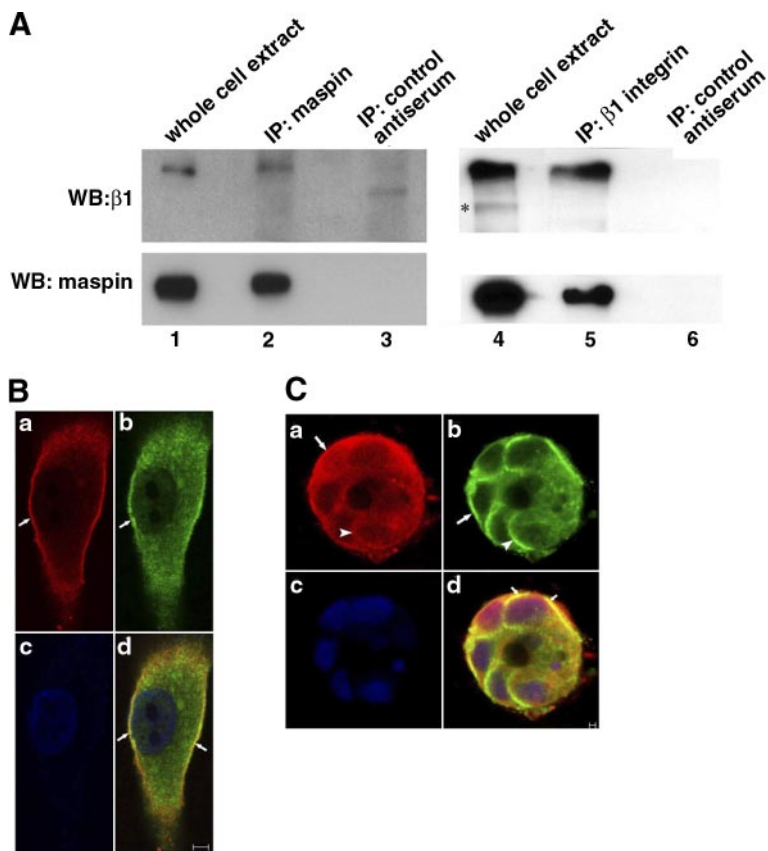


Figure 6. Maspin and $\beta 1$ integrin coimmunoprecipitate and colocalize in MCF-10A cells grown in monolayers and in 3D culture. *A*) MCF-10A cells were chemically cross-linked and lysed, and 500 μg of protein extracts were immunoprecipitated with the indicated antibodies. An irrelevant rabbit antiserum was used as a negative control (lanes 3 and 6). Immunoprecipitates were separated in SDS-PAGE gels as detailed in Materials and Methods and analyzed by Western blot as indicated. *Pre- $\beta 1$ integrin. *B*) MCF-10A cells were plated on coverslips, fixed, permeabilized, and stained with a mouse mAb anti-human maspin (*a*) and a rabbit polyclonal anti- $\beta 1$ integrin (*b*). Nucleus is shown in blue (*c*) and merged image is shown in (*d*). Arrows indicate that maspin and $\beta 1$ integrin are located in the periphery of the cell (*a* and *b*, respectively) and are colocalized in (*d*, $R_{\text{obs}}=0.704/R_{\text{rand}}=0.060\pm 0.012$); Bar 10 μm . *C*) MCF-10A cells were embedded in the Matrigel and allowed to form acini for 15 d. Cryosections (8 μm) were prepared and stained for maspin (*a*) and $\beta 1$ integrin (*b*), using the same antibodies mentioned above. Nuclei are shown in blue (*c*) and merged image is shown in (*d*). Arrow and arrowhead in (*a*) and (*b*) indicate localization on the basal membrane and in sites of cell-cell contact, respectively. Arrows in (*d*) indicate sites of maspin and $\beta 1$ integrin colocalization ($R_{\text{obs}}=0.580/R_{\text{rand}}=0.115\pm 0.019$); Bar, 20 μm .

right lower panel, lane 5), further supporting the association between maspin and $\beta 1$ integrin. Although we observed maspin on the cell surface (Fig. 1A), there is a possibility that maspin may associate with $\beta 1$ integrin in the intracellular or intramembrane space. For this reason, we chose to perform chemical cross-linking with DTSSP and DSS simultaneously, two cross-linking reagents that are membrane-impermeable and -permeable, respectively. DTSSP would cross-link maspin and $\beta 1$ integrin if their interaction occurs extracellularly. DSS would cross-link them if their interaction occurs in the intramembrane or intracellular space. With this approach we assured ourselves that maspin and $\beta 1$ integrin interaction would be cross-linked and detected. Notably, under reducing conditions maspin and $\beta 1$ integrin migrated at their respective MW. No high MW complex was observed in Western blot analysis (data not shown), indicating that they were preferentially cross-linked by DTSSP, which can be cleaved by reducing agents, and not by DSS, which cannot be reduced and therefore can't be cleaved. Since DTSSP cannot cross the plasma membrane, and thus it can only cause cross-linking among cell surface proteins, these data together suggest that maspin associates with $\beta 1$ integrin extracellularly.

Since maspin interacts with $\beta 1$ integrin, we reasoned that these proteins would also localize in the same place in MCF-10A cells. To determine if there was colocalization, we carried out indirect double-immunofluorescence to analyze the staining patterns of maspin and $\beta 1$ integrin by confocal microscopy (Fig. 6B). Cells were

first cultivated as monolayers on coverslips, fixed, permeabilized, and stained with a mAb anti-maspin (Fig. 6B, *a*) and a polyclonal anti- $\beta 1$ integrin (Fig. 6B, *b*). Strong staining along the cell perimeter was detected for both maspin and $\beta 1$ integrin (Fig. 6B, *a* and *b*, arrows). Importantly, visual inspection of the merged image showed some sites of their colocalization on the membrane (Fig. 6B, *d*, arrows), which was quantitatively confirmed by the Pearson's correlation coefficient (17).

Epithelial cells grown in monolayers can display several of their normal functions. They can proliferate, migrate, differentiate, and die. However, under these conditions they cannot recapitulate the epithelial architecture, which depends on the hallmark properties of epithelial cells, i.e., their ability to polarize, forming apical and basolateral surfaces with vectorial deposition of ECM elements. Since $\beta 1$ integrin expression is altered in cells plated on plastic and proper signal transduction depends on the spatial organization of the cell (29, 30), we were interested in confirming whether maspin and $\beta 1$ integrin colocalize in polarized epithelial cells in 3D structure compared to that which were cultivated in monolayers. To verify the colocalization of maspin and $\beta 1$ expression and distribution in MCF-10A acini. MCF-10A cells were seeded in the Matrigel and allowed to develop an acinar-like structure for 15 d. Acinar structures were analyzed on frozen section by confocal microscopy. MCF-10A cells organized to form a single polarized layer surrounding a hollow lumen

(Fig. 6C). Analysis by immunofluorescence revealed maspin localized predominantly to the basal membrane (Fig. 6C, *a*, arrow) and, to a lesser extent, to sites of cell-cell contact (Fig. 6C, *a*, arrowhead). This finding contrasts with maspin staining done on monolayer cultures, where no maspin was observed on sites of cell junctions. It is possible that in nonpermeabilized cells the access of the Ab to cell junction sites is limited. Alternatively, maspin localization in cell-junction depends on proper cell polarization, which is not achieved in monolayer cultures. β 1 integrin appeared to be equally distributed between cell-matrix and cell-cell contacts (Fig. 6C, *b*, arrow and arrowhead, respectively). The merged image indicated that maspin and β 1 integrin colocalize in some sites in polarized MCF-10A acini as well (Fig. 6C, *d*, arrows), which was further confirmed by quantitative analysis. All together, these data indicate that maspin increases cell adhesion via a β 1 integrin-dependent mechanism.

Maspin is associated with the Triton X-100 insoluble cytoskeleton fraction

Binding of integrin receptors to extracellular ligands is a complex process involving receptor-ligand interactions at the cell-substrate interface and assembly of cytoskeletal and adhesion plaque proteins (31). The rapid cellular response to recombinant maspin indicates that maspin acts on the initial steps of cell-substrate interaction and could be connected to the underlying cytoskeleton framework. To address this question, we first determined maspin's solubility in a nonionic detergent. Accordingly, MCF-10A cells were first extracted with Triton X-100 buffer (Triton-soluble fraction) and sequentially extracted with RIPA buffer (Triton-insoluble fraction). Western blot analysis confirmed that maspin was present in both fractions (Fig. 7A, *upper panel*). Purity of fractions was confirmed by reprobating the membrane with an anti-bax Ab, a Triton-soluble cytosolic protein (Fig. 7A, *lower panel*). To localize the cytoskeleton-associated maspin in the cell immunostaining was performed on cells that had been treated for 3 min with CSK buffer before fixation (see Material and Methods for details). In agreement with the cellular fractionation, maspin staining was resistant to Triton extraction and was found at the periphery of the cell (Fig. 7B, *a*). Notably, β 1 integrin staining had a very similar distribution (Fig. 7B, *b*). Quantitative analysis indicated some degree of colocalization, although it is difficult to visually determine sites of colocalization (Fig. 7B, *d*). These results suggest that maspin is connected to the cortical membrane cytoskeleton and may be part of the supramolecular structure of the adhesion plaque.

DISCUSSION

This study was designed to uncover the mechanisms underlying a maspin-induced increase in cell adhe-

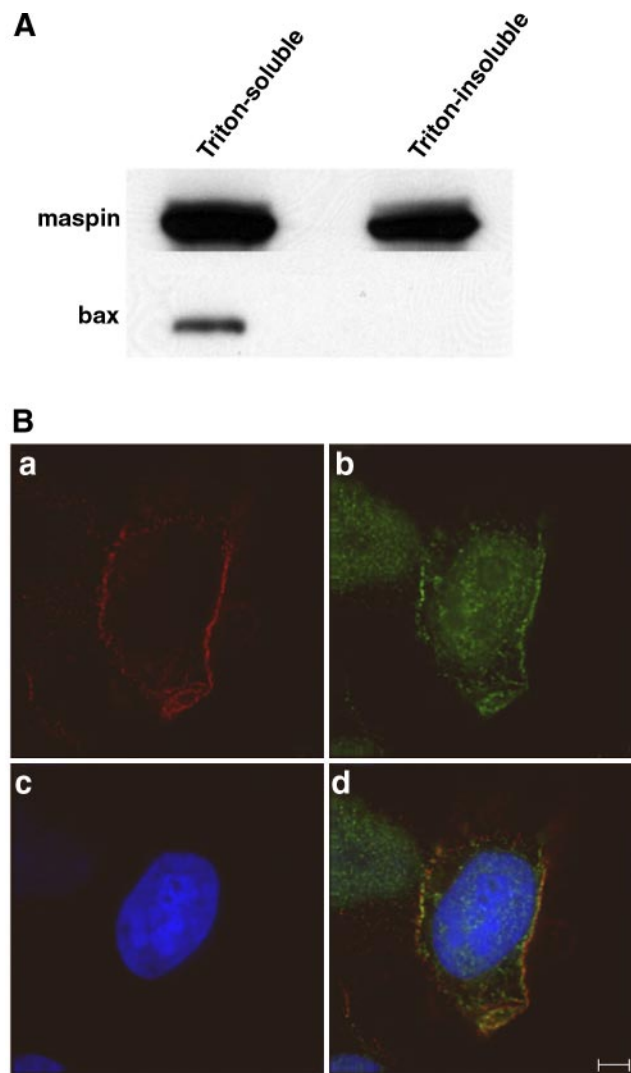


Figure 7. Maspin is associated with the Triton X-100-insoluble cytoskeleton fraction. *A*) Triton X-100 soluble and insoluble fractions were prepared as described in Materials and Methods. Protein extracts (50 μ g) were separated in a 10% SDS-PAGE, and maspin was detected by Western blot analysis (*upper panel*). Purity of fractions was controlled by reprobating the blot for bax, a Triton-soluble protein (*lower panel*). *B*) MCF-10A cells were plated on coverslips and pretreated for 3 min with CSK buffer in order to extract Triton X-100 soluble proteins. Cells were then fixed and stained with a mouse mAb anti-human maspin (*a*) and a rabbit polyclonal anti- β 1 integrin (*b*). Nuclei are shown in blue (*c*) and the merged figure is shown in (*d*). Although not possible to visualize, quantitative analysis indicated colocalization (Robs=0.507/Rrand=0.050 \pm 0.013). Bar 10 μ m.

sion. We have provided strong evidence that maspin acts rapidly on the cell surface, stimulating cell adhesion via integrin receptors. Maspin is physically and functionally associated with β 1 integrin. In particular, maspin and β 1 integrin are colocalized on the cell surface, and maspin is also found to be associated with cytoskeleton elements, suggesting that it is part of the supramolecular structure of adhesion plaques.

Maspin increases cell adhesion independently of its RSL domain

In contrast to most published results, we have used nontransformed mammary epithelial cells (MCF-10A) that express high endogenous concentration of maspin. In our adhesion assays we found that maspin increased cell adhesion independently of its RSL domain. This result is in apparent contrast with previous reports, which showed that maspin's effect on tumor and stromal cells was dependent on its RSL domain (7, 23, 32). While this difference may be due to the use of different cellular models, it may also indicate that maspin acts differently in normal vs. tumor or stromal cells. In MDA-MD-231 breast tumor cells, maspin appears to increase cell adhesion via a mechanism, which leads to increased $\alpha 5\beta 1$ integrin mRNA expression (22). However, in these studies cells were treated with maspin for 18 to 24 h, suggesting that maspin acted indirectly in the cell adhesion process. The fact that we observed maspin acting rapidly and independently of the RSL domain indicates that maspin is acting via a different mechanism in the MCF-10A model to regulate cell adhesion. Similarly, maspin inhibition of angiogenesis is also independent of the RSL domain (4, 5). In addition, a third distinct domain in maspin appears to mediate maspin interaction with type I and III collagen (33). All together these data suggest that maspin is a multifunctional protein that can exert its effects via different molecular mechanisms. We have found that a region spanning amino acids 139–225 is responsible for the maspin's positive effect on adhesion. Based on the X-ray crystal structure of maspin (24, 34), this region contains several structural motifs including s3A, s3C, and s1B. For strand s3A, due to its structure, many of the side chains are buried inside the protein. However, strands s3C and s1B (aa 180–210) are surface-exposed. It is likely that amino acids in strands s3C and s1B may mediate cell-adhesion effect. There is also a possibility that a deletion of this region may affect the folding and structure of other regions of maspin. Thus, we plan to perform "domain-swapping" experiment using different serpins and site-specific mutagenesis to identify the specific amino acids that are functionally important for adhesion.

Maspin can overcome the inhibiting effect of anti- $\beta 1$ integrin

We observed an overall decrease in cell adhesion when cells are first incubated with anti- $\beta 1$ integrin. Interestingly, this effect was abrogated when cells were incubated with maspin before the Ab addition. Although we cannot completely explain this observation yet, we envision at least four possible interpretations. 1) When added before maspin, anti- $\beta 1$ integrin could act as a dominant-negative effector in the adhesion process, since it inhibits adhesion by itself; 2) anti- $\beta 1$ integrin could be interfering in the maspin binding per se, perhaps competing for the same site. AIIB2 binds to a

small region on the $\beta 1$ integrin subunit (residues 207–218), which is the same region where activating anti- $\beta 1$ integrin mAb binds (35). In this model maspin could be acting like an activating anti- $\beta 1$ integrin; 3) maspin could polymerize and form a lattice around the adhesion site, which would prevent AIIB2 from binding to its epitope. In fact maspin has been shown to form polymers in vitro (10, 24, 36) and in vivo (Latha *et al.*, unpublished data); 4) maspin interaction to its ligand on the cell surface could result in a conformational change of the $\beta 1$ integrin, which would hide or mask AIIB2 binding site. Finally, these possibilities are not mutually exclusive, so it remains possible that two or more of these events might be taking place.

Maspin is linked to the cytoskeleton and is physically associated with $\beta 1$ integrin

We have demonstrated here that maspin associates with $\beta 1$ integrin in MCF-10A cells by reciprocal coimmunoprecipitation and colocalization under confocal microscope. Since they were cross-linked by the membrane impermeable agent DTSSP, it is possible that the extracellular portions of $\beta 1$ integrin and maspin are close enough to be involved in a direct interaction. However, an indirect interaction between maspin and $\beta 1$ integrin, via a third molecule, is equally possible.

Maspin biological effects have been previously assigned to the cell surface environment (1, 7, 26). That maspin associates with $\beta 1$ integrin and that it is detected on the cell surface further support this hypothesis. However, in our adhesion assays, maspin has been incubated with cells at 37°C, and therefore it remains possible that maspin was internalized and acted in the intracellular compartment. We observed that maspin stained to the cortical membrane cytoskeleton and was detected in the Triton-insoluble fraction. Since extracellular molecules, which interact with true membrane proteins, remain bound to skeleton framework on nonionic detergent extraction, it is possible that the Triton-resistant maspin is located outside the cell (37–39). Maspin appears to be a peripheral rather than an integral membrane protein (1). The observation that a fraction of cellular maspin is Triton-resistant argues that maspin at times can behave like a fibrous or structural protein. Interestingly, cytoskeleton proteins acquire Triton insolubility very early or concomitantly to the process of adhesion (39). Therefore, we postulate that maspin can modulate cell adhesion as being part of the supramolecular adhesion structures.

The ECM has been proven vital to mammary gland development, and differentiation (40). Particularly, $\beta 1$ integrin signaling is essential for optimal prolactin-induced milk protein gene expression (41), mammary cell proliferation (42), and cell survival (43). In addition, $\beta 1$ integrin is critical for initiation and maintenance of mammary tumor growth (44), and function-blocking anti- $\beta 1$ integrin was able to reverse the malignant phenotype (45). In accordance with $\beta 1$ integrin studies, targeted maspin expression in the

mammary gland disrupted lobular-alveolar structure and milk protein production (12) and, in tumor models, maspin can block tumor growth and invasion (3), processes that depend on cell-extracellular matrix interaction. Besides, maspin and $\beta 1$ -deficient mice displayed remarkably similar phenotypes, further supporting their functional link in vivo (46, 47). All together, these data suggest that maspin and $\beta 1$ integrin may be part of a common signaling machinery, which plays a crucial role in mammary gland function and in tumor initiation and progression. **EJ**

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