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G-helix of Maspin Mediates Effects on Cell Migration and Adhesion*[S]

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Maspin is a member of the serine protease inhibitor (serpin) superfamily that lacks protease inhibitory ability, although displaying tumor metastasis-suppressing activity resulting from its influence on cell migration, invasion, proliferation, apoptosis, and adhesion. The molecular mechanisms of these actions of maspin are as yet undefined. Here, we sought to identify critical functional motifs by the expression of maspin with point mutations at sites potentially involved in protein-protein interactions: the G α-helix (G-helix), an internal salt bridge or the P1 position of the reactive center loop. Our findings indicate that only mutations in the G-helix attenuated inhibition of cell migration by maspin and that this structural element is also involved in the effect of maspin on cell adhesion. The action of maspin on cell migration could be mimicked by a 15-mer G-helix peptide, indicating that the G-helix is both essential and sufficient for this effect. In addition, we provide evidence that the effects of the G-helix of maspin are dependent on β1 integrins. These data reveal that the major extracellular functions associated with the tumor suppressive action of maspin likely involve interactions in which the G-helix plays a key role.

Maspin (*SERPINB5*) is a member of the serpin family of serine protease inhibitors that acts as a type II tumor metastasis suppressor, decreasing tumor growth and metastasis *in vivo* (1, 2) and invasion *in vitro* (3, 4). It is down-regulated in cancers including those of the breast (1) and prostate (5). Exogenous maspin decreases proliferation and increases cell adhesion *in vitro* (6). It inhibits angiogenesis *in vivo* (7) and causes apoptosis when expressed in endothelial cells (8). In addition, we have shown that maspin can inhibit the migration of vascular smooth muscle cells (VSMCs)[3] (9), which has potential ramifications for conditions resulting from vascular injury such as atherosclerosis.

Maspin is expressed by epithelial cells and is essential for normal development because maspin-null mice die at the peri-implantation stage due to a failure of early differentiation events, resulting from aberrant adhesion and cell migration (10). However, the mechanism of action of maspin remains largely unresolved. Although early evidence suggested that maspin was an inhibitory serpin able to block plasminogen activation by urokinase plasminogen activator and tissue-type plasminogen activator (11–13), we demonstrated that this was not the case in a number of conditions where the serpin PAI-1 was inhibitory (9). That maspin is a noninhibitory serpin is supported by crystal structure data revealing that its RCL does not correspond with those found in inhibitory serpins (14, 15). It remains possible that maspin influences protease activity indirectly by noninhibitory interactions with the plasminogen activators (16, 17) and protection of matrix from degradation by cathepsin D (18).

In common with the serpin PAI-2, maspin lacks an authentic signal sequence, but is found outside the cell as well as in the cytoplasm and nucleus. Extracellular maspin interacts with β1 integrins to influence cell adhesion and migration directly (19, 20). We identified α5β1 as being critical for the effects of extracellular maspin on cell migration through a mechanism involving rapid modulation of the activation state of β1 (20). Binding of maspin to β1 integrins on the surface of mammary epithelial cells also modulates early adhesion events (19). Intracellular maspin-binding partners have also been identified, providing direct links to cell proliferation and apoptosis control (4, 8, 21–23).

In this study we aimed to dissect structural motifs of maspin essential for specific aspects of cell function, focusing on regions that were likely to be involved in the extracellular actions of maspin and that we hypothesized would be of potential importance based on crystal structure information (15). These were the unusual "G" α-helix of maspin, an internal salt bridge that causes a unique bulge in the region of the D and E helices, and the RCL, which has been implicated in the effects of maspin on cell adhesion (6, 14) and apoptosis (22, 24). We found that the G-helix was critical for the effect of maspin on cell migration and adhesion. Significantly, we show that the G-helix is necessary and sufficient for maspin effects on migration because a 15mer peptide encompassing this region was able to replicate the effects of the full protein. Finally, our data indicate that the G-helix is involved in the previously reported interactions of maspin with β1 integrins.

EXPERIMENTAL PROCEDURES

Cell Lines, Antibodies, and Peptides—MCF-7, DU145, PC3, LNCaP and HT-29 cell lines were obtained from ATCC. MCF-7 cells were grown in minimal essential medium, supple-

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[3] The abbreviations used are: VSMC, vascular smooth muscle cell; DMSO, dimethyl sulfoxide; ECM, extracellular matrix; RCL, reactive center loop; serpin, serine protease inhibitor.

mented with 10% (v/v) fetal calf serum (FCS), 1% (v/v) nonessential amino acids and 1% (v/v) sodium pyruvate. DU145, PC3, and LNCaP cells were maintained in RPMI 1640 medium supplemented with 10% (v/v) FCS. HT-29 cells were maintained in DMEM with 10% (v/v) FCS. Primary aortic smooth muscle cells (referred to as VSMCs) were cultured as detailed previously (20). All cell culture reagents including extracellular matrix (ECM) components were from Invitrogen. Monoclonal antibodies 12G10 and mAb13 were kindly provided by Prof. Martin Humphries (University of Manchester, UK). Commercially available mouse monoclonal antibodies were used to detect maspin (BD Biosciences), V5 (Invitrogen), and E-cadherin (BD Biosciences). Integrin αv function blocking antibody (mAb1980) was from Millipore. Control IgG and secondary antibodies were from Dako (Ely, UK) and Invitrogen. Maspin peptides were synthesized with biotin-aHx on their N terminus and an amide on their C terminus; the G-helix peptide consisted of the wild-type sequence (EDESTGLEKIEKQLN), for comparison with two peptides containing the G-helix mutations E244A (EDESTGLAKIEKQLN) and E247A (EDESTGLEKIAKQLN), in addition to a control peptide (EDESTGELKILKQEN) (Alta Bioscience, University of Birmingham, UK). Peptides were prepared to 80% purity and stock solutions prepared in dimethyl sulfoxide (DMSO).

Plasmids and Transfection—Generation and transfection of maspin plasmid referred to as pcDNA3.2-Maspin and the control empty vector pcDNA3.2 were described previously (20).

Site-directed Mutagenesis—Maspin constructs containing point mutations were generated using the QuikChange Site-directed Mutagenesis kit according to the manufacturer's instructions (Stratagene). Mutants made were K90A (CTTTTACTCACTGAAACTAATCGCGCGGCTCTACGTAGACAAATC), E115A (GAAGAGACCCTATGCAAAGGCGTTGGAAACTGTTGACTTC), R340A (CATAGAGGTGCCAGGAGCAGCGATCCTGCAGCACAAGGATG), E244A (GATGAGTCCACAGGCTTGGCGAAGATTGAAAAAGAACTC), and E247A (CACAGGCTTGGAGAAGATTGCGAAACAACTCAACTCAGAG). Mutations were confirmed by DNA sequencing. Plasmids were referred to as pcDNA3.2-K90A, -E115A, -R340A, -E244A, and -E247A.

Preparation of Cell Lysates and Western Blotting—Cell lysates were prepared by the method reported previously (25). For Western blotting, samples separated by SDS-PAGE on a 10% resolving gel were transferred to polyvinylidene difluoride membranes (Bio-Rad). Protein bands were detected by incubation with the appropriate antibody followed by HRP-conjugated secondary antibodies (0.65 μg/ml).

Trichloroacetic Acid Precipitation of Conditioned Media—Conditioned, serum free media was collected from transiently transfected cells (1 ml of medium from 2×10^5 cells) after 24-h incubation and concentrated 20-fold by trichloroacetic acid precipitation. One-third volume of 50% (v/v) trichloroacetic acid was added to conditioned medium and incubated for 5 min at 4 °C. Samples were centrifuged at 2,000 $\times$ *g* for 5 min at 4 °C, and pellets were washed twice in cold acetone before being resuspended in reducing Laemmli sample buffer.

Generation of Stable Cell Lines—To generate MCF-7 cell lines stably expressing pcDNA3.2, pcDNA3.2-Maspin, and pcDNA3.2-E244A, cells were transfected with FuGENE 6 and selected with 0.5 mg/ml G418 for ~3 weeks, when all nontransfected cells were judged to be dead. Single cells were selected and amplified to give clonally identical cultures. Expression of stably transfected targets was determined by Western blotting.

Cell Migration Assays—Cell migration was determined using time lapse video microscopy as described previously (20).

Immunofluorescence Microscopy—Subconfluent monolayers were fixed with 4% formaldehyde and washed with PBS. For actin staining, 0.125 unit of Alexa Fluor 568-labeled phalloidin (Invitrogen) was added to each sample and incubated in PBS for 40 min at room temperature. This was followed by two PBS washes. Mouse E-cadherin antibody was used at 0.1 μg/ml with a secondary Alexa Fluor 488 goat anti-mouse IgG at 2 μg/ml (Invitrogen); incubations and washes were as reported previously (25). Slides were mounted with hydromount (National Diagnostics). Cells were visualized and captured with an LSM confocal or CCD upright microscope (Carl Zeiss Ltd., Hertfordshire, UK) and images analyzed using Axiovision 4.5 software. Quantification of β1 conformation-specific antibodies was as reported previously (20).

siRNA Knockdown—Two siRNAs directed against human maspin and Silencer® Negative Control #1 were obtained from Applied Biosystems (Warrington, UK). siRNA were referred to as #3 targeted exon 4 (GGAUCUCACAGAUGGCCACTTGUGGCCAUCUGUGAGAUCCTT) and siRNA #4 targeted exon 7 (GGUGGAAAAGAUGAUUGAUTTAUCAAUCAUCUUUUCCACCTT). PC3 cells seeded at 2×10^5/ml were transfected with 100 nM #3 or 200 nM #4 siRNA (or control) using Oligofectamine, according to the manufacturer's instructions (Invitrogen). After 4 h, 0.5 volume of PC3 complete medium was added. 24 h after transfection with siRNA, cells were used to seed a 24-well plate at 1.4×10^4/ml/well and incubated for a further 24 h before use in experiments. Maspin knockdown was verified by Western blotting for each experiment.

Adhesion Assays—The method used for cell adhesion assays was based on that described by Messent *et al.* (26). To coat with fibrillar matrix, HT-29 cells were grown to 80% confluence in 96-well plates prior to washing with PBS. Following this, 20 mM NH_4OH was added for 5 min to remove cells; wells were then washed with sterile water (method adapted from Ref. 19). Following washing with PBS, wells were blocked with 1% bovine serum albumin (BSA)/PBS for 30 min at 37 °C. Wells were washed again with PBS prior to plating the cells at a density of 1×10^5/well in serum-free medium. Cells were incubated at 37 °C for 30 min, washed with PBS, fixed with 4% (v/v) formaldehyde for 10 min at room temperature, and stained with methylene blue in 10 mM sodium borate buffer for 30 min at room temperature. Wells were washed extensively with water, the cell-bound dye was released in 50% (v/v) ethanol/0.1% (v/v) HCl, and absorbance was measured at 650 nm in a ThermoMAX microplate reader (Molecular Devices).

Aggregation Assay—Aggregation was assessed using the method of Tan *et al.* (27). MCF-7 cells that had been serum-starved for 24 h were used to seed each well of a 24-well plate with 2.5×10^4 cells in 500 μl of complete medium containing 0.5% (w/v) BSA. Plates were incubated at 37 °C, with rotation for 90 min. Aggregates were fixed with 2% (v/v) glutaraldehyde,

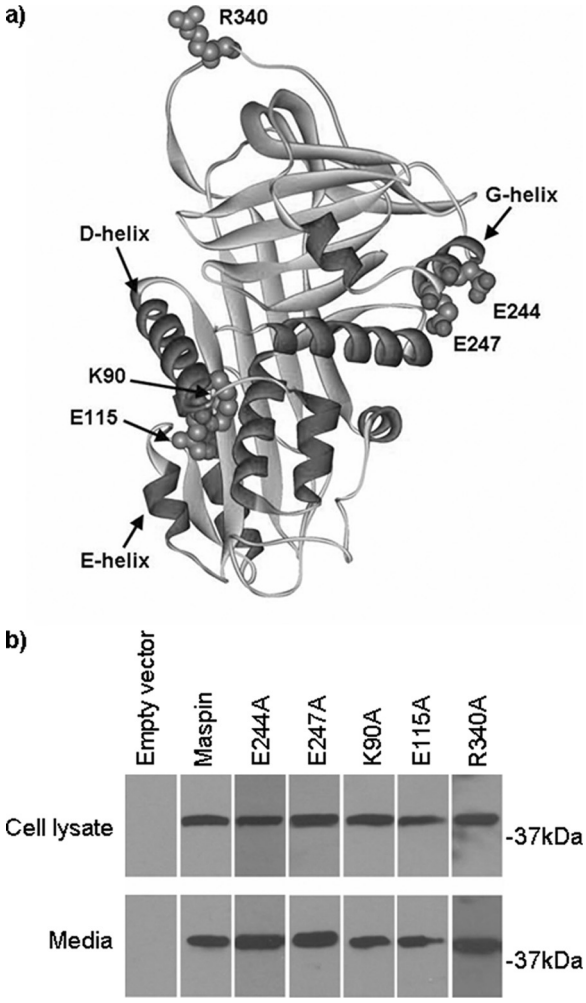

FIGURE 1. **Point mutations of maspin.** *a*, *ribbon diagram* of maspin showing the positions of the mutations generated (PDB entry 1XU8, Chain B (15)). *b*, Western blots of maspin protein expression in DU145 cell lysate and conditioned media after transient transfection, detected with 2 μg/ml anti-maspin. Media samples were concentrated 20-fold by trichloroacetic acid precipitation. No additional bands were detected on the blot.

and six views per condition were analyzed immediately with a light microscope.

Statistical Analysis—Data are presented as means ± S.E. Significance was judged using Student's *t* test.

RESULTS

Maspin Point Mutations—The diversity of roles and interactions ascribed to maspin suggests that different parts of its multidomain structure could be responsible for interacting with different cellular effectors. To address this directly, we made point mutations in domains that we theorized could be important in the function of maspin, based on its three-dimensional structure, replacing charged residues with alanine (Fig. 1*a*). The mutations E244A and E247A were made in the G α-helix of maspin, as it has been proposed that this helix can undergo a unique conformational change that increases the exposure of these residues (15), allowing the potential for controllable cofactor binding at this site. The K90A and E115A mutations disrupt either end of an internal salt bridge in a region of the molecule that is involved in cofactor regulation in other serpins. R340A is a mutation of the P1 position of the RCL which has differentially been ascribed to be involved in the functions of maspin.

Transient transfection of wild-type and mutated maspin expression constructs showed that all yielded maspin proteins that could be detected in both the cell lysate and media (Fig. 1*b*), suggesting that the proteins were folded and secreted correctly. This is shown using DU145 prostate cancer cells which do not express maspin endogenously as an example (Fig. 1*b*), but the same was observed with other cell types.[4] In the experiments that follow we sought to determine whether the mutations changed the impact of maspin on the behavior of several maspin-expressing or maspin-null cell types by transient transfection analysis. The cells involved were prostate cancer lines (maspin-null DU145, maspin-positive PC3), MCF-7 breast cancer cells (maspin-null), and vascular smooth muscle cells (maspin-null). To extend our observations, we also generated MCF-7 stable cell lines expressing empty vector (pcDNA3.2), wild-type maspin (pcDNA3.2-Maspin), or E244A maspin (pcDNA3.2-E244A).

G-helix Mutations Affect How Maspin Influences Migration—The effects of wild-type and mutated maspin on cell migration were determined by time lapse video microscopy of transfected cell lines (Fig. 2). Transfection of maspin-null DU145 with wild-type maspin reduced migration to <70% of the empty vector control (Fig. 2*a*). Transfection with proteins containing the salt bridge (K90A and E115A) or RCL (R340A) mutations had the same effect as wild-type maspin (Fig. 2*a*). These data show that the region around the D and E helices, which is suggested to be influenced by the Lys^{90}-Glu^{115} salt bridge (15), and the RCL of maspin are not required for the mechanism by which maspin inhibits cell migration. However, the mutations in the G-helix (E244A and E247A) abolished the effect of maspin on cell migration, both in transiently transfected prostate cell lines and stably transfected MCF-7 cells (Fig. 2*b*). In all cases equal expression of maspin proteins was verified. This therefore suggests that the interactions between maspin and the cellular effectors that lead to a reduction in cell migration occur through its G-helix.

The influence of maspin on cell migration was reflected by changes in the actin cytoskeleton, exemplified by the stably transfected MCF-7 (Fig. 2, *c*–*e*). MCF-7 pcDNA3.2 cells have a cytoskeletal architecture indicative of a more motile phenotype, in that the cells have multiple membrane spikes and short, thin actin filaments (Fig. 2*c*). MCF-7 pcDNA3.2-Maspin have a phenotype commensurate with a comparative reduction in motility; the cells are round with thicker actin filaments around the periphery of the cells, but a clear leading edge (Fig. 2*d*). MCF-7 pcDNA3.2-E244A have some membrane spikes, but the thicker actin filaments that indicate that the cells are less motile (Fig. 2*e*). Similar observations were made with transiently transfected DU145.[4]

The reduction in the migration of PC3 transiently transfected with wild-type maspin was of the same magnitude as that observed for the transiently transfected DU145 (>30%). How-

[4] L. Ravenhill, L. Wagstaff, D. R. Edwards, V. Ellis, and R. Bass, unpublished data.

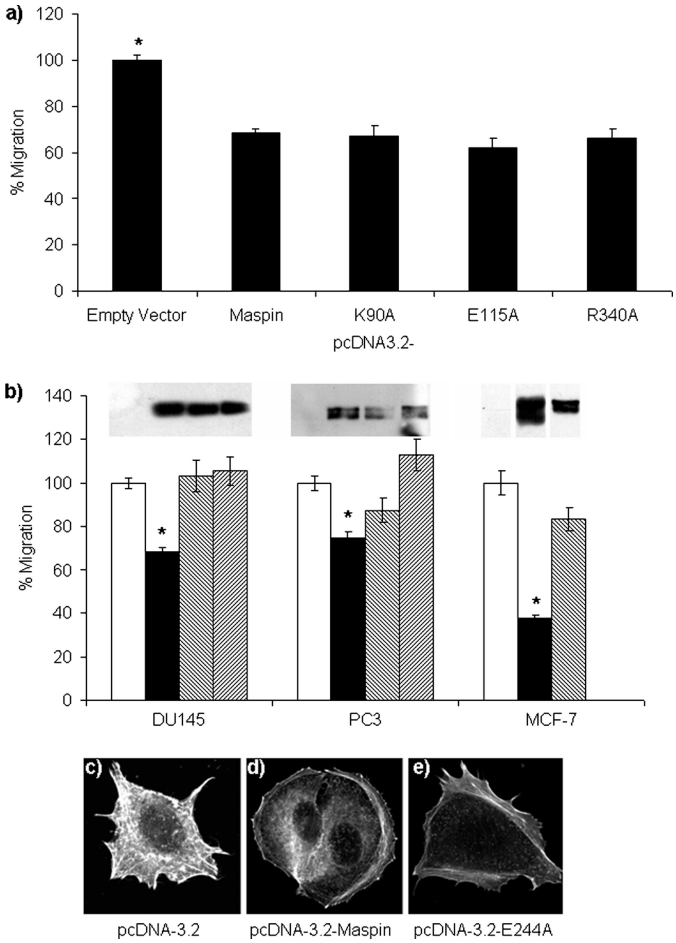

FIGURE 2. **Influence of wild-type and mutated maspin on cell migration was determined by time lapse video microscopy of transfected cells.** *a*, DU145 cells were transiently transfected with empty vector, wild-type maspin, or mutated maspin as indicated (see supplemental Movies 1–7). *b*, DU145 and PC3 cells were transiently transfected and MCF-7 cells were stably transfected with pcDNA3.2 (*open bars*), pcDNA3.2-Maspin (*black bars*), pcDNA3.2-E244A (*left-hatched bars*), or pcDNA3.2-E247A (*right-hatched bars*). *Insets* show expression of maspin proteins detected with 1 μg/ml antibody to V5. Data shown represent the means ± S.E. (*error bars*) of at least three independent experiments performed over 13 h. To allow direct comparison between differently motile cell lines, data are presented as percentage of the migration of the control cells transfected with empty vector in each case. Average migration of controls: DU145, 2.3 ± 1.3 μm/h; PC3, 17.6 ± 10.7 μm/h; MCF-7, 4.9 ± 3.6 μm/h. Statistical significance was determined using Student's *t* test. *, $p < 0.0005$. *c–e*, actin cytoskeleton was visualized with Alexa Fluor 568 phalloidin in MCF-7 cells stably transfected with pcDNA3.2 (*c*), pcDNA3.2-Maspin (*d*), and pcDNA3.2-E244A (*e*).

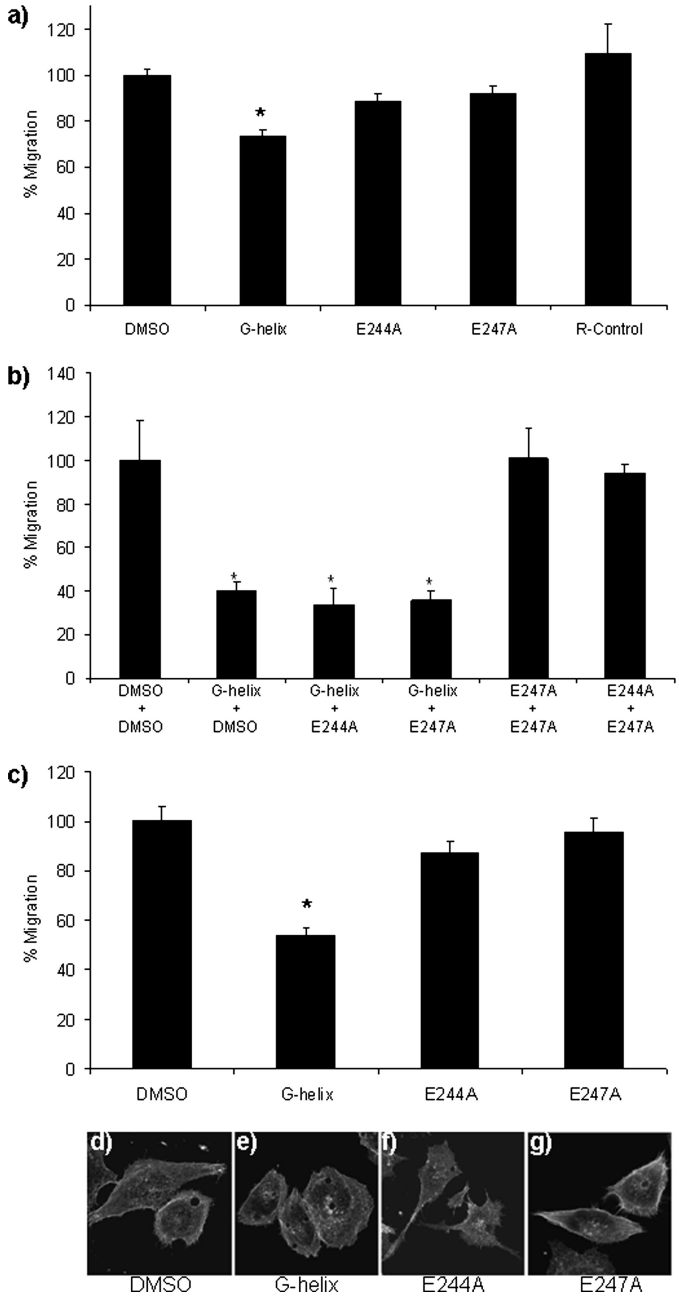

FIGURE 3. **G-helix peptides affect cell migration.** *a–c*, cell migration was determined by time lapse video microscopy of VSMC incubated with 10 μM peptide or DMSO (*a*) (see supplemental Movies 8–12), VSMC incubated with 10 μM + 10 μM peptide or DMSO (*b*), and DU145 incubated with 5 μM peptide or carrier control (DMSO) (*c*). Data shown represent the means ± S.E. (*error bars*) of at least three independent experiments, presented as percentage of the migration of the cells incubated with DMSO in each case. Average migration of controls: VSMCs, 35.6 ± 9.6 μm/h; DU145, as detailed in Fig. 3. Statistical significance was determined using Student's *t* test. *, $p < 0.02$. *d–g*, actin cytoskeleton was visualized with Alexa Fluor 568 phalloidin in DU145 incubated with DMSO (*d*), G-helix (*e*), E244A (*f*), or E247A (*g*) peptides for 24 h. The peptides used were 15-mers of the wild-type G-helix of maspin (G-helix), those containing the Glu to Ala mutations at residues 244 and 247 (E244A and E247A, respectively) and a rearranged control peptide (*R-Control*). Peptides were optimized for individual cell lines (supplemental Fig. 1) with 10 μM being optimal for VSMCs and 5 μM for DU145. Time lapse was performed for 17 h for VSMC and 13 h for DU145.

ever, MCF-7 cells stably transfected with wild-type maspin show a >60% reduction in migration, which may reflect the increased (~2-fold) level of expression of maspin achieved following stable transfection (Fig. 2*b*, *inset*). Two clones of MCF-7 stably expressing wild-type maspin were isolated and amplified; their maspin expression levels were similar, and they behaved in the same way in this assay. Data from one set of isolates are shown here.

G-helix Peptides Mimic the Effect of Full-length Maspin on Cell Migration—The preceding data suggest that the G-helix is critical for the modulation of cell migration by maspin; we investigated whether this part of maspin in isolation would exert similar effects. A 15-mer peptide spanning the sequence of the G-helix of maspin (residues 236–250) was used in migration assays, compared with peptides with the E244A and E247A mutations and also a rearranged G-helix control peptide (Fig. 3*a*). Using VSMCs the wild-type G-helix peptide was found to

significantly reduce migration, whereas the rearranged control, the E244A and the E247A mutant peptides did not. The wild-type and mutant peptides were added to VSMCs in combination to investigate whether the mutant peptides could compete with the wild-type peptide (Fig. 3*b*). The wild-type G-helix peptide was able to influence VSMC migration in the presence of equimolar mutant peptide, suggesting that the mutant peptides were not able to compete with the wild-type peptide. Overall these data suggest that the intact G-helix is both essential and sufficient for mediating the effects of maspin on cell migration. These findings were replicated with DU145 cells (Fig. 3*c*) and all other cell lines tested, including MCF-7 and PC3 (see below), indicating that the G-helix may be the common basis for the effects of maspin on cell migration.

The influence of the peptides on cell migration was reflected in changes in cell morphology and the actin cytoskeleton (Fig. 3, *d–g*). DU145 cells incubated with the G-helix peptide are markedly different from those incubated with DMSO, E244A, or E247A. DU145 cells incubated with the G-helix peptide are rounder in shape with longer/thicker actin filaments, which is consistent with the observed reduction in migration. In all of the other incubation conditions the cells have more membrane protrusions and short actin filaments, suggestive of a more motile phenotype. Similar trends were observed with VSMCs.[4]

G-helix Peptide Has Most Significant Effects on Cells with Reduced Maspin Expression—To explore the actions of the G-helix peptide, we examined its effects on the migration of maspin-null cells engineered to overexpress maspin (MCF-7; Fig. 4*a*) or maspin-positive cells in which endogenous expression had been knocked down by siRNA (PC3; Fig. 4*b*). As previously, maspin-transfected MCF-7 cells showed reduced migration compared with control cells. Addition of the G-helix peptide did not elicit any further reduction in the migration of maspin-transfected cells, suggesting that the mechanism through which the G-helix peptide acts is saturated by the maspin expressed by these cells. However, as with other cell types, the G-helix peptide reduced the migration of the control MCF-7 cells by 52% (Fig. 4*a*). Using the alternative strategy, in PC3 cells that have endogenous maspin expression, knocking down maspin expression by siRNA increased their migration by greater than 40% (Fig. 4*b*). When the G-helix peptide was added to PC3 expressing maspin (treated with control siRNA) it decreased migration by 29%. When the basal level of maspin expression by PC3 cells was reduced by siRNA there was a relative enhancement in the response to the G-helix peptide; in this case migration was reduced by at least 56%. These data show that the G-helix peptide has the greatest effect on cells which do not express endogenous maspin. Further, they indicate that the actions of the G-helix peptide and full-length maspin are interchangeable.

G-helix of Maspin Influences Migration through β1 Integrins on the Cell Surface—We have recently demonstrated that maspin can inhibit the migration of VSMCs by binding to β1 integrins on the cell surface (20). To determine whether the observed effects of the G-helix of maspin were reliant on this mechanism, the effect of the β1 function-blocking antibody mAb13 on the migration of these cells was investigated (Fig. 5). mAb13 was compared with the αv function-blocking antibody

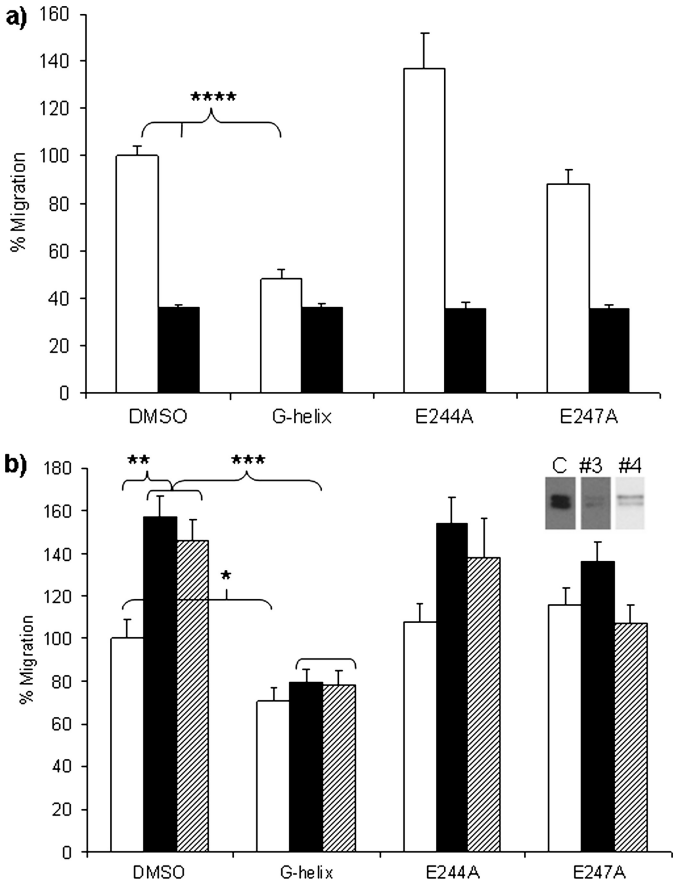

FIGURE 4. **Impact of G-helix peptides on cell migration is related to maspin expression.** Time lapse video microscopy of MCF-7 stably expressing pcDNA3.2-Maspin (*filled bars*) or pcDNA3.2 (*open bars*) incubated with 5 μM peptide or DMSO (*a*); PC3 treated with maspin targeting siRNA (#3, *filled bars*; #4, *hatched bars*) or control siRNA (*open bars*) incubated with 10 μM peptide or DMSO (*b*). *Inset*, maspin Western blot showing knockdown by #3 and #4 siRNA compared with control (*C*). The peptides as detailed were found to be optimal for MCF-7 at 5 μM and for PC3 at 10 μM. Time lapse was performed for 13 h. Data shown represent the means ± S.E (*error bars*) of at least three independent experiments, presented as percentage of the migration of the cells incubated with DMSO in each case. Average migration of controls is as detailed in Fig. 3. Statistical significance was determined using Student's *t* test. *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0005$; ****, $p < 0.00005$.

mAb1980 and control IgG (Fig. 5*a*). We found that inactivation of β1 abolished the effect of the G-helix peptide, as the peptide had no effect on VSMC migration in the presence of mAb13 (Fig. 5*a*). In contrast, neither mAb1980 nor control IgG affected the ability of the G-helix peptide to reduce the migration of VSMCs. Consistent with the observations with MCF-7 cells, the E244A mutant peptide did not reduce the migration of VSMCs. This suggests that the G-helix peptide is reliant on β1 for its influence on cell migration.

We observed previously that the effect of maspin binding to β1 integrins on cell migration was related to a reduction in integrin activation status by using conformation-dependent integrin antibodies (20). To determine whether the G-helix peptide functions by a similar mechanism, we incubated VSMCs with the maspin peptides and detected total (Fig. 5, *b–d*), active (Fig. 5, *e–g*), and inactive (Fig. 5, *h–j*) β1 by immunofluorescence using the same conformation-specific antibodies. We found that the G-helix peptide caused a 4.5-fold increase in inactive β1 on the cell surface, whereas the E244A

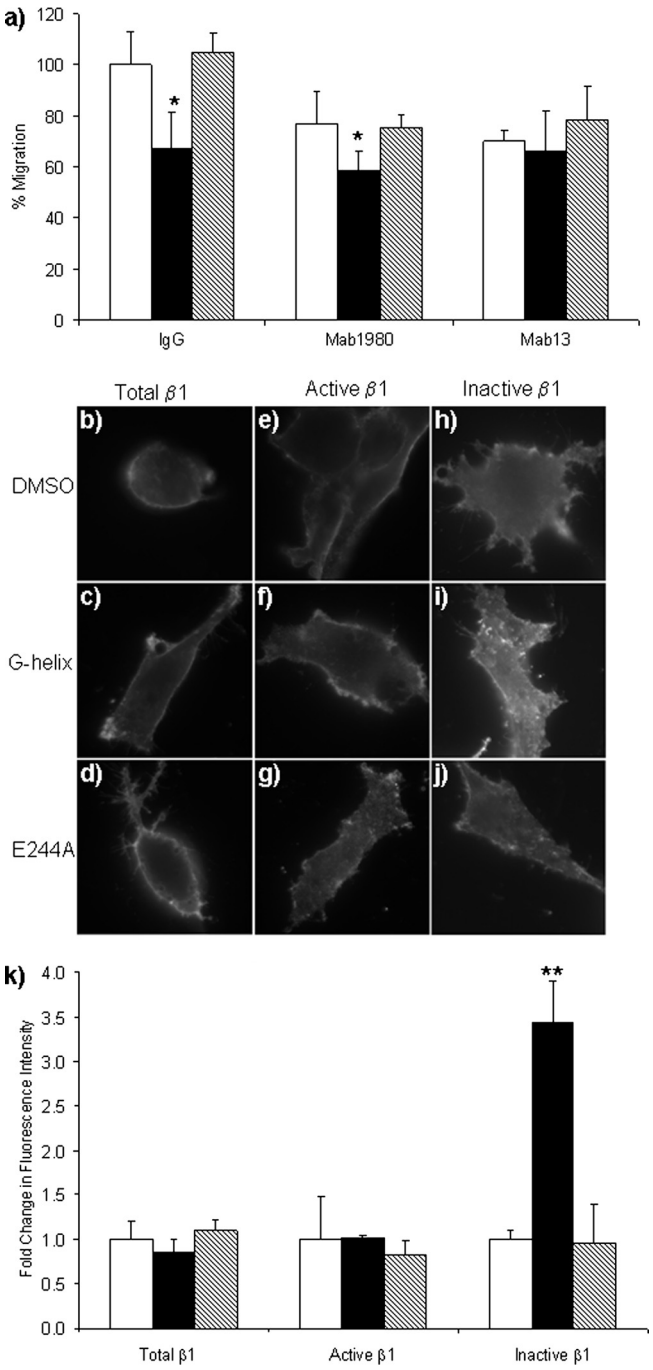

FIGURE 5. **G-helix of maspin influences cell migration through β1 integrins.** *a*, migration of VSMCs in the presence of DMSO (*open bars*), G-helix peptide (*filled bars*), or E244A peptide (*hatched bars*), and β1 function-blocking mAb13, αv function-blocking mAb1980 or control IgG as indicated. Antibodies were added to 5 μg/ml and peptides to 10 μM. Data shown represent the means ± S.E. (*error bars*) of at least three independent experiments, presented as percentage of the migration of cells incubated with DMSO and control IgG (24.1 ± 3.1 μm/h) (see supplemental Movies 13–21). *b–j*, activation state of β1 on the surface of VSMCs incubated with 10 μM peptide for 1 h at 37 °C. DMSO (*b*, *e*, and *h*), G-helix (*c*, *f*, and *i*), E244A (*d*, *g*, and *j*). This was done with a panel of conformation specific antibodies; to total β1 (1:50 dilution; *b–d*), active β1 (12G10, 5 μg/ml; *e–g*), and inactive β1 (mAb13, 5 μg/ml; *h–j*). The secondary antibody was Alexa Fluor 488 at 2 μg/ml. *k*, intensity of Alexa Fluor 488 staining in the presence of the different β1 antibodies measured to allow an assessment of the activation state of β1 on the surface of VSMC incubated with DMSO (*open bars*), G-helix peptide (*filled bars*), or E244A peptide (*hatched bars*). Data represent the mean ± S.E. of three independent experiments. Statistical significance was determined using Student's *t* test. *, $p < 0.05$; **, $p < 0.005$.

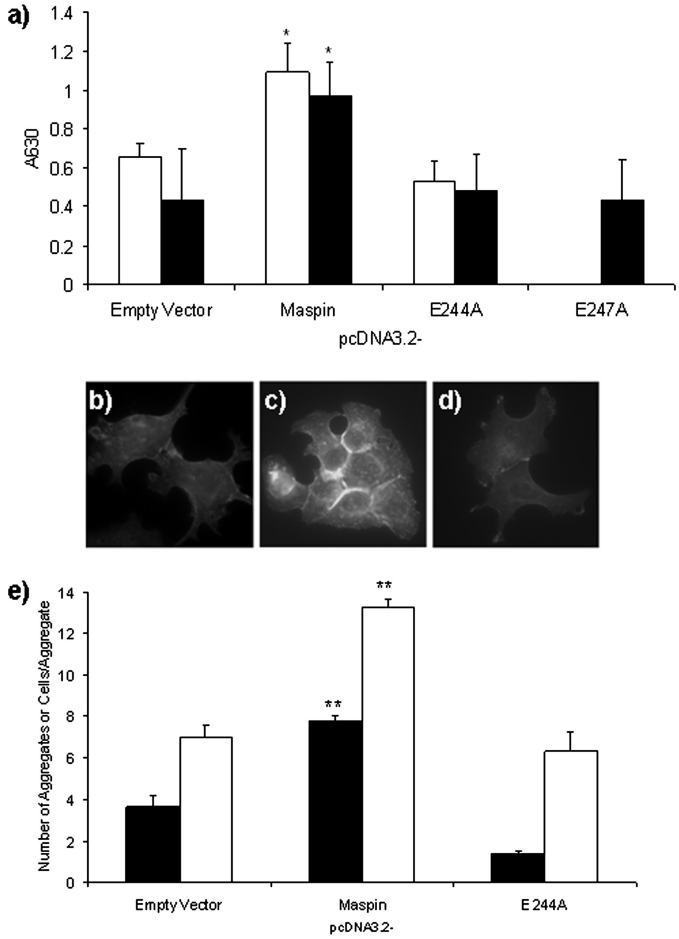

FIGURE 6. **Intact G-helix is required for the enhancement of cell-matrix and cell-cell adhesion by maspin.** *a*, adhesion of MCF-7 (*open bars*) and DU145 (*filled bars*) transfected to express wild-type or G-helix mutant maspin as indicated, to HT-29 cell matrix. *b–d*, E-cadherin expression detected by mouse antibody at 0.1 μg/ml in MCF-7 cells stably transfected with pcDNA3.2 (*b*), pcDNA3.2-Maspin (*c*), or pcDNA3.2-E244A (*d*). Secondary antibody was Alexa Fluor 488 at 2 μg/ml. *e*, aggregation of MCF-7 stably transfected as indicated, presented as number of aggregates (*filled bars*) and number of cells per aggregate (*open bars*). Data represent mean ± S.E. of three independent experiments. Statistical significance was determined using Student's *t* test. *, $p < 0.05$.

peptide had no effect on the activation state of β1 integrin (Fig. 5*k*). We have demonstrated previously that an increase in the proportion of inactive β1 is correlated with a reduction in cell motility (20). This therefore provides an insight into how the G-helix peptide reduces cell migration and further suggests that the actions of the G-helix peptide and full-length maspin are identical. All of these data are consistent with a model in which the G-helix of maspin exerts its effects on cell migration via a β1 integrin-dependent mechanism.

Effects of Maspin on Cell Adhesion Require an Intact G-helix—We investigated the effects of maspin on the adhesion of cells to endogenous ECM deposited by a range of cell types. HT-29 colon cancer cells were most frequently used, but similar results were obtained when we determined the adhesion of cells to ECM from MCF-7, LNCaP, or PC3 cells.[4] Both MCF-7 and DU145 cells expressing maspin demonstrated a 2-fold increase in adhesion to fibrillar matrix compared with control and E244A/E247A-expressing cells (Fig. 6*a*). This confirmed previ-

ous reports on the involvement of maspin in cell adhesion, but for the first time indicated the importance of the G-helix of maspin. We observed no differences in cell adhesion to isolated ECM components.

Maspin was also found to enhance cell-cell adhesion as indicated by the 2-fold up-regulation of E-cadherin in maspin-transfected MCF-7 cells compared with control cells (Fig. 6, *b* and *c*). Expression of the E244A mutant did not have this effect (Fig. 6*d*), indicating that the G-helix of maspin is involved in the mechanism by which maspin alters E-cadherin expression and hence cell-cell adhesion. This is functionally demonstrated by an aggregation assay, where maspin expressing MCF-7 forms twice as many aggregates, containing twice as many cells, as the control and E244A maspin-expressing cells (Fig. 6*e*). Taken together, these data suggest that, as is the case for its migration inhibitory actions, expression of maspin has effects on cell-cell and cell-matrix interactions that are dependent on the presence of the G-helix.

DISCUSSION

Maspin is a noninhibitory serpin with multiple cellular effects consistent with its role as a tumor metastasis suppressor. Maspin decreases cell migration, invasion, proliferation, and angiogenesis, while increasing apoptosis and adhesion. This diversity indicates the potential for engagement with multiple cellular effectors. In this study we aimed to identify structural elements critical for the extracellular effects of maspin and for the first time demonstrate the importance of the G α-helix. An intact G-helix is absolutely required for the effect of maspin on cell migration, and the effect of the maspin protein can be mimicked by a short peptide corresponding to this structural element. Maspin and the G-helix in isolation are reliant on β1 integrins for their effects on cell migration. In addition we have found that cell adhesion is altered by maspin in a way that also requires an intact G-helix.

The G-helix of maspin was targeted because the crystal structure suggested that the region around glutamic acid residues 244 and 247 was capable of a unique conformational change (15). This would allow regulation of access to these charged residues, which are of potential importance in protein-protein and protein-ligand interactions and therefore allow alterations in putative cofactor binding. The acidic patch that contains these glutamic acid residues is highly conserved in maspin orthologues and is not found in any other serpins, indicating the potential for involvement in cellular processes specific to maspin. Our data provide direct experimental evidence for the importance of the G-helix in the modulation of cell behavior by maspin. The other regions of maspin targeted in this study did not prove critical for the modulation of migration.

We suggest that the importance of the G-helix of maspin lies in an extracellular mechanism of action associated with control of migration and adhesion. Both of the G-helix mutations abolished the influence of maspin on cell migration, whereas maspin with mutations in the salt bridge and RCL inhibited cell migration as effectively as wild-type maspin. A 15-mer peptide of the G-helix was able to mimic the effects of full-length, wild-type maspin on cell migration, indicating that the G-helix is both essential and sufficient for mediating the effects of maspin on cell migration. The 15-mer G-helix peptide constitutes all of the G-helix of maspin with two additional residues at either end. The peptides are predicted to be unstructured in aqueous solution, but could attain secondary structure when bound to their cell surface target, for example, in the same way that urokinase plasminogen activator receptor inhibitory peptides only have a helical structure in complex with urokinase plasminogen activator receptor (28). Replacement of glutamic acid residues with alanine in the G-helix peptides results in a charge difference between the wild-type and G-helix mutant peptides. However, a control peptide with the two glutamic acid residues relocated in an otherwise identical sequence had no effect on cell migration, indicating that the effects of the mutations are not due to simple charge alterations. Importantly, the G-helix peptide influenced the migration of cells both with and without maspin expression, indicating that it influences a fundamental cellular mechanism. In fact the most significant effects of the peptide were observed with cells with reduced or absent maspin expression, which suggests competition between the G-helix peptide and full-length maspin.

We investigated whether the mutant peptides could compete with the wild-type G-helix peptide with regard to functional effects on cell migration. The inhibitory actions of the G-helix peptide on cell migration were maintained in the presence of the mutant peptides, indicating that the glutamic acid residues at 244 and 247 are critical for the interaction between the G-helix peptide (and by extrapolation, maspin itself) and its cell surface effector(s).

The reduction in cell migration caused by full-length maspin or the G-helix in isolation was reflected in changes in the cytoskeleton, indicating that both protein and peptide act via the same mechanism. Maspin or G-helix peptide caused a cytoskeletal architecture indicative of a less motile phenotype; there was a reduction in the number of membrane spikes/lamelliopodia, and the actin filaments were increased in terms of length and thickness. This is in agreement with previous studies suggesting that maspin inhibits cell migration by inhibiting Rac1 and cdc42 (29, 30).

In this study we found that the G-helix peptide could influence the activation state of β1 integrin on the cell surface. This is in good agreement with previous studies identifying interactions between maspin and β1 integrins (19, 20); we showed that maspin binds to integrins α3β1 and α5β1 on the surface of VSMCs, but not αvβ3, which is also abundant on the surface of these cells (20). We observed a rapid inhibition of cell migration by maspin and also a dynamic regulation of the activation state of integrins (9, 20), rather than a change in integrin expression. The findings presented here indicate that the G-helix of maspin is also reliant on the presence of active β1 on the cell surface for its action, as the peptide fails to influence cell migration when β1 is functionally blocked.

An intact G-helix was also found to be required for the enhancement of adhesion by maspin, as cells expressing maspin mutated at E244A did not show an increase in adhesion in the same way as wild-type maspin. The differences in the adhesive properties of the MCF-7 cell lines used here were not due to posttranscriptional changes in integrin expression, as the pro-

file of integrin mRNA was virtually identical between MCF-7 stably expressing empty vector, maspin or E244A.[4] Maspin has been shown to bind directly to collagen I and III (31), which could suggest the G-helix as the collagen binding site of maspin. However, we found no evidence of direct binding of wild-type or mutant G-helix peptides to collagen.[4] Maspin expressing MCF-7 also demonstrated enhanced cell-cell adhesion and had increased E-cadherin expression; again, this mechanism required an intact G-helix. In contrast to the effects of maspin on migration, the influence of maspin on adhesion could not be recapitulated with the G-helix peptides, even at enhanced concentrations.

Here, we have begun to understand how maspin, through its G-helix, is able to alter cellular behavior. The effects of maspin on migration and adhesion could involve independent mechanisms or represent separate consequences of the interaction of maspin through its G-helix to the same cell surface receptor. Although the former is consistent with a study reporting that adhesion and migration are both highly regulated but independent processes (32), we consider it likely that cross-talk between maspin and β1 integrins is involved in both cell adhesion and migration. General support for a key role of integrin-mediated adhesion in maspin function is the attribution of the embryonic lethality observed in maspin-knock-out mice to impaired ability of endodermal cells to adhere to the surrounding ECM (10). However, we have been unable to demonstrate directly a role for β1 integrins in the promotion of cell adhesion by maspin using the experimental approaches adopted here, both as the G-helix peptide did not recapitulate the effect of maspin and the β1 function-blocking antibodies reduced the adhesion of all of the MCF-7 cell lines $>$80%.[4]

This work shows for the first time that the G-helix of maspin is essential and sufficient for the inhibition of cell migration and is involved in cell adhesive effects. We propose a mechanism involving interactions between β1 integrin on the cell surface and maspin through its G-helix, resulting in an inactivation of β1 and concomitant downstream effects. The effects of the G-helix on migration and adhesion have been demonstrated on multiple cell lines, suggesting that it is fundamental to the molecular action of maspin. In the future it will be important to characterize this mechanism fully so it can be exploited in therapeutic applications. Tumor cells show a reduction in maspin expression as they become more metastatic; as the G-helix peptide had the greatest impact on cells with little or no maspin expression, we suggest that the G-helix peptide would principally target maspin-null tumor cells. This has the potential for anti-cancer therapies in a similar way to other peptides that have been used to target angiogenesis (33).

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