Evidence for a direct interaction between the tumour suppressor serpin, maspin and types I and III collagen

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SUMMARY

Maspin (mammary serine protease inhibitor) was originally identified as a tumour suppressor protein in human breast epithelial cells and is a member of the serpin superfamily. It inhibits tumour cell motility and angiogenesis, and although predominantly cytoplasmic, it is also localised to the cell surface.

In this study we have investigated the use of the yeast-two-hybrid interaction trap to identify novel maspin targets. A target human fibroblast cDNA library was screened and the alpha-2 chain of type 1 collagen was identified as a potential interactant. Binding studies with isolated proteins showed interaction between recombinant maspin and types I and III collagen but not other collagen subtypes, a profile strikingly similar to mouse pigment epithelium-derived factor (caspin) which is similarly downregulated in murine adenocarcinoma tumours and is a potent inhibitor of angiogenesis. Kinetic analysis using IAsys resonant mirror biosensor determined the dissociation constant of maspin for collagen type I to be 0.63 µM. Further two-hybrid interactions with maspin truncation constructs suggest that collagen binding is localised to amino acids 84-112 of maspin, which aligns with the collagen binding region of the serpin colligin. A direct interaction between exogenous or cell surface maspin and extracellular matrix collagen may contribute to a cell adhesion role in the prevention of tumour cell migration and angiogenesis.
INTRODUCTION

Maspin (mammary serine protease inhibitor) was identified by subtractive hybridization as a candidate tumour suppressor protein in normal mammary epithelial cells [1]. A number of findings support a role as a tumour suppressor; levels of maspin expression show an inverse correlation with progression of breast cancer; mammary carcinoma cells transfected with maspin showed reduced tumour growth and metastasis in nude mice [1, 2]; addition of recombinant maspin (rMaspin) decreased the migration potential of breast and prostate tumour cells across a reconstituted basement membrane [3]; and more recently maspin has been shown to inhibit angiogenesis by blocking in vitro migration of endothelial cells and by in vivo inhibition of rat cornea neovascularization [4].

Maspin belongs to the serpin (serine proteases inhibitor) superfamily of proteins, and can be included in the ovalbumin subfamily which appear to be mostly intracellular [5, 6]. The maspin gene has been localised to chromosome 18q21.3 where it is clustered with PAI-2 and the squamous cell carcinoma antigens [7]. Initial studies suggested that maspin may be a non-inhibitory serpin [8], and although an unusual biphasic inhibitory and activatory effect with single chain tPA has been reported [9], this does not appear to be a target for maspin in mammary gland extracts [10]. The predicted P1 residue of maspin is arginine and trypsin cleavage of the reactive site loop demonstrated that the loop sequence was necessary for inhibition of cell invasion [3]. In contrast, inhibition of angiogenesis was found to be independent of reactive site loop integrity [4].

Tissue distribution studies have shown expression of maspin in other organs, including placenta, prostate, and small intestine. Subcellular localisation studies show maspin to be predominantly a soluble cytoplasmic protein (95%) but it is also associated with secretory vesicles and is present at the cell surface [11]. Functional studies suggest

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1 Abbreviations: rMaspin, recombinant maspin; PAI-1 and PAI-2, plasminogen activator inhibitor types 1 and 2; tPA, tissue type plasminogen activator; PEDF, pigment epithelium-derived factor; ECM, extracellular matrix; PCR, polymerase chain reaction.
that this surface bound maspin is responsible for inhibition of cell invasion [12].

Addition of rMaspin to MDA-MB-435 breast cancer cells was found to alter the integrin profile by inducing $\alpha_5$ and $\alpha_3$ integrins and downregulating other integrins. This was accompanied by a decreased ability to migrate through a fibronectin matrix and may provide a mechanism whereby maspin can suppress the invasive phenotype of these cells [13]. More recently, studies on human cornea suggest a role for maspin in regulation of stromal cell adhesion to the extracellular matrix. Late-passage stromal cells which had lost the ability to produce maspin, responded to exogenous recombinant maspin as measured by increased cell adhesion to fibronectin, type I collagen, type IV collagen type I and laminin [14].

An increasing number of serpins have been found to bind ligands other than target proteases. Interaction with heparin resulting in enhanced serpin inhibitory activity has been well-documented for antithrombin III and heparin cofactor II [15]. Inhibition of cell migration by PAI-1 is mediated by specific binding of vitronectin [16], which competes with vitronectin VNR $\alpha_v\beta_3$ receptor binding and blocks smooth muscle cell migration. This binding is independent of plasminogen activator inhibition, but formation of a complex with uPA decreases affinity for vitronectin and restores cell migration [17]. The dual localisation of some partially secreted ovalbumin-type serpins such as PAI-2 and maspin suggests the possibility of more than one function or ligand. This is borne out in the case of PAI-2, which in addition to inhibition of uPA, can protect cells against TNF mediated apoptosis [18] and can also bind annexins via the C-D interhelical region [19].

In this report we have investigated the yeast-two-hybrid LexA interaction trap [20] as a potential method for detecting novel serpin targets, and have found an interaction between maspin and type I collagen. Verification with protein-protein interactions indicated that maspin preferentially binds collagen subtypes I and III. The similarity of this profile to that of the anti-angiogenic serpin, pigment epithelial derived growth factor (PEDF) [21], the expression of which correlates inversely with metastatic potential in colon adenocarcinoma cells [22], suggests that collagen
association may be a factor in serpin inhibition of angiogenesis.

EXPERIMENTAL PROCEDURES

Materials

All components of the two hybrid system including vectors pEG202 and pJG4-5, WI-38 fetal fibroblast cDNA ‘target’ library in pJG4-5, *Saccharomyces cerevisiae* EGY48, *Escherichia coli* strain KC8 and the polyclonal anti-LexA antibody were kindly donated by Luke O’Neill (Trinity College Dublin). *E. coli* strains XL-1 Blue and BL21 (DE3) were obtained from Stratagene (La Jolla, CA). The bacterial expression vector pRSETC was obtained from Invitrogen. Q Sepharose, Iminodiacetic acid (metal affinity matrix), collagen agarose, collagen subtypes I, II, III, IV and V, and HRP-linked goat anti-mouse IgM secondary antibody were purchased from Sigma. The IgM mouse monoclonal antibody to maspin was obtained from Transduction Laboratories (Lexington, KY USA). Immunoblots were developed by enhanced chemiluminescence (Boehringer Mannheim). All oligonucleotide primers were obtained from Genosys (Cambridge, UK).

**PCR amplification and cloning of serpins into pEG202**

Full length human maspin cDNA was amplified by reverse transcription-PCR. Total RNA was isolated from HeLa cells using a phenol/guanidinium thiocyanate extraction method [23] and first strand cDNA was synthesised from total RNA (1 µg) using MMLV-reverse transcriptase and random hexanucleotide primers. PCR amplification of first strand cDNA was performed with the following primer pair specific to 5 and 3 ends of the maspin gene; primer A, 5'-GGGGAATTCATGGATGCCCTGCAACTA-3' (sense oligonucleotide corresponding to nucleotides 1-18 of maspin coding sequence, and incorporating an EcoR1 restriction site), and primer B, 5'-AAAAGTCGACTGCCACTTAAGGAGAA-3' (antisense oligonucleotide corresponding to nucleotides 1119-1128 of maspin coding sequence plus nucleotides 1-6 of 3’ untranslated sequence, and incorporating a Sal1 restriction site). Amplification was carried out with 1.5 mM MgCl2 for 35 cycles of (94°C x 1
min, 45°C x 1 min, 72°C x 2 min). The product was cloned into the yeast-two-hybrid bait vector pEG202 using EcoR1 and Sal1 restriction sites and propagated in E.coli XL-1 Blue cells. Dideoxy sequencing was performed to verify correct in frame insertion.

**Immunoblot analysis of pEG202 constructs**

Yeast (*S.cerevisiae* EGY48) was transformed with the pEG202 plasmid containing maspin using a lithium acetate protocol [24]. Following overnight growth of transformants at 30°C in complete minimal medium with supplemented amino acids, fresh cultures were inoculated at OD600 0.15 and grown in enriched media (YEPD) to an OD600 of 0.6-1.0. Yeast total protein extract was prepared and the expression of the maspin-LexA fusion protein was examined by immunoblotting using a mouse polyclonal antibody to LexA (1:1500) and a peroxidase-linked goat anti-mouse secondary antibody (1:2000).

**Yeast-two hybrid library screening**

Maspin fused to the LexA binding domain was used as a bait to screen a human fibroblast cell cDNA library fused to the activation domain of LexA. The bait-containing vector, pEG202, and the library-containing target vector, pJG4-5, were sequentially transformed into the two-hybrid yeast strain EGY48, and grown on media lacking leucine to select for potential interactors. Approximately 2x10^6 clones, representing 1/3 library equivalent, were screened against the maspin bait. Transformants with the ability to grow on leucine deficient media were subsequently screened for β-galactosidase reporter gene expression. Yeast colonies passing the dual selection screen were carried forward as potential interactor clones. Unique clones within this pool were detected by PCR amplification of the target inserts using the following primers to the vector arms of pJG4-5 (primer C, 5’-CCAGCCTCTTGGCTGAGTGGAGATG-3’ and primer D, 5’-GACAAGCCGACAACCTTGATTGGAG-3’). Dideoxy sequencing was performed
on products of unique insert size and/or restriction pattern to identify the target maspin interacting protein in each case. Plasmid DNA was prepared from the clone(s) of interest and transformed into *E. coli* KC8, a bacterial strain which is auxotrophic for tryptophan and therefore selectively propagates pJG4-5. Interaction studies of target and bait vectors against non-recombinant pEG202 and pJG4-5 were conducted to eliminate false positives, which have activator function (β-galactosidase activity) in the absence of an interacting partner.

**Expression and purification of recombinant maspin protein in *E. coli***

Full length maspin cDNA was amplified by PCR from recombinant pEG202/maspin (described above) using the following primer pair; primer E, 5’-GGGGAATTCAATGGATGCCCTGCAAC-3’ (sense oligonucleotide containing nucleotides 1-16 of maspin coding sequence and incorporating an EcoR1 restriction site), and primer F, 5’-AAAAGCTTTTAAGGAGAACCAGAATT-3’ (antisense oligonucleotide containing nucleotides 1112-1128 of maspin coding sequence and incorporating a HindIII restriction site). The PCR was carried out with 1.5 mM MgCl2 for 30 cycles of (94°C x 1 min, 48°C x 1 min, 72°C x 2 min). Amplified product was subsequently cloned into the expression vector pRSETC at EcoR1 and HindIII restriction sites.

Recombinant maspin was overexpressed from pRSETC (Invitrogen) in *E. coli*. BL21(DE3), producing a recombinant fusion protein with a polyhistidine metal binding tail at the N-terminus. Cells grown to an OD600 = 0.6 were induced with 1 mM IPTG and grown for a further 5 hours. Following harvesting by centrifugation (12,000 g x 15 min), cells were resuspended and sonicated in 30 ml of 50 mM Tris-HCl, pH 8, 50 mM NaCl, 5 mM β-mercaptoethanol. The cell lysate was centrifuged at 19,000 g x 20 min. and the soluble fraction was applied to a Q-Sepharose anion exchange column. Column elution was performed with a 50 to 200 mM NaCl gradient in 50 mM Tris-HCl pH 8, and maspin containing fractions were further purified by immobilised metal affinity chromatography using a fast flow chelating Sepharose column pre-charged
with 5 mg/ml nickel chloride and equilibrated in 500 mM NaCl, 40 mM sodium phosphate, pH 8. Column elution was performed stepwise by decreasing pH, but maspin remained tightly bound to the column at pH 4.5, and was subsequently eluted with 50 mM EDTA in 50 mM Tris-HCl pH 8. Removal of the nickel and EDTA from the maspin fraction was achieved by ultrafiltration and dialysis.

**Binding of maspin to collagen-agarose**

Type 1 collagen immobilised on agarose was equilibrated with 50 mM Tris-HCL, pH 7.5, 150 mM NaCl, 0.05% Brij35. rMaspin (0.1 mg/ml) was applied and following extensive column washing with the above buffer bound material was eluted with 125 mM Tris-HCL, pH 6.8, 2% SDS, 5% β-mercaptoethanol. Control experiments examining binding of rPAI-2 and rOvalbumin, which also contained polyhistidine N-terminal tags [25], were performed under identical conditions.

**Native and SDS-PAGE immunoblot analysis of interaction**

Maspin (4µg) was incubated with collagen type 1 (0.75 4 µg) for 30 min at 37°C. The samples were electrophoresed on a 10% native gel in addition to a 10% SDS-PAGE gel. Following transfer to nitrocellulose, membranes were incubated for 2 hours with an IgM monoclonal antibody to human maspin (1:1000) followed by peroxidase-linked anti-IgM secondary antibody (1:2000). Membranes were developed by enhanced chemiluminescence and autoradiography.

**Immunoblot analysis of maspin interaction with collagen subtypes**

Collagen subtypes I, II, III, IV and V, boiled collagen and gelatin were applied at varying concentrations to a nitrocellulose membrane. Dot blots were blocked overnight at 4°C with 4% BSA and then incubated for 2 hours with a solution of recombinant maspin (20 µg/ml in 150 mM NaCl, 50 mM Tris pH 7.6). Following stringent washing with 150 mM NaCl, 50 mM Tris-Cl pH 7.6 to remove unbound ligand, membranes were subsequently immunoblotted for maspin as above.
**IAsys kinetic analysis**

IAsys affinity sensor analysis experiments were conducted on the IAsys Plus apparatus (Affinity Sensors Ltd., Saxon Hill, Cambridge, UK). Binding reactions were carried out in an IAsys resonant mirror biosensor at 25°C using planar aminosilane surfaces. Collagen type I was chemically immobilised to the surface according to the manufacturer’s instructions. Collagen was made up in 10mM sodium acetate, pH 5.0, and applied to the cuvette at 1 mg/ml. Type I collagen was coupled to the cuvette until saturation was achieved (approximately 300 arc seconds).

Interaction analysis was conducted with varying concentrations of maspin, ranging from 0.25-3 µM, made up in PBS. In all experiments BSA (30 µM in PBS) was used as a control. Regeneration of the cuvette was achieved by repeated washes with 100 mM HCL. Results were analysed with Fastfit software from IAsys.

**Yeast two-hybrid one-on-one interactions**

To identify the region of the maspin protein that interacts with collagen, truncated constructs of maspin cDNA were fused to LexA in pEG202 and tested for positive interaction against the collagen-containing pJG4-5 plasmid. In total, seven maspin cDNA fragments were amplified from recombinant pEG202/maspin. Four of these were amplified using a common forward oligonucleotide, primer A (described above), in conjunction with each of the reverse primers G, H, I and J (see list below).

| Primer name | Primer sequence | Primer properties |
|-------------|-----------------|-------------------|
| G           | 5’-AAAGGATCTCTCAGAT TGGAGAGAAGAGGAC-3’ | Antisense, corresponding to nucleotides 82-101 of maspin |
| H           | 5’-AAAGGATCTTTAGGG TCTCTTGTAGAGCTG-3’ | Antisense, corresponding to nucleotides 315-335 of maspin |
| I           | 5’-AAAGGATCTTTAGTAGG CAGCATTAACCACAAGG-3’ | Antisense, corresponding to nucleotides 477-499 of maspin |
| J           | 5’-AAAGGATCTCAATTG ATACTGTCAATGTTTCC-3’ | Antisense, corresponding to nucleotides 619-641 of maspin |
| K           | 5’-AAAGAATTTCCTGTCACTTGCTAAGTGG-3’ | Sense, corresponding to nucleotides 115-133 of maspin |
| L           | 5’-AAAGAATTCTCACCTGAACTAATCAGCG-3’ | Sense, corresponding to nucleotides 253-271 of maspin |
| M           | 5’-AAAGAATTCTGCAAGGAATTTGGAAC-3’ | Sense, corresponding to nucleotides 337-354 of maspin |
The remaining three maspin fragments were amplified using a common reverse oligonucleotide, primer L, in conjunction with each of the forward primers M, N and O (see table above). Reverse primers G, H, I, and J contain flanking BamH1 restriction sites, and forward primers K, L and M contain flanking EcoR1 restriction sites. In addition, reverse primers G, H, I and J contain reverse complimentary stop codons immediately preceding the BamH1 restriction site. Amplification was carried out with 1.5 mM MgCl₂ for 30 cycles of (94°C x 1 min, 55°C x 1 min, 72°C x 3 min). Amplified products were cloned by standard procedures into the EcoR1 and BamH1 restriction sites of pEG202. Recombinant pEG202 containing maspin truncations were each co-transformed with the collagen containing pJG4-5 into S. cerevisiae EGY48. Collagen-maspin interactions were detected by both β-galactosidase and Leu2 reporter gene expression.

RESULTS

Expression of maspin in the yeast-two-hybrid system

Expression of maspin cloned into the pEG202 vector was expected to yield a LexA fusion protein of 64kDa. However, immunoblot analysis of transformed S. cerevisiae EGY48 cells with anti-LexA antibody, indicated a fusion protein of approximately 47kDa (Figure 1). Sequence analysis of the entire pEG202 insert ruled out the possibility of an incorporated stop codon during or subsequent to the initial PCR amplification step. The presence of protease inhibitors during preparation of yeast protein extracts also failed to produce full length bait on immunoblots. This processing problem, which is most likely due to intracellular proteolysis, was not resolved and would result in a fusion with 43% of the C-terminal, including the reactive site loop, absent from the bait. However, additional work showing the expression of a fully intact lexA-PAI-2 fusion in S. cerevisiae EGY48 indicates that
truncation of serpin baits within the lexA yeast two hybrid system is not necessarily a general serpin phenomenon.

**Detection of a maspin interaction with collagen using the yeast two-hybrid interaction trap**

Despite the apparent lack of full length maspin protein we proceeded with a yeast two-hybrid screen to detect possible interactants. Prior to screening, the truncated maspin bait was shown to be transcriptionally inert using the β-galactosidase assay (result not shown). A target human fibroblast cDNA library in the pJG4-5 plasmid was screened for expression of the Leu2 reporter gene and initially produced 50 positive interactor yeast clones. Of these 10 failed to show transcription of the second reporter gene lacZ on selection with X-gal. The remaining positive clones were amplified from the target vector by PCR using primers specific to the vector arms. Four different sized inserts were selected, and dideoxy sequencing of the target plasmids was performed to identify the maspin interacting partner in each case (Figure 2). The sequences identified were transketolase (U55017), metallothionein (BC008408), human elongation factor (BC018641) and type 1 collagen (Z74616). Of these type I collagen was further investigated due to the fact that collagen binding serpins have been reported previously. The collagen insert in the target vector pJG4-5 consisted of 216 amino acids in the collagen alpha 2 (I) chain and this was in the correct reading frame as a fusion with the transactivating domain of lexA. This sequence corresponds to bases 2261 – 2909 of prepro-alpha 2 (I) collagen (accession number Z74616) and the corresponding amino acids (708-923) are within the repetitive (Gly-X-Y)\(_n\) sequence of the collagen chain.

**Protein-protein analysis of the maspin-collagen interaction**

To further investigate the relevance of a collagen interaction with maspin, recombinant full length maspin was produced for direct protein-protein interaction studies. Recombinant maspin produced in *E.coli* has been shown previously to retain
its function as an inhibitor of tumour cell migration in vitro [3]. The maspin cDNA open reading frame sequence was cloned into the bacterial expression vector pRSETC, and the protein was subsequently produced in E.coli with an N-terminal polyhistidine tag. Maspin was purified by anion exchange and metal ion affinity chromatography (Figure 3A).

Binding of rMaspin to type 1 collagen immobilised to agarose was demonstrated (Figure 3B). rMaspin bound to this affinity matrix in the presence of 150 mM NaCl, whereas recombinant PAI-2 produced in the same expression system [25] and also containing a polyhistidine tail did not bind under identical conditions (Figure 3C). Recombinant ovalbumin produced similarly also failed to bind (result not shown).

Native and SDS-PAGE gels were performed following incubation of maspin and collagen type 1 to examine the nature of the interaction. Immunoblot analysis shows complex formation on native gels where maspin is significantly retarded in the presence of collagen (Figure 4). No complexes are seen on SDS-PAGE analysis however, suggesting that the interaction is non-covalent.

To investigate binding to other collagen subtypes, dot blots were performed by applying decreasing amounts of collagen subtypes I, II, III, IV and V to nitrocellulose, incubating the membrane with recombinant maspin at a concentration of 0.44 µM, and using an anti-maspin IgM antibody to detect collagen bound maspin. Collagen subtypes I and III were found to bind recombinant maspin, with 250 ng of each binding detectable amounts of maspin, whereas 2 µg of immobilized types II, IV, and V failed to bind maspin (Figure 5). In addition, binding to thermally denatured collagen and gelatin (acid denatured collagen) was examined, but in both cases maspin failed to bind (result not shown).

**Kinetic analysis of the maspin-collagen interaction using IAsys technology**

The interaction between maspin and type I collagen was analysed by fast association kinetics using IAsys affinity sensor technology. Collagen type I was coupled covalently to an aminosilane cuvette surface, and varying concentrations of
recombinant maspin (0.25-3 µM) were applied. In a parallel control experiment, BSA (30 µM) was added to a collagen-coated aminosilane cuvette. All binding events were monitored in real time, and the rate of association of maspin to type I collagen increases with increasing maspin concentration (Figure 6A). To determine a dissociation affinity constant (K_D) for the maspin-collagen type I interaction, a plot of k_on against ligand concentration was drawn (Figure 6B). The slope of the line corresponds to the association rate constant, k_a (3.2 x 10^3 M^-1 s^-1), and the Y-intercept corresponds to the dissociation rate constant, k_d (0.002 s^-1). The K_D is described by k_d/k_a, and is calculated to be 0.63 x10^-6 M.

**Identification of the collagen binding region within maspin**

The specific protein region of maspin that interacts with collagen was investigated using yeast two-hybrid technology. A number of bait constructs were cloned in pEG202 to contain varying lengths of maspin cDNA, corresponding to amino acids 1-34, 1-112, 1-166, 1-214, 38-214, 84-214 and 112-214 of the maspin protein. Since the original screening bait appeared to be missing the C-terminal half of maspin, baits longer than 214 amino acids were not constructed. To perform one-on-one interactor analysis, the isolated collagen containing pJG4-5 plasmid was co-transformed with each of the maspin fragment pEG202 vectors into the two-hybrid yeast strain EGY48. Resulting transformants were subsequently assayed for β-galactosidase reporter gene expression to identify a maspin-collagen interaction. As expression of the collagen target from pJG4-5 is under the control of a Gal promotor, true interactor clones will form blue colonies when grown on galactose containing media and white colonies when grown on glucose containing media. In contrast, false positive interactor clones form blue colonies on glucose containing media and denote a bait that can activate the reporter gene systems on its own. Results show that all maspin fragments except 112-214 interact with collagen (Figure 7) and assays for the second reporter gene Leu2 were in complete agreement (data not shown). However, the shortest maspin construct, 1-34, was found to be transcriptionally active in the absence
of an interacting partner. Taken together, these results suggest that the interaction with collagen occurs within amino acids 84-112 of the maspin protein.

DISCUSSION

The use of the yeast-2-hybrid protein interaction trap to identify serpin targets has not previously been reported and the ability of the system to carry out post-translational modifications and correct folding of proteins such as serpins is uncertain. The lexA system gives relatively high expression of the binding domain fusion, allowing detection of bait fusion, and our results suggest that there may be a lack of expression of the full length maspin. The product detected for the maspin construct indicates that the fusion protein lacks the C-terminal 43% of the serpin including the reactive loop, and if due to proteolysis, this cleavage site would correspond to residues in the serpin structure lying between strands 1 and 2 of β-sheet B. However, PI-6 has been successfully expressed in an active form in Pichia pastoris [26] and these findings may be unique to the vector or the EGY48 yeast strain used in this study. Also, although no full length product was detected on immunoblots, there may be a proportion of the entire fusion protein in vivo which could potentially pick up serpin reactive site loop interactions. Alternatively the system may be generally useful to detect novel protein interactions with serpin N-terminal sequences or other selected regions. The two hybrid system has previously been used to successfully detect interactions between extracellular matrix components, demonstrating an association between collagen types IV and VI [27].

The positive result suggesting a potential interaction with collagen type 1 was further investigated in view of previous reports of other serpins binding to collagen. Colligin or heat shock protein 47 can bind procollagens 1 and IV and gelatin, but not collagen type III [28,29]. Colligin is localised to the endoplasmic reticulum and appears to act as a molecular chaperone in the protection of procollagen from intracellular degradation [30,31]. Protease nexin-1 activity with thrombin is enhanced with binding of heparin sulphate glycoproteins, but its specificity is significantly altered
by binding to collagen type IV, which decreases affinity for uPA and plasmin while leaving thrombin inhibition unaffected [32]. More relevant is caspin (collagen associated serpin), which is identical to murine PEDF (mPEDF) and can preferentially bind collagen types I and III [22]. In mouse colon this protein is expressed at high levels in non-metastatic clones and at low levels in high metastatic clones of adenocarcinoma cells, which is similar to loss of maspin expression in mammary carcinoma. Human PEDF is a non-inhibitory serpin with neurotrophic and neuronal survival activity [33,34], which is independent of an intact reactive site loop. It can bind glycosaminoglycans [35] and both recombinant and tissue purified PEDF were potent inhibitors of angiogenesis [21].

The yeast-2-hybrid results were corroborated with protein-protein interactions with full length maspin binding to type 1 collagen agarose where other similarly expressed recombinant serpins failed to do so. Most interestingly, binding studies with different collagen subtypes showed maspin binding types I and III but not II, IV and V, a profile identical to mPEDF. Collagen types I and III are widely distributed representing 70% and 25% respectively of total collagen content in normal breast tissue, and being abundant neomatrix components in invasive breast carcinomas [36], they present a physical barrier to metastasising cells. The physiological significance of this finding is further supported by findings that maspin is present at the cell surface of mammary myoepithelial cells [11], a localisation which would automatically facilitate a contact with these extracellular matrix components. The dissociation affinity constant of maspin for collagen type I was estimated in this study to be 0.63 µM, which is within than the range (0.3 - 3 µM) used to show mPEDF binding to collagen by surface plasmon resonance [22]. It is also similar to the concentration of maspin (0.44 µM) used to alter the integrin profile of MDA-MB-435 cells [13], to achieve inhibition of metastasis (0.17 µM) in in vitro invasion assays [3] and to inhibit angiogenesis (0.2 - 0.3 µM) [4].

The fact that the interaction was detected in the intracellular yeast system indicates that glycosylation of maspin or collagen is not required for binding, in contrast
to some serpin-glycosaminoglycan interactions. At this stage we have narrowed down the collagen binding region of maspin to amino acids 84-112 using the two hybrid system. This corresponds with evidence that colligin binding is localised to amino acids 104 to 169 [29], which aligns with residues 65 to 116 of maspin, and suggests the existence of a general collagen binding motif in serpins which remains to be well-defined. In contrast to cell migration effects, inhibition of angiogenesis by maspin was found to be independent of the C-terminal sequence but appeared to require the N-terminal 139 amino acids, suggesting that this function may coincide with the collagen binding site.

The finding that maspin can alter the integrin profile of MDA-MB-435 cells provides evidence that maspin may prevent invasion by increasing anchoring of cells to the extracellular matrix. This has been more directly demonstrated in corneal stromal cells where exogenous recombinant maspin has been found to restore cell adhesion in stromal cells as measured by increased adhesion to collagen type I and other extracellular matrix components [14]. A direct adherence to both collagen and an uncharacterised cell surface receptor for maspin may be an explanation for these findings. As found in other serpin-ligand interactions, it is possible that collagen binding may alter the structural conformation of maspin, either being necessary for stability, receptor binding or for active presentation of the maspin reactive site loop.

The possibility that intracellular maspin may be acting as a chaperone for collagen similar to colligin, seems unlikely given that maspin and collagen do not appear to be expressed in the same cell types, and the role of intracellular maspin remains unclear.

In conclusion, using yeast-two-hybrid technology, we provide evidence that the N-terminal region of maspin can bind collagen types I and III. This interaction may contribute to the function of maspin as a tumour suppressor and angiogenesis inhibitor, either by direct adherence between cell surface maspin and extracellular matrix collagen, or by altering the ability of maspin to interact with other proteins.

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FIGURE LEGENDS

Figure 1: Expression of pEG202 LexA/serpin fusion protein products
Immunoblot analysis of total protein from transfected yeast EGY48 strains separated on 10% SDS-PAGE gels, and detected with anti-lexA polyclonal antibody.
A. lane 1, extract from control yeast transformed with native pEG202 showing native lexA binding domain at the expected size of 22 kDa.; lane 2, protein extract from EGY48 transformed with a pEG202-PAI-2 construct.
B. lane 1: lexA binding domain as above; lane 2, pEG202-maspin construct. The expected molecular size of each LexA fusion protein is between 65-70 kDa but both run considerably lower at 47-49 kDa.

Figure 2: Positive maspin interactants identified from a yeast two hybrid screen of a human fibroblast library.
Positive interactor yeast clones that yielded expression of both reporter genes were isolated.
A. Agarose gel of inserts amplified from the pJG4-5 target library plasmid using oligonucleotide primers directed against vector arms flanking the multiple cloning site. Insert size and restriction analysis indicated at least four different target inserts contained within the pool of 26 positive interactor yeast clones.
B. Identity of the four target inserts, as revealed by exact database sequence homology.

Figure 3: Isolation of rMaspin and binding to type 1 collagen-agarose
A. Coomassie stained SDS-PAGE gel (10%) of pooled fractions from purification stages of recombinant human maspin expressed in E.coli. BL21 (DE-3) cells transformed with pRSETC-maspin. Lane 1, protein molecular weight markers; lane 2,
20 µg of crude extract from IPTG induced cultures; lane 3, 10 µg of pooled maspin-containing Q-Sepharose fractions; lane 4, 5 µg rMaspin following metal ion affinity chromatography.

B. Binding of rMaspin to type I collagen agarose matrix pre-equilibrated with 50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.05% Brij35. Lane 1, unbound material; lane 2, column wash with equilibration buffer; lane 3, Fraction eluted with 125 mM Tris-HCl, pH 6.8, 2% SDS, 5% β-mercaptoethanol.

C. Recombinant PAI-2 control: Lane 1, unbound recombinant PAI-2; lane 2, column wash; lane 3 eluted fraction.

**Figure 4**: Electrophoresis of maspin-collagen interaction

(A) Native 10% PAGE and (B) 10% SDS-PAGE immunoblot analysis of maspin binding to type 1 collagen. Lane 1, maspin alone (4 µg); lane 2, with 4 µg collagen; lane 3, with 2 µg collagen. Maspin was detected using mouse IgM anti-maspin with enhanced chemiluminescence.

**Figure 5**: Dot blot analysis of rMaspin binding to collagen subtypes.

Nitrocellulose membrane spotted with decreasing amounts of collagen types I, II, III, IV, and V, blocked with 4% BSA, and incubated with rMaspin (20 µg/ml). Collagen bound maspin was detected by immunostaining with anti-maspin as described.

**Figure 6**: Kinetic analysis of maspin interaction with collagen type I using Iasys technology

A. Iasys binding curves for the interaction of maspin to collagen type I immobilised to the surface of a planar aminosilane biosensor surface. The binding of different concentrations of maspin (0.5-3.9 µM) to immobilised collagen was followed in real time for approximately 3 minutes at 25°C, with binding of BSA (30 µM) to collagen as a negative control.

B. Using Fastfit software, the $k_{on}$ of maspin for collagen type I at each concentration
of maspin was determined. A plot of $k_{on}$ against maspin concentration yields a straight line, the slope of which corresponds to the association rate constant, $k_a = 3.2 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$, and the $y$-intercept of which corresponds to the dissociation rate constant, $k_d = .002 \text{ s}^{-1}$. The dissociation affinity constant $K_D$, described by $k_d/k_a$, is calculated to be $0.63 \times 10^{-6} \text{ M}$.

**Figure 7: Yeast-two-hybrid one-on-one interactions with maspin N-terminal fragments.**

Maspin truncation constructs (sizes indicated in amino acids) were individually analysed for collagen interaction. The isolated target plasmid, pJG4-5, containing the $\alpha_2$ chain of type I collagen was cotransformed into *S.cerevisiae* EGY48 with pEG202 bait plasmid containing either no insert or maspin truncation constructs corresponding to amino acids 1-34, 1-112, 1-166, 1-214, 55-214, 84-214 and 112-214. Yeast harbouring a positive interaction were identified by their ability to activate the $\beta$-galactosidase reporter system. True interactors will show $\beta$-galactosidase expression when grown on a carbon source of galactose/raffinose (Gal), but not glucose (Glu), since the collagen target expression is under the control of a Gal1 promoter. All of the maspin fragments except 112-214 show $\beta$-galactosidase activity. The shortest fragment (1-34) possesses an intrinsic ability to activate transcription of the reporter gene in the absence of a collagen partner.
| Clone name | Insert length (bp) | Insert Identity                        |
|------------|--------------------|----------------------------------------|
| hF-3       | 500                | Metallothionein                        |
| hF-7       | 1050               | Elongation factor 1 alpha              |
| hF-13      | 1350               | Transketolase                          |
| hF-18      | 650                | Collagen type 1 (alpha 2 chain)        |
| Collagen subtype | Collagen (μg) |
|------------------|--------------|
|                  | 2 | 1 | .5 | .25 |
| I                |   |   |    |    |
| II               |   |   |    |    |
| III              |   |   |    |    |
| IV               |   |   |    |    |
| V                |   |   |    |    |
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