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Identification of novel peptide motifs in the serpin maspin that affect vascular smooth muscle cell function

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ABSTRACT

Maspin is a non-inhibitory member of the serpin family that affects cell behaviours related to migration and survival. We have previously shown that peptides of the isolated G α-helix (G-helix) domain of maspin show bioactivity. Migration, invasion, adhesion and proliferation of vascular smooth muscle cells (VSMC) are important processes that contribute to the build-up of atherosclerotic plaques. Here we report the use of functional assays of these behaviours to investigate whether other maspin-derived peptides impact directly on VSMC; focusing on potential anti-atherogenic properties. We designed 18 new peptides from the structural moieties of maspin above ten amino acid residues in length and considered them beside the existing G-helix peptides. Of the novel peptides screened those with the sequences of maspin strand 4 and 5 of beta sheet B (S4B and S5B) reduced VSMC migration, invasion and proliferation, as well as increasing cell adhesion. A longer peptide combining these consecutive sequences showed a potentiation of responses, and a 7-mer contained all essential elements for functionality. This is the first time that these parts of maspin have been highlighted as having key roles affecting cell function. We present evidence for a mechanism whereby S4B and S5B act through ERK1/2 and AMP-activated protein kinase (AMPK) to influence VSMC responses.

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1. Introduction

The non-inhibitory serpin maspin (SerpinB5) affects cell behaviours that are consistent with its initial identification as a tumour metastasis suppressor in breast carcinoma [1]. Since then numerous studies have demonstrated that it decreases tumour growth, invasion, metastasis and angiogenesis (recently reviewed [2]). Maspin affects a range of cell types including those that do not express it. Commonly expressed by epithelial cells and lost in carcinogenesis, it has also been shown to be expressed by and influence endothelial cells [3], lymphocytes [4] and smooth muscle cells [5]. We have shown that maspin affects the functions of vascular smooth muscle cells (VSMC) that impact on the development of atherosclerosis; also referred to as atherogenesis [6–8].

VSMC migration and proliferation are important in the response to injury and the pathogenesis of vascular disease [9,10]. VSMC surrounding normal blood vessels are differentiated which means that they do not proliferate or migrate [11]. One of the responses to vascular injury is that VSMC dedifferentiate, becoming motile and proliferative [11]. Whether migration and proliferation of VSMC are beneficial or not depends on the stage of the disease. Migration of VSMC to sites of vascular injury is involved in the early stages of atherosclerosis and in restenosis following angioplasty; the VSMC then proliferate *in situ*. The balance between proliferation and apoptosis of VSMC is important in atherosclerotic plaque development. Once an atherosclerotic plaque is established it is dependent on VSMC for stability – plaque rupture triggers thrombus formation, leading to heart attack or stroke. The VSMC functions contributing to these behaviours can be influenced by maspin which prevents migration, invasion and proliferation, while increasing adhesion – consequences that can generally be thought of as anti-atherogenic.

We have previously demonstrated that bioactive peptides from the maspin structure can influence VSMC behaviour. We found that a peptide of the G α-helix in isolation (G-helix) replicated the effect of full-length maspin in reducing the migration of a variety of cell types including VSMC, and contributed to the action of maspin on cell adhesion [8]. Subsequently other maspin derived peptides have been shown to have biological effects. Peptides from beta sheets strand 1A (S1A) and strand 2C (S2C) of maspin were reported to cause epithelial cell adhesion [12]. In support of our demonstration of the influence of the G-helix on cell

Abbreviations: VSMC, vascular smooth muscle cells; S4B, strand 4 of beta sheet B; S5B, strand 5 of beta sheet B; RCL, reactive centre loop; AMPK, AMP-activated protein kinase; G-helix, G α-helix; S1A, strand 1 of beta sheet A; S2C, strand 2 of beta sheet C.

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migration and adhesion, this region of maspin has independently been shown to affect these functions in endothelial cells, resulting in the inhibition of angiogenesis when incorporated into supramolecular nanostructures [13].

How maspin derived peptides affect cell function is an intriguing question. It is possible that they replicate a subset of the protein-protein interactions of the whole protein. Like PAI-2 [14], maspin does not have a defined signal sequence but is found in both intra- and extra-cellular locations [8,12,15], with binding partners characterized in both contexts. We have been interested in how maspin in the extracellular environment can influence VSMC behaviour, since discovering that it acts without being able to directly inhibit serine protease activity [6]. We found that maspin directly bound β1 integrins on the VSMC surface affecting adhesion and migration [7]. Maspin – integrin interactions have also been demonstrated on the surface of epithelial and endothelial cells [3,16]. Overall these studies imply that there are both direct and indirect contributions of maspin to major cellular processes.

The cell-signalling pathways associated with maspin are not well understood, and have not been reported for maspin peptides previously. There is some evidence for an overlap with integrin-associated signalling pathways. The interaction of maspin with small GTPases Rac-1 and Cdc42 has been reported to modulate PI3K and ERK1/2 [17] and JNK kinase and AP-1 [18] in breast carcinoma and FAK in endothelial cells [3]. FAK along with Akt has been implicated in how maspin influences apoptosis and angiogenesis in prostate cancer [19]. Multiple signals have been associated with VSMC proliferation and migration, with AMPK (AMP-activated protein kinase), ERK 1/2 and Akt commonly reported [20], however there have been no previous reports of maspin interacting with these signalling pathways in VSMC.

In this study we designed eighteen new peptides from the structural moieties of maspin, in addition to the G-helix, and functionally screened them to determine how they influenced the adhesion and proliferation of VSMC. The initial screens indicated that six peptides could alter cellular activity. We designed specific peptide controls for each of these six peptides and subsequently identified two peptides which displayed significant bioactivities. Strand 4 & 5 of maspin beta sheet B (S4B and S5B) decreased VSMC proliferation, migration, and invasion, while increasing adhesion. As the sequences for these regions are consecutive we tested a long peptide of the sequences of S4B and S5B together – which showed enhanced effects in comparison to the isolated peptides. Sequential removal of residues from either end of S5B allowed the identification of a minimal active region comprising a 7-mer. Insights into how these sequences influence VSMC function were provided by studies demonstrating changes in ERK1/2 and AMPK signalling in response to S4B and S5B.

2. Materials and methods

2.1. Peptides, antibodies and materials

19 peptides corresponding to discrete structural moieties of maspin were synthesized by Pepceuticals (Leicestershire, UK), with biotin-aHx on their N terminus and an amide on their C terminus. Peptides were prepared at 95% purity and dissolved in dimethyl sulfoxide (DMSO) to a concentration of 10 mM. Test peptides are detailed in Table 1. The G-helix control peptide was used as a comparison for the initial screens [8]; additional control peptides were subsequently designed to match the charge and composition of their respective test peptides (Table 2). A long maspin peptide and matching control were synthesized; this comprised the total length of peptides S4B and S5B, which are adjacent in the maspin amino acid sequence (Table 1). Attenuated S5B peptides are detailed in Table 3 Peptides were usually used at a final concentration of 10 μM in assays, which had previously been defined as optimal [8]. Anti-β1 (Mab17781, 1:1000) integrin was from R&D Systems (Abingdon, UK). β3 integrin (MAB20232, 1:1000) MAb was purchased from Millipore (Hertfordshire, UK). Antibodies to ERK1/2 (9102S, 1:1000) and ERK1/2P (9101, 1:1000) were from New England Biolabs UK (Hertfordshire, UK). Anti-AMPKa (GTX50863, 1:1000) and AMPKaP (GTX63165, 1:1000) were from Source Bioscience (Nottingham, UK). Fluorescent dye DilC16 was from Life Technologies (Paisley, UK). WST-1 was purchased from Roche (Burgess Hill, UK). All other reagents were purchased from Sigma Aldrich.

Table 1
Sequences of maspin derived peptides.
Peptides were designed from the discrete structural moieties of maspin (sequences <10 residues were excluded). The starting position of each peptide in the maspin sequence is indicated. Nomenclature used such that S2A is strand 2 of beta sheet A.

Peptide	Sequence
RCL	329-DGGDSIEVPGARIL
Helix A	4-LQLANSAFAVDLFKQLCEKE
Helix B	33-ICLSTSLSLAQVGA
Helix C	48-DTANEIGQVLH
Helix D	65-DIPFGFQTVTSDVNKL
Helix G	236-EDESTGLEKIEKQLN
Helix F	126-EETKGQINNSIKDLTD
Helix H	252-NSESLSQWTN
Helix I	285-KACLENLGLK
S2A	74-YSLKLIKRLYVDKS
S3A	158-KILVVNAAYFVG
S5A	317-NVIHKVCLEIT
S1B	190-TDTKPVQMMNMEA
S2B	213-NCKIIELPFQN
S3B	225-HLSMFILLPKD
S4B	354-FIYIIRHNKT
S5B	364-RNIIFFGKFC
S4B/S5B Long	354-FIYIIRHNKTRNIIFFGKFC
S2C	260-NPSTMANAKVKLSIP
S3C	181-TKECPFRLNK

2.2. Cell culture

Primary aortic smooth muscle cells (referred to as VSMC), were cultured as described previously [7] in 231 medium supplemented with smooth muscle cell growth supplement containing 5% (v/v) FBS. For some experiments, VSMC were transferred into phenol red free serum-free MEM. VSMC were used between passages 3 and 8. HT29 cell line was purchased from ATCC and cultured in DMEM, supplemented with 10% (v/v) FBS, L-glutamine and penicillin/streptomycin. All cell culture reagents including extracellular matrix (ECM) components were from ThermoFisher Scientific (Paisley, UK).

2.3. Kinetic adhesion and proliferation assay

To screen the 19 maspin peptides for their ability to alter adhesion and proliferation of VSMC, an xCELLigence DP real time cell analyser

Table 2
Sequences of control peptides.
Controls were matched for size and charge to the corresponding maspin peptide.

Peptide	Sequence
G Helix Control (C-Pep)	EDESTGELKILKQEN
RCL Control	EAAESIDVPAGRIL
S2A Control	YSIRILRKIYVDRS
S3A Control	KIVLLNGGYFLA
S5A Control	TLIHKLCVEIN
S4/5B Control	FIKNRHTYCG
S4B/S5B Long Control	FIKNRHTYCGFIKNRHTYCG

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