Explant (e14, e18, e21, e22)

<table>
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<th>GAG-release (μg/mg tissue)</th>
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(54) Title: NOVEL PEPTIDES AND USES THEREOF

(57) Abstract: The present invention provides novel polypeptide comprising the amino acid sequence of SEQ ID NOS: 1, 2, 3, 4 or 5, variants, fusions or derivatives thereof, and pharmaceutical formulations of the same. The peptides and formulations of the invention are useful for treating diseases or conditions capable of being treated by an agent which modulates the function of an integrin and/or a metzincin, such as arthritic diseases, inflammatory diseases and conditions and cancer. Also provided are diagnostic methods for such diseases and conditions, and methods of screening for candidate compounds with efficacy in the treatment of such diseases and conditions.

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NOVEL PEPTIDES AND USES THEREOF

Field of Invention

The present invention relates to novel polypeptides capable of binding to an integrin I-domain, and to pharmaceutical compositions comprising the same. The invention further provides uses of such polypeptides and pharmaceutical compositions in the treatment of diseases and conditions associated with abnormal function of cellular processes involving integrins and metzincins. In particular, the present invention provides novel agents for the treatment of arthritic diseases (such as osteoarthritis and rheumatoid arthritis), inflammatory diseases and cancer.

Introduction

Integrins

Integrins are widely expressed receptors that function primarily as mediators of cell-extracellular matrix (ECM) interactions. Integrins consist of one α subunit and one β subunit, which together form a non-covalently bound heterodimer (Hynes 2002). To date, eight β subunits and 18 α subunits have been characterised in man, forming twenty-four different heterodimers. Four of the integrin heterodimers, namely αlβ1, α2β1, αQβ1 and αlβ1, are receptors for collagens, the most abundant of ECM proteins, and are known as the 'collagen-binding integrins'.

The collagen-binding integrin heterodimers are glycoproteins having masses ~220 kDa (α-130, β ~90) for the polypeptides, plus additional glycosylation. The α-subunit ectodomains consist of the N-terminal head region and the leg or stalk region. The α-subunit head regions are formed from a seven-bladed β-propeller domain, with the α-domain looping out from between blades 2 and 3. The three
leg region domains, thigh, upper calf and lower calf, all have β-sandwich folds. The β-subunit head region includes the PSI, hybrid and I-like (βl) domains, and interacts extensively with the β-propeller domain of the α-subunit.

The collagen-binding integrins contain an αl-domain (von Willebrand factor A-like domain) inserted into the α-subunit β-propeller domain between blades 2 and 3. The αl-domain is the main collagen-binding site. The βl-domains inserted into integrin β-subunit hybrid domains share homology with αl-domains. The αl-domains and βl-domains have a central β-sheet surrounded with α-helices. Metal binding sites are present in αl-domains and βl-domains, and divalent cations (specifically Mg$^{2+}$, Ca$^{2+}$ or Mn$^{2+}$), are required for high affinity binding to collagens.

The αl-domains of the collagen-binding integrins exhibit structural differences giving rise to their different ligand preferences. Structural differences that enable discrimination between the α-subunits are also useful in design of αl-βl- and αl1βl-selective peptides capable of binding to the αl-domains.

In man, all four collagen-binding integrins have different expression patterns. Integrin α1β1 is expressed in mesenchymal cell types including smooth muscle cells, endothelial cells, fibroblasts and chondrocytes (Voigt et al. 1995). It is also found on certain lymphocytes and monocytes. Integrin α2β1 is expressed on epithelial cells, platelets, endothelial cells, fibroblasts, chondrocytes (Zutter and Santoro 1990), lymphocytes, mast cells, and neutrophilic granulocytes. In comparison, expression of the integrins α1β1 and α11β1 is more restricted. The α1β1 integrin is mainly expressed by chondrocytes but has also been detected in the heart valves and in the fibrous lining of skeletal muscle, tendon and in periostium and perichondrium. The α11β1 integrin is expressed on specialised fibroblasts in tendon, periodontal ligaments, perichondrium, periostium, and synovium. (Camper et al. 2001; Tigeret al. 2001).

Structural and functional aberrations of these and other members of the integrin family, illustrated by their knockout models, result in a number of pathologies.
It is therefore not surprising that these integrins have great potential as therapeutic targets. For example, the restricted distribution of the integrins αlβl and αlβl, mainly to tissues in the joint, bone and cartilage, tendon and synovium, implicates a potential role for these integrins as therapeutic targets for diseases associated with such tissues, for example osteoarthritis and rheumatoid arthritis (Iannone and Lapadula 2003). In addition, the αlβl and αlβl integrins are believed to play a role in other pathological conditions involving tissues in which the expression of these integrins is altered, such as atherosclerosis (WO 03/105886), carcinoma (Wang et al. 2002), tumour metastasis (Melendez et al. 2005) (see also http://bioinfo.cnio.es/data/oncochip/content_12.html), melanoma, periodontitis, osteoporosis, as well as in diseases and other conditions involving elevated levels of MMPs which associate with collagen-binding integrins, such as multiple sclerosis, periodontitis, osteoporosis, osteosarcoma, osteomyelitis, bronchiectasis, chrome pulmonary obstructive disease, and skin and eye diseases (Mohammed et al. 2003).

A comprehensive review of integrins as therapeutic targets is given in (EbL e 2005) and (Rose et al. 2000).

Metzincins

Metzincins (zinc-dependent metallopeptidases) are a superfamily of proteases that includes the matrixin family of matrix metalloproteinases (MMPs), which are well known regulators of extracellular matrix (ECM) proteolysis in the microenvironment. Other members of the metzincin superfamily include the transmembrane proteases ADAMs (a disintegrin and metalloproteinases) and the secreted ADAMTSs (ADAM with thrombospondin domain). Although not all ADAMs are proteolytically active, they are recognised for their ability to process or shed cell surface molecules, whereas ADAMTS enzymes can cleave matrix and other proteins and are implicated in diseases such as arthritis (Clark and Parker 2003).
**Integrin and MMP interactions**

Matrix metalloproteinases (MMPs) constitute a superfamily of 25 zinc-dependent and calcium-dependent proteinases in mammalian systems, and are thought to be the major proteolytic enzymes that facilitate tissue remodeling in both physiological and pathological situations (Nagase and Woessner, Jr. 1999) (Elliott S. and Cawston 2001;Vu and Werb 2000). MMPs have the combined ability to degrade the major components of the extracellular matrix (ECM) (Birkedal-Hansen 1995). Moreover, the MMP family contains the only mammalian proteinases which can specifically degrade triple helical collagens, the ligands for the α<sub>1</sub>β<sub>1</sub> and α<sub>1</sub>β<sub>3</sub> integrins, at neutral pH. These so-called collagenases specifically cleave a single locus in all three collagen chains at a point three-quarters from the N-terminus of the molecule. The 'classical' collagenases (MMP-I, -8 and -13) have differing substrate specificities for collagens I, II and III, with MMP-13 showing a preference for type II collagen (Knauper et al. 1996). More recently, gelatinase A (MMP-2) and MT1-MMP (MMP-14) have also been shown to cleave collagen in a specific manner, though with less catalytic efficiency than the classical collagenases, at least in vitro (Vincenti and Brinckerhoff 2002). The enzyme(s) responsible for cartilage collagen cleavage in the arthritides remains open to debate, but the dogma has been that MMP-I, produced in the synovium, is the primary collagenase in rheumatoid arthritis (RA), whilst MMP-13, produced by the chondrocyte, is the foremost collagenase in OA. Several other members of the MMP family have been localised to cartilage or synovium in the arthritides (Pap et al 2000).

Due to their destructive nature in pathological diseases such as OA and RA, the synthesis and activation of the MMPs are very tightly controlled in normal tissues. Cells release most of the MMPs as pro-enzymes which are later activated in the extracellular matrix. Additionally, MMPs are often trapped in a deactivating complex with tissue inhibitors of metalloproteinases (TIMP).

Increasing evidence now shows that integrins can bind to MMPs and that ligand binding to an integrin can also regulate MMP expression (Lee and Murphy 2004). The induction of MMP expression upon collagen stimulation has been reported.
from several different cell lines, including keratinocytes, smooth muscle and osteoprogenitor cells (Riikonen et al. 1995). Major functions of the α2β1 receptor include the induction of MMP1, MMP13 and MT1-MMP as well as activation of proMMP2 (Langholz et al. 1995; Heino 2000; Eckes et al. 2000; Zigrino et al. 2001). Collagen-induced ligation of α2β1 was also reported to up-regulate collagen levels, thereby antagonising α1β1 function (Ivaska et al. 1999).

The formation of a direct complex between a collagen-binding integrin and an MMP has recently been shown in keratinocytes plated on native type I collagen (Dumin et al. 2001). In this complex, the collagen-binding epitope of the α2β1 integrin has been mapped to the hemopexin domain of pro-MMP1. While binding to the I-domain of the α2 subunit, pro-MMP1 physically competes with binding of collagen to the same site (Strieker et al. 2001). Furthermore, recent results suggest that MT1MMP mediated cellular invasion of ovarian cancer cells through a collagen matrix depends on α2β1 ligation and pro-MMP2 processing (Ellerbroek et al. 2001). Integrin α1 knockout mice provide additional evidence for integrin-regulated MMP expression with over-expression of MMP7 and MMP9 (Pozzi et al. 2000).

All these data indicate that collagen-binding integrins are capable of regulating MMP activity via binding of both their natural ligand (e.g. collagen) and/or by binding the MMP at the cell surface for further processing and/or activation (Lee and Murphy 2004).

MMPs in cartilage pathology

Normal cartilage ECM is in a state of dynamic equilibrium, with a balance between proteinases that degrade the ECM and their inhibitors. The ECM of cartilage is made up of two main macromolecules, type II collagen and aggrecan, the latter being a large aggregating proteoglycan (Hardingham and Fosang 1992; Prockop 1998). The type II collagen scaffold endows the cartilage with its tensile strength, whilst the aggrecan, by virtue of its high negative charge, swells against the collagen network as it draws water into the tissue, enabling it to resist
compression. Quantitatively less abundant components (e.g., type IX, XI and VI collagens, biglycan, decorin, COMP etc.) also have important roles in controlling matrix structure and organisation (Heinegard et al. 2003).

In OA and other joint diseases, it is thought that a disruption of this balance in favour of proteolysis leads to pathological cartilage destruction. Progressive degradation of the ECM that comprises the tissue of articular joints, including articular cartilage, bone and even intraarticular ligaments and tendons, is a major feature of the arthritic diseases, leading to permanent loss of function. Although proteinases of all mechanistic classes play a role in the degradation of connective tissue macromolecules, it has long been thought that the major activities involved in this process belong to the family of MMPs. These enzymes are secreted by both the resident cells of joint tissues (chondrocytes), neighboring cells in the synovium as well as by invading cells, they are active around neutral values of pH, and they have the combined ability to degrade all the components of the ECM (Murphy et al. 2002). MMPs play significant roles in both developmental and repair processes, and it appears that aberrant regulation, which can occur at many levels, leads to their hyperactivity in diseases such as rheumatoid arthritis (RA) and osteoarthritis (OA). Significant evidence now exists for the over-expression of MMPs in tissues derived from patients with arthritic disease (Kevorkian et al. 2004; Konttinen et al. 1999).

**MMPs in OA and RA**

The identity of the individual MMPs responsible for cartilage collagen cleavage in the arthritides is still questionable and is proposed to differ between OA and RA. Cartilage degradation in OA and RA is primarily mediated by the chondrocytes, the synovial fibroblasts and on occasions by osteoclasts (Vincenti and Brinckerhoff 2002).

While all of the collagenolytic MMPs (MMPs 1, 8, 13, and 14) produced by chondrocytes can cleave collagen II at Glu975-Leu976 (Sandy 2003) and have been localised in synovial tissue (Clark and Parker 2003), there is now an accumulating body of evidence, both direct and indirect, which implicates
MMP13 as the actual collagenase responsible for initiating collagen destruction in OA. This evidence comes from studies with proteinase inhibitors, immunolocalisation of MMP proteins, and in situ hybridisation for a range of MMP transcripts (Sandy 2003). Studies on normal and OA human articular cartilage explants using MMP inhibitors indicate that MMP13 or MMP8, but not MMP1, are responsible for the generation of the Glu975-Leu976 cleavage (Billinghurst et al. 2000).

MMP13 is transcriptionally regulated in areas of degraded cartilage matrix in osteoarthritic tissue by cytokines such IL-1 and TNFα (Iannone and Lapadula 2003;Mitchell et al. 1996) or alternatively by biomechanical factors (Sun and Yokota 2001;Shlopopov et al. 1997;Shlopopov et al. 1997). This increase in supply of the pro-enzyme results in high activity upon activation of the pro-MMP13. Such activation most likely involves a cell surface process requiring MTI-MMP (MMP14) (Cowell et al. 1998;Murphy et al. 1999) so that the level of active MMP13 is determined, in part, by the production and regulation of MTI-MMP. MMP13 levels are also regulated by the abundance of TIMPs 1, 2 and 3 (tissue inhibitor of metalloproteinases), the protective effects of which are overwhelmed in osteoarthritic cartilage. Further support for the central role of MMP13 has been obtained from a transgenic mouse over-expressing MMP13 (Neuhold et al. 2001).

In summary, high levels of MMP13 mRNA and protein appear to be localised to areas of high collagenolytic activity. Moreover, hMMP13 is 5-10 times as active as hMMP1 in its ability to digest collagen type II, suggesting that MMP13 has a unique role in collagen II-rich tissue such as articular cartilage (Knauper et al. 1996). This therefore makes MMP13 production and regulation a suitable target for therapeutic control in diseases such as, but not limited to, the arthritides.

In RA, a disease that affects multiple synovial joints and involves inflammation of the synovial membrane, the synovium proliferates to form an invasive pannus and the cartilage pannus junction is a site of enhanced cartilage degradation. Inflammation is accompanied by an influx of immune-competent cells, which constitute not only the resident fibroblast-like synoviocytes, but also
macrophages, T-lymphocytes and mast cells (Kobayashi and Okunishi 2002). Fibroblasts contribute to joint destruction both directly, through enhanced production of MMPs, and indirectly, through excessive release of cytokines that boost the immune system and in turn stimulate the MMP production. Targeting the proliferative fibroblast and its MMP production could facilitate regeneration of synovial joints.

A large body of evidence now exists that shows that MMP1 and MMP3 are the most important MMPs for joint destruction in RA (Poole 2001), although almost all the MMPs have been detected in RA synovium (Pap et al. 2000). MMP3's role appears to be restricted to invasiveness, probably permitting transmigration of inflammatory cells from the blood vessels into the synovium, leaving MMP1 as the main MMP responsible for cartilage collagen degradation. Cytokines such as IL-1 and TNFα produced by the invading inflammatory cells (Arend 2001;Goldring 2003) stimulate the resident synovial fibroblasts to produce MMP1 (and MMP3). Furthermore, both MMP1 and MMP3 are also induced by the recently discovered extracellular matrix metalloproteinase inducer (EMMPRIN) (Tomita et al. 2002).

MMPs in other diseases

(i) Cancer

Degradation of extracellular matrix is crucial for malignant tumour growth, invasion, metastasis and angiogenesis. Elevated levels of distinct MMPs can be detected in tumour tissue or serum of patients with advanced cancer, and their role as prognostic indicators in cancer has been widely examined. It is conceivable, that degradation of collagenous ECM by the secreted collagenases (MMP1, MMP8 and MMP13) is essential for invasion of malignant cells. These MMPs often colocalise with other MMPs, i.e. MT1MMP, MMP2, indicating that a proteolytic cascade is required for activation. It is evident, that not only malignant cells but also stromal fibroblasts and inflammatory cells produce MMPs in response to stimulation by different exogenous factors, like cytokines and tumor promoters. In addition, this may contribute to proteolytic remodeling.
of the peritumoral ECM. Numerous studies have demonstrated over-expression of collagenases in malignant tumors in comparison to normal tissue, suggesting a role for them in cancer cell invasion (Hofmann et al. 2005; Ala-aho and Kahari 2005; Vihinen et al. 2005).

(ii) Inflammation

MMPs regulate extracellular matrix and connective tissue turnover. In a number of inflammatory diseases, the affected tissue is remodelled in a manner that is detrimental to the patient. The contribution of MMPs to the pathology and resulting debility is exemplified by the role of these enzymes in diseases such as arthritis, COPD, atherosclerosis and inflammatory bowel disease (IBD) (Whelan 2004).

In contrast to MMP-mediated tissue damage during inflammation, increasing evidence suggests that MMPs can function independently of their ECM degrading activity. Recent reports suggest that specific MMPs may serve as proinflammatory mediators whereas others serve to dampen inflammation through the control of chemoldne regulation (Mohammed et al. 2003).

*Integrins and MMP inhibitors*

Much work has been focused on the design of small molecules as metalloproteinase inhibitors for the treatment of arthritic diseases (Henrotin et al. 2002; Borkakoti 2004; Clark and Parker 2003; Smolen and Sterner 2003; Wieland et al. 2005). Although inhibitors for MMPs have been developed, testing of such agents is still mostly at the experimentation stage and no clinically acceptable inhibitor for MMPs exists as a therapeutic or prophylactic drug for any of the pathological states and diseases potentially connected with MMPs. All MMP inhibitors to that have been tested in clinical studies to date have failed due to toxic side effects.

As an alternative to developing inhibitors that directly bind to the MMP, the present inventors focused upon regulating the binding of the MMP to the
collagen-binding integrins. This approach can circumvent the problems with toxic side effects of direct MMP binders and allow a more tissue (site) specific regulation of the MMPs.

Given the restricted distribution of the integrins $\alpha_1\beta_1$ and $\alpha_1\beta_1$ in adult organisms, targeting MMPs via their interaction with these integrins allows more site-specific effects and in addition can limit any potential treatment-related toxicity. Of particular interest is the pathological elevation of MMPs over the body's endogenous anti-proteinase shield, which is seen in a variety of diseases such as osteoarthritis, rheumatoid arthritis as well as in other diseases such as atherosclerosis, cancer, metastasis, multiple sclerosis, periodontitis, osteoporosis, osteosarcoma, osteomyelitis, bronchiectasis, chronic pulmonary obstructive disease, and skin and eye diseases. Proteolytic enzymes, especially MMPs, are believed to contribute to the tissue destruction damage associated with these diseases (Mohammed et al. 2003).

An alternative to rational molecular design is to screen libraries of random peptides or other small molecules to find lead compounds which are capable of binding to specific targets. In particular, peptide libraries displayed on the surface of bacteriophage have often yielded valuable binding peptides to target proteins, including integrins. The identified peptides can either be developed as drugs themselves or be used in screening of chemical substances that bind to the same site or be used in co-crystallisation together with the target for subsequent drug design.

Accordingly, the present invention seeks to provide novel treatment for diseases and conditions involving abnormal function of integrins and/or metzincins.
Summary of Invention

A first aspect of the invention provides an isolated polypeptide capable of binding to an integrin I-domain wherein the polypeptide comprises an amino acid sequence selected from the following group:

- GIWFENEW [SEQ ID NO:1];
- WIWPDSGW [SEQ ID NO:2];
- WENWDGWG [SEQ ID NO:3];
- WEDGWLHA [SEQ ID NO:4]; and/or
- WCWPDS CW [SEQ ID NO:5];

or a variant, fusion or derivative thereof, or a fusion of a said variant or derivative thereof.

By 'I-domain' we mean an inserted (I-) domain of an integrin α-subunit and/or an integrin β-subunit, located in the N-terminus of the subunit and typically comprising approximately 200 amino acids in length. The αI-domain (von Willebrand factor A-like domain) is an independent, autonomously folding domain inserted into the α-subunit β-propeller domain between blades 2 and 3. βI-domains share homology with αI-domains. Both the αI-domains and βI-domains have a central β-sheet surrounded with α-helices. By 'I-domain' we include related A-domains, for example the A-domain of von Willebrand factor.

Preferred I-domains are detailed in the Examples below.

By 'capable of binding' we mean that binding of the said polypeptide to the said I-domain can be detected using techniques known in the art, for example as described in Examples below. Convenient methods include phage display, affinity chromatography, surface plasmon resonance, yeast two-hybrid interactions, co-purification, ELISA, co-immunoprecipitation methods and cellular response assays.
In a preferred embodiment of the first aspect of the invention, the polypeptide is capable of inhibiting (at least in part) the degradation of collagen in cartilage tissue. Thus, by "inhibiting" we include a reduction of collagen degradation by at least 10% compared to collagen degradation in cartilage in the absence of the polypeptide of the invention, for example a reduction of at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80% or at least 90%. Most preferably, the polypeptide of the invention inhibits the degradation of collagen in cartilage completely.

The degradation of collagen in cartilage may be measured using methods well known in the art, for example by measuring glycosaminoglycan (GAG) release in human cartilage explants (see Examples).

The term 'polypeptide' as used herein means a plurality of amino acids that are linked together via a peptide bond.

In the formulas representing polypeptide embodiments of the present invention, the amino- and carboxy-terminal groups, although often not specifically shown, will be understood to be in the form they would assume at physiological pH values, unless otherwise specified. Thus, the N-terminal H⁺ and C-terminal O' at physiological pH are understood to be present though not necessarily specified and shown, either in specific examples or in generic formulas. In the polypeptide notation used herein, the left-hand end of the molecule is the amino terminal end and the right-hand end is the carboxy-terminal end, in accordance with standard usage and convention. The basic and acid addition salts including those which are formed at non-physiological pH values are also included in the polypeptides of the invention.

The term 'amino acid' as used herein includes the standard twenty genetically-encoded amino acids and their corresponding stereoisomers in the 'D' form (as compared to the natural 'L' form), omega-amino acids other naturally-occurring amino acids, unconventional amino acids (e.g. α, α-disubstituted amino acids, N-alkyl amino acids, etc.) and chemically derivatised amino acids (see below).
When an amino acid is being specifically enumerated, such as 'alanine' or 'Ala' or 'A', the term refers to both L-alanine and D-alanine unless explicitly stated otherwise. Other unconventional amino acids may also be suitable components for polypeptides of the present invention, as long as the desired functional property is retained by the polypeptide. For the peptides shown, each encoded amino acid residue, where appropriate, is represented by a single letter designation, corresponding to the trivial name of the conventional amino acid.

Preferably, the polypeptides of the invention comprise or consist of L-amino acids

In a preferred embodiment of the first aspect of the invention, the polypeptide is less than 50 amino acids in length. Preferably, the polypeptide is less than 40 amino acids in length, for example less than 30, less than 20, less than 15, less than 14, less than 13, less than 12, less than 11, less than 10, less than 9, less than 8, or less than 7 amino acids in length. More preferably, the polypeptide is between 8 and 10 amino acids in length. Most preferably, the polypeptide of the invention is 10 amino acids in length.

It will be appreciated by persons skilled in the art that the first aspect of the invention encompasses variants, fusions and derivatives of the defined polypeptides, as well as fusions of a said variants or derivatives, provided such variants, fusions and derivatives retain the capability of binding to an integrin I-domain.

Variants (whether naturally-occurring or otherwise) may be made using the methods of protein engineering and site-directed mutagenesis well known in the art using the recombinant polynucleotides (see example, see Molecular Cloning: a Laboratory Manual, 3rd edition, Sambrook & Russell, 2001, Cold Spring Harbor Laboratory Press).

By 'fusion' of said polypeptide we include a polypeptide fused to any other polypeptide. For example, the said polypeptide may be fused to a polypeptide such as glutathione-S-transferase (GST) or protein A in order to facilitate purification of said polypeptide. Examples of such fusions are well known to
those skilled in the art. Similarly, the said polypeptide may be fused to an oligo-
histidine tag such as His6 or to an epitope recognised by an antibody such as the
well-known Myc tag epitope. Fusions to any variant or derivative of said polypeptide are also included in the scope of the invention. It will be appreciated that fusions (or variants, derivatives or fusions thereof) which retain desirable properties, such as I-domain binding properties are preferred. It is also particularly preferred if the fusions are ones which are suitable for use in the methods and screening assays described herein.

For example, the fusion may comprise a further portion which confers a desirable feature on the said polypeptide of the invention; for example, the portion may be useful in detecting or isolating the polypeptide, or promoting cellular uptake of the polypeptide. The portion may be, for example, a biotin moiety, a radioactive moiety, a fluorescent moiety, for example a small fluorophore or a green fluorescent protein (GFP) fluorophore, as well known to those skilled in the art. The moiety may be an immunogenic tag, for example a Myc tag, as known to those skilled in the art or may be a lipophilic molecule or polypeptide domain that is capable of promoting cellular uptake of the polypeptide, as known to those skilled in the art.

By 'variants' of the polypeptide we include insertions, deletions and substitutions, either conservative or non-conservative. In particular we include variants of the polypeptide where such changes do not substantially alter the activity of the said polypeptide. In particular, we include variants of the polypeptide where such changes do not substantially alter the I-domain binding activity of the said polypeptide.

It will be appreciated that a variant that comprises substantially all of the sequences shown above may be particularly useful. By 'substantially all' is meant at least six amino acids of the defined sequence, for example at least six consecutive amino acids. More preferably, the variant may comprise at least seven amino acids or, most preferably, all eight of the amino acids of the defined sequence.
By 'conservative substitutions' is intended combinations such as Gly, Ala; Val, He, Leu; Asp, Glu; Asn, Gln; Ser, Thr; Lys, Arg; and Phe, Tyr.

It is particularly preferred if the polypeptide variant has an amino acid sequence which has at least 75% identity with the amino acid sequence given above, more preferably at least 87.5%, and most preferably 100% identity with the amino acid sequence given above.

The percent sequence identity between two polypeptides may be determined using suitable computer programs, for example the GAP program of the University of Wisconsin Genetic Computing Group and it will be appreciated that percent identity is calculated in relation to polypeptides whose sequences have been aligned optimally.

The alignment may alternatively be carried out using the Clustal W program (as described in Thompson *et al.*, 1994, *Nuc. Acid Res.* 22:4673-4680).

The parameters used may be as follows:

- Fast pairwise alignment parameters: K-tuple(word) size; 1, window size; 5, gap penalty; 3, number of top diagonals; 5. Scoring method: x percent.

- Multiple alignment parameters: gap open penalty; 10, gap extension penalty; 0.05.

- Scoring matrix: BLOSUM.

Alternatively, the BESTFIT program may be used to determine local sequence alignments.

Preferably, the variant polypeptides do not comprise the tetrapeptide motif D/E-D/E-G/K-W.
The polypeptides of the first aspect of the invention may comprise one or more amino acids which have been modified or derivatised.

Chemical derivatives of one or more amino acids may be achieved by reaction with a functional side group. Such derivatised molecules include, for example, those molecules in which free amino groups have been derivatised to form amine hydrochlorides, p-toluene sulphonyl groups, carboxybenzoyl groups, tert-butyloxycarbonyl groups, chloroacetyl groups or formyl groups. Free carboxyl groups may be derivatised to form salts, methyl and ethyl esters or other types of esters and hydrazides. Free hydroxyl groups may be derivatised to form O-acyl or O-alkyl derivatives. Also included as chemical derivatives are those peptides which contain naturally occurring amino acid derivatives of the twenty standard amino acids. For example: 4-hydroxyproline may be substituted for proline; 5-hydroxylysine may be substituted for lysine; 3-methylhistidine may be substituted for histidine; homoserine may be substituted for serine and ornithine for lysine. Derivatives also include peptides containing one or more additions or deletions as long as the requisite activity is maintained. Other included modifications are amidation, amino terminal acylation (e.g. acetylation or thioglycolic acid amidation), terminal carboxylamidation (e.g. with ammonia or methylamine), and the like terminal modifications.

It will be further appreciated by persons skilled in the art that peptidomimetic compounds may also be useful. Thus, by 'polypeptide' or 'peptide' we include peptidomimetic compounds which are capable of binding to an integrin I-domain. The term 'peptidomimetic' refers to a compound that mimics the conformation and desirable features of a particular peptide as a therapeutic agent.

For example, the polypeptides of the invention include not only molecules in which amino acid residues are joined by peptide (-CO-NH-) linkages but also molecules in which the peptide bond is reversed. Such retro-inverso peptidomimetics may be made using methods known in the art, for example such as those described in Meziere et al. (1997) J. Immunol. 159, 3230-3237. This approach involves making pseudopeptides containing changes involving the backbone, and not the orientation of side chains. Retro-inverse peptides, which
contain NH-CO bonds instead of CO-NH peptide bonds, are much more resistant to proteolysis.

In an alternative preferred embodiment, the polypeptide of the invention is a peptidomimetic compound wherein one or more of the amino acid residues are linked by a -CH_2NH- bond in place of the conventional amide linkage.

Similarly, the peptide bond may be dispensed with altogether provided that an appropriate linker moiety which retains the spacing between the carbon atoms of the amino acid residues is used; it is particularly preferred if the linker moiety has substantially the same charge distribution and substantially the same planarity as a peptide bond.

It will be appreciated that the peptide may conveniently be blocked at its N- or C-terminus so as to help reduce susceptibility to exoproteolytic digestion.

A variety of uncoded or modified amino acids such as D-amino acids and N-methyl amino acids have also been used to modify mammalian peptides. In addition, a presumed bioactive conformation may be stabilised by a covalent modification, such as cyclisation or by incorporation of lactam or other types of bridges, for example see Veber et al., 1978, Proc. Natl. Acad. Sd. USA 75:2636 and Thursell et al., 1983, Biochem. Biophys. Res. Comm. 111:166.

A common theme among many of the synthetic strategies has been the introduction of some cyclic moiety into a peptide-based framework. The cyclic moiety restricts the conformational space of the peptide structure and this frequently results in an increased affinity of the peptide for a particular biological receptor. An added advantage of this strategy is that the introduction of a cyclic moiety into a peptide may also result in the peptide having a diminished sensitivity to cellular peptidases.

Thus, preferred polypeptides of the invention comprise terminal cysteine amino acids. Such a polypeptide may exist in a heterodetic cyclic form by disulphide bond formation of the mercaptide groups in the terminal cysteine amino acids or
in a homodetic form by amide peptide bond formation between the terminal amino acids. As indicated above, cyclising small peptides through disulphide or amide bonds between the N- and C-terminus cysteines may circumvent problems of affinity and half-life sometime observed with linear peptides, by decreasing proteolysis and also increasing the rigidity of the structure, which may yield higher affinity compounds. Polypeptides cyclised by disulphide bonds have free amino and carboxy-termini which still may be susceptible to proteolytic degradation, while peptides cyclised by formation of an amide bond between the N-terminal amine and C-terminal carboxyl and hence no longer contain free amino or carboxy termini. Thus, the peptides of the present invention can be linked either by a C-N linkage or a disulphide linkage.

Cyclic peptides may have longer half-lives in serum (see, for example, (Picker and Butcher 1992;Huang et al. 1997). Moreover, the side-effects from cyclic peptide therapy are minimal, since anaphylaxis and immune responses against the small peptide occur only rarely (Ohman et al. 1995;Adgey 1998). In addition, cyclic peptides have been shown to be effective inhibitors in vivo of integrins involved in human and animal disease (Jackson et al. 1997;Cuthbertson et al. 1997;Lefkovits and Topol 1995;Lefkovits and Topol 1995;Lefkovits and Topol 1995;Goligorsky et al. 1998;Ojima et al. 1995;Noûr et al. 1994). The present invention is not limited in any way by the method of cyclisation of peptides, but encompasses peptides whose cyclic structure may be achieved by any suitable method of synthesis. Thus, heterodetic linkages may include, but are not limited to formation via disulphide, alkylene or sulphide bridges. Methods of synthesis of cyclic homodetic peptides and cyclic heterodetic peptides, including disulphide, sulphide and alkylene bridges, are disclosed in US 5,643,872. Other examples of cyclisation methods are discussed and disclosed in US 6,008,058. Cyclic peptides can also be prepared by incorporation of a type H1 β-turn dipeptide (Doyle et al. 1996).

A further approach to the synthesis of cyclic stabilised peptidomimetic compounds is ring-closing metathesis (RCM). This method involves steps of synthesising a peptide precursor and contacting it with an RCM catalyst to yield a conformationally restricted peptide. Suitable peptide precursors may contain two
or more unsaturated C-C bonds. The method may be carried out using solid-phase-peptide-synthesis techniques. In this embodiment, the precursor, which is anchored to a solid support, is contacted with a RCM catalyst and the product is then cleaved from the solid support to yield a conformationally restricted peptide.

Another approach, disclosed by D. H. Rich in *Protease Inhibitors*, Barrett and Selveson, eds., Elsevier (1986), has been to design peptide mimics through the application of the transition state analogue concept in enzyme inhibitor design. For example, it is known that the secondary alcohol of staline mimics the tetrahedral transition state of the scissile amide bond of the pepsin substrate.

In summary, terminal modifications are useful, as is well known, to reduce susceptibility by proteinase digestion and therefore to prolong the half-life of the peptides in solutions, particularly in biological fluids where proteases may be present. Polypeptide cyclisation is also a useful modification and is preferred because of the stable structures formed by cyclisation and in view of the biological activities observed for cyclic peptides.

Thus, in one embodiment the polypeptide of the first aspect of the invention is linear. However, in a preferred alternative embodiment, the polypeptide is cyclic.

The present invention also includes compositions comprising pharmaceutically acceptable acid or base addition salts of the polypeptides of the present invention. The acids which are used to prepare the pharmaceutically acceptable acid addition salts of the aforementioned base compounds useful in this invention are those which form non-toxic acid addition salts, *i.e.* salts containing pharmacologically acceptable anions, such as the hydrochloride, hydrobromide, hydroiodide, nitrate, sulphate, bisulphate, phosphate, acid phosphate, acetate, lactate, citrate, acid citrate, tartrate, bitartrate, succinate, maleate, fumarate, gluconate, saccharate, benzoate, methanesulphonate, ethanesulphonate, benzensulphonate, p-toluenesulphonate and pamoate [*i.e.* l,l'-methylene-bis-(2-hydroxy-3 naphthoate)] salts, among others.
Pharmaceutically acceptable base addition salts may also be used to produce pharmaceutically acceptable salt forms of the compounds according to the present invention.

The chemical bases that may be used as reagents to prepare pharmaceutically acceptable base salts of the present compounds that are acidic in nature are those that form non-toxic base salts with such compounds. Such non-toxic base salts include, but are not limited to those derived from such pharmacologically acceptable cations such as alkali metal cations (e.g. potassium and sodium) and alkaline earth metal cations (e.g. calcium and magnesium), ammonium or water-soluble amine addition salts such as N-methylglucamine-(meglumine), and the lower alkanolammonium and other base salts of pharmaceutically acceptable organic amines, among others.

It will be appreciated by persons skilled in the art that the polypeptides of the invention may exist in monomeric form or in the form of a multimer thereof (e.g. dimer, trimer, tetramer, pentamer, etc.).

In a preferred embodiment of the first aspect of the invention, there is provided a polypeptide comprising the amino acid sequence of SEQ ID NO:1. Particularly preferred is a polypeptide comprising or consisting of the amino acid sequence CGIWFENEWC [SEQ ID NO:6].

In an alternative preferred embodiment of the first aspect of the invention, there is provided a polypeptide comprising the amino acid sequence of SEQ ID NO:2. Particularly preferred is a polypeptide comprising or consisting of the amino acid sequence CWTWPDSGWC [SEQ ID NO:7].

In a further alternative preferred embodiment of the first aspect of the invention, there is provided a polypeptide comprising the amino acid sequence of SEQ ID NO:3. Particularly preferred is a polypeptide comprising or consisting of the amino acid sequence CWENWDGWGC [SEQ ID NO:8].
In yet another alternative preferred embodiment of the first aspect of the invention, there is provided a polypeptide comprising the amino acid sequence of SEQ ID NO:4. Particularly preferred is a polypeptide comprising or consisting of the amino acid sequence CWEDGWLHAC [SEQ ID NO:9].

In yet another alternative preferred embodiment of the first aspect of the invention, there is provided a polypeptide comprising the amino acid sequence of SEQ ID NO:5. Particularly preferred is a polypeptide comprising or consisting of the amino acid sequence CWCWPSCWC [SEQ ID NO:10].

Most preferably, the polypeptide comprising or consisting of the amino acid sequence of SEQ ID NOS: 5, 6, 7, or 8 contains a disulphide bond between the two cysteine amino acids.

A characterising feature of the polypeptides of the present invention is their ability to bind to an I-domain of an integrin subunit.

Advantageously, the I-domain is from an integrin which is expressed on chondrocytes, fibroblasts and/or cancer cells.

Preferably, the integrin is a collagen-binding integrin. More preferably, the I-domain is from an integrin selected from the group consisting of α1β1, α2β1, αδΩβ1 and αδlβ1. Most preferably, the integrin is αδΩβ1 or αδlβ1 integrin.

Alternatively, the integrin may be selected from the group consisting of αDβ2, αMβ2, αLβ2, αXβ2 and αEβ7.

The polypeptides of the invention may be capable of binding specifically to the I-domain of a single integrin species or may be capable of binding to the I-domain of a number of different integrin species.
In a preferred embodiment, the polypeptides of the invention are capable of inhibiting a function or property of an integrin. For example, the polypeptide may be capable of inhibiting the binding of collagen to an integrin.

Alternatively, or in addition, the polypeptide is capable of inhibiting the binding of a metzincin to an integrin. By 'metzincin' we include matrix metalloproteinases (MMPs), disintegrin and metalloproteases (ADAMs) and disintegrin and metalloproteases with thrombospondin motifs (ADAMTSs), and pro-forms thereof.

In one embodiment, the metzincin is a disintegrin and metalloprotease (ADAM) (Clark and Parker 2003).

In an alternative embodiment, the metzincin is a disintegrin and metalloprotease with thrombospondin motifs (ADAMTS). For example, see Jones and Riley, 2005, Arthritis Research & Therapy 7(4): 160-9.

In a preferred embodiment, the metzincin is a matrix metalloproteinase (MMP) or a pro-form thereof. More preferably, the metalloproteinase is selected from the group consisting of the collagenases MMP 1, MMP 8 and MMP 13, and pro-MMPs thereof.

Most preferably, the metalloproteinase is MMP 13 or pro-MMP13.

Thus, novel polypeptides provided by the present invention may inhibit one or more of the following:

(a) MMP binding to the I-domain of an integrin (such as a collagen-binding integrin);
(b) MMP activity;
(c) MMP expression; and/or
(d) MMP regulation (i.e. control of the rate of MMP activity or expression).
While not being limited by way of theory, it is believed that polypeptides of the invention have efficacy in the prophylactic and therapeutic treatment of diseases and conditions associated with abnormal function of integrins and/or MMPs. As discussed in detail below, the polypeptides of the invention are particularly suited for the treatment of arthritic diseases, inflammatory diseases and cancer.

In determining whether a given polypeptide may be useful in the treatment of such conditions, any of a number of assays, both in vitro and in vivo, are available to the persons skilled in the art.

*In vitro* assays suitable for identifying and evaluating polypeptides of the invention are described in the Examples below.

Suitable *in vivo* methods for identifying and evaluating polypeptides of the invention include several well known animal models in mice, rats, rabbits, guinea pigs and dogs. For example, suitable *in vivo* models for arthritic diseases include:

**Meniscectomy-induced osteoarthritis**

This model has been described previously in, for example, Sabatini et al (Sabatini et al. 2005) and is applicable to both guinea pigs and rabbits.

In brief, partial medial meniscectomy is performed under anaesthetic on the knee of a group of guinea pigs. A group of animals undergo sham surgery, in which the joint capsule is opened by transection of the medial collateral ligament and the meniscus is left intact. After the operation, the collateral ligament is sutured and the skin incision closed and disinfected.

AU animals are randomly allocated into different groups: 1) sham operated animals; 2) control meniscectomized animals receiving a vehicle made of a suspension e.g. 0.2% hydroxypropyl cellulose; 3) meniscectomized animals treated with polypeptides at, for example, 10 mg/kg (bid) twice per day; and 4) meniscectomized animals treated with polypeptides at a range of concentrations from 1mg/kg - 1000mg/kg bid. Oral bid treatment administered by gavage is
started 1 day after surgery and continued for 12 weeks, at which time the animals were killed.

During the study, the weight and the food consumption of the guinea pigs is recorded weekly. Animals are killed by exsanguinations under anaesthesia, and the proximal part of the tibias (from the operated knees) are removed and stripped of tendon and musculature. Immediately after dissection, the joints are placed in 10% formalin for further analyses.

**Anterior cruciate ligament transection (ACLT)-induced osteoarthritis**

This model was originally described in detail in dogs by Pond and NuId (Pond and Nuki 1973) and is well-established as a rabbit model where it is described in, for example, Elmali et al (Elmali et al. 2002).

In brief, rabbits of age 7-8 months old are operated on for unilateral anterior cruciate ligament transection (ACLT) to create degenerative changes in the articular cartilage. These rabbits are skeletally mature, with closed epiphyses as seen by roentgenogram. Each animal is anaesthetised and a hind limb is shaved and disinfected. Medial parapatellar incision and arthrotomy are performed. The patella is dislocated laterally and the knee placed in full flexion. The ACL is visualised and transected with a surgical blade. The contralateral non-operated knee serves as control (sham).

After ACLT, the joint is washed and the capsule and then skin is closed with sutures. After surgery, the rabbits are returned to cage activity and the limbs are not immobilised.

Four weeks after ACLT, the rabbits are divided into two groups. The knees of the test group are injected intra-articularly with peptides in the range 0.1-1000mg/kg in a suitable suspension once daily for 2 weeks, and the knees of the control group (nonoperated) are injected with the same amount of suspension without peptide. During intra-articular injection, the animals were anaesthetised.
A U rabbits are killed 2 weeks after the last injection and histological examination of the cartilage tissue is performed.

Collagenase-induced osteoarthritis

This model has been described in detail previously by van der Kraan et al (van der Kraan et al. 1990) and van Osch et al (van Osch et al. 1993).

In brief, mice are injected intra-articularly in the right knee with highly purified bacterial collagenase followed by a second injection three days later. The first ligament damage and synovial inflammation occurs one day after the first injection. Mice are then sacrificed after six weeks. Both right and left knee are fixed in formalin for 3 days, followed by decalcification and embedding in paraffin. Both joints are then sectioned for histology staining. The development of osteoarthritis in the joints is visually scored by light microscopy for cartilage lesions in four locations in the joint as well as for the size of osteophyte formation. Digital image analysis of the histological sections is also performed.

Polypeptides to be tested are administrated during the experiments for a four-week period starting approximately at day 8. Peptides will be injected intraperitoneally (i.p.) in a volume of 0.2 ml at a range of concentration 0.01 mg to 10 g or more per day, preferably about 0.1 mg to about 1 g per day. Administration may be from one to four times per day.

Collagen-induced arthritis (CIA)

This model has been described in detail previously by Holmdahl et al. (Holmdahl et al. 1990).

Collagen type II (100 microgram) emulsified in adjuvant is injected subcutaneously (s.c.) in a volume of 0.1 ml. Thirty-five days after the first injection the mice are boosted with an additional injection of half the amount of collagen. The clinical signs of arthritis are scored using a scoring system where every
affected finger or knuckles gets 1 point and ankle 5 points (maximum of 60 points).

Mice are treated i.p. with test polypeptides at four occasions; 1, 5, 9 and 13 days after antigen-injection. Polypeptides are injected in a volume of 0.2 ml at a range of concentration 0.01 mg to 10 g or more per day, preferably about 0.1 mg to about 1 g per day. Administration may be from one to four times per day.

_**Antigen-induced arthritis (AIA)**_

This model has been described in detail previously by Brackertz et al (Brackertz et al. 1977).

Mice are sensitised by injections of methylated bovine serum albumin (mBSA). An acute arthritis reaction is induced by intra-articular (i.a.) injection of 60 µg mBSA. The mice were then followed for 15 days. The swelling of the knee is measured at day 3 after i.a. injection, in the very acute phase, and at 10 days using. Mice were sacrificed at day 15 and inflammation, cartilage pathology and osteophyte formation was scored in histology sections of the knees as above.

Mice are treated i.p. with test polypeptides at four occasions; 1, 5, 9 and 13 days after antigen-injection. Polypeptides are injected in a volume of 0.2 ml at a range of concentration 0.01 mg to 10 g or more per day, preferably about 0.1 mg to about 1 g per day. Administration may be from one to four times per day.

_**Cancer Models**_

Suitable _in vivo_ methods for evaluating the polypeptides of the invention include several well known models (Hirst and Balmain 2004; Khanna and Hunter 2005) and the means for testing peptide agents for anti-cancer activity in such models are well known in the art (Koivunen et al. 1999a; Medina et al. 2001; Bjorklund et al. 2004; Stefanidakis and Koivunen 2004).
A second aspect of the invention provides an isolated nucleic acid molecule encoding a polypeptide according to the first aspect of the invention.

Thus, the isolated nucleic acid molecule is suitable for expressing a polypeptide of the invention. By 'suitable for expressing' is meant that the nucleic acid molecule is a polynucleotide that may be translated to form the polypeptide, for example RNA, or that the polynucleotide (which is preferably DNA) encoding the polypeptide of the invention is inserted into an expression vector, such as a plasmid, in proper orientation and correct reading frame for expression. The polynucleotide may be linked to the appropriate transcriptional and translational regulatory control nucleotide sequences recognised by any desired host; such controls may be incorporated in the expression vector.

The nucleic acid molecule of the invention may be DNA or RNA, preferably DNA. The nucleic acid molecule may or may not contain introns in the coding sequence; preferably the nucleic acid molecule is a cDNA.

The nucleic acid molecule (or polynucleotide) may be expressed in a suitable host to produce a polypeptide of the invention. Thus, the polynucleotide encoding the polypeptide of the invention may be used in accordance with known techniques, appropriately modified in view of the teachings contained herein, to construct an expression vector, which is then used to transform an appropriate host cell for the expression and production of the polypeptide of the invention (for example as described in Sambrook & Russell, supra.)

The nucleic acid molecule encoding the polypeptide of the invention may be joined to a wide variety of other polynucleotide sequences for introduction into an appropriate host. The companion polynucleotide will depend upon the nature of the host, the manner of the introduction of the polynucleotide into the host, and whether episomal maintenance or integration is desired.

Generally, the nucleic acid molecule is inserted into an expression vector, such as a plasmid, in proper orientation and correct reading frame for expression. If necessary, the nucleic acid molecule may be linked to the appropriate
transcriptional and translational regulatory control nucleotide sequences recognised by the desired host, although such controls are generally available in the expression vector. The vector is then introduced into the host through standard techniques. Generally, not all of the hosts will be transformed by the vector. Therefore, it will be necessary to select for transformed host cells. One selection technique involves incorporating into the expression vector a polynucleotide sequence, with any necessary control elements, that codes for a selectable trait in the transformed cell, such as antibiotic resistance. Alternatively, the gene for such selectable trait can be on another vector, which is used to co-transform the desired host cell.

Host cells that have been transformed by the recombinant nucleic acid molecule of the invention are then cultured for a sufficient time and under appropriate conditions known to those skilled in the art in view of the teachings disclosed herein to permit the expression of the polypeptide, which can then be recovered.

Many expression systems are known, including bacteria (for example *E. coli* and *Bacillus subtilis*), yeasts (for example *Saccharomyces cerevisiae*), filamentous fungi (for example *Aspergillus*), plant cells, animal cells and insect cells.

The vectors typically include a prokaryotic replicon, such as the CoIE1 ori, for propagation in a prokaryote, even if the vector is to be used for expression in other, non-prokaryotic, cell types. The vectors can also include an appropriate promoter such as a prokaryotic promoter capable of directing the expression (transcription and translation) of the genes in a bacterial host cell, such as *E. coli*, transformed therewith. Promoter sequences compatible with exemplary bacterial hosts are typically provided in plasmid vectors containing convenient restriction sites for insertion of a polynucleotide of the present invention.

Typical prokaryotic vector plasmids are pUC18, pUC19, pBR322 and pBR329 available from Biorad Laboratories, (Richmond, CA, USA) and pTrc99A and pKK223-3 available from Pharmacia, Piscataway, NJ, USA.
A typical mammalian cell vector plasmid is pSVL available from Pharmacia, Piscataway, NJ, USA. This vector uses the SV40 late promoter to drive expression of cloned genes, the highest level of expression being found in T antigen-producing cells, such as COS-I cells.

An example of an inducible mammalian expression vector is pMSG, also available from Pharmacia. This vector uses the glucocorticoid-inducible promoter of the mouse mammary tumour virus long terminal repeat to drive expression of the cloned gene.

Useful yeast plasmid vectors are pRS403-406 and pRS413-416 and are generally available from Stratagene Cloning Systems, La Jolla, CA 92037, USA. Plasmids pRS403, pRS404, pRS405 and pRS406 are Yeast Integrating plasmids (Yips) and incorporate the yeast selectable markers HIS3, TRP1, LEU2 and URA3. Plasmids pRS413-416 are Yeast Centromere plasmids (YCps).

The present invention also relates to a host cell transformed with a polynucleotide vector construct of the present invention. The host cell can be either prokaryotic or eukaryotic. Bacterial cells are preferred prokaryotic host cells and typically are a strain of *E. coli* such as, for example, the *E. coli* strains DH5 available from Bethesda Research Laboratories Inc., Bethesda, MD, USA, and RR1 available from the American Type Culture Collection (ATCC) of Rockville, MD, USA (No ATCC 31343). Preferred eukaryotic host cells include yeast, insect and mammalian cells, preferably vertebrate cells such as those from a mouse, rat, monkey or human fibroblastic and kidney cell lines. Yeast host cells include YPH499, YPH500 and YPH501 which are generally available from Stratagene Cloning Systems, La Jolla, CA 92037, USA. Preferred mammalian host cells include Chinese hamster ovary (CHO) cells available from the ATCC as CCL61, NTH Swiss mouse embryo cells NIH/3T3 available from the ATCC as CRL 1658, monkey kidney-derived COS-I cells available from the ATCC as CRL 1650 and 293 cells which are human embryonic kidney cells. Preferred insect cells are Sf9 cells which can be transfected with baculovirus expression vectors.
Transformation of appropriate cell hosts with a nucleic acid molecule of the present invention is accomplished by well known methods that typically depend on the type of vector used. With regard to transformation of prokaryotic host cells, see for example Sambrook & Russell (supra). Transformation of yeast cells is described in numerous reviews, for example see Gietz & Woods (2001) *Biotechniques* 30:816-228. With regard to vertebrate cells, reagents useful in transfecting such cells, for example calcium phosphate and DEAE-dextran or liposome formulations, are available from Stratagene Cloning Systems, or Life Technologies Inc., Gaithersburg, MD 20877, USA.

Electroporation is also useful for transforming and/or transfecting cells and is well known in the art for transforming yeast cell, bacterial cells, insect cells and vertebrate cells. For example, many bacterial species may be transformed by the methods described in Luchansky et al. (1988) *Mol. Microbiol.* 2:637-646. Methods for transformation of yeast by electroporation are disclosed in Becker & Guarente (1990) *Methods Enzymol.* 194:182.

Successfully transformed cells, *i.e.* cells that contain a nucleic acid molecule of the present invention, can be identified by well known techniques. For example, cells resulting from the introduction of an expression construct of the present invention can be grown to produce the polypeptide of the invention. Cells can be harvested and lysed and their DNA content examined for the presence of the DNA using a method such as that described by Sambrook & Russell (supra.). Alternatively, the presence of the protein in the supernatant can be detected using antibodies.

In addition to assaying directly for the presence of recombinant DNA, successful transformation can be confirmed by well known immunological methods when the recombinant DNA is capable of directing the expression of the protein. For example, cells successfully transformed with an expression vector produce proteins displaying appropriate antigenicity. Samples of cells suspected of being transformed are harvested and assayed for the protein using suitable antibodies.
Thus, a third aspect of the invention provides a vector comprising a nucleic acid molecule according to the second aspect of the invention. In a preferred embodiment, the vector is an expression vector.

Advantageously, the vector is suitable for replication in a eucaryotic cell, such as a mammalian cell.

Preferred vectors may be selected from the group consisting of pTWIN, pShuttle, pUC18, pUC19, pBacPAK, pBR322, pBR329, pTrec99A, pKK223-3, pSVL, pMSG, pRS403 to 406 and pRS413 to 416.

A fourth aspect of invention provides a host cell comprising a nucleic acid molecule according to the second aspect of the invention or a vector according to the third aspect of the invention. Conveniently, the cell is a eucaryotic cell, for example a mammalian cell.

Preferably, the host cell is selected from the group consisting of E. coli strain DH5, RRI, ER2566, CHO cells (e.g. CCL61), NIH Swiss mouse embryo cells (NIH/3T3), COS-I cells (e.g. CRL 1650 and 293), Sf9 cells and yeast cell lines YPH499 to 501.

In addition to the transformed host cells themselves, the present invention also contemplates a culture of those cells, preferably a monoclonal (clonally homogeneous) culture, or a culture derived from a monoclonal culture, in a nutrient medium.

A fifth aspect of the invention provides a method for making a polypeptide according to the first aspect of the invention, the method comprising culturing a host cell according to the fourth aspect of the invention which expresses the polypeptide, and isolating the polypeptide therefrom. Methods of cultivating host cells and isolating recombinant proteins are well known in the art.

A sixth aspect of the invention provides a method for making a polypeptide according to the first aspect of the invention comprising solid phase synthesis of
the polypeptide. For example, the polypeptides may be synthesized as described in *Solid-Phase Peptide Synthesis* (1997) Fields, Abelson & Simon (Eds), Academic Press (ISBN: 0-12-182190-0).

A seventh aspect of the invention provides a pharmaceutical formulation comprising a polypeptide according to the first aspect of the invention in admixture with a pharmaceutically or veterinarily acceptable adjuvant, diluent or carrier.

As used herein, 'pharmaceutical formulation' means a therapeutically effective formulation according to the invention.

A 'therapeutically effective amount', or 'effective amount', or 'therapeutically effective', as used herein, refers to that amount which provides a therapeutic effect for a given condition and administration regimen. This is a predetermined quantity of active material calculated to produce a desired therapeutic effect in association with the required additive and diluent, *i.e.* a carrier or administration vehicle. Further, it is intended to mean an amount sufficient to reduce and most preferably prevent, a clinically significant deficit in the activity, function and response of the host. Alternatively, a therapeutically effective amount is sufficient to cause an improvement in a clinically significant condition in a host. As is appreciated by those skilled in the art, the amount of a compound may vary depending on its specific activity. Suitable dosage amounts may contain a predetermined quantity of active composition calculated to produce the desired therapeutic effect in association with the required diluent. In the methods and use for manufacture of compositions of the invention, a therapeutically effective amount of the active component is provided. A therapeutically effective amount can be determined by the ordinary skilled medical or veterinary worker based on patient characteristics, such as age, weight, sex, condition, complications, other diseases, *etc.*, as is well known in the art.

Thus, in a preferred embodiment, the present invention provides a pharmaceutical formulation comprising an amount of a polypeptide of the invention effective to reduce the activity, function and/or expression of one or more MMPs, especially
of MMP-13 and/or INIMP-I, and a pharmaceutically and biochemically acceptable carrier.

It will be appreciated by persons skilled in the art that the polypeptides of the invention will generally be administered in admixture with a suitable pharmaceutical excipient diluent or carrier selected with regard to the intended route of administration and standard pharmaceutical practice (for example, see Remington: The Science and Practice of Pharmacy, 19th edition, 1995, Ed. Alfonso Gennaro, Mack Publishing Company, Pennsylvania, USA). Suitable routes of administration are discussed below, and include topical, intravenous, oral, pulmonary, nasal, aural, ocular, bladder and CNS delivery.

For example, the polypeptides, molecules and pharmaceutical formulations of the present invention may be delivered using an injectable sustained-release drug delivery system, such as a microsphere. These are designed specifically to reduce the frequency of injections. An example of such a system is Nutropin Depot which encapsulates recombinant human growth hormone (rhGH) in biodegradable microspheres that, once injected, release rhGH slowly over a sustained period.

Alternatively, the polypeptides, molecules and pharmaceutical formulations of the present invention can be administered by a surgically implanted device that releases the drug directly to the required site.

Electroporation therapy (EPT) systems can also be employed for the administration of proteins and polypeptides. A device which delivers a pulsed electric field to cells increases the permeability of the cell membranes to the drug, resulting in a significant enhancement of intracellular drug delivery.

Proteins and polypeptides can also be delivered by electroincorporation (EI). EI occurs when small particles of up to 30 microns in diameter on the surface of the skin experience electrical pulses identical or similar to those used in electroporation. In EI3 these particles are driven through the stratum corneum and into deeper layers of the skin. The particles can be loaded or coated with drugs or
genes or can simply act as "bullets" that generate pores in the skin through which the drugs can enter.

An alternative method of protein and polypeptide delivery is the thermo-sensitive ReGeI injectable. Below body temperature, ReGeI is an injectable liquid while at body temperature it immediately forms a gel reservoir that slowly erodes and dissolves into known, safe, biodegradable polymers. The active drug is delivered over time as the biopolymers dissolve.

Protein and polypeptide pharmaceuticals can also be delivered orally. One such system employs a natural process for oral uptake of vitamin B12 in the body to co-deliver proteins and polypeptides. By riding the vitamin B12 uptake system, the protein or polypeptide can move through the intestinal wall. Complexes are produced between vitamin B12 analogues and the drug that retain both significant affinity for intrinsic factor (IF) in the vitamin B12 portion of the complex and significant bioactivity of the drug portion of the complex.

Preferably, the pharmaceutical formulation of the present invention is a unit dosage containing a daily dose or unit, daily sub-dose or an appropriate fraction thereof, of the active ingredient.

The polypeptides and pharmaceutical formulations of the present invention will normally be administered orally or by any parenteral route, in the form of a pharmaceutical formulation comprising the active ingredient, optionally in the form of a non-toxic organic, or inorganic, acid, or base, addition salt, in a pharmaceutically acceptable dosage form. Depending upon the disorder and patient to be treated, as well as the route of administration, the compositions may be administered at varying doses.

In human therapy, the polypeptides of the invention can be administered alone but will generally be administered in admixture with a suitable pharmaceutical excipient diluent or carrier selected with regard to the intended route of administration and standard pharmaceutical practice.
For example, the compounds, *i.e.* polypeptides, of the invention can be administered orally, buccally or sublingually in the form of tablets, capsules, ovules, elixirs, solutions or suspensions, which may contain flavouring or colouring agents, for immediate-, delayed- or controlled-release applications. The compounds of invention may also be administered via intracavernosal injection.

Such tablets may contain excipients such as microcrystalline cellulose, lactose, sodium citrate, calcium carbonate, dibasic calcium phosphate and glycine, disintegrates such as starch (preferably corn, potato or tapioca starch), sodium starch glycollate, croscarmellose sodium and certain complex silicates, and granulation binders such as polyvinylpyrrolidone, hydroxypropylmethylcellulose (HPMC), hydroxy-propylcellulose (HPC), sucrose, gelatin and acacia. Additionally, lubricating agents such as magnesium stearate, stearic acid, glyceryl behenate and talc may be included.

Solid compositions of a similar type may also be employed as fillers in gelatin capsules. Preferred excipients in this regard include lactose, starch, cellulose, milk sugar or high molecular weight polyethylene glycols. For aqueous suspensions and/or elixirs, the compounds of the invention may be combined with various sweetening or flavouring agents, colouring matter or dyes, with emulsifying and/or suspending agents and with diluents such as water, ethanol, propylene glycol and glycerin, and combinations thereof.

The compounds of the invention can also be administered parenterally, for example, intravenously, intra-articularly, intra-arterially, intraperitoneally, intrathecellly, intraventricularly, intrasternally, intracranially, intra-muscularly or subcutaneously, or they may be administered by infusion techniques. They are best used in the form of a sterile aqueous solution which may contain other substances, for example, enough salts or glucose to make the solution isotonic with blood. The aqueous solutions should be suitably buffered (preferably to a pH of from 3 to 9), if necessary. The preparation of suitable parenteral formulations under sterile conditions is readily accomplished by standard pharmaceutical techniques well known to those skilled in the art.
Formulations suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example sealed ampoules and vials, and may be stored in a freeze-dried (lyophilised) condition requiring only the addition of the sterile liquid carrier, for example water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described.

For oral and parenteral administration to human patients, the daily dosage level of the compounds of the invention will usually be from 1 to 1000 mg per adult (i.e. from about 0.015 to 15 mg/kg), administered in single or divided doses.

Thus, for example, the tablets or capsules of the compound of the invention may contain from 1 mg to 1000 mg of active compound for administration singly or two or more at a time, as appropriate. The physician in any event will determine the actual dosage which will be most suitable for any individual patient and it will vary with the age, weight and response of the particular patient. The above dosages are merely exemplary of the average case. There can, of course, be individual instances where higher or lower dosage ranges are merited and such are within the scope of this invention.

The compounds of the invention can also be administered intranasally or by inhalation and are conveniently delivered in the form of a dry powder inhaler or an aerosol spray presentation from a pressurised container, pump, spray or nebuliser with the use of a suitable propellant, e.g. dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoro-ethane, a hydrofluoroalkane such as 1,1,2-tetrafluoroethane (HFA 134A3 or 1,1,1,2,3,3,3-heptfluoropropane (HFA 227EA3), carbon dioxide or other suitable gas. In the case of a pressurised aerosol, the dosage unit may be determined by providing a valve to deliver a
metered amount. The pressurised container, pump, spray or nebuliser may contain a solution or suspension of the active compound, e.g. using a mixture of ethanol and the propellant as the solvent, which may additionally contain a lubricant, e.g. sorbitan trioleate. Capsules and cartridges (made, for example, from gelatin) for use in an inhaler or insufflator may be formulated to contain a powder mix of a compound of the invention and a suitable powder base such as lactose or starch.

Aerosol or dry powder formulations are preferably arranged so that each metered dose or 'puff' contains at least 1 mg of a compound of the invention for delivery to the patient. It will be appreciated that the overall daily dose with an aerosol will vary from patient to patient, and may be administered in a single dose or, more usually, in divided doses throughout the day.

Alternatively, the compounds of the invention can be administered in the form of a suppository or pessary, or they may be applied topically in the form of a lotion, solution, cream, ointment or dusting powder. The compounds of the invention may also be transdermally administered, for example, by the use of a skin patch. They may also be administered by the ocular route.

For ophthalmic use, the compounds of the invention can be formulated as micronised suspensions in isotonic, pH adjusted, sterile saline, or, preferably, as solutions in isotonic, pH adjusted, sterile saline, optionally in combination with a preservative such as a benzylalkonium chloride. Alternatively, they may be formulated in an ointment such as petrolatum.

For application topically to the skin, the compounds of the invention can be formulated as a suitable ointment containing the active compound suspended or dissolved in, for example, a mixture with one or more of the following: mineral oil, liquid petrolatum, white petrolatum, propylene glycol, poloxymethylene polyoxypropylene compound, emulsifying wax and water. Alternatively, they can be formulated as a suitable lotion or cream, suspended or dissolved in, for example, a mixture of one or more of the following: mineral oil, sorbitan
monostearate, a polyethylene glycol, liquid paraffin, polysorbate 60, cetyl esters wax, cetearyl alcohol, 2-octyldecanol, benzyl alcohol and water.

Formulations suitable for topical administration in the mouth include lozenges comprising the active ingredient in a flavoured basis, usually sucrose and acacia or tragacanth; pastilles comprising the active ingredient in an inert basis such as gelatin and glycerin, or sucrose and acacia; and mouth-washes comprising the active ingredient in a suitable liquid carrier.

Generally, in humans, oral or parenteral administration of the compounds of the invention is the preferred route, being the most convenient.

It will be appreciated by persons skilled in the art that such an effective amount of the polypeptide or formulation thereof may be delivered as a single bolus dose (i.e. acute administration) or, more preferably, as a series of doses over time (i.e. chronic administration).

It will be further appreciated by persons skilled in the art that the polypeptides and pharmaceutical formulations of the present invention have utility in both the medical and veterinary fields. Thus, the methods of the invention may be used in the treatment of both human and non-human animals (such as horses, dogs and cats). Preferably, however, the patient is human.

For veterinary use, a compound of the invention is administered as a suitably acceptable formulation in accordance with normal veterinary practice and the veterinary surgeon will determine the dosing regimen and route of administration which will be most appropriate for a particular animal.

In an eighth aspect of the invention, there is provided a polypeptide according to the first aspect of the invention or a pharmaceutical formulation according to the seventh aspect of the invention for use in medicine.

An ninth aspect of the invention provides the use of a polypeptide according to the first aspect of the invention or a pharmaceutical formulation according to the
seventh aspect of the invention in the preparation of a medicament for treating a
disease or condition capable of being treated by an agent which modulates the
function of an integrin and/or a metzincin (e.g. an MMP, ADAM or ADAMTS).

Thus, the polypeptides and formulations of the present invention may be used to
treat patients or subjects who suffer from or are at risk of suffering from the
following conditions or disease states; arthritic diseases (including osteoarthritis,
rheumatoid arthritis and reactive arthritis), inflammatory conditions (including
atherosclerosis and multiple sclerosis), cancer and metastasis progression
(including melanoma), oral diseases (including periodontitis; see Sorsa et al
(2004) Oral Diseases 10:311-318), osteoporosis, tissue remodeling, angiogenesis,
angio genesis, lung diseases (bronchiectasis and chronic obstructive pulmonary
diseases and other lung diseases), wounds, burns, fractures, lesions and ulcers.

In a preferred embodiment of the ninth aspect of the invention, the disease or
condition capable of being treated by an agent which modulates the function of an
integrin and/or a metzincin (e.g. a matrix metalloproternase) is an arthritic disease
or an inflammatory disease or condition.

By 'arthritic disease' we include osteoarthritis, rheumatoid arthritis and reactive
arthritis.

By 'inflammatory disease or condition' we include Alzheimer's disease,
psoriasis, asthma, atherosclerosis, sarcoidosis, atopic dermatitis, systemic lupus
erythematosus, bullous pemphigoid, type I diabetes mellitus, chronic obstructive
pulmonary disease, ulcerative colitis, gout, Helicobacter pylori gastritis*,
inflammatory bowl disease, Hepatitis C*, ischaemia-reperfusion injury, multiple
sclerosis, Neisserial or pneumococcal meningitis*, tuberculosis and periodontitis
(* indicated diseases of infectious origin in which the host inflammatory reaction
is as important to its pathology as the microbial infection itself).

Most preferably, the disease or condition is osteoarthritis or rheumatoid arthritis.
In an alternative preferred embodiment of the ninth aspect of the invention, the disease or condition capable of being treated by an agent which modulates the function of an integrin and/or a metzincin is a proliferative disease (i.e. cancer). For example, proliferative disease may be selected from the group consisting of sarcomas (e.g. bone and connective tissues including synovium), carcinomas (e.g. mammary, pancreatic, colon, squamous cell, adeno, large cell), myelomas, leukaemias, lymphomas (e.g. Hodgkins disease, non-Hodgkins lymphomas, T-cell lymphomas), melanoma and small cell lung cancer.

A tenth aspect of the invention provides a method of treating a patient in need of modulation of the function of an integrin and/or a metzincin (e.g. an MMP, ADAM or ADAMTS), the method comprising administering to the patient an effective amount of a polypeptide according to the first aspect of the invention or a pharmaceutical formulation according to the seventh aspect of the invention.

By 'treatment' we include both therapeutic and prophylactic treatment of the patient. The term 'prophylactic' is used to encompass the use of a polypeptide or formulation described herein which either prevents or reduces the likelihood of a condition or disease state in a patient or subject.

As discussed above, the term 'effective amount' is used herein to describe concentrations or amounts of compounds according to the present invention which may be used to produce a favourable change in a disease or condition treated, whether that change is a remission, a favourable physiological result, a reversal or attenuation of a disease state or condition treated, the prevention or the reduction in the likelihood of a condition or disease state occurring, depending upon the disease or condition treated. Where polypeptides of the invention are used in combination, each of the polypeptides may be used in an effective amount, wherein an effective amount may include a synergistic amount.

Preferably, the method of the tenth aspect of the invention comprises treating a patient suffering from an arthritic disease, an inflammatory disease or condition and/or cancer (see above).
It will be appreciated by persons skilled in the art that the polypeptides and formulations of the invention may be co-administered in combination with one or more known or conventional agents for the treatment of the particular disease or condition. By 'co-administer' it is meant that the present polypeptides are administered to a patient such that the polypeptides as well as the co-administered compound may be found in the patient's body (e.g. in the bloodstream) at the same time, regardless of when the compounds are actually administered, including simultaneously.

For example, where the patient is suffering from an arthritic disease and/or an inflammatory disease or condition, the polypeptide or formulation of the invention comprises may be administered in combination with one or more conventional anti-inflammatory agents, including corticosteroids, non-steroidal anti-inflammatory drugs (NSAIDs), disease modifying anti-rheumatic drugs (DMARDs) and antibody therapies (e.g. anti-TNFα antibodies). Such combination therapies are disclosed in US 5,780,426.

Likewise, where the patient is suffering from cancer, such as cancer, the polypeptide or formulation of the invention comprises may be administered in combination with one or more conventional anti-cancer agents, including cytotoxic drugs (e.g. alkylating drugs, cytotoxic antibiotics, antimetabolites, vinca alkaloids and etoposide), drugs affecting the immune response (e.g. antiproliferative immunosuppressants, corticosteroids and other immunosuppressants, rituximab and alemtuzumab), and sex hormones and hormone antagonists (e.g. estrogens, progestogens, androgens).

It will also be appreciated by skilled persons that the polypeptides and formulations of the invention may be co-administered in combination with one or more agents that inhibit the function of integrins and/or MMPs.

In an eleventh aspect of the invention, there is provided the use of a polypeptide according to the first aspect of the invention to detect the presence of an integrin in a sample.
Thus, the present invention provides the use of a polypeptide of the invention in the diagnosis of a disease or condition associated with abnormal level of an integrin (such as osteoarthritis, rheumatoid arthritis, or cancer).

It will be appreciated by persons skilled in the art that the polypeptide of the invention may be used to detect the presence of an integrin in a sample in vitro or in vivo. Conveniently, the polypeptide of the invention comprises a detectable moiety (i.e. label) or indicating means, such as a fluorescent or radioactive label.

As used herein, the terms 'label' and 'indicating means' refer to single atoms and molecules that are either directly or indirectly involved in the production of a detectable signal to indicate the presence of the polypeptide. Any label or indicating means can be linked to or incorporated in a polypeptide of the present invention, or used separately, and those atoms or molecules can be used alone or in conjunction with additional reagents. Such labels are themselves well known in clinical diagnostic chemistry and the linking of these labels to polypeptides and proteins is well known in the art. Examples of labels for polypeptides include, but are not limited to, the following: radioisotopes (e.g. $^3$H, $^{14}$C, $^{35}$S, $^{123}$I, $^{125}$I, $^{131}$I, $^{99}$Tc, $^{111}$In, $^{90}$Y, $^{188}$Re), radionuclides (such as carbon-11, fluorine-18 or copper-64.), fluorescent labels (e.g. FITC, rhodamine, lanthanide phosphors, carbocyanine), enzymatic labels (e.g. horseradish peroxidase, $\beta$-galactosidase, luciferase, alkaline phosphatase), chemiluminescent, biotinyl groups, predetermined polypeptide epitopes recognised by a secondary reporter (e.g. leucine zipper pair sequences, binding sites for secondary antibodies, metal binding domains, epitope tags). In some embodiments, labels are attached by spacer arms of various lengths to reduce potential stearic hindrance.

The technique of labelling polypeptides and their use in diagnostics is well known to the skilled man in the art (Li and Anderson 2003; Haubner et al. 2003). Means for performing peptide-based imaging, including suitable labels and linkers, are also known in the art, for example see WO 03/006491 to Amersham Health A/S; EP 1 272 507 B1 to Amersham Health A/S, and (Medina et al. 2005; Medina et al. 2005).
In a preferred embodiment, the sample is selected from the group consisting of tissue samples (e.g., cartilage, bone, synovium, atherosclerotic plaque, or tumour samples), synovial fluid, blood samples and plasma samples.

More preferably, the sample is a connective tissue sample.

Most preferably, the sample is a cartilage tissue sample, synovium sample or tumour sample.

Advantageously, the integrin is detected in vivo by X-ray, MR, ultrasound, scintigraphy, PET, SPECT, electrical impedance, light or magnetometric imaging modalities.

A twelve aspect of the invention provides a method for diagnosing in a patient a disease or condition associated with abnormal amount and/or function of an integrin, the method comprising:

(a) providing a sample from the patient to be tested;
(b) contacting the sample with a polypeptide of the invention under conditions which permit the binding of the polypeptide to an integrin (if present in the sample); and
(c) detecting the binding of the polypeptide to an integrin (if present in the sample)

wherein the amount of polypeptide bound to the sample from the patient to be tested provides an indication of the amount and/or function of integrin in the sample.

Preferably, the method further comprises step (d) of comparing the amount of polypeptide bound to the sample from the patient to be tested with the amount of the polypeptide bound to a sample from a healthy individual (or population of individuals).
In a preferred embodiment of the eleventh and twelfth aspects of the invention, the integrin is a collagen-binding integral, for example α₁β₁ or α₁lβ₁.

Thus, in a preferred embodiment the method comprises

(a) contacting a tissue sample suspected of harbouring the disease to be diagnosed with a polypeptide of the invention;
(b) determining the level of binding of the polypeptide to an integrin α₁β₁ or α₁lβ₁ in the tissue sample; and
(c) comparing the level of binding in (b) to a standard level of binding of said polypeptide to a cell extract from a healthy tissue sample.

An altered level of binding of the polypeptide to the 'diseased' tissue sample may indicate the likely presence of a chondrocyte-mediated disease state, a fibroblast-mediated state, an osteoclast or osteoblast-mediated disease state, a smooth-muscle cell disease state, a macrophage-mediated disease state, a stem cell-mediated disease state or cancerous cells.

Such a method may also be used to detect diseases in which the integrin levels are changed, such as OA, RA, atherosclerosis and cancer.

It will be appreciated by skilled persons that the diagnostic methods of the present invention may be of a competitive or non-competitive nature, as those terms are understood in the art.

A thirteenth aspect of this invention provides a kit for performing a method according to the twelfth aspect of the invention comprising a polypeptide of the invention.

Preferably, the diagnostic kit comprises, in an amount sufficient for at least one assay, a peptide of this invention as a separately packaged reagent. Instructions for use of the packaged reagent are also typically included. Such instructions typically include a tangible expression describing the reagent concentration or at least one assay method parameter such as the relative amounts of reagent and
sample to be mixed, maintenance time periods for reagent/sample admixtures, temperature, buffer conditions and the like.

A fourteenth aspect of the invention provides the use of a polypeptide according to the first aspect of the invention to improve targeting of liposomes to target cells (useful in drug delivery and for delivery of gene expression vectors). Such uses are well known in the art and described in detail in WO 02/076491, Koivunen et al (Koivunen et al. 1999a);(Medina et al. 2001) and (Medina et al. 2004).

A fifteenth aspect of the invention provides a polypeptide complex comprising a polypeptide according to the first aspect of the invention and an integrin subunit, or fragment thereof comprising an I-domain. Such complexes are of particular use in molecular modelling and drug design.

Preferably, the complex is in crystalline form.

The novel peptides developed are useful lead compounds to design peptidomimetics and to develop more selective inhibitors for MMPs. Methods for using the peptides of the invention co-crystallised with their ligand for drug design are well known in the art and described in (Blundell et al. 2002;Hruby 2002;Kitchen et al. 2004;Williams et al. 2005)

A sixteenth aspect of the invention provides a method of making a complex according to the fifteenth aspect of the invention comprising a peptide of the invention and its ligand. For example, methods for the co-crystallisation of peptides and antibody fragments with collagen-binding integrins are described in the literature (Karpusas et al. 2003);(Emsley et al. 2004).

A seventeenth aspect of the invention provides a method for identifying a candidate compound for the treatment of a disease or condition capable of being treated by an agent which modulates the function of an integrin and/or a metzincin the method comprising determining if a compound to be tested alters or interferes (e.g. competes) with the binding of a polypeptide according to the first aspect of the invention to an integrin I-domain.
The ability of candidate compounds to bind to an integrin and/or MMP may be assessed by a competitive binding assay. Candidate compounds may be peptide or non-peptide compounds. Binding to an integrin and/or MMP is quantified by the ability to displace a peptide of the present invention, including the peptides of the present invention. The displaced peptide can be assayed by a number of techniques. For example, radiolabeled peptide can be synthesized using commercial available radiolabeled amino acids precursors. Peptides radiolabeled with $^3$H, $^{14}$C or $^{35}$S can be quantified by routine liquid scintillation techniques. Alternatively, a fluorescent labelled peptide can be synthesized. For example, lysine can be inserted in a non-critical position and labelled with fluorescein isothiocyanate (FITC). In addition to FITC, the peptide may be labelled with any suitable fluorophore. A carboxy fluorescein derivative of one or more of the peptides of the present invention may be prepared. Alternatively, peptides cyclised with an amide peptide linkage have free sulfhydryl groups available for linkage to fluorescent compounds such as thiocyanates. Separation of bound from unbound peptide and quantitation of displaced peptide can be performed by routine techniques known to one of skill in the art.

This embodiment of the invention is not limited by the method used to quantify the displaced peptide, and it will be appreciated that any suitable analytical technique may be used.

The candidate compound may be a drug-like compound or lead compound for the development of a drug-like compound for each of the above methods of identifying a compound. It will be appreciated that the said methods may be useful as screening assays in the development of pharmaceutical compounds or drugs, as well known to those skilled in the art.

The term 'drug-like compound' is well known to those skilled in the art, and may include the meaning of a compound that has characteristics that may make it suitable for use in medicine, for example as the active ingredient in a medicament. Thus, for example, a drug-like compound may be a molecule that may be synthesized by the techniques of organic chemistry, less preferably by
techniques of molecular biology or biochemistry, and is preferably a small molecule, which may be of less than 5000 daltons molecular weight. A drug-like compound may additionally exhibit features of selective interaction with a particular protein or proteins and be bioavailable and/or able to penetrate cellular membranes, but it will be appreciated that these features are not essential.

The term 'lead compound' is similarly well known to those skilled in the art, and may include the meaning that the compound, whilst not itself suitable for use as a drug (for example because it is only weakly potent against its intended target, non-selective in its action, unstable, difficult to synthesize or has poor bioavailability) may provide a starting-point for the design of other compounds that may have more desirable characteristics.

It will be appreciated that the compound may be a polypeptide that is capable of competing with the polypeptide of the invention for binding to an integrin. Thus, it will be appreciated that a screening method as described above may be useful in identifying polypeptides that may also interact with an integrin, for example a receptor molecule.

It will be further appreciated that it will be desirable to identify compounds that may modulate the activity of the polypeptide(s) in vivo. Thus, it will be understood that reagents and conditions used in the method may be chosen such that the interactions between the said polypeptide and the integrin are substantially the same as between the said polypeptide or a fragment thereof and a naturally occurring interacting polypeptide in vivo.

The 'drug-like compounds' and 'lead compounds' identified in the screening assays of the invention are suitably tested in further screens to determine their potential usefulness in treating arthritic diseases, inflammatory conditions, proliferative disorders, etc.

In a preferred embodiment of the seventeenth aspect of the invention, the method further comprises the step of mixing the compound thus identified with a pharmaceutically acceptable carrier.
An eighteenth aspect of the invention provides a kit for performing a method according to the seventeenth aspect of the invention comprising a polypeptide of the invention and an integrin subunit or fragment comprising or consisting of an I-domain thereof, or means for producing the same.

A nineteenth aspect of the invention provides a compound identified by a method according to the seventeenth aspect of the invention.

A twentieth aspect of the invention provides a compound according to the nineteenth aspect of the invention for use in medicine.

A twenty-first aspect of the invention provides a pharmaceutical formulation comprising a compound according to the nineteenth aspect of the invention in admixture with a pharmaceutically or veterinarily acceptable adjuvant, diluent or carrier.

Preferred aspects of the invention are described in the following non-limiting examples, with reference to the following figures:

Figure 1. Stereoview (above) and tube drawing (below) of predicted structure of (A) peptide 'p617' (corresponding to the cyclic form of SEQ ID NO:6) and (B) peptide 'p618' (corresponding to the cyclic form of SEQ ID NO:5).

Figure 2. Binding of phageAl (expressing the cyclic peptide CGIWFENEWC) and phageA2 (expressing the cyclic peptide CWTWPDSGWC) to immobilised integrin I-domains (α1, α10 and all), to GST-control or to BSA. Bound phages were detected using an anti-M13 antibody.

Figure 3. Binding of phageAl (A) and phageA2 (B) to immobilised integrin α11 I-domain in the presence of synthetic cyclic peptides (p617, p618 and control peptide (ctrl-pep). Binding of phageA2 (C) to integrin α10 I-domain in the
presence of synthetic cyclic peptides. Bound phages were detected using an anti-Mi3 antibody.

**Figure 4.** (A) Binding of phage Al to an anti-αl l coated surface with or without cell extract (CE) from integrin all transfected cells. Blocking by synthetic peptide p618 and p617. Bound phages were detected using an anti-Mi3 antibody.

**Figure 5.** (A) Solid-phase interaction to collagen VI, of recombinant GST-tagged I-domains (αl, αO and all and GST-control) in the presence or absence of EDTA. (B) I-domain interaction to collagen VI in the presence of synthetic cyclic peptides (p617, p618 or control-peptide). (C) Titration of the p618 effect on I-domain interaction to collagen VI. (**: pO.Ol, ***: pO.OOl). Bound I-domain was detected using an anti-GST antibody.

**Figure 6.** (A) Integrin αl O and all I-domain binding to pro-MMP 13. Titration of I-domain (αl O, all and GST-control) (20-32OnM) with a fixed concentration of pro-MMP13 (33 nM). (B) Binding between all I-domain and pro-MMP13 in the presence or absence of EDTA. Controls include a GST-control, a control without I-domain and a control without pro-MMP13. Black bars represent anti-his coating and grey bars represent BSA coating. (C) all I-domain and GST-control interaction to pro-MMP13 or active MMPl 3. In figures A-C wells are coated with anti-his antibody that bind the C-terminally his-tagged pro-MMP 13. Interacting I-domain is detected using an anti-GST antibody. (D) I-domain (αl M, αO, all and GST-control) solid-phase interaction to pro-MMP9. Wells were pre-coated with pro-MMP9 and bound GST-fusion was detected using an anti-GST antibody. Values are corrected for unspecific binding to BSA.

**Figure 7.** Interaction between pro-MMP-13 and the I-domains of integrin αl O (A) and integrin all (B) in the presence of peptides (p617, p618, or the control peptide (ctrl-pep)). Wells are coated with anti-his antibody that bind the C-terminally his-tagged pro-MMP13. Interacting I-domain (or GST-control) is detected using an anti-GST antibody. One-way ANOVA followed by Tukey’s multiple comparison test, N=6 ((B): data from two independent experiments). (**: pO.OOl).
**Figure 8.** Interaction between pro-MMP-13 and whole integrin αlOBl in the absence or presence of peptides (p617, p618 or the control peptide (ctrl-pep). Cell-extract from αlO transfected HEK 293-cells was preincubated for 10 minutes with peptides. After pre-incubation his-tagged proMMP-13 was added and incubation was continued for an additional 1h. Samples were added to anti-His coated wells and detection was carried out using a polyclonal anti-αlO antibody.

**Figure 9.** Human chondrocytes cultured in high-density monolayer induce MMP-activity after 2 days of interleukin-113 (IL-1β) stimulation. Dexamethasone (Dex) inhibits the IL-1β induced MMP-activity. MMP production was measured in the APMA activated culture supernatants using a quenched fluorescence assay.

**Figure 10.** Effects of peptides (p617, p618 and control-peptide (contr.)) and dexamethasone (Dex) on IL-1β induced total MMP activity in cell culture supernatants of human chondrocytes. MMP-activity measured in APMA activated culture supernatants using a quenched-fluorescence assay. Kruskal-Wallis test followed by Dunn's multiple comparison test, N=6. (**: p<0.001).

**Figure 11.** Effect of peptides p617 and p618 on IL-1β induced mRNA expression of (A) MMP1, (B) MMP13 and (C) MMP14 from both αlO (Adeno5-αlO) and all (Adeno5-all) Adeno5 transfected human chondrocytes. Measured using real-time RT-PCR and MMP-specific primers. Values relative to a housekeeping gene (GAPDH). (D) Effect of peptides p617 and p618 on IL-1β induced total MMP activity in the supernatants of transfected compared to un-transfected human chondrocytes. MMP-activity measured in APMA activated culture supernatants using a quenched-fluorescence assay. Dex: Dexamethasone.

**Figure 12.** Effect of exemplary polypeptides of the invention on glycosaminoglycan (GAG) release in human cartilage explants. Exemplary polypeptides of the invention tested were p617 [SEQ ID NO:7], p617:2 [SEQ ID NO:10], p618 [SEQ ID NO:6], p619 [SEQ ID NO:8] and p620 [SEQ ID NO:9] (p620N indicates a new batch of p620). One control peptide was tested, p618:1 [SEQ ID NO: 23]. The following additional controls were included; BMP2 with IGF-I and IL1β with OSM.
EXAMPLES

Example 1

Objective

The objective with this example was to present a model structure of the peptides of the invention.

Methods for Peptide Modelling

Model structures of the peptides were created by means of molecular dynamics simulations. The peptides were cyclised prior to simulation and built such that residues within the ring adopt a β-turn conformation. Molecular dynamics simulations were performed without solvent using the AMBER 7 FF02 force field (Ponder and Case 2003). The dielectric constant was set to 80 and the temperature was 300 K. Simulations were sampled with a time step of 1 fs for a total length of 1 ns for exemplary polypeptide of the invention 'P617' (corresponding to the cyclic form of SEQ ID NO:7) and for 80 ps for exemplary polypeptide of the invention 'P618' (corresponding to the cyclic form of SEQ ID NO:6). The final structure of each simulation was used for the drawings and is represented in Figure 1.

Results

The results of the molecular dynamics simulations in figure 1 show predicted stable 3-dimentional structures of peptides p617 (CWTWPDSGWC; SEQ ID NO:7) and p618 (CGIWFENECWC; SEQ ID NO:6).
Example 2

Objective

The objective with this example was to demonstrate binding of I-domain selected phages to different recombinant I-domains by phage ELISA.

Materials and methods

The integrin αl (Genbank acc#: NP_852478 amino acids (aa): 167-364 (CST-LEA)) αl O (Genbank acc#: NP_003628 aa: 162-359 (CPT-LEG)) and all (Genbank acc#: NP_036343 aa: 159-354 (CQT-LEG)) I-domains were cloned in fusion with a N-terminal GST-tag in the pCEP4-vector (Invitrogen). The constructs also contain a c-myc tag (sequence: MEQKLISEEDLGKL; SEQ ID NO: 11) located between the GST-tag and the I-domain. HEK-293 cells were transfected with the expression constructs using GenePORTER™ Transfection Reagent (Gene Therapy Systems, San Diego, CA, USA) according to the manufacturers recommendations and stably transfected cells were selected using 260 mM Hygromycin B (Ducheta, Haarlem, the Netherlands). HEK-293 cells stably expressing GST-αl I-domain, GST-αl O I-domain, GST-αl 1 I-domain or GST-only (GST-control) were maintained in DMEM:F12 medium (GIBCO BRL) supplemented with 260 mM Hygromycin B (Ducheta, Haarlem, the Netherlands), 50 U/ml penicillin / 50 µg/ml streptomycin (PEST) (GIBCO BRL) and 10 % foetal bovine serum (FBS) (GIBCO BRL) until confluent. Before purification of recombinant secreted proteins, the medium was replaced with DMEM:F12 supplemented with 130 mM Hygromycin B and 50 U/ml PEST. After 48h the culture supernatants were harvested and Complete EDTA-free protease inhibitor (Roche, Mannheim, Germany) (1:200) was added. After dialysis against PBS, 5mM di-thio-threitol (DTT) was added and the supernatants were rotated at 4°C for 24h with GST-sepharose beads 4 fast flow (Amersham Biosciences, Uppsala, Sweden). The GST-sepharose beads were collected on chromatography columns, washed and bound proteins were eluted with 40mM Tris / HCl (pH 8.0), 150 mM NaCl, 5 mM DTT and, 10 mM glutathione. The eluted samples were concentrated and dialysed against PBS in Amicon® Ultra-4, 10 000 NWML filter tubes.
(Millipore, Molsheim, France) following the manufacturer's instructions. Size and quality of the purified proteins were analysed on SDS-PAGE and samples were aliquoted and stored at -80°C for later use.

I-domain binding phages were selected from a phage library expressing 6-10 amino acid long cyclic peptides (C-X_{6-10}-C) using panning as described elsewhere (Koivunen et al. 1994; Koivunen et al. 1999b). Briefly described, the phage libraries were pre-selected in tris-buffered-saline with 0.05% Tween-20 (TBS-T) for 2h at room temperature on 96-well plates pre-coated with GST-control (20 μg/ml) and blocked with 2% bovine serum albumin (BSA). To select I-domain binding phages the un-bound phages were transferred to 96-well plates, pre-coated with recombinant GST-α (20 μg/ml) or all (20 μg/ml) I-domains and blocked for 1h at room temperature with tris-buffered-saline (TBS) + 1% BSA + ImM Mg squared. After shake-incubation for 2h at the wells were washed for 10 x 1 minute with TBS + ImM Mg + 0.5% Tween 20 and incubated with 100 μl elution buffer (0.1M glycine / HCl with 0.1% BSA and 0.05% phenol-red, pH 2.2) for 10 minutes with gentle shaking. The eluate was neutralised in IM Tris/HCl buffer (pH 9.0). Eluted phages were amplified in starved K91kan bacteria, the amplified phages were precipitated using polyethylene glycol and redissolved in TBS as described (Smith and Scott 1993). The selected and amplified phages were subjected to a second and third round of panning as described above. Selected individual phages were sequenced using BigDye (Applied BioSystems, UK) and the primers A and B (primer A: TAATACGACTCTATAGGGCAAGCTGATAAACCGATAAAC [SEQ ID NO: 12] and B: CCCTCATAAGTCGAACGATCTG [SEQ ID NO: 13] (15 pmol / ml) for 35 cycles of the following conditions; 95°C - 5 min, 92°C - 20 sec, 60°C - 20 sec, 72°C 1 min,, followed by 72°C for 4 minutes.

96-well MaxiSorb plates (Nunc) was coated with I-domain-GST fusions or the GST-control (2nM) at 4°C for 20h. Wells were blocked with TBS-T+1% BSA for 1h at room temperature. After blocking plates were washed three times with phosphate-buffered-saline with 0.05% Tween-20 (PBS-T) and incubated (shaking) for 1h at room temperature with 1x10⁹ plaque forming units (pfu) of phages. Plates were washed three times with PBS-T and bound phages detected
with HRP-anti-M13 (1:3000 in TBS-T+0.1% BSA) (Calbiochem) staining for 1h at room temperature. After staining plates were washed three times with PBS-T and developed using SIGMA-FAST™ o-phenylenediamine dihydrcIloride tablets (SIGMA-Aldrich, St Louis, MO, USA) according to manufactures instructions.

**Results**

The results in figure 2 demonstrate that phageA1 expressing the cyclic peptide CGIWFENEWC [SEQ ID NO:6] and A2 expressing the cyclic peptide CWIWPDSGWC [SEQ ID NO:7] in fusion with the pill phage protein, bind immobilised integrin I-domains in a solid-phase assay. PhageA1, expressing the p618 peptide, binds αl and αl1 equally well but not αl0. PhageA2 expressing the p617 peptide binds to αl, all and weaker to αl0. Neither phageA1 nor phageA2 bind to the GST control or to BSA coated wells.

**Example 3**

**Objective**

The objective with this example was to demonstrate that synthetic cyclic peptides with sequences derived from the selected phages could inhibit the phage interaction to I-domains.

**Materials and methods**

96-well MaxiSorb plates (Nunc) were coated with I-domain-GST fusions (2nM) or the GST control (2nM) (see example 2) at 4°C for 20h. Wells were blocked with 1% bovine serum albumin (BSA) in tris-buffered-saline with 0.05% Tween-20 (TBS-T+1% BSA) for 1h at room temperature. After blocking plates were washed three times with phosphate-buffered-saline with 0.05% Tween-20 (PBS-T) and incubated at room temperature with peptides (80 µM) in PBS-T+0.1%
BSA. After Ih, \(1 \times 10^9\) pfu of phages (see example 2) were added (in 10 µl) and incubation was continued for an additional Ih at room temperature with agitation. Plates were washed three times with PBS-T and bound phages detected with HRP-anti-M13 (1:3000 in TBS-T+0.1% BSA) (Calbiochem) staining for Ih at room temperature. After staining, plates were washed three times with PBS-T and developed using SIGMA-FAST™ o-phenylenediamine dihydrachloride tablets (SIGMA-Aldrich, St Louis, MO, USA) according to manufacture's instructions.

Cyclic peptides were synthesized at the Department of Bioscience (Helsinki, Finland) as described (Koivunen et al. 1994; Koivunen et al. 1999b). Peptide sequences were as follows:

\[
\begin{align*}
p617: & & \text{CWTWPDSGWC} & & [\text{SEQ ID NO:7}] \\
p618: & & \text{CGIWFENEWC} & & [\text{SEQ ID NO:6}] \\
\text{control peptide (ctrl-pep):} & & \text{CILWMKKGGWC} & & [\text{SEQ ID NO:14}] 
\end{align*}
\]

**Results**

The results in figure 3 demonstrate that the synthetic peptides p617 and 618 inhibit \(\alpha_l\)O and all I-domain interaction of phages A1 and A2 expressing cyclic peptides. P617 completely inhibits interaction of phageA1 (figure 3A and C) and phageA2 (figure 3B) to both \(\alpha_l\)O and all I-domains. P618 completely inhibits phageA1 interaction to all I-domain (figure 3A) and to a lower degree the phageA2 interaction to \(\alpha_l\)O (figure 3C) and \(\alpha_l\)1 (figure 3B).

These results demonstrate that the peptides derived from the selected phages are functional and bind to the I-domains thereby blocking the phage interaction to the I-domains.
Example 4

Objective

The objective with this example was to demonstrate that phages and peptides could interact with whole integrin.

Materials and methods

96-well MaxiSorb plates (Nunc) were coated with polyclonal antibodies (5 µg/ml) recognising the cytoplasmic tail of αl1 integrins, over night at 4°C in phosphate buffered saline (PBS)+Mg2+/Ca2+ (GIBCO BRL) followed by washing in tris buffered saline with 0.05% Tween-20 (TBS-T) and blocking with 2 % bovine serum albumin (BSA) in TBS-T with 1 mM Mg2+ and 10 µM Ca2+ at room temperature for Ih. After washing, wells were incubated for Ih at room temperature with cell-extract from HEK 293-cells transfected with αl1-integrins, in cell lysis buffer (1% NP40, 20 mM Tris pH 7.5, 150 mM NaCl, 1 mM MgCl2, 10 µM CaCl2, 1:50 Complete proteinase inhibitor). After washing, phages (see example 2) (109 plaque forming units (pfu)/ml in TBS + ImM Mg2+ and 10 µM Ca2+ with 1 % BSA and peptides (see example 3)(200 µg/ml in TBS + ImM Mg2+ and 10 µM Ca2+ with 1 % BSA) were added. After incubation for one hour at room temperature, the plates were washed and bound phages detected with HRP conjugated anti-M13 phage antibody (diluted 1:3000) (Calbiochem) for Ih at room temperature. After staining plates were washed three times with TBS-T and developed using SIGMA-FAST™ o-phenylenediamine dihydrachloride tablets (SIGMA-Aldrich, St Louis, MO, USA) according to the manufacturer's protocol and the absorbance at 450 nm was measured in a SpectraMax plate reader using the Softmax pro software.

Results

The results in figure 4A demonstrate that phageAl, expressing the p618 peptide, binds to an anti-αl1 cytoplasmic tail coated surface pre-incubated with cell
extract (CE) from integrin all transfected C2C12 cells (used as an example). PhageAl interaction is blocked by synthetic peptide p618 and to a lesser extent by p617. Binding of phageAl is dependent on CE from the integrin all transfected cells. These data suggest that the synthetic cyclic peptides bind the I-domains of the intact integrin α-chains.

Example 5

Objective

The objective with this example is to demonstrate that peptides p617 and p618 are functional modulators of integrin αl, αlO and/or all I-domain interaction to collagen.

Materials and Methods

96-well MaxiSorb plates were coated with 10 mg/ml collagen type VI in phosphate buffered saline (PBS) at 4°C for 48 hours followed by blocking with 1% bovine serum albumin (BSA) in tris-buffered-saline with 0.05% Tween-20 (TBS-T), 1mM Mg²⁺ and 10 μM Ca²⁺, for 1 hour at room temperature. The wells were washed 3 x 300 µl with TBS-T+Mg/Ca and the recombinant I-domains (100nM) (see example 2), when indicated preincubated for 30 min with peptides (see example 3) (160µM or as indicated in figure), were added in TBS-T+Mg/Ca with or without 10 mM EDTA. After 1 h incubation at room temperature the wells were washed again as described and incubated for 1h at room temperature with 50 µl goat-anti-GST antibody (Amersham Bioscience) diluted 1:1000 in 0.1% BSA in TBS-T+Mg/Ca, washed and incubated for 45 min at room temperature with HRP-conjugated rabbit-anti-goat antibody (Dakopatts A/S, Copenhagen Denmark) diluted 1:5000. After final washing, detection was carried out using SIGMA-FAST™ o-phenylenediamine dihydrachloride tablets (SIGMA-Aldrich, St Louis, MO, USA) according to the manufacturers' description. 50 µl 2 M H₂SO₄ was added and the absorbance at 492 nm was measured in a

**Results**

Recombinant GST-tagged I-domains (10OnM) were allowed to bind collagen VI or BSA pre-coated wells and bound protein was detected using an anti-GST antibody. Binding was allowed in the presence or absence of peptides p617 or p618. The results in figure 5A demonstrate that GST-tagged αl, αlO and all I-domains interact with collagen type VI in a cation dependent manner, i.e. EDTA clearly reduce binding. There is no binding to BSA coated wells and no binding of the GST control to collagen type VI. The results in figure 5B demonstrate that the synthetic cyclic peptides inhibit I-domain interaction to collagen VI. P617 significantly inhibits αlO (p<0.01) interaction to collagen. P618 significantly inhibits αl (p<0.001), αlO (p<0.001) and all (p<0.001) interaction to collagen. The inhibitory effect of p618 on collagen interaction is strongest on αl I-domain interaction, then αlO I-domain interaction. This could reflect a different affinity/specificity of p618 for the different integrin α-chains. The results in figure 5C demonstrate that p618 inhibits αl, αlO and all I-domain interaction to collagen type VI in a concentration dependent manner. Taken together, these data shows that the peptides are functional modulators of integrin function, since they can reduce I-domain interaction to a natural ligand (collagen) in a concentration dependant manner. As a consequence it is likely that the peptides would interfere with e.g. integrin dependent focal adhesion and migration.
Example 6

Objective

The objective with this example is to demonstrate that pro-MMP13 but not pro-MMP9 interacts with αlO and all I-domains.

Materials and Methods

Sandwich ELISA (figures 6A, B and C): MaxiSorb plates were coated with 10 µg/ml anti-his-antibody (MAB050 R&D Systems) diluted in tris-buffered-saline (TBS) over night at 4°C followed by blocking with 1% bovine serum albumin (BSA) in TBS-T (TBS+0.05% Tween 20) for 1 hour and washing (x3) with TBS-T. His-tagged proMMP-13 (33nM) was allowed to interact with the GST-tagged I-domains (see example 2) (20 - 320 nM) in 100 µl TBS-T with ImM Mg²⁺, 100µM Ca²⁺ and 0.1% BSA for 1 hour. The samples were then diluted and added to parallel wells of the anti-His coated plate and incubated for 1 hour at room temperature. After incubation, plates were sequentially stained with; goat-anti-GST antibody (Amersham Bioscience) (50 µl/well diluted 1:1000 in TBS-T+Mg/Ca+0.1%BSA) for 1h at room temperature, donkey-anti-goat-biotin (Dakopatts A/S, Copenhagen, Denmark) (50 µl/well diluted 1:100 000 in TBS-T+Mg/Ca+0.1%BSA) for 1h at room temperature and Streptavidin-HRP (Dakopatts A/S, Copenhagen, Denmark) (50 µl/well diluted 1:2000 in TBS-T+Mg/Ca+0.1%BSA) for 30 minutes at room temperature. Wells were washed with TBS-T+Mg/Ca between each incubation. After final washing, detection was carried out using SIGMA-FAST™ o-phenylenediamine dihydrochloride tablets (SIGMA-Aldrich, St Louis, MO, USA) according to the manufacturers' description. Absorbance was measured in a SpectraMax plate reader using the Softmax pro software. In figure C pro-MMP13 was activated for 1.5h at 37°C with ImM APMA in TBS-T+Mg/Ca.

Solid phase assay (figure D): MaxiSorb plates were coated over night at 4°C with 20 ng/ml proMMP9 (Calbiochem, San Diego, CA, USA) in TBS followed by
blocking with TBS-T+1% BSA for 1 hour at room temperature. Plates were washed 3x with TBS-T and incubated with recombinant GST-tagged I-domains (2OIIvI) in TBS-T+Mg/Ca+0.1% BSA for 2h at room temperature. After incubation and washing (x3) with TBS-T+Mg/Ca plates were incubated for 1h at room temperature with 50 µl goat-anti-GST antibody (Amersham Bioscience) diluted 1:1000 in TBS-T+Mg/Ca+0.1% BSA, washed and incubated for 45 min at room temperature with HRP-conjugated rabbit-anti-goat antibody (Dakopatts A/S, Copenhagen Denmark) diluted 1:5000. After final washing, detection was carried out using SIGMA-F AST™ o-phenylenediamine dihydrachloride tablets (SIGMA-Aldrich, St Louis, MO 5 USA) according to the manufacturer's description. 50 µl 2 M H₂SO₄ was added and the absorbance at 492 nm was measured in a SpectraMax plate reader using the Softmax pro software.

Results

The results in figure 6A demonstrate that αlO and all I-domains bind pro-MMP13 in a concentration dependent manner. Increased concentrations (20-32OnM) of I-domain, but not the GST control, results in increased binding to a fixed concentration of pro-MMP13 (33 nM), I-domain was allowed to interact with recombinant bis-tagged pro-MMP13 in solution. The complex was then added to wells pre-coated with anti-His-antibody. Bound GST-fusions was detected using a monoclonal anti-GST antibody. Values are corrected for unspecific binding to BSA.

The results in figure 6B demonstrate that the interaction between αl I-domain and pro-MMP13 is not cation dependent. AlphaI I-domain (16OnM) was allowed to interact with recombinant his-tagged pro-MMP13 (33 nM) in the presence of 1 nM Mg²⁺ AO0 µM Ca²⁺ or 10 mM EDTA. The complex was then added to wells pre-coated with anti-His-antibody. Bound GST-fusions was detected using a monoclonal anti-GST antibody. Data also demonstrate that that the binding of pro-MMP13 is I-domain specific since there is low binding to the GST control and since excluding pro-MMP13 or I-domain from the assay results in low signal.
The results in figure 6C demonstrate that pro-MMP13, but not activated MMP13, interacts with I-domain. These data suggest a model where proMMP (e.g. proMMP13) is bound to the collagen binding integrins (e.g. integrin αI0/betal or α1l/betal) on the cell surface (e.g. a chondrocyte), i.e. the inactive proMMP is localised to the cell surface and presented for activation by e.g. the membrane bound MMP14. After activation the active MMP is released from the integrin to digest its substrate. This way the integrin could be involved in both localisation of MMP and regulation of MMP activity. Breaking or prohibiting (see example 7) this interaction could be a way of blocking matrix degradation.

After activation of the proAll I-domain (80 nM) or GST-control (80 nM) was allowed to interact with recombinant bis-tagged pro-MMP13 (33 nM) or APMA activated MMP13 (33 nM) in solution. The complex was then added to wells pre-coated with anti-His-antibody. Bound GST-fusions was detected using a monoclonal anti-GST antibody. Values are corrected for unspecific binding to BSA.

The results in figure 6D demonstrate that αM I-domain, but not αd0, all or the GST-control, interacts with proMMP9. This shows selectivity in I-domain-MMP interaction where certain MMPs interacts with certain I-domains. We show as an example of this that the collagen type II degrading proenzyme pro-MMP13, but not pro-MMP9, could interact with the I-domains of integrin αd O and αl1 but that the I-domain of αM could interact with pro-MMP9. Interaction was analyzed using a solid phase assay where GST-fused I-domains were allowed to bind to wells coated with pro-MMP9 (20 ng/well). Bound GST-fusions was detected using a monoclonal anti-GST antibody. Values are corrected for unspecific binding to BSA.
Example 7

Objective

The objective with this example was to demonstrate that the synthetic cyclic peptide p618 could inhibit pro-MMP13 interaction to integrin αlβ1 and all I-domains.

Materials and Methods

MaxiSorb plates were coated with 10 µg/ml anti-his-antibody (MAB050 R&D Systems) diluted in tris-buffered-saline (TBS) over night at 4°C followed by blocking with 1% bovine serum albumin (BSA) in TBS-T (TBS+0.05% Tween 20) for 1 hour and washing (x3) with TBS-T. His-tagged proMMP-13 (33nM) was allowed to interact with the GST-tagged I-domains (see example 2) (160 nM) in 100 µl TBS-T with 1mM Mg²⁺, 100µM Ca²⁺ and 0.1% BSA in the presence or absence of 160 µM peptide (see example 3) for 1 hour. The samples were then diluted and added to parallel wells of the anti-His coated plate and incubated for 1 hour at room temperature. After incubation, plates were sequentially stained with; goat-anti-GST antibody (Amersham Bioscience) (50 µl/well diluted 1:1000 in TBS-T+Mg/Ca+0.1%BSA) for 1h at room temperature, donkey-anti-goat-biotin (Dakopatts A/S, Copenhagen, Denmark) (50 µl/well diluted 1:100 000 in TBS-T+Mg/Ca+0.1%BSA) for 1h at room temperature and Streptavidin-HRP (Dakopatts A/S, Copenhagen, Denmark) (50 µl/well diluted 1:2000 in TBS-T+Mg/Ca+0.1%BSA) for 30 minutes at room temperature. Wells were washed with TBS-T+Mg/Ca between each incubation. After final washing, detection was carried out using SIGMA-FAST™ o-phenylenediamine dihydrachloride tablets (SIGMA-Aldrich, St Louis, MO, USA) according to the manufacturers’ description. 2 M H2SO4 was added and the absorbance at 492 nm was measured in a SpectraMax plate reader using the Softmax pro software.
Results

The interaction between pro-MMP-13 and the recombinant GST-tagged I-domains of integrin αlO- and all was studied in a sandwich-ELISA in the presence of the peptides; p617, p618, or a control peptide. The results in figure 7A and 7B demonstrate that p618 inhibits the binding of the GST-tagged αlO and all I-domains to proMMP-13. The p617 or the control peptide had no effect on pro-MMP13/I-domain interactions. GST represent background interaction between proMMP13 and the GST control protein. Statistics: One-way ANOVA followed by Tukey's multiple comparison test, N=6 (data from two independent experiments).

Example 8

Objective

The objective with this example is to demonstrate that the synthetic peptide p618 inhibits pro-MMP13 interaction to the whole integrin using integrin αlO as an example.

Materials and Methods

MaxiSorb plates were coated with 10 µg/ml anti-his-antibody (MAB050 R&D Systems) diluted in phosphate-buffered-saline (PBS) over night at 4°C followed by blocking with 1 % heat-inactivated bovine serum albumin (BSA) in TBS-T (TBS+0.05% Tween 20) for 1 hour and washing (x3) with TBS-T. Cell-extract from HEK 293-cells, transfected with αlO-integrin in cell lysis buffer (1% NP40, 20 mM Tris pH 7.5, 150 mM NaCl, 1 mM MgCl₂, 10 µM CaCl₂, 1:50 Complete proteinase inhibitor) was preincubated at room temperature with peptides (see example 3)(160 µM). After 10 minutes of pre-incubation bis-tagged proMMP-13 (22nM) was added and incubation was continued for an additional 1h at room temperature. The samples were then diluted and added to parallel wells of the
anti-His coated plate and incubated for 1 hour at room temperature. After incubation plates were sequentially stained with; polyclonal anti-α1O antibody (specific for α1O cytoplasmic tail)(100 µl/well diluted 1:1000 in TBS-T with ImM Mg2+, 100µM Ca2+ and 1%BSA for 1h at room temperature and donkey-anti-rabbit-HRP (Dakopatts AJS, Copenhagen, Denmark) (100 µl/well diluted 1:1000 in TBS-T+Mg/Ca+1%BSA) for 1h at room temperature. Wells were washed with TBS-T+Mg/Ca between each incubation. After final washing, detection was carried out using SIGMA-FAST™ o-phenylenediamine dihydrachloride tablets (SIGMA-Aldrich, St Louis, MO, USA) according to the manufacturers' description. 2 M H2SO4 was added and the absorbance at 492 nm was measured in a SpectraMax plate reader using the Softmax pro software.

Results

The interaction between pro-MMP-13 and solubilised intact integrin α1O/betal was studied in a sandwich-ELISA in the presence of the peptides; p617, p618 or a control peptide. The results in figure 8 demonstrate that p618 have an inhibitory effect on the binding between the whole α1O integrin and pro-MMP13. The p617 had no or only weak effects compared to the control peptide.

Example 9

Objective

The objective with this example is to show that human chondrocytes cultured in high-density monolayer up-regulate MMP production after IL-1β stimulation and that this induction is prohibited in the presence of dexamethasone.

Material and Methods

Human chondrocytes from femur condyle cartilage of a 56-year old donor was cultured in DMEM/F12 (GIBCO BRL) supplemented with 10% FCS, PEST and
ascorbic acid (50 µg/ml) for 10 days before onset of experiment. On day 1, cells were plated (100,000 cells/well) in 96-well culture plates in DMEM/F12 supplemented with 10% FCS, PEST and ascorbic acid (50 µg/ml). On day 2 and 3 medium was changed to DMEM high glucose without phenol red (GIBCO BRL) supplemented with PEST and ascorbic acid (50 µg/ml). On day 2 and 3 cells were stimulated with IL-IB (R&D Systems) (5ng/ml) or IL-IB plus dexamethasone (Sigma)(10 µM). On day 4 supernatants were transferred to a black 96 well plate, and MMP activity was measured as described by (Knight et al. 1992) using a quenched fluorescence assay. Briefly, the supernatants were treated with 1 mM amino-phenyl-mercuric-acetate (APMA) in assay-buffer (50mM Tris/HCl, 5mM CaCl₂, pH 7.5) for one hour at 37°C. After addition of 2 µl MMP-substrate (Calbiochem), the increase in fluorescence (ex 328 nm and em 393 nm) due to substrate cleavage was measured during one hour at 37°C. The Vmax / second was calculated using Softmax PRO.

Results

The results in figure 9 demonstrate that IL-IB induce MMP-synthesis from human chondrocytes cultured in high-density monolayer after 2 days of stimulation. The presence of dexamethasone during IL-IB stimulation inhibits the MMP-production. MMP production was measured, using a quenched fluorescence assay, in APMA activated culture supernatants after 48 hours stimulation.
Example 10

Objective

The objective with this example was to demonstrate that cyclic peptides p617 and p618 could inhibit IL-IB induced increased MMP protein production.

Materials and methods

Human chondrocytes were prepared from femoral condyle cartilage of a 63-year-old donor as described in Example 9 above. The chondrocytes were cultured in DMEM-FI2 (GIBCO BRL) supplemented with 10% foetal calf serum (FCS), PEST (50 U/ml penicillin and 50 µg/ml streptomycin) and ascorbic acid (50 µg/ml), for ten days post isolation. On day 1 of the experiment the cells were detached and seeded in a 96 well culture plate at high cell density (100 000 cells/well) and left to adhere over night (37°C, 5% CO₂). On day 2 the cells were gently washed with PBS and stimulated with IL-1β (5 ng/ml), IL-1β plus dexamethasone (10 µM) or IL-1β plus peptides (see example 3) (160 µM) in DMEM-high glucose medium (without indicator) (GIBCO BRL) supplemented with PEST and ascorbic acid and the stimulation was repeated on day 3. On day 4 (48h after the first stimulation) 40 µl of the supernatant was transferred to a black micro titre plate (NUNC) in duplicate wells. The detection of MMP-present in the supernatant was performed as described (Knight et al. 1992) using a quenched fluorescence assay. Briefly, the supernatants were treated with 1 mM amino-phenyl-mercuric-acetate (APMA) in assay-buffer (50 mM Tris/HCl, 5 mM CaCl₂, pH 7.5) for one hour at 37°C. After addition of 2 µl MMP-substrate (Calbiochem), the increase of fluorescence (ex 328 nm and em 393 nm) was measured during one hour at 37°C. The Vmax / second was calculated. Statistics: Kruskal-Wallis test followed by Dunn's multiple comparison test, N=6.
Results

The results in figure 10 demonstrate that the cyclic peptide p617 reduce IL-IB induced total MMP activity in the APMA activated cell culture supernatants of human chondrocytes (p<0.001 p617 vs. ctrl-peptide and p<0.001 p617 vs. IL-IB). Also, p618 inhibited MMP production but to a lower degree. The control peptide had no effect on MMP activity.

Example 11

Objective

The objective with this example was to demonstrate that the altered MMP gene expression profile and increased total MMP protein production induced by over-expression of integrin αlβ0 and all αlβ integrins in human chondrocytes could be inhibited by treatment with peptides p617 and p618.

Materials and methods

Human chondrocytes were prepared from tibial plateau of a 45-year-old donor as described (Goldring et al. 1986; Archer et al. 1990). The chondrocytes were cultured in DMEM-F12 (GIBCO BRL) supplemented with 10% foetal calf serum (FCS), PEST (50 U/ml penicillin and 50 µg/ml streptomycin) and ascorbic acid (50 µg/ml), for ten days post isolation. On day 1 of the experiment the chondrocytes were detached and infected in suspension (100 µl of DMEM-F12 supplemented with 10% FCS and PEST) with 100 multiplicity of infection (MOI) of adenovirus (Adeno5) encoding human αlβ0 or all αlβ integrins (He et al. 1998). After 2h of infection, the cells seeded in a 96 well culture plate at high cell density (100 000 cells/well) and allowed to adhere over night (37°C, 5% CO2). On day 2 the cells were gently washed with PBS and stimulated with IL-1β (5 ng/ml), IL1-β plus dexamethasone (10 µM) or IL-1β plus peptides (see example 3) (160µM) in DMEM-high glucose medium (without indicator) (GIBCO BRL).
supplemented with PEST and ascorbic acid and the stimulation was repeated on
day 3. On day 4 (48h after the first stimulation) 40 µl of the supernatant was
transferred to a black micro titre plate (NUNC) in duplicate wells. The detection
of MMP-present in the supernatant was performed as described (Knight et al.
1992) using a quenched fluorescence assay. Briefly, the supernatants were treated
with 1 mM amino-phenyl-mercuric-acetate (APMA) in assay-buffer (50mM
Tris/HCl, 5mM CaCl2, pH 7.5) for one hour at 37°C. After addition of 2 µl
MMP-substrate (Calbiochem), the increase of fluorescence (ex 328 nm and em
393 nm) was measured during one hour at 37°C. The Vmax / second was
calculated. The cells were gently washed with PBS and medium changed to
DMEM-high glucose medium (without indicator) (GIBCO BRL) supplemented
with PEST and ascorbic acid. Four days after infection 40 µl of the supernatant
was transferred to a black micro titre plate (NUNC) in duplicate wells. The
detection of MMP-present in the supernatant was performed as described (Knight
et al. 1992) using a quenched fluorescence assay. Briefly, the supernatants were
treated with 1 mM amino-phenyl-mercuric-acetate (APMA) in assay-buffer
(50mM Tris/HCl, 5mM CaCl2, pH 7.5) for one hour at 37°C. After addition of 2
µl MMP-substrate (Calbiochem), the increase of fluorescence (ex 328 nm and em
393 nm) was measured during one hour at 37°C. The Vmax / second was
calculated.

On day 4 after infection total RNA was isolated from the chondrocytes using a kit
(RNeasy, Qiagen) with the addition of a DNase treatment step according to the
manufacturers' instructions. 1 µg total RNA was reverse transcribed using 200
units Superscript II (Invitrogen) and 200 ng random hexamer oligonucleotides.
The reaction parameters were: 10 minutes at 25°C, 30 minutes at 37°C,
30 minutes at 42°C and 15 minutes at 65°C. cDNA (20 ng) was subjected to real-
time RT-PCR analyses to determine the relative mRNA levels of MMP1,
MMP13 and MMP14 to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).
The primer sequences used were as follows:

For \textit{MMP1}: CCCTGAAGGTGATGAAGCAG [SEQ ID NO:15], and  
CTTGGTGATGTCAGAGGTG [SEQ ID NO: 16];

For \textit{MMP1S}: CTCAGCAGGTGATGCGGAG [SEQ ID NO:17], and  
CTCAAAGTGAACAGCTGCAC [SEQ ID NO:18];

For \textit{MMP14}: CTTCAAGGAGCGCTGGTTCTG [SEQ ID NO:19], and  
AGAGCAGCATCAATCTTGTC [SEQ ID NO:20]; and

For \textit{GAPDH}: GGAGGGGAGATTCAGTGTGGT [SEQ ID NO:21] and  
ACCAGCGACACCCACTCCTC [SEQ ID NO:22].

The mRNA expression levels were quantified with Real-Time reversed PCR (RT-PCR) using FastStart DNA Master SYBR Green I (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufactures instructions and analysed on a LightCycler (Roche Diagnostics). The amplification parameters were: 10s at 95°C, 5s at 65°C and 12s at 65°C. Products were checked using the melting curve function of the LightCycler. The relative ratio of \textit{MMP} to \textit{GAPDH} was calculated.

\textbf{Results}

The results in figure 11 A-C demonstrate that both p617 and p618 decrease IL-IB induced mRNA expression of both \textit{MMP1} and \textit{MMP13} but to a lesser extent \textit{MMP14} from both \(\alpha\)O and all transfected human chondrocytes. The results in figure HD shows that adenovirus based transfection of integrin \(\alpha\)O or a11 results in increased total \textit{MMP} activity compared to non-transfected cells and that IL-IB treatment do not further increase the total \textit{MMP} activity in the supernatants of the transfected cells. However, the p617 peptide as well as dexamethasone can reduce the total \textit{MMP} activity caused by over-expression of \(\alpha\)O and all in human chondrocytes.
Example 12

Objective

The objective with this example was to test how exemplary peptides of the invention affect reduction of collagen degradation in human explants.

Material and Methods

Cartilage explant samples

Four independent experiments were performed, and data pooled, using cartilage explants from four subjects:

- Subject 1: 42 years old (normal healthy control)
- Subject 2: 73 years old (early osteoarthritis sufferer)
- Subject 3: 59 years old (supposed early osteoarthritis sufferer)
- Subject 4: 63 years old (supposed early osteoarthritis sufferer)

Peptides

The following exemplary peptides of the invention were tested

- p617 [SEQ ID NO: 7]
- p617:2 [SEQ ID NO: 10]
- p618 [SEQ ID NO: 6]
- p619 [SEQ ID NO: 9]
- p620 [SEQ ID NO: 8]

(p620N has the same amino acid sequence as p620)
The following control peptide was used:

\[ \rho 618:1 \quad \text{CGIWFKNKWC} \quad \text{[SEQ ID NO: 23]} \]

5 **Experimental protocol**

Day 0. Cartilage cut into ~1-2mm\(^3\) pieces. Add ~80 mg (~7-9 pieces) of cartilage per well to 48-well plates containing 1 ml DMEM-F12 w/o phenol red (Invitrogen) (+ 1:100 PEST + 1:250 fungizone (Invitrogen) + 0.01 % BSA (Invitrogen) + non-essential amino acids (Invitrogen) + 5% FCS).

Day 1: Change to DMEM-F12 w/o phenol red (+ 1:100 PEST + 1:250 fungizone + 0.01 % BSA + non-essential amino acids. + 25 mM HEPES (Invitrogen)) without peptides/growth factors.

Day 2: Change to DMEM-F12 w/o phenol red (+ 1:100 PEST + 1:250 fungizone + 0.01 % BSA + non-essential amino acids. + 25 mM HEPES) plus peptides or growth factors.

Peptide concentration: 50 \(\mu\)g/ml; growth factor concentrations: BMP2 200ng/ml, IGF-I 200ng/ml, IL1\(\beta\) 0.02ng/ml, OSM 50 ng/ml.

Days 2-6: Incubate explant samples in presence of peptides or growth factors four 4 days. Change of medium every second day.

Statistical analysis

One-way ANOVA with Tukey's multiple comparison test:

\[ \text{CI: 99\%} \]
\[ n: \text{ 8 replicate / 4 experiment} \]
\[ \text{Data: Mean+SD} \]

Results

Results are shown in Figure 12.

Test peptides p617, P617:2, p618 & p620 all significantly reduced GAG-release compared to the two control peptides used (p619 & p618:1). Peptide P619 also reduced GAG-release but to a lesser extent

Conclusions

Test peptides p617, P617:2, p618 & p620 (and, to a lesser extent, p619) are able to reduce collagen degradation in human cartilage explants.
REFERENCES


Clark IM, Parker AE (2003) Metalloproteinases: their role in arthritis and potential as therapeutic targets. Expert Opin Ther Targets 7:19-34


Dumin JA, Dickeson SK, Strieker TP, Bhattacharyya-Pakrasi M, Rohy JD, Santoro SA, Parks WC (2001) Pro-coUagenase-1 (matrix metalloproterase-1) binds the alpha(2)beta(1) integrin upon release from keratinocytes migrating on type I collagen. J Biol Chem 276:29368-29374


Ref Type: Serial (Book,Monograph)


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CLAIMS

1. An isolated polypeptide capable of binding to an integrin I-domain wherein the polypeptide comprises an amino acid sequence selected from the following group:

   GIWFENEW [SEQ ID NO: 1];
   WTPDSCGW [SEQ ID NO: 2];
   WENWGDGWG [SEQ ID NO: 3];
   WEDGWLHA [SEQ ID NO: 4] and/or
   WCWPDSCW [SEQ ID NO: 5];

   or a variant, fusion or derivative thereof, or a fusion of a said variant or derivative thereof.

2. A polypeptide according to Claim 1 wherein the polypeptide is capable of inhibiting the degradation of collagen in cartilage tissue.

3. A polypeptide according to Claim 1 or 2 wherein the polypeptide is capable of inhibiting glycosaminoglycan (GAG) release in human cartilage explants.

4. A polypeptide according to any one of the preceding claims wherein the polypeptide is less than 50 amino acids in length, for example less than 40, less than 30, less than 20, less than 15, less than 14, less than 13, less than 12, less than 11, less than 10, less than 9, less than 8, or less than 7 amino acids in length.

5. A polypeptide according to Claim 4 wherein the polypeptide is between 8 and 10 amino acids in length.

6. A polypeptide according to any one of the preceding claims wherein the polypeptide comprises or consists of L-amino acids.
7. A polypeptide according to any one of the preceding claims wherein one or more amino acids are modified or derivatised.

8. A polypeptide according to any one of the preceding claims wherein the polypeptide is linear.

9. A polypeptide according to any one of the preceding claims wherein the polypeptide is cyclic.

10. A polypeptide according to any one of the preceding claims wherein the polypeptide exists in the form of a multimer.

11. A polypeptide according to any one of the preceding claims comprising the amino acid sequence of SEQ ID NO:1.

12. A polypeptide according to Claim 11 comprising or consisting of the amino acid sequence of SEQ ID NO:5.

13. A polypeptide according to any one of the preceding claims comprising the amino acid sequence of SEQ ID NO:2.

14. A polypeptide according to Claim 13 comprising or consisting of the amino acid sequence of SEQ ID NO:6.

15. A polypeptide according to any one of the preceding claims comprising the amino acid sequence of SEQ ID NO:3.

16. A polypeptide according to Claim 15 comprising or consisting of the amino acid sequence of SEQ ID NO:7.

17. A polypeptide according to any one of the preceding claims comprising the amino acid sequence of SEQ ID NO:4.
18. A polypeptide according to Claim 17 comprising or consisting of the amino acid sequence of SEQ ID NO:8.

19. A polypeptide according to any one of the preceding claims comprising the amino acid sequence of SEQ ID NO:5.

20. A polypeptide according to Claim 19 comprising or consisting of the amino acid sequence of SEQ ID NO:10.

21. A polypeptide according to Claim 12, 14, 16, 18 or 20 wherein the polypeptide contains a disulphide bond between the two cysteine amino acids.

22. A polypeptide according to any one of the preceding claims wherein the integrin is expressed on chondrocytes, fibroblasts and/or cancer cells.

23. A polypeptide according to any one of the preceding claims wherein the integrin is a collagen-binding integrin.

24. A polypeptide according to Claim 23 wherein the integrin is selected from the group consisting of αl/β1, α2/β1, αθ/β1 and αl1/β1.

25. A polypeptide according to Claim 24 wherein the integrin is αl0/β1.

26. A polypeptide according to Claim 24 wherein the integrin is αl1/β1.

27. A polypeptide according to any one of Claims 1 to 23 wherein the integrin is selected from the group consisting of αDβ2, αMβ2, αLβ2, αXβ2 and αEβ7.

28. A polypeptide according to any one of the preceding claims wherein the polypeptide exhibits specificity for a single integrin I-domain.

29. A polypeptide according to any one of the preceding claims wherein the polypeptide is capable of inhibiting a function of an integrin.
30. A polypeptide according to any one of the preceding claims wherein the polypeptide is capable of inhibiting the binding of collagen to an integrin.

31. A polypeptide according to any one of the preceding claims wherein the polypeptide is capable of inhibiting the binding of a metzincin to an integrin.

32. A polypeptide according to Claim 31 wherein the metzincin is selected from the group consisting matrix metalloproteinases (MMPs), disintegrin and metalloproteases (ADAMs) and disintegrin and metalloproteases with thrombospondin motifs (ADAMTSs), and pro-forms thereof.

33. A polypeptide according to Claim 32 wherein the metzincin is a metalloproteinase (MMP) or pro-form thereof.

34. A polypeptide according to Claim 33 wherein the metalloproteinase is selected from the group consisting of the collagenases MMPI, MMP8 and MMP13.

35. A polypeptide according to Claim 34 wherein the metalloproteinase is MMP13.

36. An isolated nucleic acid molecule encoding a polypeptide according to any one of Claims 1 to 35.

37. A vector comprising a nucleic acid molecule according to Claim 36.

38. A vector according to Claim 37 wherein the vector is an expression vector.

39. A vector according to Claims 37 or 38 wherein the vector is suitable for replication in a eukaryotic cell.
40. A vector according to Claims 39 wherein the vector is suitable for replication in a mammalian cell.

41. A vector according to any one of Claims 37 to 40 wherein the vector is selected from the group consisting of pTWIN, pShuttle, pUC18, pUC19, pBacPAK, pBR322, pBR329, pTrc99A, pKK223-35, pSVL, pMSG, pRS403 to 406 and pRS413 to 416.

42. A host cell comprising a nucleic acid molecule according to Claim 36 or a vector according to any one of Claims 37 to 41.

43. A host cell according to Claim 42 wherein the cell is a eukaryotic cell.

44. A host cell according to Claim 43 wherein the cell is a mammalian cell.

45. A host cell according to any one of Claims 42 to 44 wherein cell is selected from the group consisting of E. coli strain DH5, RRL, ER2566, CHO cells (e.g. CCL61), NIH Swiss mouse embryo cells (NIH/3T3), COS-I cells (e.g. CRL 1650 and 293), S© cells and yeast cell lines YPH499 to 501.

46. A method for making a polypeptide according to any one of Claims 1 to 35, the method comprising culturing a host cell according to any one of Claims 42 to 45 which expresses the polypeptide, and isolating the polypeptide therefrom.

47. A method for making a polypeptide according to any one of Claim 1 to 35 comprising solid phase synthesis of the polypeptide.

48. A pharmaceutical formulation comprising a polypeptide according to any one of Claims 1 to 35 in admixture with a pharmaceutically or veterinarily acceptable adjuvant, diluent or carrier.

49. A polypeptide according to any one of Claims 1 to 35 or a pharmaceutical formulation according to Claim 48 for use in medicine.
50. Use of a polypeptide according to any one of Claims 1 to 35 or a pharmaceutical formulation according to Claim 48 in the preparation of a medicament for treating a disease or condition capable of being treated by an agent which modulates the function of an integrin and/or a metzincin.

51. The use according to Claim 50 wherein the disease or condition capable of being treated by an agent which modulates the function of an integrin and/or a metzincin is selected from the group consisting of arthritic diseases, inflammatory diseases and conditions, cancer and metastasis progression, oral diseases, osteoporosis, tissue remodeling, angiogenesis, angiogenesis, lung diseases, wounds, burns, fractures, lesions and ulcers.

52. The use according to Claim 50 or 51 wherein the disease or condition capable of being treated by an agent which modulates the function of an integrin and/or a metzincin is an arthritic disease.

53. The use according to Claim 48 wherein the arthritic disease is selected from the group consisting of osteoarthritis, rheumatoid arthritis and reactive arthritis.

54. The use according to Claim 50 or 51 wherein the disease or condition capable of being treated by an agent which modulates the function of an integrin and/or a metzincin is an inflammatory disease or condition.

55. The use according to Claim 54 wherein the inflammatory disease or condition is selected from the group consisting of Alzheimer's disease, psoriasis, asthma, atherosclerosis, sarcoidosis, atopic dermatitis, systemic lupus erythematosus, bullous pemphigoid, type I diabetes mellitus, chronic obstructive pulmonary disease, ulcerative colitis, gout, Helicobacter pylori gastritis, inflammatory bowel disease, Hepatitis C, ischaemia-reperfusion injury, multiple sclerosis, Neisserial or pneumococcal meningitis, tuberculosis and periodontitis.
56. The use according to Claim 50 or 51 wherein the disease or condition capable of being treated by an agent which modulates the function of an integrin and/or a metzincin is cancer.

57. The use according to Claim 56 wherein the cancer is selected from the group consisting of sarcomas, carcinomas, myelomas, leukaemias, lymphomas, melanoma and small cell lung cancer.

58. A method of treating a patient in need of modulation of the function of an integrin and/or a metzincin, the method comprising administering to the patient an effective amount of a polypeptide according to any one of Claims 1 to 35 or a pharmaceutical formulation according to Claim 48.

59. A method according to Claim 58 wherein the patient has a disease or condition selected from the group consisting of arthritic diseases, inflammatory diseases and conditions, cancer and metastasis progression, oral diseases, osteoporosis, tissue remodeling, angiogenesis, angiogenesis, lung diseases, wounds, burns, fractures, lesions and ulcers.

60. A method according to Claim 59 wherein the patient has an arthritic disease.

61. A method according to Claim 60 wherein the method further comprises administering one or more conventional anti-arthritic agents.

62. A method according to Claim 59 wherein the patient has an inflammatory disease or condition.

63. A method according to Claim 62 wherein the method further comprises administering one or more conventional anti-inflammatory agents.

64. A method according to Claim 59 wherein the patient has cancer.
65. A method according to Claim 64 wherein the method farther comprises administering one or more conventional anti-cancer agents.

66. A method according to any one of Claims 58 to 65 wherein the patient is human.

67. Use of a polypeptide according to any one of Claims 1 to 35 to detect the presence of an integrin in a sample.

68. The use according to Claim 67 in the diagnosis of a disease or condition associated with an abnormal amount and/or function of an integrin.

69. The use according to Claim 67 or 68 to detect the presence of an integrin in a sample in vitro.

70. The use according to any one of Claims 67 to 69 wherein the sample is selected from the group consisting of tissue samples, blood samples, plasma samples and synovial fluid.

71. The use according to Claim 70 wherein the sample is a connective tissue sample.

72. The use according to Claim 70 or 71 wherein the sample is a cartilage tissue sample.

73. The use according to Claim 70 or 71 wherein the sample is a synovium sample.

74. The use according to any one of Claims 67 or 73 to detect the presence of an integrin in a sample in vivo.

75. The use according to Claim 74 wherein the integrin is detected in vivo by X-ray, MR, ultrasound, scintigraphy, PET, SPECT, electrical impedance, light or magnetometric imaging modalities.
76. The use according to any one of Claims 67 to 75 wherein the integrin is a collagen-binding integrin.

77. The use according to Claim 76 wherein the collagen binding integrin is αl0βl or αl1βl integrin.

78. A method for diagnosing in a patient a disease or condition associated with abnormal level of an integrin, the method comprising:

(a) providing a sample from the patient to be tested;
(b) contacting the sample with a polypeptide according to any one of Claims 1 to 35 under conditions which permit the binding of the polypeptide to an integrin (if present in the sample); and
(c) detecting the binding of the polypeptide to an integrin (if present in the sample)

wherein the amount of polypeptide bound to the sample from the patient to be tested provides an indication of the amount of integrin in the sample.

79. A method according to Claim 78 further comprising step (d) of comparing the amount of polypeptide bound to the sample from the patient to be tested with the amount of the polypeptide bound to a sample from a healthy individual (or population of individuals).

80. The method according to Claim 78 wherein the integrin is a collagen-binding integrin.

81. The method according to Claim 80 wherein the collagen-binding integrin is αl0βl or αl1βl integrin.

82. A kit for performing a method according to any one of Claims 78 to 81 comprising a polypeptide according to any one of Claims 1 to 35.
83. Use of a polypeptide according to any one of Claims 1 to 35 to improve targeting of liposomes to target cells.

84. A polypeptide complex comprising a polypeptide according to any one of Claims 1 to 35 and an integrin subunit, or fragment thereof comprising an I-domain.

85. A complex according to Claim 84, wherein the complex is in crystalline form.

86. A method of making a complex according to Claim 85 comprising co-crystallising a polypeptide according to any one of Claims 1 to 35 and an integrin subunit, or fragment thereof comprising an I-domain.

87. A method for identifying a candidate compound for the treatment of a disease or condition capable of being treated by an agent which modulates the function of an integrin and/or a matrix metalloproteinase, the method comprising determining if a compound to be tested competes with the binding of a polypeptide according to any one of Claims 1 to 35 to an integrin I-domain.

88. A method according to any of Claim 87 further comprising the step of mixing the compound thus identified with a pharmaceutically acceptable carrier.

89. A kit for performing a method according to Claim 87 or 88 comprising a polypeptide according to any one of Claims 1 to 35 and an integrin subunit or fragment thereof comprising or consisting of an I-domain, or means for producing the same.

90. A compound identified by the method of Claim 87 or 88.

91. A compound according to Claim 90 for use in medicine.
92. A pharmaceutical formulation comprising a compound according to Claim 91 in admixture with a pharmaceutically or veterinarily acceptable adjuvant, diluent or carrier.

93. A polypeptide capable of binding to an integrin I-domain substantially as defined herein with reference to the description.

94. A pharmaceutical formulation substantially as defined herein with reference to the description.

95. A method of treating a patient in need of modulation of the function of an integrin and/or a metzincin substantially as defined herein with reference to the description.

96. A method for identifying a candidate compound for the treatment of a disease or condition capable of being treated by an agent which modulates the function of an integrin and/or a metzincin substantially as defined herein with reference to the description.
FIGURE 2
FIGURE 3(A)

Binding (Abs. 492nm)

- Alpha11
- GST
- BSA

PhageA1 + ctrl-pep + p617 + p618
FIGURE 3(B)

Binding (Abs. 492nm)

PhageA2  +ctrl-pep  +p617  +p618

- alpha11
- GST
- BSA
FIGURE 3(C)

Phage binding (Abs.)

- alpha10
- GST
- BSA

PhageA2 +ctrl-pep +p617 +p618
FIGURE 5(A)

[Graph showing binding levels for different samples: CVI, CVI+EDTA, BSA for Alpha1, Alpha10, Alpha11, and GST.]

- CVI
- CVI+EDTA
- BSA
FIGURE 5(B)
FIGURE 6(B)

Binding (Abs. 490nm)

- anti-His
- BSA

compounds: alpha11+MMP13, alpha11+MMP13+EDTA, GST+MMP13, GST+MMP13+EDTA, No alpha11, No MMP-13
FIGURE 7(A)

Alpha10/MMP-13 binding (OD 492nm)

alpha10
alpha10+p617
alpha10+p618
alpha10+ctl
GST
FIGURE 7(B)
FIGURE 8

Alpha10 integrin/MMP13 binding (OD 492nm)

No pep. p617 p618 ctrl pep.
FIGURE 9

Bar graph showing the fold change in V-max with different conditions:
- No stim.
- IL1β
- IL1β + Dex

The y-axis represents the fold change, ranging from 0 to 14, with error bars indicating variability.
FIGURE 10

[Graph showing MMP activity (Vmax/DNA) for different conditions involving IL-1β and other treatments.]
FIGURE 11(C)

- Adeno5-alpha10
- Adeno5-alpha11

MMP14 mRNA (MMP14/GAPDH)

- No stim.
- IL1β
- IL1β+p617
- IL1β+p618
- IL1β+dex
FIGURE 11(D)

- Non-transfected
- Adeno5-alpha10
- Adeno5-alpha11

MMP activity (Vmax)

- No stim.
- IL-1β
- IL-1β+p617
- IL-1β+p618
- IL-1β+dex
FIGURE 12

Explant (e14, e18, e21, e22)