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(54) Title: INHIBITORS OF THE LEUKOCYTE PROMMP-9/BETA(2) INTEGRIN COMPLEX

(57) Abstract: The present invention concerns peptide compounds, which are inhibitors of integrin-MMP complex. The compounds comprise the peptide motif, D/E-/D/E-G/L-W, and they specifically bind to the  $\alpha$ M integrin I domain and inhibit its complex formation with proMMP-9. The compounds thus prevent neutrophil migration, as well as leukocyte migration. The compounds can be used in the treatment of inflammatory conditions and leukaemia.

## Inhibitors of the leukocyte proMMP-9/beta(2) integrin complex

### Field of the Invention

5 The present invention concerns peptide compounds, which are inhibitors of integrin-MMP complex. In specific, the compounds bind to the  $\alpha_M$  integrin I domain and inhibit its complex formation with proMMP-9. The compounds thus prevent neutrophil migration, as well as leukocyte migration. The compounds can be used in the treatment of inflammatory conditions, and leukaemia.

10

### Background of the Invention

The leukocyte integrin family consists of four heterodimeric glycoproteins with specific  $\alpha$ -chains ( $\alpha_L$ ,  $\alpha_M$ ,  $\alpha_X$ , or  $\alpha_D$ ) and a common  $\beta_2$ -chain (CD18). They play an 15 essential role in mediating adhesion of cells in the immune system (1). The major ligand-binding site locates to an 200 amino acid long sequence within the  $\alpha$ -chain called I or inserted domain, which is homologous to the A domains of von Willebrand factor, repeats of cartilage matrix protein and collagen (2).

20 Among the  $\beta_2$  integrins,  $\alpha_M\beta_2$  is the most promiscuous binder being able to interact with a multitude of unrelated ligands. These include ICAM 1 to 5, complement fragment iC3b, fibrinogen, uPAR, E-selectin and various extracellular matrix proteins (see (3) and references therein). The integrin has also been shown to have a capacity to bind certain enzymes, but whether this is important for leukocyte adhesion or immune 25 reactions is unclear. Such enzymes showing integrin-binding activity are catalase (4), myeloperoxidase (5) and the proteinases elastase (6) and urokinase (7).

Extensive work has been done to identify the ligand binding sites in  $\beta_2$  integrin I-domains, but less is known about the interacting ligand regions (8, 9). Recently, the 30 structure of an  $\alpha_L$  I domain/ICAM-1 complex was reported (10). Low molecular weight peptides binding to the  $\beta_2$  integrins are useful reagents to study integrin function, and such peptides have been derived from ICAM-2 (11), fibrinogen (12), and Cyr61 (13). We have used phage display libraries to study the peptide binding specificity of integrins and to develop potential drug leads. In our previous study, we isolated the

biscyclic peptide CPCFLLGCC (LLG-C4)(unpublished observations of the present inventors) as the most active binder to the purified  $\alpha_M\beta_2$  integrin (14). Leukocytes can efficiently adhere to the immobilized LLG-C4 peptide via the  $\alpha_M\beta_2$  and  $\alpha_X\beta_2$  integrins.

## 5 Summary of the Invention

We have now extended phage display screenings to the purified  $\alpha_M$  I domain. This has resulted in the identification of a novel I domain-binding tetrapeptide motif D/E-D/E-G/L-W, which is found on some of the known  $\beta_2$  integrin ligands and interestingly also 10 on the catalytic domain of MMPs. We show that the D/E-D/E-G/L-W motif mediates binding between an MMP and  $\beta_2$  integrin, and proMMP-9 gelatinase, which is known to be the major MMP of leukocytes, occurs in complex with the  $\alpha_M\beta_2$  and  $\alpha_L\beta_2$  integrins 15 in leukaemic cell lines following cellular activation. The peptide inhibitors of the integrin-MMP complex prevent leukaemia cell migration, suggesting a role for the complex in cell motility. The compounds of the invention also attenuated PMN migration *in vitro* and *in vivo*, suggesting a role for the MMP-integrin complex in PMN motility.

## Detailed description of the Invention

20

Analysis of  $\alpha_M$  I domain-binding peptides led to the finding that MMPs, particularly the MMP-9 and MMP-2 progelatinases, are potent  $\beta_2$  integrin ligands. Our studies show that proMMP-9, the major MMP of activated leukocytes, is co-localized with the  $\beta_2$  integrin on the cell surface. Cell surface labelling and co-immunoprecipitation further 25 demonstrates the occurrence of the complex in leukemic cell lines. Finally, we have found evidence that this proteinase-integrin complex plays a role in migration of the leukemic cells.

Although phage display has been extensively used with whole integrins, to the best of 30 our knowledge, this is the first successful phage display selection on an isolated integrin I domain. We could enrich only one binding motif even though the  $\alpha_M$  I domain can bind a variety of ligands. The peptide motif we isolated could not compete with the ICAM-1, fibrinogen, or LLG-C4 ligands. The success of phage display depends on the libraries used and the biopanning conditions. Our method favoured “high affinity”

interactions with the cyclic peptides yielding the D/E-D/E-G/L-W motif. Interestingly, this motif shows a high degree of similarity to the CWDD(G/L)WLC peptide isolated by phage display as an RGD sequence-binding peptide (15). By recognizing the RGD ligand sequence, CWDDGWLC structurally and functionally behaves like a minimal 5 integrin. Here, we have identified the DDGW peptide in a reverse situation, as a ligand to integrin. However, the RGD sequence does not compete with the  $\alpha_M$  I domain as the GRGDSP peptide at a 1 mM concentration was unable to inhibit proMMP-9 binding to the I domain (unpublished observations of the present inventors).

10 Some hints for the binding site of DDGW come from the interaction of iC3b with  $\alpha_M\beta_2$  integrin. Our pepspot analysis showed that the iC3b peptide ARSNLDEDIIAEENI, but not the control peptide ARSNLDAAIIAEENI, bound the  $\alpha_M$  I domain and the DDGW peptide blocked this binding. The DEDIIAEENI sequence with multiple adjacent negative charges is required for efficient binding of iC3b to  $\alpha_M\beta_2$  integrin. The binding 15 site of complement protein iC3b in the I domain has been mapped indicating a role for the positively charged amino acid residue K<sup>245</sup> for iC3b binding. Mutation of this residue does not affect the binding of the fibrinogen recognition peptide. This may account for the inability of the DDGW peptide to inhibit ICAM-1 and fibrinogen-mediated cell adhesion. These findings suggest that the K<sup>245</sup> residue is the positively 20 charged contact site for the DDGW peptide and the D/E-D/E-G/L-W motif as well.

The pepspot analysis indicates that a class of  $\beta_2$  integrin ligands contains an active D/E-D/E-G/L-W motif. These include the previously identified  $\alpha_M\beta_2$  ligands iC3b, thrombospondin-1, and the enzymes myeloperoxidase and catalase. In our experiments, 25 the peptides derived from several secreted MMPs, but not membrane-bound MT1-MMP, were also active. It is notable that the D/E-D/E-G/L-W motif is relatively conserved in the secreted members of the MMP family.

Finding of a dominant integrin-binding site in the catalytic domain of proMMP-9 was 30 unexpected, because previous studies suggested an essential role for another MMP domain, the hemopexin domain, in integrin binding. The hemopexin domains mediated MMP-2 binding to the  $\alpha_V\beta_3$  integrin and MMP-1 binding to the  $\alpha_2\beta_1$  integrin. The cleaved hemopexin domain of MMP-2 has also been shown to occur *in vivo* and to inhibit angiogenesis. Understandably, phage peptide display and pepspot techniques

have limitations and only linear peptide sequences can be analysed, not protein conformations. Thus, in the present study we cannot make conclusions of the function of separate MMP-9 domains in integrin binding and it remains to be seen whether cleavage products of MMP-9, if present *in vivo*, can act as  $\beta_2$  integrin ligands. Our 5 studies suggest that the peptide sequence from the catalytic domain is essential for the binding of full-length proMMP-9 to the  $\beta_2$  integrin, as the synthetic DDGW peptide could completely inhibit the integrin binding. It was important to use natural proMMP-9 because  $\alpha_M\beta_2$  is known to bind to denatured proteins and this may sometimes be the case for bacterially expressed proteins. Furthermore, we did not observe binding of an 10 active MMP-2 or MMP-9 to the integrin, although the DELW(T/S)LG sequence should remain unchanged in the active enzyme. We rather found that AMPA and trypsin, activators of proMMP-9, released MMP-9 from THP-1 cells, apparently affecting the integrin complex (unpublished observations of the present inventors). These results suggest that the proenzyme presents the integrin binding site more efficiently than the 15 active enzyme and the  $\beta_2$  integrin may even control the activation of the proenzyme.

In the three-dimensional structures of proMMP-2 and -9, the I domain binding site is located in the vicinity of the zinc-binding catalytic sequence HEFGHALGLDH between the catalytic domain and the fibronectin type II repeats. This location suggests a 20 mechanism for evading proMMP-9 inhibition by tissue inhibitors of MMPs (TIMPs) or  $\alpha_2$ -macroglobulin. In the absence of inhibitors, the cell surface-localized proMMP-9 would be readily susceptible for activation and substrate hydrolysis, which may also occur in the presence of intact propeptide. On the other hand, because the binding site of the I domain is located in the vicinity of the catalytic groove, it also suggests an 25 explanation for the blocking of MMP-9/ $\beta_2$  integrin interaction by the small molecule MMP inhibitors such as CTT and Inh1.

The activity of the DDGW peptide in the THP-1 cell migration assay suggests an important function for the integrin-progelatinase complex in leukocyte migration. 30 Obviously, we cannot exclude the possibility that the DDGW peptide blocks binding of other ligands than gelatinases and in this way inhibits the leukocyte migration. However, as the specific gelatinase inhibitor CTT also blocks the THP-1 cell migration, these results strongly suggest that the proMMP-9/ $\beta_2$  integrin complex is the main target for DDGW. Interestingly, the DDGW peptide blocked THP-1 cell migration although it

increased the level of proMMP-9 in the medium, which suggests that cell-surface bound rather than total MMP-9 level is a critical factor in cell migration.

DDGW and HFDDDE (see below) had potent activities *in vivo* in the mouse peritonitis 5 model, but it is unclear to what extent this was due to inhibition of proMMP-9 as both peptides can potentially inhibit other  $\beta_2$  integrin ligands as well. A subset of  $\beta_2$  integrin ligands have a DGDW-like sequence and these include, in addition to MMPs, at least complement iC3b and thrombospondin-1. The excellent *in vivo* activity of DGDW makes it a useful tool to study the components involved in leukocyte migration and the 10 peptide may be considered as a lead to develop anti-inflammatory compounds. Our results suggest that the proMMP-9/ $\alpha_M\beta_2$  complex may be part of the neutrophil's machinery for a specific  $\beta_2$  integrin-directed movement.

The present invention is thus directed to new peptide compounds, in specific to peptide 15 compounds comprising the tetrapeptide motif D/E-D/E-G/L-W. Said compounds can be used as pharmaceuticals, which inhibit leukocyte migration, as well as neutrophil migration. The inhibitory activity was shown both in *in vitro* and *in vivo* experiments. Consequently, the compounds can be used to treat leukaemia and prevent and treat inflammatory conditions.

20

One embodiment of the invention is the use of the compounds of the invention for the manufacture of a pharmaceutical composition for prophylaxis and treatment of conditions dependent on neutrophil migration.

25 Another embodiment of the invention is the use of the compounds of the invention for the manufacture of a pharmaceutical composition for the treatment of conditions dependent on leukocyte migration.

A further embodiment of the invention is a pharmaceutical composition comprising as 30 an active ingredient a compound of the invention, and a pharmaceutically acceptable carrier.

A still further embodiment of the invention is a method for therapeutic or prophylactic treatment of conditions dependent on leukocyte or neutrophil migration, comprising

administering to a mammal in need of such treatment a leukocyte or neutrophil migration inhibiting compound of the invention in an amount which is effective in inhibiting migration of leukocytes or neutrophils. Specific embodiments of the invention include methods for treatment of leukaemia and inflammatory conditions.

5

### Abbreviations used

APMA, aminophenylmercuric acetate;  
α<sub>M</sub>β<sub>2</sub>, CD11b/CD18, Mac-1 integrin;  
10 CTT, CTTHWGFTLC peptide;  
DDGW, ADGACILWMDDGWCGAAG peptide;  
GPA, glycolphorin A;  
GST, glutathione-S-transferase;  
HMEC, human microvascular endothelial cell;  
15 ICAM, intercellular adhesion molecule;  
Inh1, matrix metalloproteinase inhibitor 1;  
KKGW, ADGACILWMKKGWCGAAG peptide;  
LLG-C4, CPCFLLGCC peptide;  
MMP, matrix metalloproteinase;  
20 NGAL, neutrophil gelatinase-associated lipocalin;  
PMN, polymorphonuclear neutrophil;  
RGD-C4, ACDCRGDCFCG peptide;  
STT, STTHWGFTLC peptide;  
TAT-2: tumor-associated trypsinogen-2;  
25 W→A CTT, CTTHAGFTLC peptide.

### Description of the Drawings

#### FIG. 1A to 1E. Identification of an I domain binding site in progelatinase.

30 **FIG. 1A**, Phage display peptide sequences specifically bound to the α<sub>M</sub> I domain. The consensus motif is shown in bold. Peptides with the strongest binding (CILWMDDGW) and the highest similarity (CPEELWWLC) are aligned with human MMPs (accession numbers shown in parenthesis).

**FIG. 1B**, Phages bearing the CILWMDDGWC peptide or a control peptide were 35 allowed to bind to immobilized α<sub>M</sub> I domain-GST fusion protein (20 ng/well) in the

absence or presence of 15  $\mu$ M DDGW peptide or LLG-C4 peptide. Bound phages were detected using a monoclonal anti-M13 phage antibody. Mean absorbance of triplicate samples  $\pm$  SD is shown.

**FIG. 1C**,  $\alpha_L$ ,  $\alpha_M$ , or  $\alpha_X$  I domain-GST fusions were coated on microtiter wells as in **B**, 5 and the binding of CILWMDDGWC peptide bearing phage or a control phage was measured.

**FIG. 1D**, Peptides covering the complete sequence of proMMP-9 were synthesized as overlapping peptides on a pepspot membrane. The  $\alpha_M$  I domain (0.5  $\mu$ g/ml) was allowed to bind to the peptides followed by immunodetection using anti- $\alpha_M$  I domain 10 antibody LM2/1. The  $\alpha_M$  I domain-binding peptide 13 (arrow) is shown in boldface and the zinc binding catalytic sequence is underlined. The prodomain (Pro), catalytic domain containing the fibronectin type II repeats (Cat) and hemopexin domain (Pex) are marked to illustrate the domain structure of proMMP-9.

**FIG. 1E**, Alanine-mutated and truncated peptides were synthesized on a pepspot filter 15 and probed with the recombinant  $\alpha_M$  I domain (5  $\mu$ g/ml). Bound I domain was measured using mAb MEM-170 (5  $\mu$ g/ml) followed by HRP-conjugated anti-mouse secondary antibody and ECL detection. The binding was quantified by densitometric scanning. The bars show  $\alpha_M$  I domain binding to single peptide spots as arbitrary optical density 20 units/mm<sup>2</sup>. Similar results were obtained in three independent experiments.

20

**FIG. 2A to 2C. Binding of progelatinases to purified integrins and I domains.**

**FIG. 2A**, proMMP-9, proMMP-2 or their trypsin-activated forms, at the concentrations indicated, were allowed to bind to  $\alpha_M\beta_2$  integrin-coated wells. Appropriate MMP 25 antibodies were used to determine binding. The results in this and other figures are represented as the means  $\pm$  SD from triplicate wells.

**FIG. 2B**, binding of proMMP-9 (80 ng/well) was examined on microtiter wells coated with an integrin ( $\alpha_L\beta_2$ ,  $\alpha_M\beta_2$ ,  $\alpha_1\beta_1$ ,  $\alpha_3\beta_1$ ) or an I domain ( $\alpha_L$ ,  $\alpha_M$ ,  $\alpha_X$ ). The binding was determined using anti-MMP-9 antibody.

**FIG. 2C**, proMMP-2 (80 ng/well) was allowed to bind to  $\alpha_L\beta_2$ ,  $\alpha_M\beta_2$ ,  $\alpha_1\beta_1$  or the I 30 domains  $\alpha_L$ ,  $\alpha_M$  or  $\alpha_X$ . The binding was determined using an anti-MMP-2 antibody.

**FIG. 3A to 3D. Inhibitors of the proMMP-9 /  $\beta_2$  integrin complex.**

**FIG. 3A**,  $\alpha_M$  and  $\alpha_L$  I domain-GST fusions were immobilized on microtiter wells. ProMMP-9 (100 ng/well) was added in the presence or absence of various peptides (200

$\mu\text{M}$ ) or lovastatin (100  $\mu\text{M}$ ) in a buffer containing 0.5% BSA. ProMMP-9 binding was detected using a monoclonal antibody against MMP-9. The results are shown as percent binding compared to binding in the absence of inhibitors (100%) and no proMMP-9 added (0%).

5 **FIG. 3B**, DDGW peptide blocks proMMP-9 binding to  $\alpha_M$  in a dose dependent manner. The assay was done similarly as in (A), except various concentrations of peptides were added to compete for binding. All samples were assayed as triplicates and results shown are means  $\pm$  SD from a representative experiment.

10 **FIG. 3C**, proMMP-9 binding to  $\alpha_M\beta_2$  and  $\alpha_L\beta_2$  was examined in the absence and presence of EDTA (5 mM), MMP inhibitor-1 (100  $\mu\text{M}$ ), CTT (200  $\mu\text{M}$ ), STT (200  $\mu\text{M}$ ), MEM170 (40  $\mu\text{g/ml}$ ), or control TL3 antibody (40  $\mu\text{g/ml}$ ). The background of primary and secondary antibodies was measured by omitting proMMP-9 from the wells or by coating with ICAM-1.

15 **FIG. 3D**, proMMP-9 binding to purified  $\alpha_M$  and  $\alpha_L$  I domain GST fusion proteins or wild type GST was studied in the absence or presence of competitors as indicated. Control shows the background when proMMP-9 was omitted.

**FIG. 4A and 4B. Coprecipitation of progelatinase with  $\beta_2$  integrin.**

20 **FIG. 4A**,  $\alpha_M\beta_2$  integrin (3  $\mu\text{g}$ ) was incubated with a 500  $\mu\text{l}$  sample of HT1080 medium containing proMMP-9 and proMMP-2 in the absence or presence of CTT or STT (200  $\mu\text{M}$ ) for 2 h. The integrin was immunoprecipitated with the OKM10 antibody, and the immunoprecipitates were analyzed by gelatin zymography. In control experiments, integrin was omitted from the medium and ICAM-1 was added instead.

25 **FIG. 4B**, a 500  $\mu\text{l}$  sample of HT1080 medium containing proMMP-9 and proMMP-2 was incubated with the  $\alpha_M$  I domain GST (3  $\mu\text{g}$ ) or LLG-C4-GST control. ICAM-1, LM2/1, CTT, STT, or LLG-C4 were used as competitors. GST was pulled down with glutathione beads, and bound proteins were analyzed by zymography. The lane 1 in the figure insert: the proMMP-2 and proMMP-9 zymogens present in non-treated HT1080 medium, lane 2: lack of gelatinases pulled down with control LLG-C4-GST, lane 3: 30 proMMP-9 and proMMP-2 coprecipitated by  $\alpha_M$  I domain GST fusion protein.

**FIG. 5A and 5B. CTT peptide binds to both latent and active MMP-9.**

**FIG. 5A**, Binding of proMMP-9 or APMA-activated MMP-9 to CTT-GST was examined in the absence or presence of competitors CTT (100  $\mu\text{M}$ ), W $\rightarrow$ A mutant CTT

(100  $\mu$ M), and Inh1 (100  $\mu$ M). GST control was LLG-C4-GST. Binding was determined as in Figs. 2 and 3. The background in the absence of proMMP-9 is shown.

**FIG. 5B**, THP-1 cells were incubated in serum-free medium containing CTT, Inh1 or W $\rightarrow$ A CTT at 200  $\mu$ M concentration. Samples from the media were collected at the 5 time points indicated and analyzed by zymography (panels 1, 3, and 4) or Western blotting (panel 2).

**FIG. 6 A to 6 C. Progelatinases occur in complex with  $\alpha_M\beta_2$  and  $\alpha_L\beta_2$  in PDBu-activated THP-1 and Jurkat cells.**

10 **FIG. 6A**, THP-1 cell surface proteins were [ $^3$ H]-labelled using periodate-tritiated borohydride and analyzed by immunoprecipitation. CTT was used as a competitor (200  $\mu$ M). The immunoprecipitated samples were resolved on a 8-16% polyacrylamide gel, and the film was exposed for 3 days. Lanes 1-4 are from non-activated cells and lanes 6-10 from PDBu-activated cells. Lane 5 shows molecular weight markers.

15 **FIG. 6B**, lysates from PDBu-activated THP-1 cells were immunoprecipitated with integrin or MMP antibodies followed by Western blotting with  $\alpha_M$  (OKM10),  $\alpha_L$  (TS2/4) or MMP-9 antibodies. Preclearings of the cell lysates were done using  $\alpha_M$  (lane 6) and  $\alpha_L$  (lane 7) antibodies.

20 **FIG. 6C**, lysates from PDBu-activated Jurkat cells were subjected to immunoprecipitation followed by blotting with the  $\alpha_L$  (MEM83) and MMP-9 antibodies.

**FIG. 7A and 7B. PDBu-induced colocalization of  $\alpha_M\beta_2$  and proMMP-9 in THP-1 cells.** Cells were preincubated for 30 min at +37°C with 50 nM PDBu.

25 **FIG. 7A**, cells were treated with anti- $\alpha_M$  OKM10 and anti-MMP-9 antibodies followed by FITC-labeled (green fluorescence) and TRITC-labeled (red fluorescence) secondary antibodies. Yellow color indicates colocalization of  $\alpha_M\beta_2$  and proMMP-9. Bars, 8.5  $\mu$ m.

30 **FIG. 7B**, immunofluorescence staining shows intense colocalization of MMP-9 (polyclonal antibody) and  $\alpha_M\beta_2$  integrin (OKM-10) on the surface of PDBu-activated THP-1 cells at higher magnification as visualized by confocal microscopy (Bars, 2.5  $\mu$ m).

**FIG. 8A to 8D. The DDGW peptide supports THP-1 cell adhesion and induces proMMP-9 release, but does not block adhesion to the major  $\beta_2$  integrin ligands fibrinogen and ICAM-1.**

FIG. 8A, THP-1 cells were allowed to bind to immobilized, glutaraldehyde 5 polymerized peptides with or without phorbol ester activation (50 nM) and the adherent cells were quantitated by phosphatase assay. THP-1 cells were allowed to bind to immobilized fibrinogen (in FIG. 8B), or recombinant ICAM-1-Fc (in FIG. 8C), in the presence or absence of 200  $\mu$ M soluble peptides. All samples were assayed as triplicates and results show means  $\pm$  SD. Identical results were obtained in two other independent 10 experiments.

FIG. 8D, THP-1 cells were incubated in the presence or absence of peptides at 200  $\mu$ M concentration for 48 hours. Aliquots of conditioned medium were analyzed by gelatin zymography. Arrows show the 92 kDa proMMP-9 and 220 kDa proMMP-9 dimer.

15 **FIG. 9A to 9C. Peptide inhibition of THP-1 cell migration.** THP-1 cells were preincubated with the peptide as indicated at a 200  $\mu$ M concentration for 1 h at room temperature and applied to transwells in the absence (FIG. 9A), or presence (FIG. 9B), of LLG-C4-GST coating. Cells were allowed to migrate for 16 hours at +37°C. Cells migrated to the lower surface of the filter were stained and counted microscopically.  
20 **FIG. 9C, HT1080 fibrosarcoma cell migration** was similarly assayed in the absence of LLG-C4-coating. The bars show means  $\pm$  SD from triplicate wells.

**FIG. 10A to 10D.  $\alpha_M$ -I domain binding to recombinant MMP-9 domains.**

FIG. 10A, Schematic representation of MMP-9 and its recombinant forms produced in 25 *E. coli*.

FIG. 10B, ProMMP-9, its recombinant forms or BSA were coated on microtiter wells (80 $\mu$ g/well) and soluble GST- $\alpha_M$  I domain was allowed to bind at the concentrations indicated. The binding was determined by anti-GST monoclonal antibody. The results are means  $\pm$  SD from triplicate wells in this and other figures.

30 **FIG. 10C, Binding of proMMP-9 to the immobilized GST- $\alpha_M$  I domain** was studied in the presence of each peptide at the concentrations indicated. The binding was determined with the anti-MMP-9 antibody GE-213.

FIG. 10D, Binding of GST- $\alpha_M$  I domain to the immobilised proMMP-8, proMMP-9, ICAM-1, and fibrinogen was studied with ICAM-1, DDGW or KKGW (50  $\mu$ M) as

competitors. In control wells, GST was added instead of GST- $\alpha_M$  I domain. The experiment was repeated three times with similar results.

**FIG. 11A to 11D. Recognition of recombinant MMP-9 domains by  $\alpha_M\beta_2$  integrin-expressing cells.** The studied cells were PMNs (11A, 11B, 11C),  $\alpha_M\beta_2$  L-cell transfectants (11D), non-transfectants (11D), and LAD-1 cells (11D). PMNs were in resting state or stimulated with PMA (11A, 11C) or C5a or TNF $\alpha$  (11B) before the binding experiment to proMMP-9 or its domains. Cells were also pretreated with each peptide (50  $\mu$ M), antibody (20 $\mu$ g/ml) or the  $\alpha_M$  I domain as indicated. Unbound cells were removed by washing and the number of adherent cells was quantitated by a phosphatase assay. The experiment was repeated three times with similar results.

**FIG. 12A to 12 D. Blockage of PMN and THP-1 cell migration *in vitro* by gelatinase and  $\beta_2$  integrin inhibitors.** PMNs ( $1 \times 10^5$  in 100  $\mu$ l) were applied on the LLG-C4-GST or GST coated surface (12A) or HMEC monolayer (12B) in the absence or presence of peptides (200  $\mu$ M) or antibodies (20 $\mu$ g/ml) as indicated. PMNs were stimulated with 20 nM PMA (12A), HMECs with 50  $\mu$ M C5a or 10ng/ml TNF $\alpha$  or left untreated (12B). THP-1 cells ( $5 \times 10^4$  in 100 $\mu$ l) were stimulated with 50 nM PMA and applied on the coated surfaces together with each peptide (200  $\mu$ M) (12C). The cells migrated through transwell filters were stained and counted microscopically. All experiments were repeated at least twice. (12D) Phorbol ester-activated THP-1 cells ( $5 \times 10^4$  in 100 $\mu$ l) were incubated for 16 h at +37°C in the presence or absence of peptides as indicated. The conditioned medium was analyzed by gelatin zymography.

**FIG. 13A to 13D. Inhibition of neutrophil migration to an inflammatory tissue.** FIG. 13A, Mice were injected with thioglycolate or PBS intraperitoneally. The peptides were applied intravenously at the amounts indicated. After 3 h, the intraperitoneal leukocytes were harvested and counted. The results show means  $\pm$  SD of 2–4 mice in a group. (\*) indicates statistical significant difference ( $p < 0.001$ ). The experiment was repeated at least 3 times. The infiltrated neutrophils of mice treated with thioglycolate (13B) or PBS (13C) were stained with anti-MMP-9 and anti- $\alpha_M$  by incubating the cells with the antibodies for 3 h. Fluorescence was studied by confocal microscopy. Bars: 9.1  $\mu$ m and 4.8  $\mu$ m, respectively.

**FIG. 13D**, Gelatinolytic activity of the supernatants from the peritoneal cavities of mice collected as in (13A). Lanes 1-4: samples are from thioglycolate-treated mice; lane 5: a sample from PBS-treated mouse. DDGW, HFDDDE, and DFEDHD were injected intravenously at doses of 0.1, 0.2 and 0.2 mg per mouse. The arrows show proMMP-9 5 dimer, proMMP-9 and proMMP-2. The experiment was repeated three times with similar results.

The publications and other materials referred to or used herein to illuminate the background of the invention, and in particular, to provide additional details with respect 10 to its practice, are incorporated herein by reference. The invention will be described in more detail in the following Experimental Section.

## EXPERIMENTAL

15 **Antibodies and Reagents**

The antibodies MEM170 and LM2/1 were against the  $\alpha_M$  and the MEM-83 and TS2/4 antibodies against the  $\alpha_L$  integrin subunit (19, 20). The monoclonal antibody 7E4 (21) reacted with the common  $\beta_2$ -chain of the leukocyte integrins. The  $\alpha_M$  antibody OKM10 was obtained from the American Type Culture Collection, ATCC, Rockville, MD (22). 20 A monoclonal antibody against ICAM-5 (TL3) (23) was used as an antibody control. The monoclonal anti-MMP-9 antibody (GE-213) and anti-MMP-2 antibody (Ab-3) were obtained from Lab Vision Corporation (Fremont CA) and from Oncogene<sup>TM</sup> research products, respectively. Affinity purified rabbit anti-MMP-9 polyclonal antibodies were from the Borregaard laboratory (24). As monoclonal antibody controls, 25 we used a mouse IgG (Silenius, Hawthorn, Australia) and anti-glycophorin A (GPA) (ATCC). Anti-trypsinogen-2 (TAT-2) antibody was a rabbit polyclonal antibody control (32). The peroxidase-conjugated anti-GST mAb was from Santa Cruz Biotechnology. The rabbit anti-mouse horseradish peroxidase-conjugated secondary antibody was from Dakopatts a/s (Copenhagen, Denmark). Inh1 (2R-2-(4- 30 biphenylylsulfonyl)amino-N-hydroxy-propionamide) was purchased from Calbiochem, La Jolla, CA. Human recombinant ICAM-1 was obtained commercially by R&D systems (Minneapolis, MN). ICAM-1-Fc fusion protein was expressed in chinese hamster ovary cells and purified as described (14). The synthetic peptides CTT, STT, LLG-C4 and RGD-4C were obtained as previously described (14, 25). W→A CTT was

ordered from Neosystem, Strasbourg, France. ProMMP-2 and proMMP-9 were obtained commercially (Roche). In zymography, the commercial proMMP-9 showed the 92kDa monomer, 200 kDa homodimer, and 120 kDa NGAL complex bands. The integrins  $\alpha_1\beta_1$  and  $\alpha_3\beta_1$  were purchased from Chemicon International (Temecula, CA). Human plasma 5 fibrinogen and lovastatin were from Calbiochem.

### Phage display

Phage display selections were made using a pool of random peptides CX<sub>7-10</sub>C and X<sub>9-10</sub>, where C is a cysteine and X is any amino acid (14, 25). Briefly,  $\alpha_M$  I domain-GST or 10 GST fusion protein was immobilized on microtiter wells at 20  $\mu$ g/ml concentrations and the wells were blocked with BSA. The phage library pool was first subtracted on wells coated with GST and then unbound phage was transferred to  $\alpha_M$  I domain-GST-coated wells in 50 mM Hepes/5 mM CaCl<sub>2</sub>/1  $\mu$ M ZnCl<sub>2</sub>/150 mM NaCl/2% BSA (pH 7.5). After three rounds of subtraction and selection, individual phage clones were tested for 15 binding specificity and the sequences of the phage that specifically bound to the I domain were determined (14).

### Peptide biosynthesis and chemical synthesis

The phage peptides were initially prepared biosynthetically as intein fusions. The DNA 20 sequences encoding the peptides were PCR cloned from 1  $\mu$ l aliquots of the phage-containing bacterial colonies that were stored at -20 C. The forward primer was 5'-CCTTCTGCTCTCCAACGCCGACGGGGCT-3' and the reverse primer 5'-ACTTTAACCTGCAGTTACCCAGCGGGCCC-3'. The PCR conditions included initial denaturation at 94°C for 2 min followed by 30 cycles of 94°C 30 sec, 55°C 30 25 sec, and 72°C 30 sec. The PCR products were purified using QIAGEN Nucleotide removal kit. They were then digested with SapI and PstI restriction enzymes and ligated to a similarly digested and phosphatase treated pTWIN vector (New England Biolabs). Correct insertions were verified by DNA sequencing. Intein fusion proteins were produced in *E. coli* strain ER2566 and affinity purified on a chitin column essentially as 30 described (26). The peptide was cleaved on the column, eluted and finally purified by HPLC. Chemical peptide synthesis was done using Fmoc-chemistry as described and the sequences were verified by mass spectroscopy (26).

**Phage binding assay**

Phage ( $10^8$  infective particles/well) in 50 mM H<sub>2</sub>Epes/5 mM CaCl<sub>2</sub>/1 μM ZnCl<sub>2</sub>/0.5% BSA (pH 7.5) were added to microtiter wells coated with I domain-GST fusion or GST (20 ng/well). The phages were allowed to bind in the absence or presence of a 5 competitor peptide (15 μM) for one hour followed by washings with PBS containing 0.05% Tween 20. The bound phage was detected using 1:3000 dilution of a peroxidase-labelled monoclonal anti-phage antibody (Amersham Biosciences) and o-phenylenediamine dihydrochloride as a substrate. The reactions were stopped by addition of 10% H<sub>2</sub>SO<sub>4</sub> and the absorbance was read at 492 nm using a microplate reader.

**Pepspot**

The peptides were synthesized on cellulose membranes as described (27). The membrane was blocked with 3% BSA in TBS containing 0.05% Tween 20, and 15 incubated with 0.5-5 μg/ml α<sub>M</sub> I domain for 2 h at room temperature. The DDGW peptide was used as a competitor at a 50 μM concentration. Bound α<sub>M</sub> I domain was detected using the monoclonal antibody LM2/1 (1 μg/ml) or MEM-170 (5 μg/ml) and peroxidase-conjugated rabbit anti-mouse antibody (1:5000 dilution) followed by chemiluminescence detection.

20

**Cell culture**

The human HT1080 fibrosarcoma and THP-1 and Jurkat leukemic lines were obtained from ATCC and maintained as described previously (20, 25, 28). OCI/AML-3, derived from the primary blasts of an AML patient (29) was maintained in 10% FBS/RPMI 25 supplemented with L-glutamine, penicillin and streptomycin. Cell viability was assessed with a MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay according to the instructions of the manufacturer (Roche).

**Purification of integrins**

30 α<sub>L</sub>β<sub>2</sub> (CD11a/CD18, LFA-1), α<sub>M</sub>β<sub>2</sub> (CD11b/CD18, Mac-1) and α<sub>X</sub>β<sub>2</sub> (CD11c/CD18) integrins were purified from human blood buffy coat cell lysates by adsorption to the anti-CD11a (TS 2/4), anti-CD11b (MEM170), or anti-CD11c (3.9) antibodies linked to protein A-Sepharose CL 4B. The integrins were eluted at pH 11.5 in the presence of 2 mM MgCl<sub>2</sub>, and 1% n-octyl glucoside as described previously (28).

### Expression and purification of GST fusion proteins

The  $\alpha_L$ ,  $\alpha_M$ , and  $\alpha_X$  I domains were produced as GST fusion proteins in *E. coli* strains BL 21 or JM109 and purified by affinity chromatography on glutathione-coupled beads

5 GST containing CTT in the C-terminus was constructed using the protocols described for LLG-C4-GST (14) and glutathione-coupled beads were employed for purification. The purity of the GST-fusion proteins was confirmed by SDS-PAGE with Coomassie Blue staining and Western blot analysis. For pepspot analysis, GST was cleaved from the  $\alpha_M$  I domain with thrombin.

10

### Binding of MMPs to purified integrins

The purified I domains (GST- $\alpha_M$ , GST- $\alpha_L$ , GST- $\alpha_X$ ), or integrins ( $\alpha_M\beta_2$ ,  $\alpha_L\beta_2$ ,  $\alpha_X\beta_2$ ,

$\alpha_1\beta_1$ ) (1  $\mu$ g/well) were immobilized in 20 mM Tris, 150 mM NaCl, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, and 1 mM MnCl<sub>2</sub>, pH 7.4. The wells were washed with PBST (10 mM

15 phosphate, 140 mM NaCl, pH 7.4, containing 0.05% Tween20) and blocked with 3% BSA in PBST. ProMMP-2, proMMP-9, or the p-aminophenyl mercuric acetate (APMA)

or trypsin-activated forms (32) were incubated for 2 h at room temperature. In the inhibition experiments, CTT and Inh1 were first preincubated with the proMMPs for 30 minutes at room temperature. The wells were washed three times and incubated with

20 anti-MMP-9 (GE-213) or anti-MMP-2 (Ab-3) antibody at a 2  $\mu$ g/ml concentration in PBST for 1 h. Bound antibodies were detected using peroxidase-conjugated rabbit anti-mouse IgG (DAKO, Glostrup, Denmark) and o-phenylenediamine dihydrochloride as a substrate.

25 **Coprecipitation of  $\beta_2$  integrin and progelatinases**

Serum-free conditioned medium containing proMMP-2 and proMMP-9 was collected from human HT-1080 fibrosarcoma cells grown in the presence of 100 nM phorbol ester 4 $\beta$ -Phorbol 12,13-dibutyrate (PDBu) (Sigma-Aldrich, St. Louis, MO) overnight at +37°C. A 500  $\mu$ l volume of the supernatant was incubated with 100 ng of GST- $\alpha_M$ ,

30 GST- $\alpha_L$ , or GST- $\alpha_X$  I domain or  $\alpha_M\beta_2$  integrin for 3 h at 25°C. GST and GST-LLG-C4 were used to determine non-specific binding. CTT, STT, LLG-C4, and ICAM-1 were used as competitors at a 200  $\mu$ g/ml concentration, and the antibodies LM2/1 and TL3 at 40  $\mu$ g/ml. After an hour incubation at +4°C, complexes of I domain and gelatinases were pelleted with Glutathione Sepharose. Integrin complexes were captured by

incubating first with the OKM10 antibody for 3 h at +4°C and then with protein G Sepharose for 1 h. After centrifugation and washing, samples were analyzed by gelatin zymography on 8% SDS-polyacrylamide gels containing 0.2 % gelatin (32).

5 **Effect of peptides on proMMP-9 release from cells**

THP-1 cells (40 000/100 µl) were incubated in serum-free RPMI medium for 48 h in the absence or presence of 200 µM peptide as described in the text. Aliquots of the conditioned media were analyzed by gelatin zymography.

10 **Interaction between CTT and proMMP-9**

CTT-GST and GST control (5 µg/well) were coated overnight on 96-well microtiter plates in 50 µl TBS followed by blocking of the wells by BSA. proMMP-9 or APMA-activated form (80 ng/well) was incubated in the absence or presence of competitors for 2 h in 50 µM Hepes buffer containing 1 % BSA, 5 mM CaCl<sub>2</sub>, and 1 µM ZnCl<sub>2</sub> (pH 15 7.5). After washing, bound MMP-9 was determined with anti-MMP-9 and HRP-conjugated anti-mouse IgG as described above. To examine complexing of CTT with proMMP-9 in cell culture, THP-1 cells were activated with PDBu for 30 min and then incubated with CTT, W→A CTT, or Inh1 (each 200 µM) at +37°C in serum-free medium. Samples were taken from the media at 0, 1, 2, 3, 4, and 5 h time points and 20 analyzed by zymography and Western blotting with polyclonal anti-MMP-9 antibodies. Experiments with HT-1080 cells were performed similarly except that the medium samples were collected after 6 h.

**Cell surface labelling, immunoprecipitation and immunoblotting**

25 Non-activated or PDBu-activated THP-1 cells (1 x 10<sup>7</sup>) were subjected to surface labelling using periodate tritiated sodium borohydride (33). The [<sup>3</sup>H]-labelled cells were lysed with 1 % (v/v) Triton X-100 in PBS, clarified by centrifugation and precleared with protein G-Sepharose. The lysate was immunoprecipitated with polyclonal anti-MMP-9, α<sub>M</sub> (OKM-10) or β<sub>2</sub> (7E4) antibodies. After one-hour incubation at +4°C 30 together with protein G-Sepharose, immunocomplexes were pelleted, washed and resolved on 8-16% SDS-PAGE gels (Bio-Rad, Hercules CA). The gels were treated with an enhancer (Amplify, Amersham Biosciences), dried and exposed. Non-labelled THP-1 cells (1 x 10<sup>7</sup>) were similarly lysed and immunoprecipitated as above. The samples were resolved on 4-15% SDS-PAGE gels and transferred to nitrocellulose

membranes. Immunodetection was performed with  $\alpha_M$  (MEM170) antibody (10  $\mu$ g/ml) followed by peroxidase-conjugated anti-mouse IgG and chemiluminescence detection (Amersham Biosciences). The membranes were stripped of bound antibodies and reprobed with monoclonal  $\alpha_L$  chain (TS2/4) or polyclonal anti-MMP-9 antibodies.

5

### Immunofluorescence

Immunofluorescence was performed on resting cells or the cells activated with PDBu for 30 min. A portion of the cells was treated with ICAM-1 or CTT to block  $\beta_2$  integrins or gelatinases, respectively. Cells were bound to poly-L-Lysine coated cover slips, fixed 10 with methanol for 10 min at -20°C or with 4% paraformaldehyde for 15 min at +4°C, and permeabilized with 0.1% Triton X-100 in PBS at room temperature for 10 min followed by several washings. The cover slips were incubated with rabbit anti-MMP-9 polyclonal and mouse anti- $\alpha_M$  (OKM-10) antibodies diluted 1:500. After washing with PBS, the secondary antibodies, rhodamine (TRITC)-conjugated porcine anti-rabbit or 15 FITC-conjugated goat anti-mouse (Fab')<sub>2</sub> (Dakopatts a/s, Copenhagen, Denmark) were incubated at a 1:1000 dilution for 30 min at room temperature. The samples were mounted with moviol, incubated in the dark for 2 days, and examined by a confocal microscope (Leica multi band confocal imagine spectrophotometer) at a 400x magnification or a fluorescence microscope (Olympus Provis 70) at a 60x 20 magnification.

### Neutrophil preparations and cell lines

PMNs were isolated from peripheral blood anticoagulated in acid-citrate dextrose. Erythrocytes were sedimented by centrifugation on 2% Dextran T-500, and the 25 leukocyte-rich supernatant was pelleted, resuspended in saline and centrifuged on a Lymphoprep (Nyegaard, Oslo, Norway) at 400g for 30 minutes to separate polymorphonuclear cells from platelets and mononuclear cells (16). PMN purity was >95% with typically <2% eosinophils. Cell viability was measured using an MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium) bromide assay as instructed by the 30 manufacturer (Roche).

Human microvascular endothelial cells (HMEC-1) (17), kindly provided by S. Mustjoki (Haartman Institute, University of Helsinki), were grown in RPMI 1640 in the presence of 10% FBS containing 2 mM glutamine, 100 IU/ml penicillin and 100  $\mu$ g/ml

streptomycin. Human monocytic THP-1 cells were maintained as described (14, 25). Leukocyte adhesion deficiency type-1 (LAD-1) cells, wild type and  $\alpha_M\beta_2$ -transfected L929 mouse fibroblastic cells were generous gifts from Dr. Jean-Pierre Cartron (INSERM, Paris, France). These cells were maintained as described previously (18) and 5 the  $\alpha_M\beta_2$  expression was examined by fluorescence-activated cell sorting (FACS, Becton Dickinson, San Jose, CA).

### Cell adhesion and migration

Fibrinogen and ICAM-1-Fc were coated at 40  $\mu$ g/ml in TBS at +4°C. Peptides (2 10  $\mu$ g/well) were coated in TBS containing 0.25% glutaraldehyde at +37°C. The wells were blocked with 1% BSA in PBS. THP-1 cells (50 000/well) with or without PDBu activation were added in 0.1% BSA-RPMI medium in the presence or absence of 200  $\mu$ M peptides or monoclonal antibodies at 50  $\mu$ g/ml. After 30-35 minutes the wells were 15 washed with PBS to remove non-adherent cells and the adhesive cells were quantitated by a phosphatase assay. The cell migration assay was conducted using transwell migration chambers (8  $\mu$ m pore size, Costar) in serum-containing medium as described (14). Briefly, the membranes were coated on the upper and lower surface with 40  $\mu$ g/ml GST, LLG-C4-GST, or left uncoated. The wells were blocked with 10% serum-containing medium for 2 h. THP-1 cells (50 000/100  $\mu$ l) or HT1080 (20 000/100  $\mu$ l) 20 were preincubated with the peptides for 1 h before transfer to the upper chamber. The lower chamber contained 500  $\mu$ l of the medium without the peptides. The cells were allowed to migrate to the lower surface of the membrane for 16 h and then stained with crystal violet and counted.

25 MMP-9 proteins (200 nM in PBS) were coated at +4°C for 16 h and the microtiter wells were blocked with 3 % BSA in PBS for 1 h at room temperature. The  $\alpha_M\beta_2$ -integrin L-cell transfectants and PMNs ( $1 \times 10^5$  cells/well) were suspended in RPMI medium supplemented with 2mM MgCl<sub>2</sub> and 0.1% BSA and activated with PMA (20nM) for 20 min, or with C5a (50nM) or TNF- $\alpha$  (10nM) for 4h at +37°C. The L926 wild type and 30 LAD-1 cells were used as controls. The cells were treated with the indicated antibody (20  $\mu$ g/ml) or peptide (50  $\mu$ M) at +37°C for 30 min, washed twice with serum-free medium and incubated in the microtiter wells at +37°C for 30 min. The wells were washed with PBS, and the number of adherent cells was quantitated by a phosphatase assay (14).

Cell migration was conducted using Costar 24-transwell migration chambers with a 3  $\mu$ m pore size for PMNs and 8  $\mu$ m for THP-1 cells. To study  $\beta_2$  integrin-directed migration, the chamber membrane was coated on both sides with LLG-C4-GST integrin ligand (40 $\mu$ g/ml) or GST as a control and blocked with 10% serum-containing medium as described above. To study transendothelial migration, confluent HMECs ( $4 \times 10^5$  cells/well) were grown on the upper side of the gelatin-coated membrane for 5 days. Culture medium was changed after 3 days. After washing the HMEC layers twice with PBS, chemotactic activation was carried out by adding C5a (50nM), TNF- $\alpha$  (10ng/ml), 10 or medium alone to the lower compartment at +37°C for 4 h. Cultures were then washed again twice to remove all agents. PMNs or THP-1 cells were preincubated with the peptide inhibitor or antibody studied for 1 h before transfer to the upper compartment (1 $\times 10^5$  cells in 100 $\mu$ l RPMI/0.1 % BSA or the complete 10 % FCS-containing medium). PMNs were allowed to migrate for 2 h through the LLG-C4-GST coated membrane and 15 for 30 min through the HMEC monolayer. THP-1 cells were allowed to migrate for 16 h. The non-migrated cells were removed from the upper surface by a cotton swab and the cells that had traversed the filters were stained with crystal violet and counted.

### Mouse inflammation model

20 Balb/c mice at the age of 31-32 weeks were injected intraperitoneally with 3% (w/v) thioglycolate in sterile saline (36). Peptides (5-500 $\mu$ g in 100 $\mu$ l) were introduced intravenously through the tail vein. Animals were euthanized after 3 h and the peritoneal cells were harvested by injecting 10 ml of sterile PBS through the peritoneal wall. Red blood cells present in the lavage fluid were removed by hypotonic lysis. Cells 25 were centrifuged and resuspended in 1 ml of sterile 0.25% BSA/Krebs-Ringer. The supernatants were also collected and analysed by gelatin zymography. The number of neutrophils was determined following staining with 0.1% crystal violet and using a light microscope equipped with a  $\times 100$  objective. For immunofluorescence staining, cells were allowed to bind to poly-L-lysine coated cover slips, fixed with 2.5% 30 paraformaldehyde in PBS at +4°C for 30 min followed by several washings. The Fc receptors were blocked in the presence of 20% of rabbit serum and 3% BSA in PBS. The cells were then incubated with anti-MMP-9 polyclonal and  $\alpha_M$  monoclonal (MCA74) antibodies for 30 min. After washing with PBS, the secondary antibodies, rhodamine (TRITC)-conjugated anti-rabbit or FITC-conjugated anti-rat (Fab')<sub>2</sub> were

incubated for another 30 min. The samples were examined with a confocal microscope. The animal studies were approved by an ethical committee of Helsinki University.

## RESULTS

5

### **Identification of the $\alpha_M$ I domain-binding peptide motif D/E-/D/E-G/L-W**

Using phage peptide display libraries, we selected peptides that interact with the  $\alpha_M$  I domain. GST-binding phage were first eliminated on GST-coated wells and the unbound phage preparations were incubated on  $\alpha_M$  I domain GST fusion protein-coated 10 wells. The  $\alpha_M$  I domain-binding phage were enriched by three rounds of panning and the peptide sequences were determined. With the exception of one linear peptide, the peptides were derived from the cyclic CX<sub>7</sub>C and CX<sub>8</sub>C libraries. The I domain-binding sequences showed only one conserved motif, a somewhat unexpected finding in terms of the known ligand binding promiscuity of the I domain. The bound peptides contained 15 two consecutive negatively charged amino acids, i.e. glutamatic and/or aspartic acids, followed by glycine and tryptophan residues (Fig. 1A). The consensus D/E-/D/E-G/L-W determined by this approach was clearly different from LLG-C4 and other  $\beta_2$  integrin-binding peptides reported so far.

20 We first prepared the phage display peptides as intein fusion proteins, from which the peptides were cleaved. This allowed us to rapidly test the peptide solubility and the binding specificity before large-scale chemical peptide synthesis. The peptides were cloned using oligonucleotide primers that amplify the peptide library insert from the phage vector. Consequently, all the peptides prepared contain the vector-derived 25 sequences ADGA and GAAG in the NH<sub>2</sub>- and COOH- termini, respectively. Phage binding experiments using soluble peptides as competitors indicated that the peptides bearing the two adjacent negative charges bound to a common site (not shown). We chose the peptide ADGA-CILWMDDGWC-GAAG (DDGW) for further experiments as this peptide showed strong binding and was highly soluble in aqueous buffers 30 (soluble in 50 mM NaOH at >10 mM concentrations). The peptide was also prepared by chemical synthesis. The phage bearing the DGDW sequence avidly bound to the  $\alpha_M$  I domain and this was readily inhibited by low concentrations of the DGDW peptide, but only marginally affected by the LLG-C4 peptide, indicating different binding sites for DGDW and LLG-C4 (Fig. 1B). Control phage bearing other peptide sequences did not

bind. The DDGW-bearing phage also showed also specific binding to the  $\alpha_L$  I domain that was inhibitable by DDGW but the interaction was weaker than with the  $\alpha_M$  I domain (Fig. 1C and data not shown). No binding was observed with the  $\alpha_X$  I domain or GST used as a control (Fig. 1C).

5

**Characterization of DELW sequence on the catalytic domain of gelatinases that mediates interaction with the  $\beta_2$  integrin I domains**

We searched protein databases for matches to the novel D/E-/D/E-G/L-W motif. One of the phage library-derived peptides, CPEELWWLC, was highly similar to the 10 DELW(S/T)LG sequence present on the catalytic domain of MMP-2 and MMP-9 gelatinases (Fig. 1A). DELW-like sequences with double negative charges are also present in other secreted MMPs but not in the membrane-type MMPs such as MMP-14.

15 No MMP has been reported to bind to the leukocyte  $\beta_2$  integrins. We therefore set out to study whether MMP-9 in particular could be a ligand of the  $\beta_2$  integrins as MMP-9 gelatinase is the major leukocyte MMP and is induced during  $\beta_2$  integrin activation. As a first step, we synthesized the whole proMMP-9 sequence as overlapping 20-mer peptides on a pepspot membrane. Binding assays with the  $\alpha_M$  I domain revealed a single active peptide that located to the MMP-9 catalytic domain (Fig. 1D). No binding was 20 observed, when the I domain was omitted and the membrane was probed with antibodies only. The sequence of the I domain-binding peptide was QGDAHFDDDELWSLGKGVVV and it contained the binding motif identified by phage display (Fig. 1D).

25 The active MMP-9 peptide contained four consecutive amino acids with negative charges, DDDE. To study the importance of these residues, the aspartic and glutamic acid residues that were closest to the tryptophan were replaced by alanines. At the same time the peptide length was shortened to 15-mer. The alanine mutagenesis significantly abrogated I domain binding on the pepspot filter; the OD value dropped from 2010 to 30 476 (Table I). To study whether the negatively charged peptide from other MMPs is also active, we synthesized the corresponding 15-mers and the double alanine mutations. Sequences from MMP-1, 2, 3, 7, 8, 9 and 13, but not the membrane-anchored MMP-14 (MT1-MMP), could bind the  $\alpha_M$  I domain. Alanine mutations always decreased the binding.

We did similar pepspot analysis for some of the known I domain ligands, which contain D/E-/D/E-G/L-W like sequences. Peptides derived from myeloperoxidase, catalase, thrombospondin-1 and complement protein iC3b strongly bound the I domain in this assay and the double alanine mutation caused a loss of binding (see Table I). Of the three iC3b peptide permutations tested, ARSNLDEDIIAEENI was the active one. The acidic residues were followed by a hydrophobic isoleucine cluster in this peptide. The soluble DDGW peptide efficiently inhibited the binding of this peptide to the I domain. Weaker I domain binding was observed with one complement factor H-derived peptide and one fibronectin-derived peptide. Peptides derived from ICAMs-1, 2 and 3, neutrophil inhibitory factor, Cyr61, fibrinogen, GP1b, factor X, or E-selectin lacked activity.

Alanine scanning mutagenesis of the DDGW peptide with the pepspot system similarly indicated the importance of the glutamic acid residues for I domain binding (Fig. 1E). Alanine mutations of the glycine or either one of the tryptophan residues also inactivated the peptide. Mutations of the isoleucine, leucine or methionine residues were tolerated. Deletion of the ADGA sequence from the N terminus had no effect on I domain binding, but removal of the C terminal GAAG sequence abolished the binding. As the peptides were immobilized via the C terminus on the filter, a sufficient linker sequence such as GAAG seemed important. We also tested a series of truncated cyclic peptides to identify the shortest active sequence. This analysis showed that ADGA-CEDGWC-GAAG, but not ADGA-CDDGWC-GAAG was the minimal peptide that supported  $\alpha_M$  I domain binding. The longer side chain of glutamate compared to aspartate is probably required to bring the negatively charged carboxyl group in the correct position for I domain binding.

#### **Progelatinases bind to purified $\alpha_M\beta_2$ and $\alpha_L\beta_2$ integrins and their I domains**

We next used a microtiter well-based sandwich assay to study gelatinase binding to purified integrins. Progelatinases bound in a concentration-dependent manner to coated  $\alpha_M\beta_2$  integrin (Fig. 2A). Curiously, MMP-2 and MMP-9 lost the integrin binding ability after activation by trypsin or APMA. The binding of proMMP-2 and proMMP-9 was observed with both  $\alpha_M\beta_2$  and  $\alpha_L\beta_2$  integrins and their corresponding I domains (Fig. 2B and 2C). No binding was detected on the  $\alpha_X$  I domain or the  $\alpha_1\beta_1$  and  $\alpha_3\beta_1$  integrins.

The DDGW peptide was an efficient inhibitor and it inhibited proMMP-9 binding to the  $\alpha_M$  I domain with an IC<sub>50</sub> of 20  $\mu$ M (Fig. 3A and 3B). To demonstrate that the negative charges of aspartic acids are essential for the peptide activity, the peptide 5 ADGACILWMKKGWCGAAG (KKGW) containing lysines in place of aspartic acids was prepared. As expected, the KKGW peptide was inactive and did not compete with proMMP-9 binding. We were also interested in testing lovastatin, as its binding site in the  $\alpha_L$  I domain is known (34, 35). Lovastatin was not able to compete with proMMP-9 even at a high concentration.

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ProMMP-9 bound like a true integrin ligand, as the cation chelator EDTA (5 mM) nearly completely prevented the binding (Fig. 3C and 3D). For background measurement in the sandwich assay, we used antibodies alone (control), or coating with ICAM-1 or wild type GST. The gelatinase-binding peptide CTT (200  $\mu$ M) inhibited 15 proMMP-9-integrin interaction with the same efficiency as EDTA did. The control peptides STTHWGFTLS (STT) and CTTHAGFTLC (W→A CTT), which lack gelatinase inhibitory activity (26), were without effect. A non-peptide chemical MMP inhibitor (Inh1) also prevented proMMP-9 binding. As EDTA inhibits both the gelatinase and the integrin, we used integrin blocking antibodies and ligand peptides to 20 demonstrate the specific binding activity of  $\beta_2$  integrin. The known ligand-binding blocking antibodies MEM 170, MEM 83, and LM2/1 inhibited proMMP-9 binding. A control antibody TL3 had no effect. The I domain binding peptide LLG-C4 showed a partial inhibitory effect. RGD-4C, a ligand of  $\alpha_V$  integrins, served as control peptide and had no effect on proMMP-9 binding. The purity of the integrins was typically more than 25 90% and that of I domains 95%, making it unlikely that progelatinases would bind to impurities in the preparations.

Progelatinase-integrin complexes were also obtained by co-precipitation experiments using HT1080 conditioned medium as a source of proMMP-9 and proMMP-2, which 30 were analyzed by zymography. The progelatinases co-precipitated with  $\alpha_M\beta_2$  integrin or  $\alpha_M$  I domain GST fusion protein when these were used as a bait. The integrin added to the medium was immunoprecipitated with the  $\alpha_M$  antibody OKM10 (Fig. 4A). The  $\alpha_M$  I domain GST protein was pulled down with glutathione-beads (Fig. 4B). CTT but not STT had an inhibitory effect. Inhibition of the I domain by LM2/1, ICAM-1 or

LLG-C4 also affected the pull-down of progelatinases. GST control did not coprecipitate the gelatinases. No active forms of gelatinases were found to coprecipitate with  $\alpha_M\beta_2$  or the I domain, when APMA-treated HT-1080 medium or APMA-activated MMP-9 was used (not shown).

5

As the gelatinase inhibitors CTT and Inh1 prevented the binding of proMMP-9 to the integrin, it can be anticipated that CTT and Inh1 avidly bind to proMMP-9. To gain more insight into this, we examined binding of proMMP-9 to immobilized CTT peptide. ProMMP-9 specifically bound to the CTT-GST fusion protein (Fig. 5A) but not to LLG-C4-GST. CTT and Inh1 at 100  $\mu$ M concentrations effectively competed in binding but W→A CTT did not. The proMMP-9 preparation did not contain detectable amounts of active MMP-9 on zymography analysis, and after proMMP-9 activation with APMA, the CTT-GST binding increased. CTT and Inh1 could also bind to proMMP-9 secreted into the medium of PDBu-activated THP-1 leukemic cells (Fig. 5B) or HT1080 fibrosarcoma cells (not shown). A time-dependent reduction in the gelatinolytic activity of proMMP-9 was observed with CTT (panel 1) and Inh1 (panel 3), but not with the W→A CTT peptide (panel 4). Western blot analysis indicated that CTT does not decrease the secretion of proMMP-9 by the cells (panel 2). Furthermore, the CTT complex was reversible and disappeared after repeated freezing and thawing of the samples.

#### **Demonstration of a cell-surface complex between progelatinases and $\beta_2$ integrins**

To study whether the progelatinases occur in a complex with the  $\beta_2$  integrins on the leukocyte surface, we performed immunoprecipitation and co-localization studies. First, we examined THP-1 monocytic leukemia cells in the resting state and after induction by PDBu, which mimics leukocyte activation *in vivo*. THP-1 cell-stimulation with PDBu led to upregulation of MMP-9 (data not shown). The cell surface glycoproteins of THP-1 cells were labelled with tritium [ $^3$ H] followed by immunoprecipitation with  $\beta_2$  integrin and MMP-9 antibodies. In the PDBu-activated cells, the  $\alpha_M$  chain antibody OKM10 and  $\beta_2$  chain antibody 7E4 immunoprecipitated two [ $^3$ H]-labelled proteins corresponding to the integrin  $\alpha_M$  chain (165 kDa) and  $\beta_2$  chain (95 kDa) (Fig. 6A, lanes 9-10). Importantly, polyclonal MMP-9 antibodies immunoprecipitated the same two integrin chains (lane 7). In non-activated cells, essentially no co-precipitation of  $\alpha_M$  and  $\beta_2$  were observed with MMP-9 antibodies, although the  $\alpha_M$  and  $\beta_2$  chains were present. The co-

precipitation of the integrin chains by MMP-9 antibodies was prevented by the CTT peptide (lane 8). The control antibody (TL3) did not precipitate any proteins.

With the [<sup>3</sup>H]-labelled cells, we did not observe any band corresponding to proMMP-9, 5 perhaps because the carbohydrates of proMMP-9 are poorly labelled. We therefore analyzed the PDBu-activated THP-1 cells by Western blotting (Fig. 6B). ProMMP-9 was readily immunoprecipitated with antibodies against MMP-9,  $\alpha_M$  or  $\alpha_L$ , but not by the control antibody. MMP-9 antibodies in turn were able to immunoprecipitate the  $\alpha_M$  but not the  $\alpha_L$  chain. MMP-2 antibodies similarly co-precipitated  $\alpha_M$  but not  $\alpha_L$ . When 10 the cell lysate was precleared with the  $\alpha_M$  antibody OKM-10, the amount of immunoprecipitated  $\alpha_M$  and proMMP-9 clearly decreased (lane 6). Preclearing with the  $\alpha_L$  antibody TS2/4 did not significantly remove  $\alpha_M$  or proMMP-9, but abolished the  $\alpha_L$  precipitation (lane 7).

15 As THP-1 cells do not express high amounts of the  $\alpha_L$  chain (20), we examined the Jurkat T cell line, which expresses more  $\alpha_L$  than  $\alpha_M$  (28). We observed a significant immunoprecipitation of  $\alpha_L$  by MMP-9 and MMP-2 antibodies after PDBu activation (Fig. 6C). Furthermore, the  $\alpha_L$  antibody co-precipitated more proMMP-9 in comparison to the  $\alpha_M$  antibody. No proMMP-9 co-precipitated with MMP-2 antibodies in Jurkat or 20 THP-1 cells.

ProMMP-9 and  $\alpha_M\beta_2$  were found to co-localize on the cell surface following PDBu-activation of THP-1 cells as studied by fluorescence and confocal microscopy (Figs. 7A and 7B, respectively). Using a higher magnification, colocalization was primarily seen 25 in cell surface clusters (Fig. 7B), and to a lesser extend on areas where cells contacted each other (not shown). We believe that the MMP-9 co-localizing with  $\alpha_M\beta_2$  is the proMMP-9, as the activated MMP-9 did not bind to  $\alpha_M\beta_2$ . Without PDBu activation, there was hardly any co-localization of proMMP-9 and  $\alpha_M\beta_2$ . The secondary antibodies did not stain the cells when the primary antibodies were omitted (data not shown). 30 When the cells were preincubated with the CTT peptide or recombinant soluble ICAM-1 to block proMMP-9 or  $\alpha_M\beta_2$ , the cell surface clusters did not form and the proMMP-9- $\alpha_M\beta_2$  colocalization was not observed (not shown). ProMMP-9- $\alpha_M\beta_2$  colocalization was also observed on Jurkat cells following phorbol ester stimulation (not shown).

**Blocking the progelatinase/β<sub>2</sub> integrin complex with DDGW releases cell-bound proMMP-9 and inhibits cell migration but not adhesion**

As the DDGW peptide is an integrin ligand, one of the questions was whether it can support adhesion of leukocytes. We studied adhesion of human myelomonocytic THP-1 cells on immobilized glutaraldehyde-polymerized peptide. Phorbol-ester activated cells efficiently bound to the DDGW peptide, whereas there was no binding in the absence of cell activation (Fig. 8A). As a positive control, the recombinant intein-produced ADGA-CPCFLLGCC-GAAG peptide supported adhesion, but unlike the DDGW peptide, it also supported adhesion in the absence of integrin activation. The acute myeloid leukemic cell line OCI/AML-3 also avidly adhered to DDGW, whereas human fibrosarcoma HT1080 cells which lack β<sub>2</sub> integrins did not (not shown). As THP-1 cells were able to adhere on DDGW, we next studied the effect of the peptide on β<sub>2</sub> integrin dependent adhesion to fibrinogen and ICAM-1. Interestingly, DDGW did not block cell adhesion to fibrinogen, whereas the LLG-C4 peptide blocked the adhesion as previously reported (Fig. 8B). Similarly, DDGW did not block the binding of recombinant α<sub>M</sub> I domain to immobilized fibrinogen (not shown). DDGW did not either block cell adhesion on ICAM-1-Fc fusion protein. As a control, the blocking antibody 7E4 against β<sub>2</sub> integrins prevented the ICAM-1 binding, indicating that the THP-1 cells bound in a β<sub>2</sub> integrin dependent manner (Fig. 8C). We also found no blocking effect of DDGW on THP-1 adhesion to LLG-C4-GST fusion protein (not shown).

The second question raised by these studies was whether the DDGW peptide can release cell-bound proMMP-9. When THP-1 cells were cultured for 48 h in the presence of DDGW, an increase of proMMP-9 level was observed in the conditioned medium as studied by gelatin zymography (Fig. 8D). The peptide increased both monomeric and dimeric proMMP-9 in the culture medium. In contrast, CTT slightly decreased or inhibited active proMMP-9. KKGGW and W→A CTT had no effect.

We also studied the role of the progelatinase/β<sub>2</sub> integrin complex in leukocyte migration using a transwell assay in which leukocyte migration can be adjusted by the choice of coated matrix or ligand protein. We tested that the CTT, LLG and DDGW peptides are not toxic to the THP-1 cells in a 48 h time frame at >200 μM concentrations using an MTT assay. Using transwells coated with 10% serum in cell culture medium, we first

studied the effect of peptides on the basal migration of THP-1 cells in the absence of any stimulus by phorbol ester or an adhesive matrix. Under such conditions, CTT, LLG-C4 or the gelatinase inhibitor Inh1 at a 200  $\mu$ M concentration had no effect on THP-1 migration indicating no active involvement of gelatinases or  $\beta_2$  integrins (Fig. 9A). We 5 have previously shown that when the transwells are coated with LLG-C4-GST fusion protein, THP-1 cells adhere and migrate in a  $\beta_2$  integrin dependent manner (14). Thus, transwells were coated with LLG-C4-GST fusion protein or GST alone. Both the DDGW and CTT peptide, but not KKGW, inhibited the migration of THP-1 cells on the 10 LLG-C4-GST substratum (Fig. 9B). The soluble LLG-C4 peptide also blocked the migration. In the presence of GST coating, cell migration was negligible. To verify that the effect of DDGW peptide was  $\beta_2$  integrin dependent, HT1080 fibrosarcoma cells lacking these integrins were allowed to migrate in the presence of CTT, DDGW, KKGW or LLG-C4. Of these peptides, only CTT was capable of inhibiting cell 15 migration (Fig. 9C).

15

#### **Peptide inhibitors of the proMMP-9/ $\alpha_M\beta_2$ complex prevent neutrophil migration**

As shown above, pepspot analysis located the integrin interactive site of proMMP-9 to a 20-amino acid long sequence present in the catalytic domain, QGDAHFDDDE-LWSLGKGVVV. Further screening by the pepspot system has indicated that sufficient 20 integrin binding activity is achieved by truncating this sequence to a hexapeptide, HFDDDE (data not shown). To confirm that such a short sequence is the bioactive site of proMMP-9, we first prepared bacterially expressed recombinant domains of MMP-9 (Fig. 10A).  $\Delta$ MMP-9 is composed of the prodomain (Pro) and the catalytic domain but lacks the hemopexin domain. The fibronectin type II repeats (FnII) were also produced 25 as a separate recombinant protein as this is an important substrate-binding region. The procatalytic domain construct  $\Delta$ MMP-9 bound the  $\alpha_M$  I domain nearly as efficiently as the wild type proMMP-9 (Fig. 10B). FnII protein almost lacked activity. The HFDDDE peptide identified by the solid-phase pepspot analysis was highly active when made by peptide synthesis and inhibited proMMP-9 binding to the  $\alpha_M$  I domain with an IC<sub>50</sub> of 30 20  $\mu$ M (Fig. 10C). The bound proMMP-9 was determined with the GE-213 antibody, which recognizes an epitope of the FnII domain (data not shown). A scrambled peptide DFEDHD with the same set of negatively charged amino acids was inactive. HFDDDE was equally potent as DDGW, the  $\alpha_M$  I domain-binding peptide discovered by phage display and described above. KKGW, the control peptide for DDGW, was without

effect. As the HFDDDE sequence is highly conserved in the members of the MMP family, we also examined the  $\alpha_M$  I domain binding to human neutrophil collagenase, MMP-8. I domain showed a similar DDGW-inhibitable binding to proMMP-8 as to proMMP-9 (Fig. 10D). ICAM-1 and fibrinogen did not compete with either proMMP, 5 implying different binding sites for the matrix proteins and proMMPs in the I domain.

After integrin activation, PMNs exhibited an ability to adhere on proMMP-9. PMA-stimulated PMNs bound to microtiter well-coated  $\Delta$ MMP-9 nearly as strongly as to proMMP-9 (Fig. 11A). Stimulation of PMNs with C5a or TNF- $\alpha$  gave similar results 10 PMN adherence increasing by 3-fold (Fig. 11B). The FnII domain did not support PMN adhesion. PMN adherence was inhibited by HFDDDE (50 $\mu$ M), DDGW (50 $\mu$ M), the soluble  $\alpha_M$  I domain and the MEM170 antibody (Fig. 11C), indicating  $\beta_2$  integrin-directed binding. The control peptides (DFEDHD, KKGW) and an irrelevant 15 monoclonal antibody (anti-GPA) had no effect. The CTT peptide, but not the W $\rightarrow$ A CTT control peptide lacking MMP inhibitory activity, binds to the MMP-9 catalytic domain (unpublished results) and also inhibited the PMN adherence. MMP-9 antibodies inhibited partially.

We also examined  $\alpha_M\beta_2$ -transfected L cells. The  $\alpha_M\beta_2$  L-cell transfectants bound to 20 proMMP-9 and  $\Delta$ MMP-9 similarly as PMNs did and the I domain ligands and MMP-9 inhibitors attenuated the binding (Fig. 11D). The transfected cells also showed a weak adherence to FnII domain, but the studied peptides and antibodies did not inhibit this binding. Wild type L cells or LAD-1 cells showed no binding to proMMP-9 or its domains.

25 The *in vitro* migration of PMNs was studied on transwell filter assays. Coating with the artificial  $\beta_2$  integrin ligand LLG-C4-GST renders cell migration dependent on the  $\beta_2$  integrins (14). The migration of PMA-activated PMNs was 5-fold in the LLG-C4-GST substratum in comparison to GST substratum (Fig. 12A). HFDDDE (200 $\mu$ M) inhibited 30 the migration of PMA-stimulated cells but not the basal migration of non-activated cells. DDGW, CTT, MEM170 (20 $\mu$ g/ml) and polyclonal anti-MMP-9 (20 $\mu$ g/ml) worked similarly, affecting the migration of the PMA-activated cells only. Control peptides and an antibody control (anti-TAT-2) had no effect. Similar results were obtained in a transendothelial migration assay (Fig. 12B). Chemotaxis with C5a or

TNF- $\alpha$  increased PMN transmigration by 5-10 fold and inhibition was obtained by DDGW, HFDDDE, and CTT but not with the control peptides. Similarly,  $\alpha_M$  and MMP-9 antibodies inhibited but an antibody control (anti-GPA) did not. We also examined the effects of peptides on THP-1 leukemia cell migration through the LLG-C4-GST coated 5 transwell filters. The results were the same as for PMNs. HFDDDE, DDGW, and CTT inhibited THP-1 migration and the control peptides did not (Fig. 12C).

We show above that the DDGW peptide can release proMMP-9 from THP-1 cells. We found that the HFDDDE peptide also released proMMP-9 but was less effective than 10 DDGW (Fig. 12D). The scrambled peptide did not induce the release of proMMP-9. Under the 16 h incubation time, the peptides had no effect on the secretion of proMMP-2.

To study neutrophil migration *in vivo*, we used a mouse model of thioglycolate-induced 15 peritonitis. The cells that infiltrated into the peritoneal cavity within 3 h after thioglycolate irritant were judged to be predominantly PMNs by crystal violet staining. The DDGW and HFDDDE peptides had potent *in vivo* activities in this inflammation model (Fig. 13A). An intravenous tail injection of DDGW or HFDDDE inhibited the intraperitoneal accumulation of PMNs. The KKGW and DFEDHD peptides used as 20 controls had no effect. The effects of DDGW and HFDDDE were concentration-dependent and up to 90 % inhibition was obtained by doses of 50 $\mu$ g and 500 $\mu$ g per mouse, respectively. DDGW was active even at 5 $\mu$ g given per mouse corresponding to an effective dose of 0.1 mg/kg mouse tissue. Approximately 20-fold more PMNs were present intraperitoneally after thioglycolate-stimulus in comparison to the PBS control. 25 The collected inflammatory PMNs stained positively for the proMMP-9/ $\alpha_M\beta_2$  complex by double immunofluorescence (Fig. 13B). The cells collected after PBS injection lacked the complex; they expressed the integrin but had no cell-surface MMP-9 (Fig. 13C). Zymography analysis of the supernatants from the collected intraperitoneal fluid showed that thioglycolate induced elevated levels of gelatinases in comparison to PBS 30 (Fig. 13D). DDGW and HFDDDE, but not the scrambled peptide, prevented the increase in gelatinase levels in accordance with the inhibition of cell migration.

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**Table I: Pepspot analysis of peptides derived from MMPs and  $\alpha_M\beta_2$  integrin ligands**

Protein	Peptide	OD/mm <sup>2</sup>	Binding positivity
MMP-1	DAHFDEDERWTNNFR	1792 +	
	DAHFDEAARWTNNFR	1417 -	
MMP-2	DSHFDDDELWTLGEG	4687 +++	
	DSHFDDAALWTLGEG	3334 ++	
MMP-3	DAHFDDDEQWTKDTT	4993 +++	
	DAHFDDAAQWTKDTT	2188 +	
MMP-7	DAHFDEDERWTDGSS	4043 ++	
	DAHFDEAARWTDGSS	2065 +	
MMP-8	DAHFDAEETWTNTSA	4295 ++	
	DAHFDAAAATWTNTSA	1258 -	
MMP-9	DAHFDDDELWSLGKG	2010 +	
	DAHFDDAALWSLGKG	476 -	
MMP-13	DAHFDDDETWTSSSK	4324 ++	
	DAHFDDAATWTSSSK	1732 +	
MMP-14	DTHFDSEAEPWTVRNE	1264 -	
	DTHFDSEAAPWTVRNE	1198 -	
MMP-1	SGDVQLDDIDGIQAI	484 -	
	SGDVQLAAIDGIQAI	441 -	
MMP-3	RFRLSQDDINGIQSL	810 -	
	RFRLSQAAINGIQSL	1541 +	
MMP-8	NYSLPQDDIDGIQAI	3348 ++	
	NYSLPQAAIDGIQAI	505 +	
MMP-13	HFMLPDDDVQGIQSL	542 -	
	HFMLPAAVQGIQSL	384 -	
MMP-14	NFVLPPDDRRGIQQL	518 -	
	NFVLPDAAARRGIQQL	609 -	
Fibronectin	HEATCYDDGKTYHVG	1271 -	
	HEATCYAAGKTYHVG	596 -	
ICAM-3	LNATESDDGRSFFCS	369 -	
	LNATESAAGRSFFCS	277 -	
Complement factor H	EEMHCSDDGFWSKEK	2972 +	
	EEMHCSAAGFWSKEK	321 -	
TSP-1	WPSDSADDGWSPWSE	4655 +++	
	WPSDSAAAGWSPWSE	1543 +	
NIF	DPVCIPDDGVCFIGS	221 -	
	DPVCIPAAGVCFIGS	114 -	
ICAM-2	NSTADREDGHRNFSC	75 -	
	NSTADRAAGHRNFSC	73 -	
Fibronectin	NYVQISEDGEQSLIL	1260 -	
	NYVQISAAGEQSLIL	453 -	
Fibronectin	VTYSSPEDGIHELP	301 -	
	VTYSSPAAGIHELP	212 -	
Cyr61	KMRFRCEDGETFSKN	317 -	
	KMRFRCAAGETFSKN	386 -	
Myeloperoxid	WLPAEYEDGFSLPYG	4603 +++	

ase	WLPAEYAAAGFSLPYG	924 -
Catalase	AVKFYTEDGNWDLVG	5045 +++
	AVKFYTAAGNWDLVG	690 -
Fibrinogen alpha	KEVVTSEDGSDCPEA	124 -
	KEVVTSAAGSDCPEA	225 -
Fibrinogen beta	RKQCSKEDGGGWWYN	483 -
	RKQCSKAAGGGWWY N	325 -
Fibrinogen alpha	GFGSLNDEGEGEFWL	732 -
	GFGSLNAAGEGEFWL	397 -
GP1b	GCPTLGDEGDTDLYD	544 -
	GCPTLGAAGDTDLYD	238 -
ICAM-1	VSVTAEDEGTQRLTC	51 -
	VSVTAEAAGTQRLTC	68 -
Factor X	DRNTEQEEGGEAVHE	379 -
	DRNTEQAAGGEAVHE	236 -
E-selectin	TCTFDCEEGFELMGA	309 -
	TCTFDCAAGFELMGA	70 -
E-selectin	SCNFTCEEGFMLQGP	138 -
	SCNFTCAAGFMLQGP	54 -
E-selectin	SCAFSCEEGFELHGS	236 -
	SCAFSCAAGFELHGS	74 -
Fibronectin	TFHKRHEEGHMLNCT	39 -
	TFHKRHAAGHMLNCT	12 -
Fibronectin	VEYELSEEGDEPQYL	3676 ++
	VEYELSAAGDEPQYL	2682 +

Range: 0-1499 OD/mm<sup>2</sup> = -, 1500-2999 = +, 3000-4499 = ++,  
>4500 = +++

	Peptide	No competitor	Soluble DDGW
DDGW peptide	ADGACILWMDDGWC GAAG	10859	0
iC3b	ARSNLDEDIIAEENI	13265	409
	ARSNLDAIIIAEENI	0	0
iC3b	EDIIAEENIVSRSEF	0	0
	EDIIAAAANIVSRSEF	0	0
iC3b	EGVQKEDIPPADLSD	0	0
	EGVQKAAIPPADLSD	0	0

## CLAIMS

1. A pharmaceutical composition comprising a compound comprising the tetrapeptide motif D/E-D/E-G/K-W, and a pharmaceutically acceptable carrier.

5

2. The pharmaceutical composition according to claim 1, wherein the tetrapeptide motif is DDGW.

10 3. Use of a compound comprising the tetrapeptide motif D/E-D/E-G/K-W for the manufacture of a pharmaceutical composition for the treatment of conditions dependent on leukocyte migration.

4. Use according to claim 3 wherein the condition dependent on leukocyte migration is leukaemia.

15

5. Use according to claim 3 for the manufacture of a pharmaceutical composition for inhibiting the adhesion of progelatinases to  $\beta_2$ -integrins.

20 6. Use of the compound comprising the tetrapeptide motif D/E-D/E-G/K-W for the manufacture of a pharmaceutical composition for prophylaxis and treatment of conditions dependent on neutrophil migration.

7. Use according to claim 6 for the manufacture of a pharmaceutical composition for prophylaxis and treatment of inflammatory conditions.

25

8. Use according to any one of claims 3 to 7, wherein the tetrapeptide motif is DDGW.

30 9. A method for therapeutic or prophylactic treatment of conditions dependent on leukocyte migration, comprising administering to a mammal in need of such treatment a compound comprising the tetrapeptide motif D/E-D/E-G/K-W in an amount which is effective in inhibiting leukocyte migration.

10. A method for therapeutic or prophylactic treatment of conditions dependent on neutrophil migration, comprising administering to a mammal in need of such treatment

a compound comprising the tetrapeptide motif D/E-D/E-G/K-W in an amount which is effective in inhibiting neutrophil migration.

11. A method for therapeutic or prophylactic treatment of leukaemia, comprising  
5 administering to a mammal in need of such treatment a compound comprising the tetrapeptide motif D/E-D/E-G/K-W in an amount which is effective in inhibiting leukaemia cell migration.
12. A method for therapeutic or prophylactic treatment of inflammatory conditions,  
10 comprising administering to a mammal in need of such treatment a compound comprising the tetrapeptide motif D/E-D/E-G/K-W in an amount which is effective in inhibiting neutrophil migration.
13. The method according any one of claims 9 to 12, wherein the tetrapeptide motif is  
15 DDGW.

**A** Phage sequences

```

CREDGMIQTC
CSEDIXXGLC
CTLMEDGAC
CTEDGMIATC
CIMMDGAC
CWDGGINFC
CDDGMMFC
CFEELWMLC
CQSDADGRC
CQGDWGDRC
CFINHDMILC
CWVDSKRC
ADWYHFWYSS
CIMNDDGMC
CFEELWMLC
KNP-2 (P09253) GDGHFDDDELWTIGEQQQIV
KNP-3 (P14700) GDAHFDDEDELNSLQKGVVV
KNP-1 (P03956) GDAHFDDEDEFTNINFRSYIN
KNP-3 (P08254) GDAHFDDEDEFTNDITGHN
KNP-7 (P09237) GDAHFDDEDEFTNDGSSLGI
KNP-8 (P22834) GDAHFDAAETWTWTSANVYH
KNP-13 (P45452) GDAHFDDEDEWTSSSSKGVN
KNP-14 (P50281) GOTHESAEIWTVTRHEDLN

```

Figure 1A

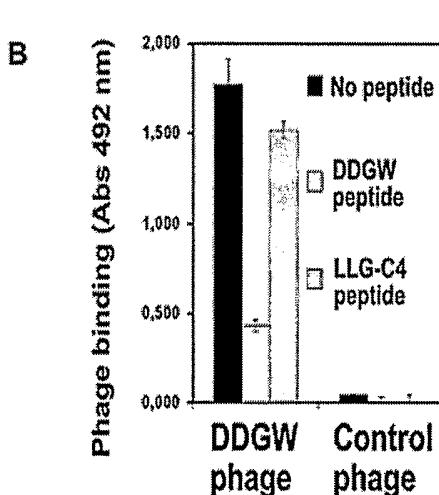


Figure 1B

Figure 1C

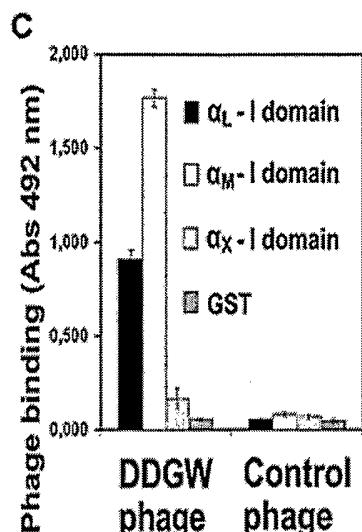


Figure 1D

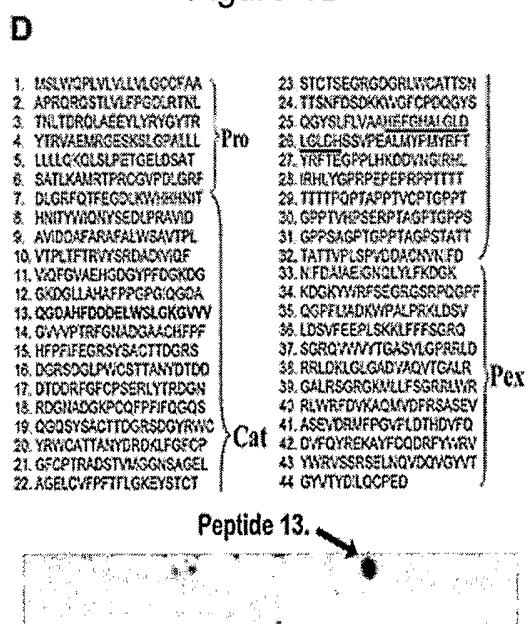
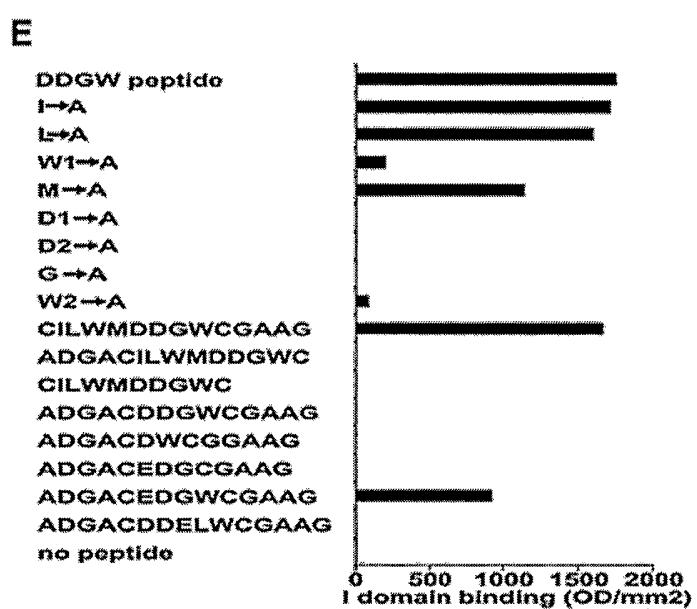


Figure 1E



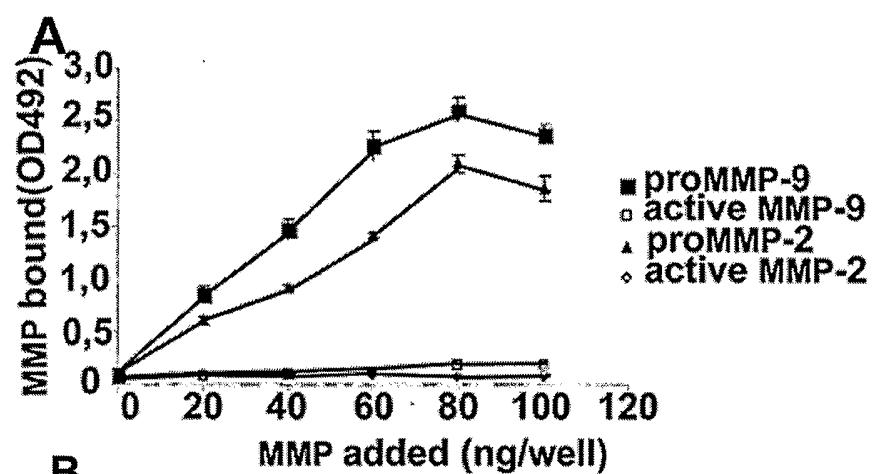


Figure 2A

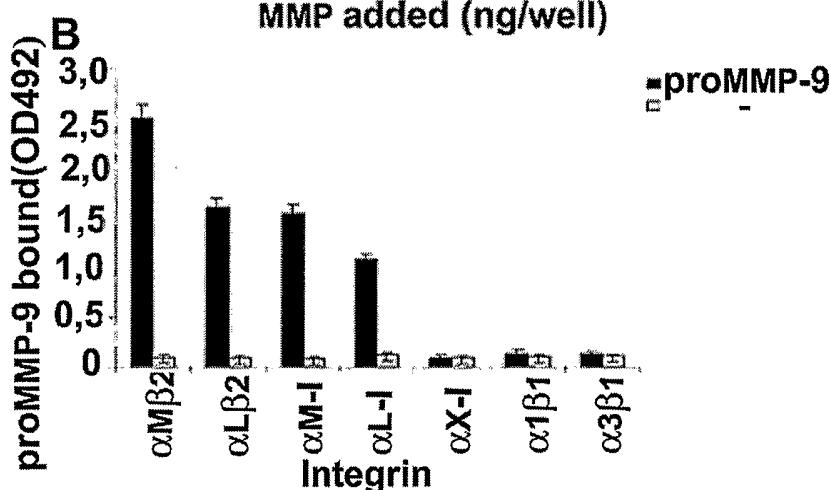


Figure 2B

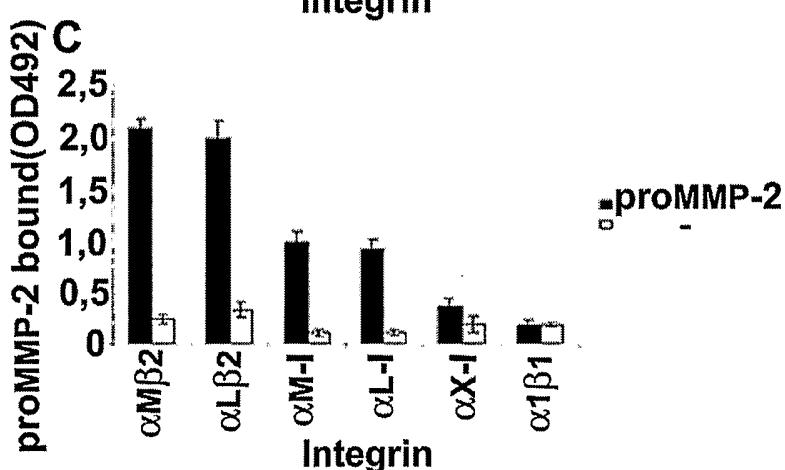


Figure 2C

Figure 3A

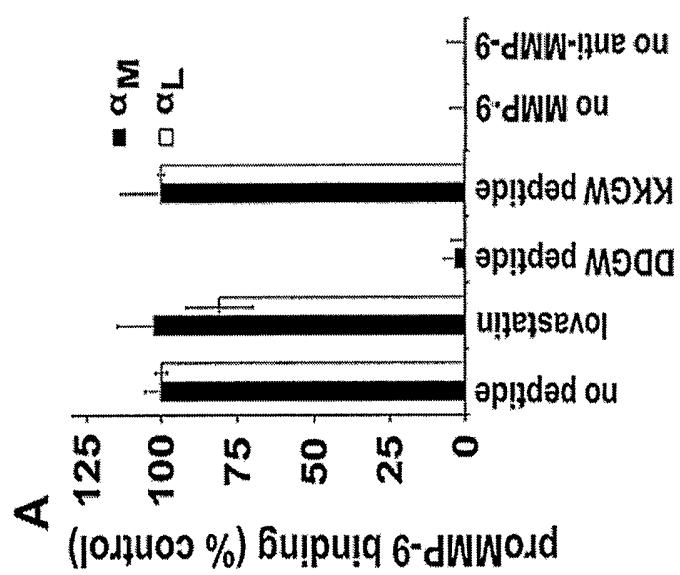


Figure 3B

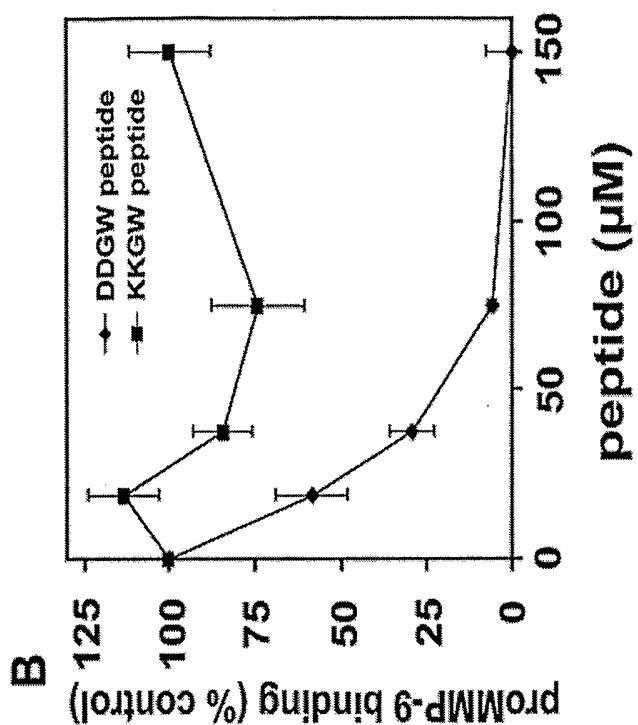


Figure 3C

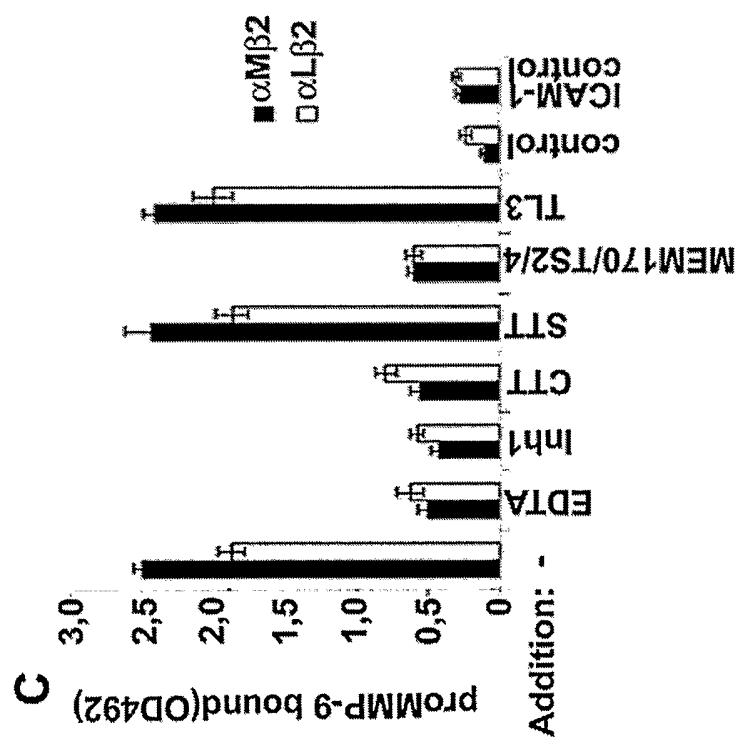
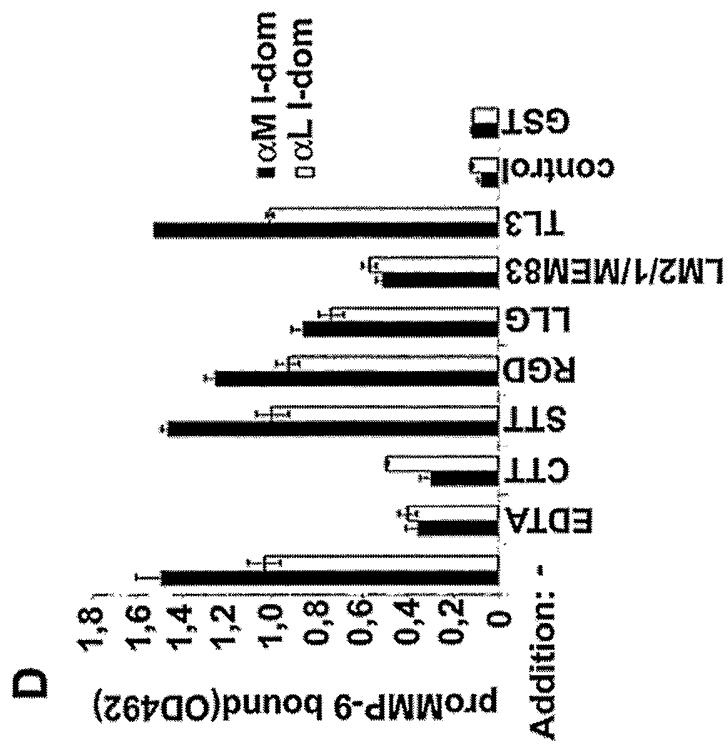


Figure 3D



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Figure 4A

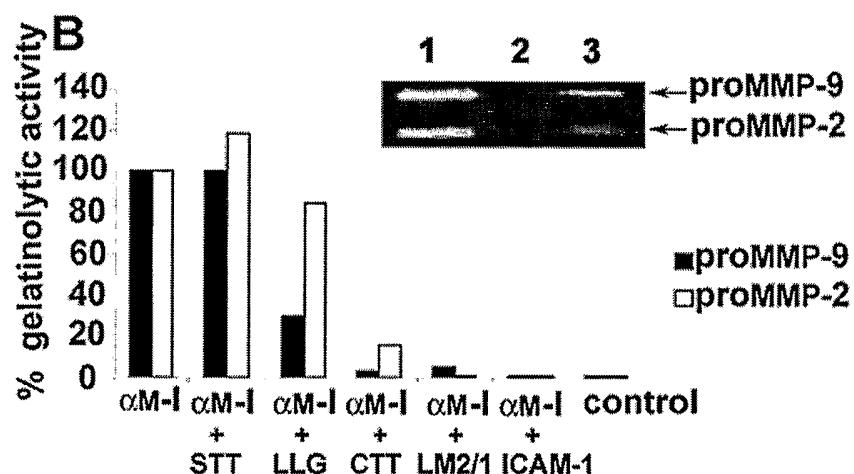
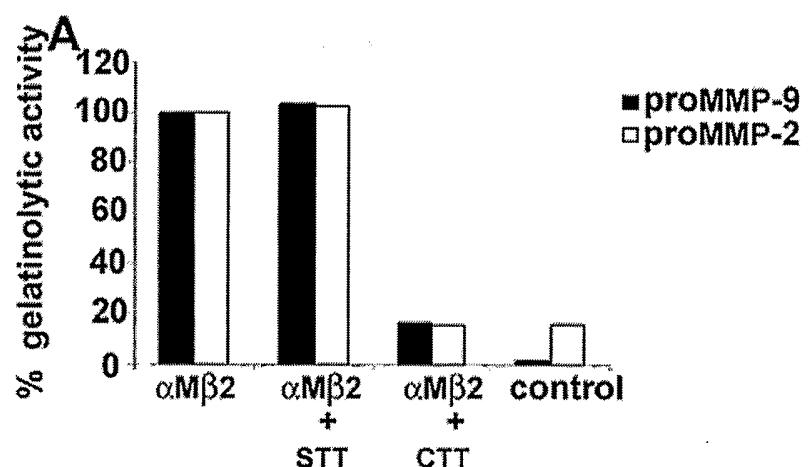


Figure 4B

Figure 5A

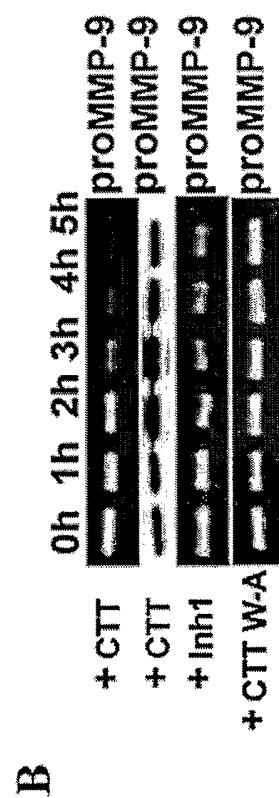
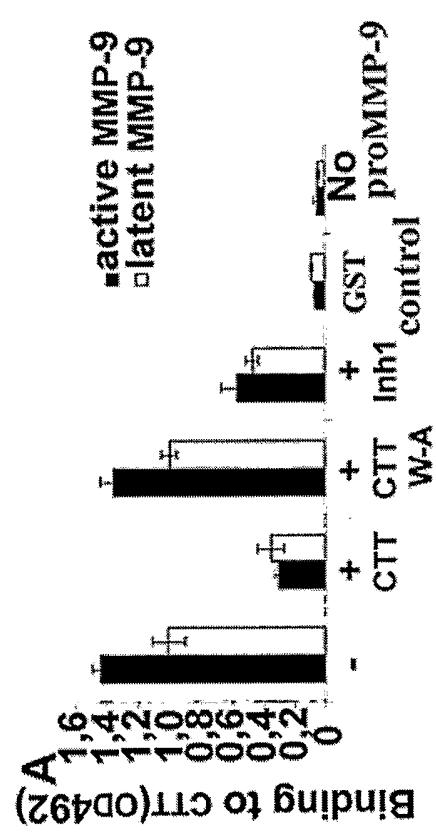


Figure 5B

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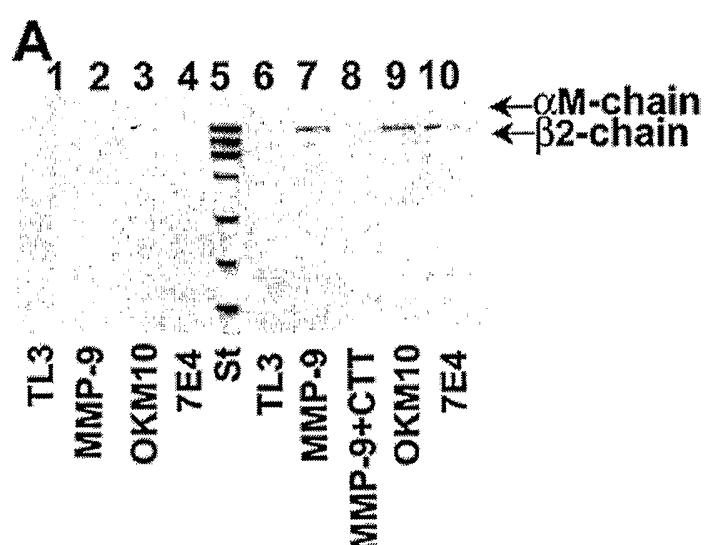


Figure 6A

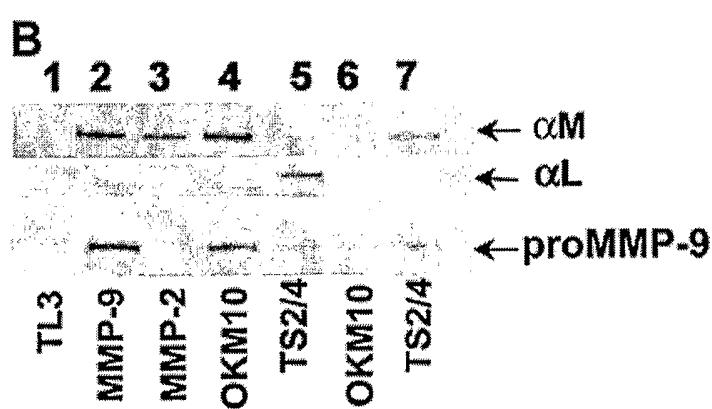


Figure 6B

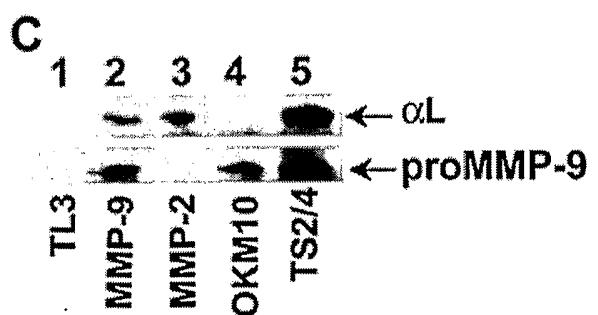


Figure 6C

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**Figure 7A**



**Figure 7B**



Figure 8A

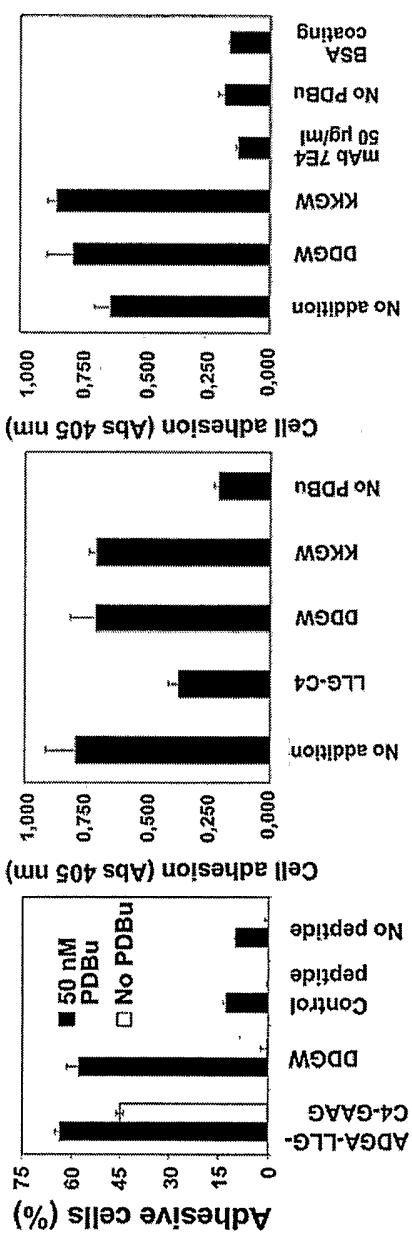


Figure 8B

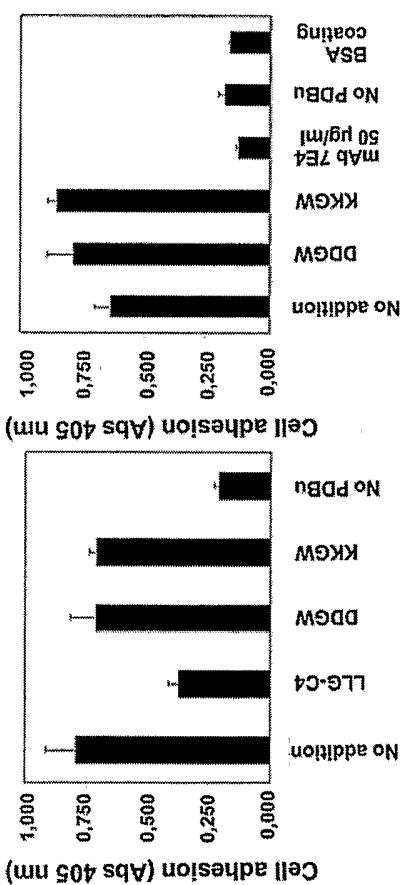


Figure 8C

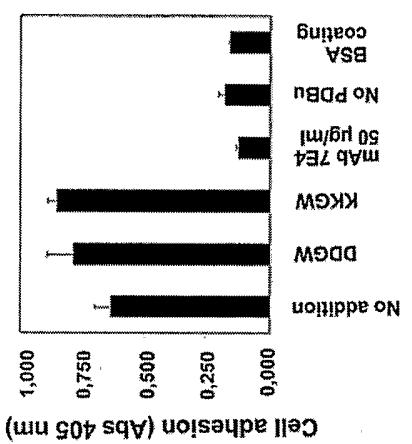
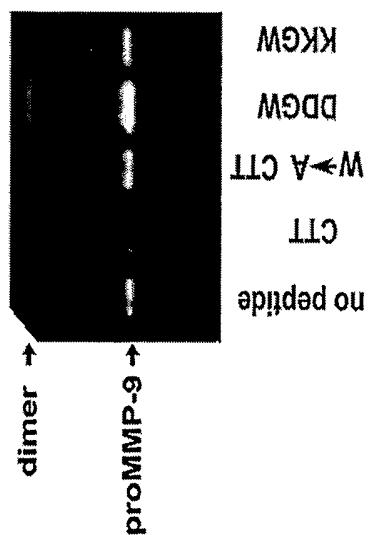


Figure 8D



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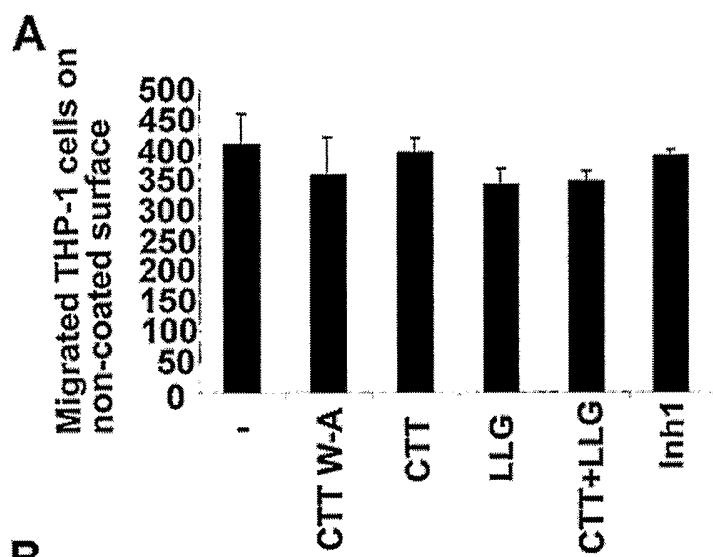


Figure 9A

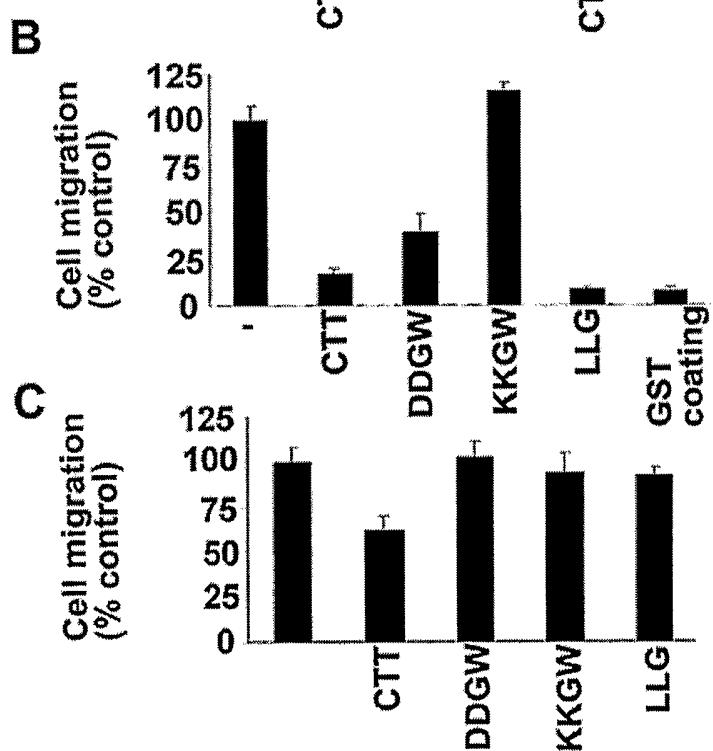


Figure 9B

Figure 9C

Figure 10A

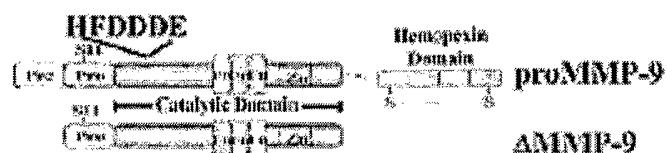
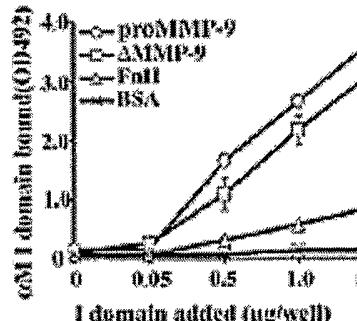


Figure 10B



FnnI repeats

C

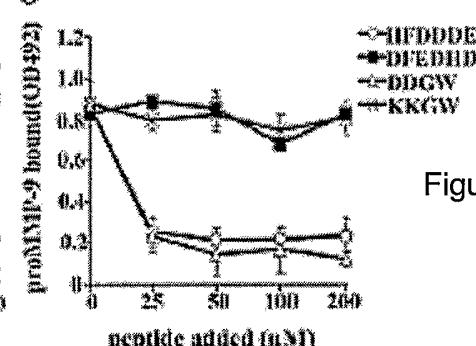


Figure 10C

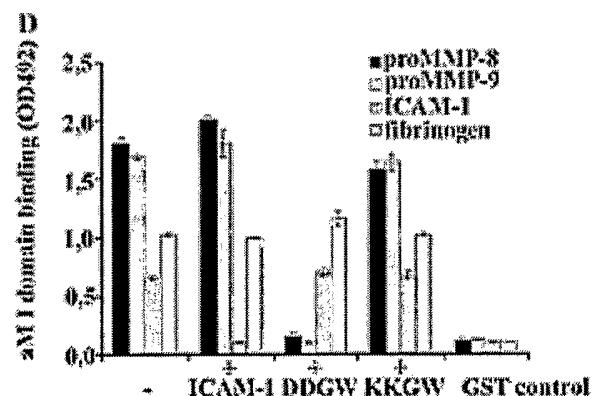


Figure 10D

Figure 11A

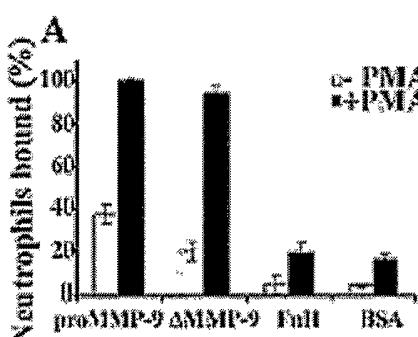


Figure 11B

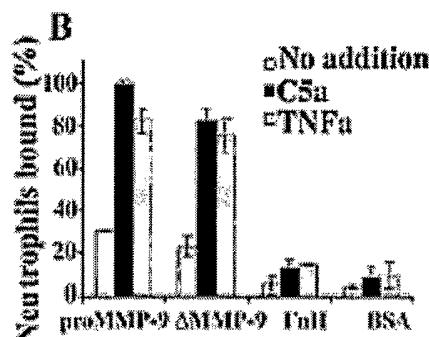


Figure 11C

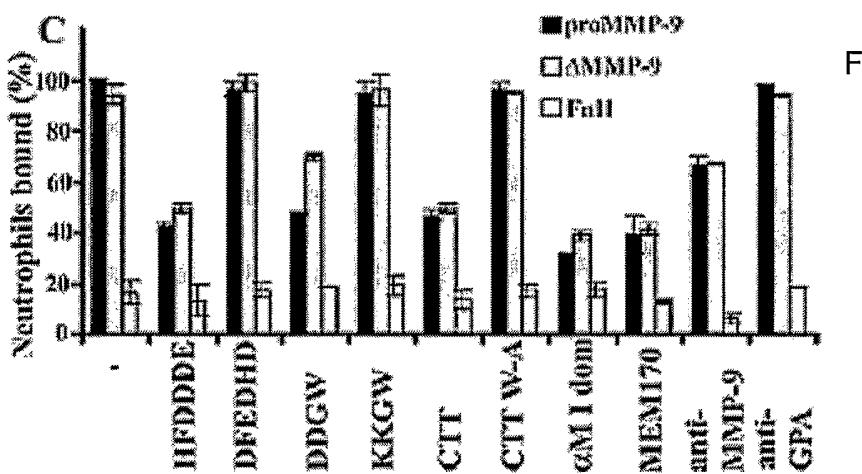
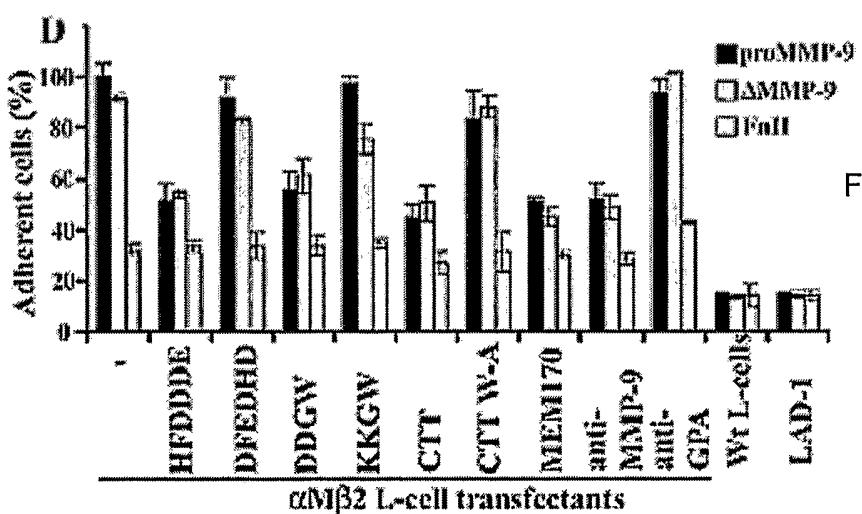


Figure 11D



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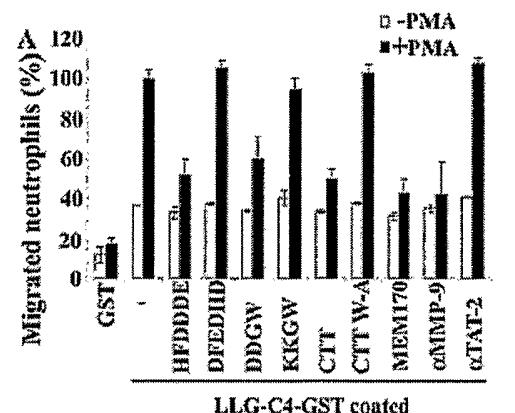


Figure 12A

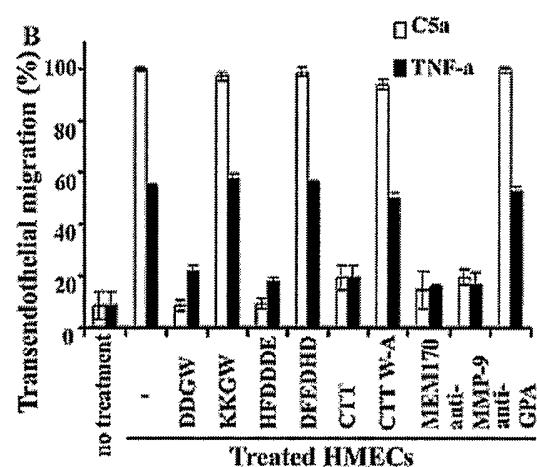


Figure 12B

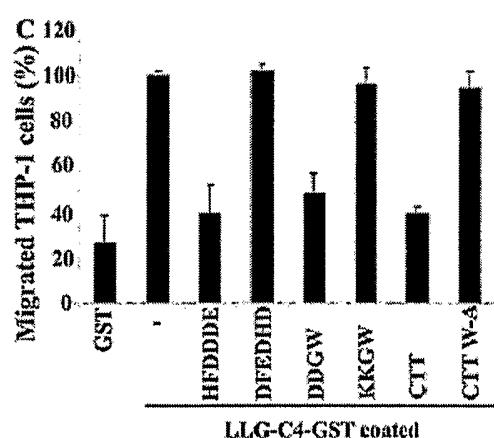


Figure 12C

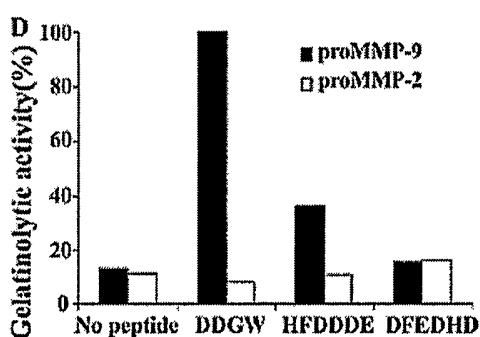


Figure 12D

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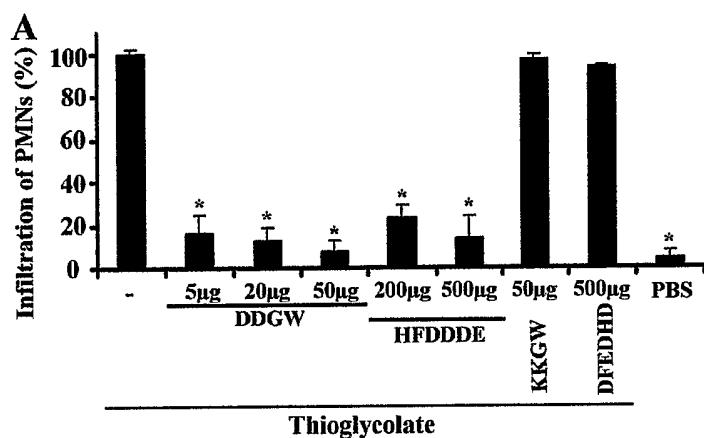


Figure 13A

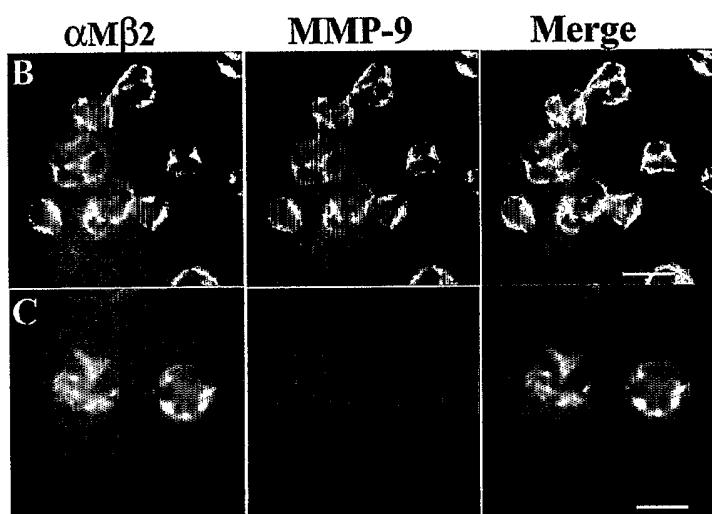


Figure 13B

Figure 13C

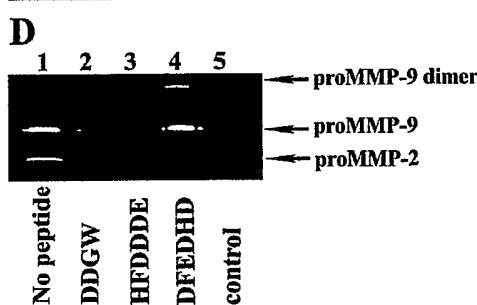


Figure 13D

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/FI 2004/000375

## A. CLASSIFICATION OF SUBJECT MATTER

**IPC7: A61K 38/48, A61K 38/02, C12N 9/50 // A61P29/00, A61P35/02**  
 According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

**IPC7: A61K**

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

**SE,DK,FI,NO classes as above**

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	<p>STN International, File CAPLUS, CAPLUS accession no. 2003:684969, Document no. 139:303787, Stefanidakis, Michael et al: "Identification of a Negatively Charged Peptide Motif within the Catalytic Domain of Progelatinases That Mediates Binding to Leukocyte Beta 2 Integrins"; &amp; Journal of Biological Chemistry (2003), 278(36), 34674-34684</p> <p>---</p>	1-5,8-9,11, 13

 Further documents are listed in the continuation of Box C. See patent family annex.

* Special categories of cited documents:	
"A" document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E" earlier application or patent but published on or after the international filing date	"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family

Date of the actual completion of the international search <b>22 November 2004</b>	Date of mailing of the international search report <b>23-11-2004</b>
Name and mailing address of the ISA / Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. + 46 8 666 02 86	Authorized officer <b>Yvonne Siösteen/EÖ</b> Telephone No. + 46 8 782 25 00

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/FI 2004/000375

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	STN International, File CAPLUS, CAPLUS accession no. 1995:781250, Document no. 123:191600, Pasqualini, Renata et al: "A peptide isolated from phage display libraries is a structural and functional mimic of an RGD-binding site on integrins"; & Journal of Cell Biology (1995), 130(5), 1189-96	1,2,6-8,10, 12-13
A		3-5,9,11
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X	WO 9708203 A1 (LA JOLLA CANCER RESEARCH FOUNDATION), 6 March 1997 (06.03.1997), abstract, see page 25, line 17	1-2,6-8,10, 12-13
A		3-5,9,11
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X	US 20030022835 A1 (JAMES D. WATSON ET AL), 30 January 2003 (30.01.2003), see claim 16, SEQ ID NO 644, 636 and 504 & STN International File Registry, see RN 491890-02-9, RN 491889-94-2, RN 491888-64-3	1-2,6-8,10, 12-13
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X	STN International, File Registry, Registry accession no. RN 359735-04-9; & WO 2001064886, abstract and SEQ ID NO 1351	1-5,8-9,11, 13
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**INTERNATIONAL SEARCH REPORT**

Information on patent family members

30/10/2004

International application No.

PCT/FI 2004/000375

WO	9708203	A1	06/03/1997	AU	6910996	A	19/03/1997
				US	5817750	A	06/10/1998
				US	5955572	A	21/09/1999
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US	20030022835	A1	30/01/2003	AU	758074	B	13/03/2003
				AU	3961499	A	16/11/1999
				CA	2327317	A	04/11/1999
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				JP	2002512798	T	08/05/2002
				NZ	507728	A	28/06/2002
				US	20030040471	A	27/02/2003
				AU	4958800	A	05/12/2000
				BR	0010563	A	19/02/2002
				CA	2374045	A	23/11/2000
				CN	1373774	T	09/10/2002
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				NZ	515388	A	28/03/2003
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				WO	0069884	A	23/11/2000
				CN	1307637	T	08/08/2001
				US	6150502	A	21/11/2000
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