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Interaction of metargidin (ADAM-15) with $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins on different haemopoietic cells

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SUMMARY

Metargidin (ADAM-15) is a type I transmembrane glycoprotein belonging to the ADAM (A Disintegrin and Metalloprotease Domain) family of proteins and is widely expressed in different tissues and cell types. Members of this family contain an amino-terminal metalloprotease domain followed by a disintegrin domain, a cysteine-rich region and a membrane proximal EGF-like domain. The disintegrin domain of metargidin contains an RGD tripeptide sequence, suggesting that it may potentially interact with the integrin family of proteins. Here we identify integrin ligands for metargidin on haemopoietic cells, by using a chimeric protein containing the extracellular domain of metargidin fused to the Fc portion of human IgG. Binding activity to a panel of human cell lines was analysed by solid-phase cell-adhesion assays. Metargidin bound to a monocytic cell line, U937, and a T cell line, MOLT-4, in a specific manner. Adhesion was divalent cation- and temperature- dependent and strongly enhanced by Mn^{2+}, all features of integrin-mediated binding. Using a panel of anti-integrin antibodies we show that $\alpha_v\beta_3$ is a ligand for metargidin on U937 cells. In contrast, for MOLT-4 cells, the integrin $\alpha_5\beta_1$ contributes to cell binding. Adhesion was mediated by the disintegrin domain of metargidin as RGD-based peptides inhibited cell binding to both cell lines. The specificity of the interaction between both $\alpha_v\beta_3$ and $\alpha_5\beta_1$ and metargidin was further confirmed by solid-phase adhesion assays using purified recombinant integrins. These results together indicate that metargidin can function as a cell adhesion molecule via interactions with $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins.

Key words: Metargidin, ADAM-15, Integrin, Disintegrin, Metalloprotease, $\alpha_v\beta_3$, $\alpha_5\beta_1$, Adhesion, Ligand

INTRODUCTION

The ADAMs (A Disintegrin and Metalloprotease domain) are a family of type I transmembrane glycoproteins thought to be important in diverse biological processes such as cell adhesion, fusion and proteolysis. Members of this family have a unique multidomain structure, comprising an amino-terminal metalloprotease domain, followed by a disintegrin domain, a cysteine-rich region and a membrane proximal EGF-like domain. They frequently have large cytoplasmic domains with potential signalling properties. ADAMs are related to soluble snake venom disintegrins, which have the ability to bind to integrins and disrupt their normal adhesion (Weskamp and Blobel, 1994; Wolfsberg et al., 1995, 1996).

There are 21 members of the ADAM family to date, expressed in both invertebrates and vertebrates such as *Caenorhabditis elegans, Drosophila melanogaster, Xenopus laevis*, mice, hamster and humans (Alfandari et al., 1997; Fambrough et al., 1996; Rooke et al., 1996; Wolfsberg and White, 1996; Blobel, 1997; Black et al., 1997; Kuno et al., 1997; Moss et al., 1997). At least seven ADAMs have been found in spermatogenic cells and three have been implicated in sperm-egg binding and fusion. Of these, the best characterized is the fertilin α/β (ADAM 1, 2) complex, which interacts with the integrin $\alpha_6\beta_1$ during fertilization (Almeida et al., 1995). In vivo and in vitro studies have shown that ADAM-12 (meltrin α) is important in myotube formation (Yagami-Hiromasa et al., 1995, Gilpin et al., 1998), ADAM-10 (Kuzbanian) plays a critical role in neurogenesis in *Drosophila* (Fambrough et al., 1996; Rooke et al., 1996; Blaumueller and Artavanasis-Tsakonas, 1997), and ADAM-17 (TNF α converting enzyme or TACE) releases the membrane-anchored cytokine tumor necrosis factor α (Black et al., 1997; Moss et al., 1997). The findings that TACE is an ADAM and that Kuzbanian can apparently activate the signalling receptor Notch by proteolysis, raise the possibility that other cell surface molecules can be processed or released by the ADAMs. This mechanism may also be important under inflammatory conditions where it is well known that several adhesion molecules are released from the surface of cells.

The integrin superfamily of proteins are a major class of cell adhesion molcules that can bind to a variety of cell surface proteins and extracellular matrix molecules (reviewed by Hynes, 1992; Springer, 1994; Schwartz et al., 1995; Yamada and Miyamoto, 1995; Humphries, 1996; Sugimori et al., 1997). ADAMs are potential ligands for integrins due to the presence of identifiable integrin binding motifs within their disintegrin domain and by their homology to snake venom disintegrins, which can bind to integrins like $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ (Paine et al., 1992; reviewed by Blobel and White, 1992a). The molecular basis of integrin-ligand interactions have been the focus of intense study and it has been found that many integrins recognise short linear amino acid sequences within their ligands. The best characterised motif is that containing the sequence Arg, Gly, Asp (RGD), which is present in several integrin ligands including fibronectin, vitronectin and von Willebrand factor. Human metargidin is the only ADAM identified to date containing an RGD motif within its disintegrin domain. It is widely expressed in various tissues and cell types including endothelial cells and smooth muscle cells. Its cytoplasmic domain contains Src homology 3 (SH3) ligand binding domains, suggesting a potential for activating intracellular signalling pathways (Krätzschmar et al., 1996).

In this study we have attempted to identify ligands for metargidin. Using a recombinant soluble form of metargidin, we analysed the binding activity of a panel of human cell lines representing different cell lineages. Our results show that metargidin can bind to $\alpha_v\beta_3$ on a monocytic cell line U937, and to $\alpha_5\beta_1$ integrin on the T cell line, MOLT-4. Furthermore, binding was found to be RGD-dependent, suggesting that metargidin can function as a cell adhesion molecule via its disintegrin domain. These results may lead to a better understanding of the function of metargidin and its involvement in physiological and pathological processes.

MATERIALS AND METHODS

Cells and reagents

Unless specified otherwise, all reagents and chemicals were obtained from Sigma (St Louis, USA). The cell lines U937 and HL-60 (monocytic line), K562 (erythroleukemic line) and MOLT-4 (T-cell line) were from the European Tissue Culture Collection**.** A375M human melanoma cells were a generous gift from Dr J. F. Marshall (Richard Dimbleby Department of Cancer Research, ICRF Laboratory, St Thomas's Hospital, London, UK). Purified human recombinant $\alpha_v\beta_3$ was a generous gift from Dr S. Goodman (Merck, A46/304, Frankfurter Str. 250, 64271 Darmstadt, Germany). Recombinant $\alpha_5\beta_1$ was purchased from Chemicon International (Harrow, UK). All haemopoietic cells were maintained in RPMI containing 10% foetal calf serum (FCS). A375M cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, USA) supplemented with 10% FCS (purchased from Globepharm, UK). Hanks Balanced Salt Solution (HBSS) was purchased from Life Technologies, USA.

Antibodies

The following anti-integrin monoclonal antibodies (mAbs) were used. Anti-α_2 integrin 5E8 (kind gift from Dr R. Bankert, Roswell Park Cancer Institute, Buffalo, NY, USA); anti-α_3 clone P1B5 (Chemicon International, Harrow, UK); anti-α_4 clone HP2/1 (Serotech Ltd, Oxford, UK); anti-α_5 clone mAb (gift from K. M. Yamada); anti-α_6 clone GoH3 (Serotech, Oxford, UK); anti-β_1 integrin clone mAb 13 (gift from K. M. Yamada). The anti-$\alpha_v\beta_3$ mAbs used were: clone LM609 (Chemicon International, Harrow, UK); 23C6 (gift from Dr M.A Horton, University College, London, UK). Polyclonal goat anti-human-Fc Ig was obtained from Sigma (St Louis, USA).

Recombinant fusion protein constructs

Construction of metargidin-Fc

A population of cDNAs was generated from RNA isolated from human peripheral blood mononuclear cells that had been stimulated for 4 hours with 50 ng/ml PMA and 500 ng/ml ionomycin. The cDNA was used as a template for amplifying the metargidin coding sequence using oligonucleotide primers based on the published sequence, and which placed a *Hin*dIII site immediately upstream from the initiating methionine codon (Krätzschmar et al., 1996). Expression of the metargidin extracellular domains, fused to the human IgG1 hinge region and heavy chain constant domains CH2 and CH3, was achieved using the eukaryotic expression vector pEE12.2. This vector contains a *Sal*I-*Eco*RI genomic fragment encoding 14 residues of the upper hinge, the hinge and the CH2 and CH3 domains of human IgG1 (Takahashi et al., 1982). The fusion was generated by ligating a 1477 bp *Hin*dIII-*Eco*RI fragment, corresponding to the 5′ end of metargidin, to a 623 bp *Eco*R1-*Sal*1 PCR fragment corresponding to the 3′ end. The PCR fragment was generated using the oligonucleotides: 5′ GACGGACCCTGTTGTCAAAATTGC 3′ and 5′ TCTTGTCGACTGTGGTCAGGGAGCTGGTTTGC 3′.

The fusion generates the sequence: $L_{694}T_{695}T_{696}$VD at the junction between metargidin and the Fc portion of human IgG1 (numbering according to Krätzschmar et al., 1996).

Construction of KDR-Fc

An Fc fusion protein consisting of the extracellular domains of the kinase domain receptor (KDR; Ferrara and Davis-Smyth, 1997) was used as a control in all experiments. The construct was generated as follows. A pEE12 plasmid containing the full-length KDR coding region (derived from human umbilical vein endothelial cell cDNA) was digested with *Tth*III and *Sal*I to remove a DNA fragment encoding the C-terminal end of the extracellular domain together with the transmembrane and intracellular domains of KDR. This was replaced with a PCR-generated *Tth*III-*Sal*I fragment that rebuilt the C-terminal end of the KDR extracellular domain up to residues $L_{764}E_{765}$ (Terman et al., 1992), followed by a *Sal*I fragment which encoded the hinge region, CH2 and CH3 domains of human IgG1, creating the junction sequence, LEVDKK.

Production of recombinant fusion proteins

Metargidin-Fc (refered to as Met-Fc) protein was prepared by transient transfection of COS-7 cells using the DEAE-dextran method as previously described (Simmons et al., 1993). In brief, COS-7 cells were transfected at 70-80% confluency using 30 μg of plasmid DNA/15-cm diameter plate. 24 hours post-transfection, the medium was changed to Dulbecco's modified Eagle's medium with 0.5% FCS that had been depleted of IgG with Protein A-Sepharose. The COS cell supernatants were harvested 5-7 days post-transfection, and insoluble material was removed by centrifugation and filtration. The control Fc protein (KDR-Fc) was purified from a stable NS0 mouse myeloma line grown in Iscove's medium containing 10% dialysed FCS. All the Fc chimeric proteins were purified by Protein A chromatography, eluted in 10 mM diethylamine, pH 10.3, and immediately neutralised by the addition of one-tenth the amount of 0.1 M glycine, pH 3.0. The purity of the protein preparations were checked by SDS-PAGE.

Cell adhesion assays

Cell adhesion assays to soluble chimeric fusion proteins were carried out as described previously (Nath et al., 1995) with the following modifications. 96-well adhesion assay plates (Immulon-4; Dynatech Research Laboratories, Chantilly, Virginia, USA) were precoated

overnight at 4°C with 15 μg/ml goat anti-human IgG-Fc antibody in 0.1 M bicarbonate buffer, pH 9.6. Non-specific sites were blocked with HBSS containing 5% non-fat milk. Recombinant Fc proteins in HBSS (with Ca^{2+} and Mg^{2+}) + 0.25% BSA were added at 3 μg/ml. Prior to binding assays, cells were resuspended in HBSS + 0.25% BSA (without or with 1 mM $MnCl_2$) to a concentration of 2×10^6 cell/ml, and 50 μl portions were added to wells of the microtiter plate containing 50 μl of the same buffer. After 1 hour at 37°C, 100 μl of 0.125% glutaraldehyde in HBSS was added, and bound cells were fixed for 5 minutes at room temperature with gentle shaking. The nonadherent cells were removed by washes in HBSS until the cells in the control wells were sufficiently removed, as monitored by visual inspection. Cell binding was calculated by staining with 1% Methylene Blue in 0.01 M borate for 30 minutes. Excess dye was washed off with water and bound cells lysed with ethanol/0.1 M HCl (1:1 ratio). The absorbance of the released dye was read at 630 nm on a multiwell-plate reader.

In experiments where mAbs were added, cells were preincubated with antibodies at a final concentration of 10 μg/ml for 20 minutes at room temperature prior to plating and included throughout the binding assay. In experiments to analyse the effect of Mn^{2+} on cell binding, cells were incubated in HBSS buffer containing 1.0 mM $MnCl_2$ for 10 minutes prior to plating.

The synthetic peptides Gly-Arg-Gly-Asp-Ser (GRGDS) and Ser-Asp-Gly-Arg-Gly (SDGRG) were purchased from Sigma (St Louis, USA). A cyclic peptide Ac-Pen-Arg-Gly-Asp-Cys-OH (hereafter referred to as cRGDC; Pen, penicillamine; Ac, acetyl) was purchased from Bachem Ltd (Essex, UK). The peptides were dissolved in HBSS and stored at −20°C prior to use. In adhesion assays, cells were preincubated with the peptides at varying concentrations for 15 minutes at room temperature, prior to plating onto wells containing the recombinant Fc proteins.

All adhesion assays were performed on at least three independent occasions. Each data point represents the mean ± s.e.m. of three replicates.

Solid-phase binding assay

Solid-phase binding assays were performed using a modification of the method of Buckley et al. (1996). Purified human $\alpha_v\beta_3$ was diluted to 5 μg/ml in HBSS, 0.02% NaN_3 and $\alpha_5\beta_1$ was diluted to 10 μg/ml, and 50 μl portions added to a 96-well Nunc-Maxisorp ELISA plate (Life Technologies, USA). Plates were incubated overnight at 4°C and blocked with 200 μl 5% BSA in HBSS for 2 hours at room temperature. Wells were then washed 3 times in HBSS, 1 mM $MnCl_2$, 1 mg/ml BSA (Buffer A). 50 μl samples of the Fc protein ligands (at 5 μg/ml) were added with or without mAbs and the plate incubated for 3 hours at 30°C. Wells were washed 3 times in Buffer A and bound Fc ligands detected by the addition of peroxidase-conjugated anti-human Fc antibody (1:4000) in Buffer A for 30 minutes at room temperature. Wells were then washed 4 times in Buffer A and colour developed using o-Phenylenediamine Dihydrochloride (OPD) substrate (Sigma, St Louis, USA). Absorbance was read at 450 nm on a multiwell microtiter plate reader.

RESULTS

Cell-chimeric adhesion assays for metargidin binding

To identify a ligand for metargidin we used a chimeric protein consisting of the entire extracellular domain of metargidin fused to the hinge region and the two constant domains of the Fc portion of human IgG1. We expressed the entire extracellular portion rather than the disintegrin domain alone, because at present it is unclear whether interactions between the disintegrin domain and other domains are required for adhesion with potential ligands (Blobel et al., 1992b, Yagami-Hiromasa et al., 1995). Also, the disintegrin domain alone may not be stable on its own, due to the presence of an odd number of cysteine residues, which may be part of an inter-domain disulphide bond or may be involved in receptor oligomerisation (Jia et al., 1997).

The metargidin-Fc (Met-Fc) chimeric protein was affinity-purifed using Protein A-Sepharose, from supernatants of

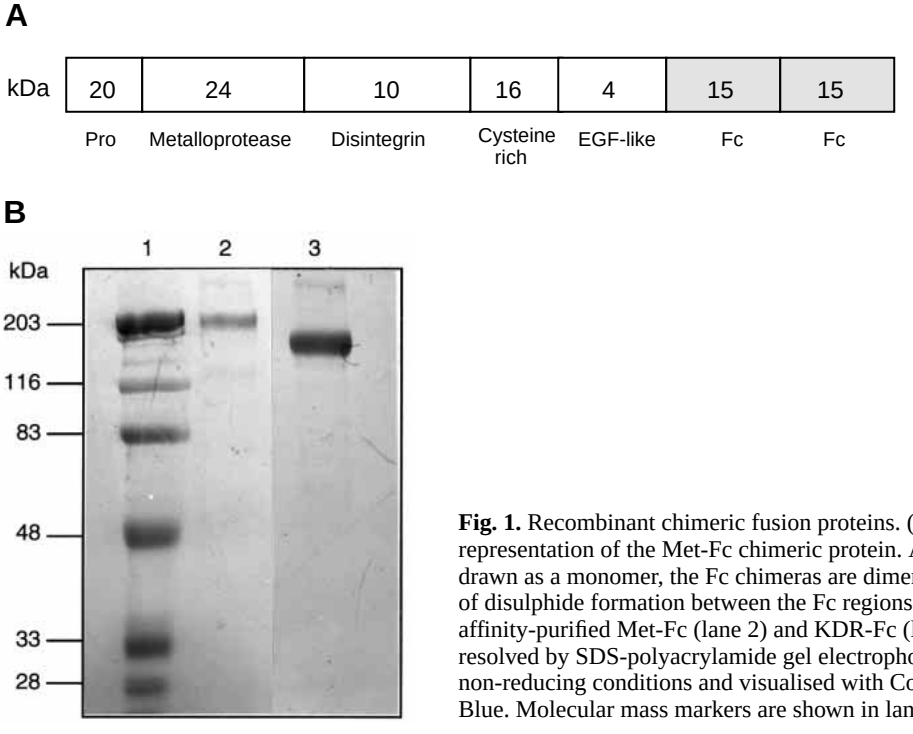

Fig. 1. Recombinant chimeric fusion proteins. (A) Schematic representation of the Met-Fc chimeric protein. Although drawn as a monomer, the Fc chimeras are dimers as a result of disulphide formation between the Fc regions. (B) 3 μg of affinity-purified Met-Fc (lane 2) and KDR-Fc (lane 3) were resolved by SDS-polyacrylamide gel electrophoresis under non-reducing conditions and visualised with Coomassie Blue. Molecular mass markers are shown in lane 1.

transiently transfected COS cells (see Materials and methods). In all our experiments we used KDR-Fc fusion protein as a control. A schematic representation of the Met-Fc chimeric construct is shown in Fig. 1A, and a Coomassie Blue-stained polyacrylamide gel run under non-reducing conditions showing purified forms of Met-Fc (lane 2) and KDR-Fc (lane 3) chimeric proteins are shown in Fig. 1B. The approximate molecular mass of the Met-Fc, deduced from electrophoretic mobility under non-reducing conditions, is 190 kDa. This corresponds to a form consisting of a dimer of the metalloprotease, disintegrin, EGF-like and cysteine-rich domains of metargidin fused to the hinge region and the two constant Fc domains of human IgG1. Under reducing conditions the construct ran as a single band of about 95 kDa (data not shown).

Mn^{2+} regulation, cation and temperature dependence of metargidin mediated cell adhesion

In order to investigate the binding properties of metargidin, we performed solid-phase cell adhesion assays with a panel of human cell lines, representing different cell lineages: MOLT-4 (T cell), U937 and HL-60 (pro-monocyte) and K562 (erythroleukemic). Of the cell lines screened, two (U937 and MOLT-4) bound constitutively to Met-Fc (Fig. 2A). Binding of metargidin was specific, since the control KDR-Fc protein did not bind to any of the cell lines tested.

To further investigate whether metargidin was binding to an integrin we included Mn^{2+} in our cell adhesion assays. This is because Mn^{2+} is a highly potent effector of several integrin-mediated adhesive events. It can 'activate' many integrins (Gailit and Ruoslahti, 1988; Karecla et al., 1995; Smith et al.,

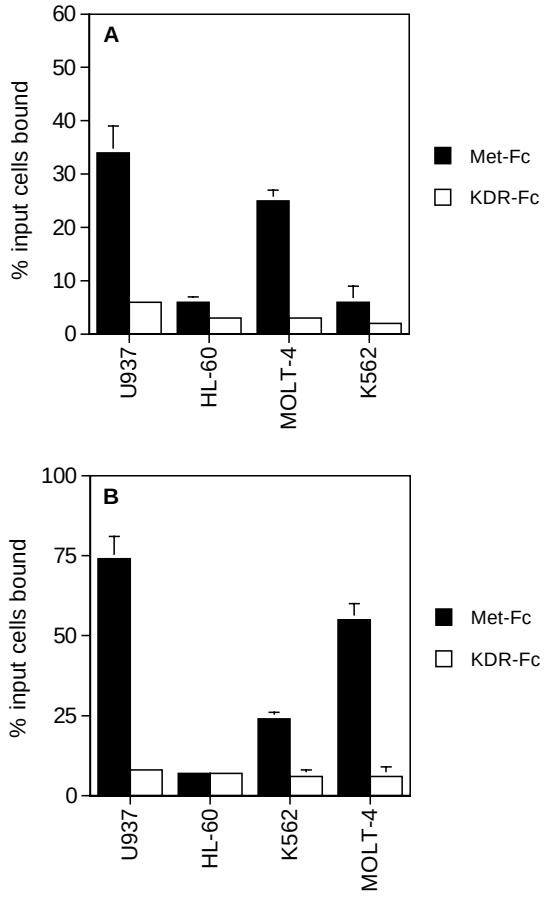

Fig. 2. Solid-phase cell binding assay. The Fc fusion proteins at 3 μg/ml, were adsorbed onto microtiter plates precoated with goat anti-human IgG. Binding assays were carried out with four haemopoietic cell lines (HL-60, U937, MOLT-4 and K562). (A) Cells were resuspended in HBSS buffer (0.5 mM Ca^{2+} and 0.5 mM Mg^{2+}) and added to wells containing immobilised Fc fusion proteins as described in Materials and methods. (B) Mn^{2+} regulation of cell binding. Cells were incubated in HBSS buffer containing $MnCl_2$ for 10 minutes prior to adding to wells containing immobilised Fc fusion proteins. Bound cells were quantified by measuring the amount of Methylene Blue incorporated by the cells. Background adhesion is to a control protein, KDR-Fc. Results are expressed as the percentage of total input cells bound ± s.d. (*n*=3).

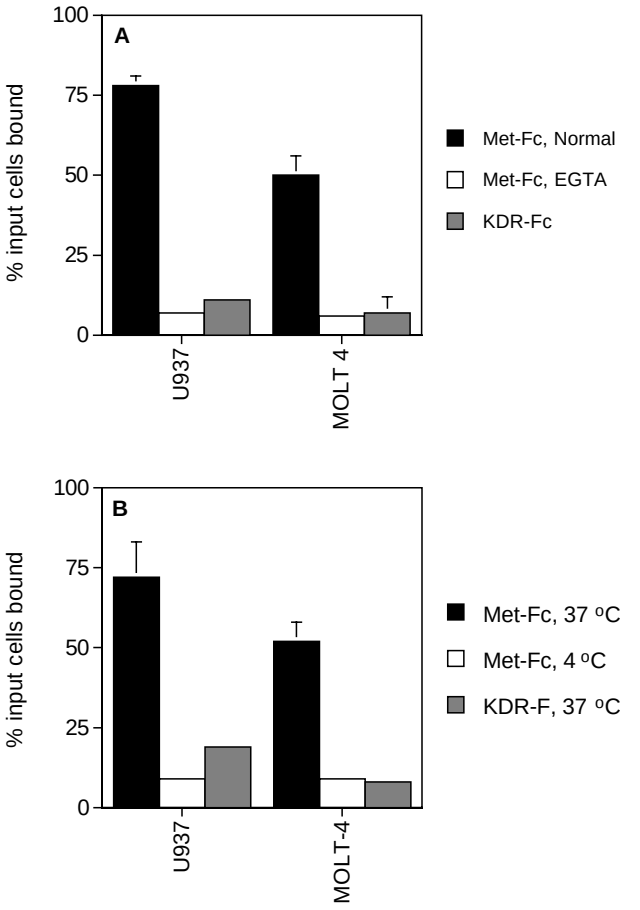

Fig. 3. Cation and temperature dependence of U937 and MOLT-4 cell binding to Met-Fc. Solid-phase cell adhesion assays were carried out as described in the legend to Fig. 2. (A) Divalent cation dependence. Cells were incubated in assay buffer alone (0.5 mM Ca^{2+}, 0.5 mM Mg^{2+}, 1.0 mM Mn^{2+}) or washed twice in assay buffer containing 2 mM EGTA and then assayed in the same buffer containing 2 mM EGTA. (B) Temperature dependence. Adhesion of U937 and MOLT-4 cells to Met-Fc either at 37°C or 4°C in assay buffer containing (0.5 mM Ca^{2+}, 0.5 mM Mg^{2+}, 1.0 mM Mn^{2+}). For both A and B, the background level of binding is indicated by adhesion to KDR-Fc. Results are expressed as the percentage of total input cells bound ± s.d. (*n*=3).

1994; Buckley et al., 1996), facilitating weak adhesive events, or in some cases, unmasking binding functions that could not be measured previously (Masumoto and Hemler, 1993). After pretreatment of the cells with Mn^{2+} there was a significant increase (two- to threefold) in binding of both cell lines to Met-Fc (Fig. 2B), whereas there was no binding to KDR-Fc control protein.

We confirmed that the binding of both cell lines (U937 and MOLT-4) was due to integrin function by demonstrating strict dependence on both divalent cations and temperature. There was complete inhibition of cell adhesion in the presence of 2 mM EGTA (Fig. 3A). In addition, there was no binding at 4°C (Fig. 3B).

Using FACS analysis we found that, of the cells studied, only the U937 line were expressing detectable levels of $\alpha_v\beta_3$. They also expressed α_5, α_6 and β_1 epitopes. MOLT-4, HL60 and K562 cells expressed significant amounts of α_5 and β_1 integrins and some α_4 integrin (data not shown).

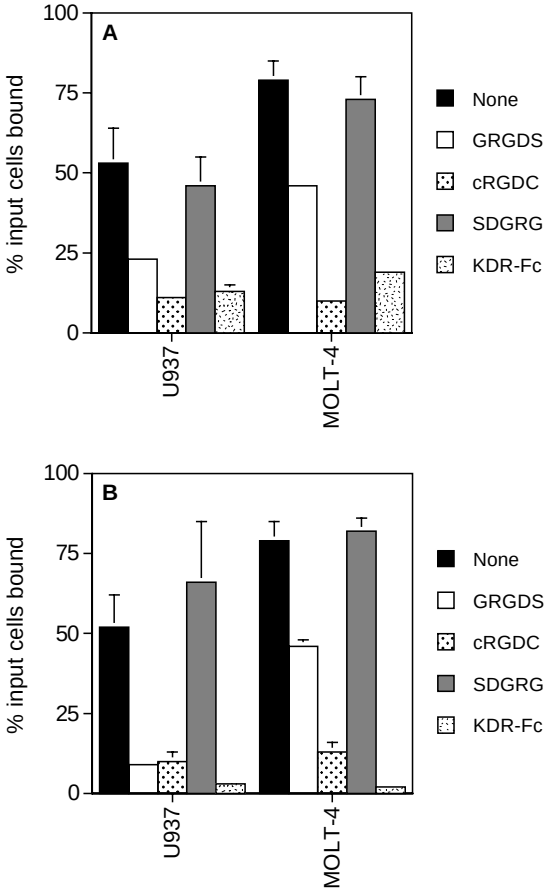

Fig. 4. RGD-dependent adhesion of U937 and MOLT-4 cells to Met-Fc. (A) Cells were treated with 1.0 mM $MnCl_2$ for 10 minutes and then incubated in the presence or absence of 0.2 mM peptides, GRGDS, SDGRG and cRGDC for 15 minutes, prior to plating onto wells coated with the Fc fusion proteins. (B) Cells were treated with 1.0 mM $MnCl_2$ for 10 minutes and then incubated in the presence or absence of 1.0 mM peptides, GRGDS, SDGRG and cRGDC. Results are expressed as the percentage of total input cells bound ± s.d. (n=3).

Adhesion of metargidin to U937 and MOLT-4 cells is dependent on the tripeptide RGD

It is well established that short synthetic peptides containing the RGD integrin-binding motif can mimic the binding activity of integrin ligands (reviewed by Ruoslahti, 1996). They can promote cell adhesion when immobilized onto a surface, and inhibit it when presented to cells in solution. Such peptides have been very useful in determining the specificities of integrin-ligand integractions and their functions in biological systems (reviewed by Humphries, 1994 and Ruoslahti, 1996). The functional RGD motif in integrin ligands is thought to exist in a favorable conformation at the apex of a long loop between two β strands. Hence, the affinity of cyclic peptides is several fold more than linear peptides.

In order to determine whether the binding of metargidin to U937 and MOLT-4 was RGD-dependent, we tested the ability of linear and cyclic RGD based synthetic peptides to inhibit cell binding. We selected the linear peptides Gly-Arg-Gly-Asp-Ser (GRGDS), and the reverse Ser-Asp-Gly-Arg-Gly (SDGRG) as a negative control. Since metargidin contains the RGDC integrin binding motif, we also tested the effect of a cyclic peptide Ac-Pen-Arg-Gly-Asp-Cys-OH (cRGDC) in our adhesion assays. We found that the GRGDS partially inhibited binding at 0.2 mM and completely inhibited it at 1 mM, whilst cRGDC peptides completely inhibited binding of U937 cells to metargidin at both concentrations (Fig. 4A,B). The binding of MOLT-4 cells was inhibited completely by the cyclic peptide cRGDC, but only partially (60% reduction) by both concentrations of the linear GRGDS peptide. The negative control peptide SDGRG had no effect on cell binding to any of the cell lines, thus confirming the specificity of the GRGDS

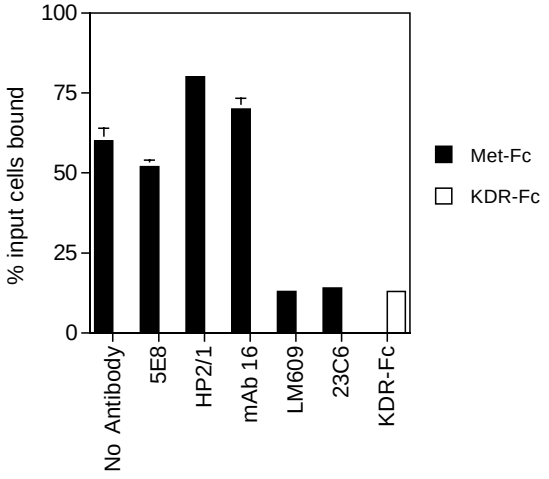

Fig. 5. Antibodies to $\alpha_v\beta_3$ block adhesion of U937 to Met-Fc. Adhesion of U937 to Met-Fc in the absence or presence of a panel of blocking mAbs to integrins. Cells were incubated in HBSS buffer containing 1.0 mM $MnCl_2$ for 10 minutes and then with mAbs at 10 μg/ml for 20 minutes at room temperature before the adhesion assay (Materials and methods). mAbs were present throughout the assay. The mAbs were: anti-α_2 integrin 5E8, anti-α_4 integrin HP2/1, anti-α_5 integrin mAb 16, anti-$\alpha_v\beta_3$ integrin LM609 and anti-$\alpha_v\beta_3$ integrin 23C6. Background binding is represented by binding to KDR-Fc. Results are expressed as the percentage of total input cells bound ± s.d. (n=3).

and cRGDC peptides. These results suggest that binding of metargidin to both cell lines was RGD-dependent and hence mediated by the disintegrin domain of metargidin.

Adhesion of U937 cells to metargidin is inhibited by antibodies to the integrin $\alpha_v\beta_3$

To identify the integrin that binds metargidin on U937 cells, we screened a panel of anti-integrin adhesion blocking antibodies for their ability to inhibit cell binding to metargidin. Only antibodies to $\alpha_v\beta_3$ (LM609 and 23C6) were able to completely inhibit cell adhesion to background levels (Fig. 5). Blocking antibodies to other integrins (alpha 2, 4 and 5) had no effect on binding. These results demonstrated that $\alpha_v\beta_3$ on U937 cells was mediating binding to Met-Fc. mAbs LM609 and 23C6 did not block binding of MOLT-4 to metargidin (data not shown), suggesting that another integrin was involved in these interactions.

A375M melanoma cells bind to metargidin via $\alpha_v\beta_3$

In order to test whether metargidin can mediate binding to non-haemopoietic cells via $\alpha_v\beta_3$ integrin, we analysed the binding activity of a human melanoma line A375M that is known to express high levels of $\alpha_v\beta_3$ (Marshall et al., 1991). As shown in Fig. 6, these melanoma cells bind to Met-Fc in a specific manner and this binding was blocked by the LM609 mAb. Thus, metargidin can bind to $\alpha_v\beta_3$ on haemopoietic and non-haemopoietic cells. To show unequivocally that metargidin can interact with $\alpha_v\beta_3$ we employed a solid-phase adhesion assay in which we examined the binding of Met-Fc to purified $\alpha_v\beta_3$. As shown in Fig. 7, metargidin can bind to $\alpha_v\beta_3$ specifically whereas a negative control KDR-Fc protein did not bind. Furthermore, the blocking $\alpha_v\beta_3$ mAb, LM609, inhibited binding, confirming the specificity of the metargidin/$\alpha_v\beta_3$ interaction.

Adhesion of MOLT-4 cells to metargidin is inhibited by antibodies to the integrin $\alpha_5\beta_1$

To identify the integrin responsible for binding to MOLT-4, we screened a panel of anti-integrin blocking mAbs in solid-phase cell adhesion assays. An antibody to β_1 integrin (mAb 13), completely abolished cell binding to metargidin (Fig. 8). We then screened different anti-alpha chain blocking antibodies and found that an anti-α_5 antibody (mAb 16) inhibited cell binding by approximately 70% (Fig. 8). Antibodies against α_2, α_3, α_4, α_v and α_6 had no effect on cell binding. We also tested these antibodies either in combinations of two or three antibodies together for their ability to completely abrogate cell binding. However, none of

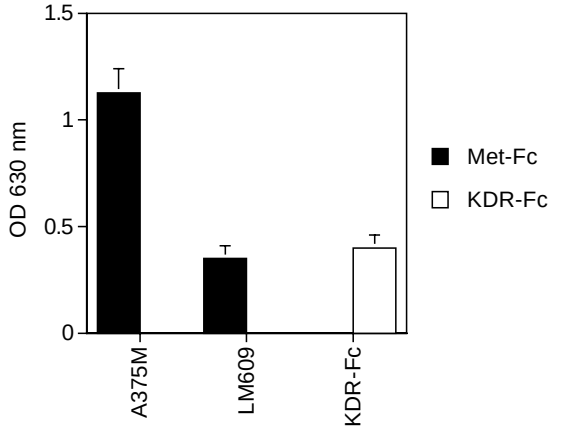

Fig. 6. Binding of Met-Fc to A375M melanoma cells via $\alpha_v\beta_3$. Cell adhesion assays to A375M cells were carried out in the presence or absence of an anti-$\alpha_v\beta_3$ mAb, LM609. Assays were carried out as described in the legend to Fig. 5. Results are expressed as the mean (OD_{630nm}) ± s.d. (n=3).

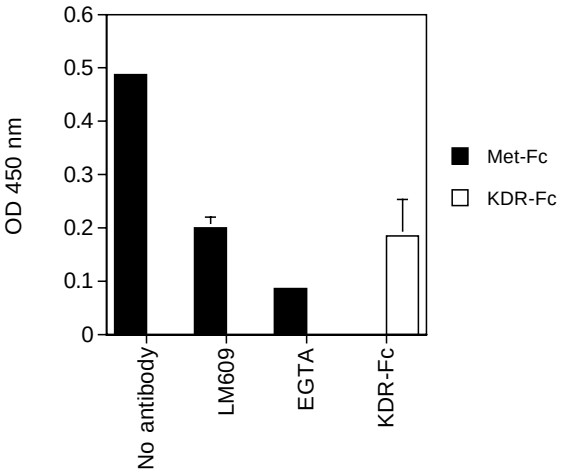

Fig. 7. Met-Fc binds to purified immobilized $\alpha_v\beta_3$. Met-Fc and the control protein, KDR-Fc, were added to wells coated with $\alpha_v\beta_3$ in the presence or absence of the $\alpha_v\beta_3$ mAb LM609 (10 μg/ml) and incubated for 3 hours at 30°C. A peroxidase-conjugated anti-human Fc antibody was used to detect bound Fc proteins. Results are expressed as the mean (OD_{450nm}) ± s.d. (n=6).

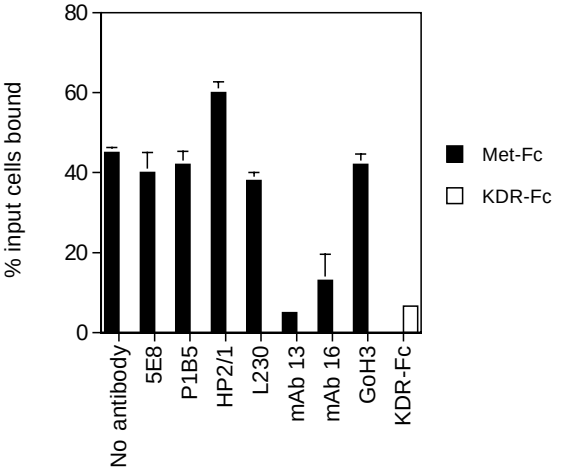

Fig. 8. Antibodies to α_5 and β_1 inhibit adhesion of MOLT-4 cells to Met-Fc. Cell adhesion assays were carried out as described in the legend to Fig. 5. The mAbs were: anti-α_2 integrin 5E8, anti-α_3 integrin P1B5, anti-α_4 integrin HP2/1, anti-α_v integrin L230, anti-β_1 integrin mAb 13, anti-α_5 integrin mAb 16 and anti-α_6 integrin GoH3. Results are expressed as the percentage of total input cells bound ± s.d. (n=3).

the combinations tested decreased binding any further than mAb 16 used alone (data not shown).

To provide additional evidence for a specific interaction between metargidin and $\alpha_5\beta_1$, we examined the binding of Met-Fc to purified recombinant $\alpha_5\beta_1$ using a solid-phase assay. As shown in Fig. 9, Met-Fc bound to $\alpha_5\beta_1$ at higher levels than the control protein KDR-Fc. A blocking antibody to α_5 reduced binding to background levels, as did 2 mM EGTA.

DISCUSSION

In this study we report that metargidin can mediate cell adhesion by binding to more than one integrin, depending on the type of cell involved. Using an Fc fusion protein of metargidin and a combination of biochemical and antibody blocking assays we show that metargidin can bind to $\alpha_v\beta_3$ on U937 cells and $\alpha_5\beta_1$ on MOLT-4 cells. Direct evidence for the specificity of the metargidin/$\alpha_v\beta_3$ and $\alpha_5\beta_1$ interaction was obtained by solid-phase adhesion assays. Furthermore, binding to both cell lines was mediated at least in part by the disintegrin domain of metargidin, as RGD-based synthetic peptides completely abrogated cell binding.

To our knowledge this is the first report describing the interaction of metargidin with both $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins. In this respect metargidin is similar to the neural cell adhesion molecule L1, which also binds to $\alpha_v\beta_3$ and $\alpha_5\beta_1$ in an RGD-dependent manner (Ruppert et al., 1995; Montgomery et al., 1996; Felding-Habermann et al., 1997). It will be interesting to see whether metargidin can bind to other integrins with RGD recognition sites and whether other ADAMs can interact with more than one integrin. Very recently, another laboratory expressed the isolated disintegrin domain of metargidin as a fusion with glutathione S-transferase and examined its binding activity to a panel of CHO cell lines expressing different integrins (Zhang et al., 1998). Using this bacterially expressed protein they demonstrated binding to $\alpha_v\beta_3$, which is in agreement with our findings. Interestingly, they failed to demonstrate binding of this construct to other integrins, including $\alpha_5\beta_1$. Since the gst fusion protein was produced in *E. coli*, evidence of its correct folding is required before concluding that it is representative of the native protein. Our recombinant construct was produced in eukaryotic cells and contained all the extracellular domains and is therefore more likely to represent the native form. It bound to $\alpha_v\beta_3$ on two cell types expressing this integrin, U937 and A375M cells. MOLT-4 cells bound at least in part through $\alpha_5\beta_1$, but other cells expressing $\alpha_5\beta_1$ (K562 and HL60) did not bind. It is not clear why there is a difference between different cellular sources of $\alpha_5\beta_1$ since the purified recombinant integrin bound to Met-Fc. It has been observed that $\alpha_6\beta_1$ integrin binding to the ADAM 1 and 2 (fertilin α and β) heterodimer, occurs when the integrin is in a form that does not bind to its natural ligand, laminin. We conclude that the integrins may adopt different conformations for binding to individual ligands. The modifications to cellular $\alpha_5\beta_1$ which allow binding to metargidin will require further analysis.

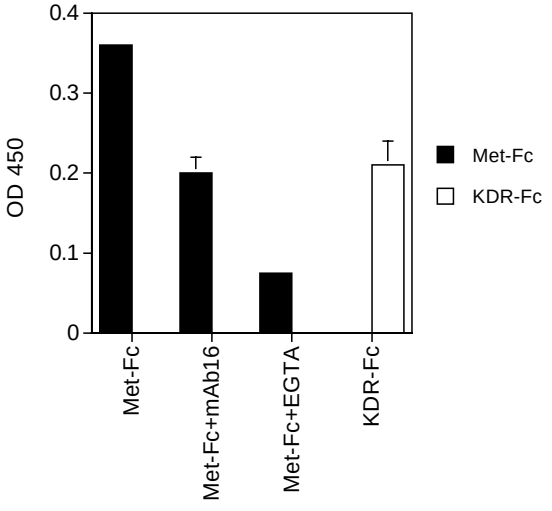

Fig. 9. Met-Fc binds to purified immobilized $\alpha_5\beta_1$. Met-Fc and the control protein were added to wells coated with $\alpha_5\beta_1$ in the presence or absence of 2 mM EGTA or the α_5 blocking antibody mAb 16 (10 μg/ml) and incubated for 3 hours at 30°C. A peroxidase-conjugated anti-human Fc antibody was used to detect bound Fc proteins. Results are expressed as the mean (OD_{450nm}) ± s.d. (n=6).

As a general rule, all integrins require divalent cations for ligand recognition and it is now well-documented that different divalent cations can influence integrin affinity for their ligands and determine ligand specificity. We observed that in the presence of Ca^{2+} and Mg^{2+}, U937 and MOLT-4 cells bound to metargidin, suggesting that $\alpha_v\beta_3$ and $\alpha_5\beta_1$ on these cells, respectively, is expressed in an already activated state. These integrins could be further activated by Mn^{2+} ions, which are a well-established external activator of integrin adhesion (Karecla et al., 1995). There is now a structural basis for Mn^{2+} binding to integrins because a novel Mn^{2+}-binding site with micromolar affinity has been identified on the α_M subunit and a homologous site in the β_3 subunit (Smith et al., 1994).

$\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins appear to bind to the RGD motif in metargidin, since RGD-based peptides inhibited cell binding in all the cell lines. We used a GRGDS peptide, based on the fibronectin domain, and a cyclic RGDC peptide, based on the metargidin sequence. Both peptides blocked cell binding in a specific manner. The conformation or stereochemical presentation of the RGD motif is a key element in dictating receptor recognition and specificity. Structural studies of RGD-containing domains, like the tenth type-III repeat from fibronectin, the disintegrin Echistatin and foot-and-mouth disease virus (FMDV), reveal that the RGD sequence is present at the apex of a long flexible loop and is thereby accessible for high-affinity interactions with integrins (Main et al., 1992; Brockel et al., 1992). Unlike the snake venom disintegrins, in the disintegrin domain of the ADAMs there is an additional cysteine residue after the integrin-binding motif (RGDC, in the case of metargidin). It is not known whether this cysteine forms an inter-or intra-domain disulphide link with another Cys. If the Cys does form a linkage, then the conformation of this region in the ADAMs will probably be more constrained than the corresponding region in the snake venom disintegrins. It is interesting to note that mouse metargidin does not contain an RGD sequence, nor do many of the mammalian and snake disintegrin domains. A number of these are known to constitute a flexible loop and have integrin binding properties. The precise interaction motif remains to be defined, however.

The interaction of metargidin with $\alpha_v\beta_3$ may be important in several biological processes, since $\alpha_v\beta_3$ has been implicated in diverse functions including angiogenesis, tumour cell metastasis and osteoporosis. $\alpha_v\beta_3$ is expressed by various cell types and has several ligands, including both ECM molecules like vitronectin, osteopontin, laminin and fibronectin and cell surface proteins like CD31 and L1 (Cheresh, 1992; Brooks et al., 1994; Piali et al., 1995; Buckley et al., 1996; Montgomery et al., 1996). The finding that metargidin can bind to melanoma cells via $\alpha_v\beta_3$ is interesting because on these cells increased expression of $\alpha_v\beta_3$ by these cells is positively correlated with increased malignancy (Albeda et al., 1991; Montgomery et al., 1994). It has been shown that metastatic cells adhere to the vascular endothelium before they can reach the subendothelial matrix, containing the ligands for $\alpha_v\beta_3$. Since metargidin is expressed on endothelial cells (Herren et al., 1997), it is therefore conceivable that metargidin/$\alpha_v\beta_3$ interactions may be important in the adhesion of tumour cells to endothelium.

Our finding that metargidin can bind to both $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins expands the potential repertoire of the adhesive interactions that metargidin can support and the range of cell types that may be involved. Though we have focussed only on haemopoietic and melanoma cells, both these integrins and metargidin are expressed on multiple cell types of diverse origins. Thus, there is a potential for several metargidin-mediated interactions and functions that have yet to be described.

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