Meltrin γ (ADAM-9) mediates cellular adhesion through $\alpha_6\beta_1$ integrin, leading to a marked induction of fibroblast cell motility

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This paper is dedicated to the memory of Dr R. M. Warn

SUMMARY

The ADAMs (<u>A</u> <u>D</u>isintegrin and <u>M</u>etalloprotease Domains) are a family of membrane-anchored proteins that play a role in fertilisation, myoblast fusion and ectodomain shedding of cell surface proteins. Meltrin γ (ADAM-9) is a widely expressed member of this family and is involved in the shedding of heparin binding epidermal growth factor. Here we report that meltrin γ can function as a cell adhesion molecule via its disintegrin domain. Using solid-phase binding assays and antibody inhibition experiments, we demonstrate that a murine meltrin γ-Fc (Melγ-Fc) fusion protein binds to the integrin $\alpha_6\beta_1$ on the surface of fibroblast cell lines, HT1080 and Wehi 164 in a specific manner. Since $\alpha_6\beta_1$ is important for the motility of several cell types on laminin, cell migration studies using time-lapse video microscopy were performed. Cells adhering to Melγ-Fc displayed a rounded morphology and a marked increase (eight- to tenfold) in their motility compared to that on laminin. Furthermore, the p160 ROCK kinase inhibitor Y-27632 specifically reduced the migration of cells on meltrin γ but had no effect on migration of cells on laminin, whilst the general tyrosine phoshorylation inhibitor, genistein, inhibited cell migration on both substrates. These results together suggest that meltrin γ may play a role in regulating the motility of cells by binding to $\alpha_6\beta_1$ integrin and this may be important during a variety of biological and pathological processes.

Key words: ADAM, Integrin, Cell migration, Fibroblast

INTRODUCTION

The ADAMs (<u>A</u> <u>D</u>isintegrin and <u>M</u>etalloprotease Domain) are a growing family of multifunctional proteins thought to be important in a wide range of biological processes such as cell adhesion, cell fusion and proteolysis. In general, they are type I transmembrane glycoproteins comprising an N-terminal metalloprotease domain, a disintegrin domain, a cysteine-rich region and an EGF-like domain (reviewed by Blobel, 1997; Black and White, 1998). They frequently have large cytoplasmic tails with putative signalling motifs. To date, there are at least 30 transmembrane ADAMs that are known and expressed throughout the animal kingdom such as *Caenorhabditis elegans*, *Drosophila melanogaster*, *Xenopus laevis*, mice, hamster and humans (Wolfsberg et al., 1993, 1995a,b; Weskamp and Blobel, 1994; Wolfsberg and White, 1996; Fambrough et al., 1996; Rooke et al., 1996; Kratzschmar et al., 1996; Black et al., 1997; Kuno et al., 1997; Blobel, 1997; Alfandari et al., 1997; Black and White, 1998; Inoue et al., 1998). The protease and disintegrin of domains of the ADAMs are homologous to snake venom metalloproteases (SVMPs), which have the ability to degrade basement membrane components and bind to integrins, hence disrupting cell-matrix interactions (reviewed by Wolfsberg and White, 1996).

Integrins can bind to components of the extracellular matrix such as laminin, fibronectin, collagen etc. as well as to other cell surface molecules such as IgSF (immunoglobulin superfamily) members and cadherins (reviewed by Hynes, 1992; Humphries, 1996).

The best characterised ADAM to date is the fertilin α/β (ADAM 1,2) complex expressed on the surface of sperm cells. During the process of fertilisation the fertilin complex interacts with the integrin $\alpha_6\beta_1$ expressed on the surface of egg cells and mediates sperm-egg adhesion and fusion (Almeida, 1995; Chen et al., 1999). This adhesive interaction is mediated by the disintegrin domain of fertilin β, since peptides containing the putative integrin binding motif ECD present within this domain inhibit sperm-egg binding (Chen and Sampson, 1999). Several in vitro and in vivo studies have shed light on the biological functions of other ADAMs. Thus, ADAM-12 is important for myoblast fusion (Yagami-Hiromasa et al., 1995), TACE (TNF α converting enzyme; ADAM-17) is responsible for the proteolytic processing of pro TNF-α to TNF-α, shedding of TRANCE, L-selectin and several other classes of cell surface proteins (Black et al., 1997; Peschon et al., 1998; Lum et al., 1999) and ADAM 10 plays a role during neurogenesis in *Drosophila melanogaster*.

$\alpha_6\beta_1$ integrin is a receptor for laminins and is expressed from early stages of mouse embryogenesis in organs such as the myocardium, developing epidermis, lens, gonads and a few epithelia (Delwel et al., 1993; Hierck et al., 1993; Salanova et al., 1995; Thorsteinsdottir et al., 1995). In the adult mouse, it is expressed in a variety of epithelial tissues, fibroblasts, Schwann cells and platelets (Hogervorst et al., 1993). There are several lines of evidence demonstrating that $\alpha_6\beta_1$ can induce motility of cells on laminin and this has been shown for Langherhan cells (Price et al., 1997), fibroblasts (Jasiulionis et al., 1996), neural precursor cells (Jacques et al., 1998), melanoma cells (Hangan et al., 1997), lymphocytes (Gimond et al., 1998) and macrophages (Shaw and Mercurio, 1994).

The ability of cells to migrate is crucial for biological processes such as embryonic development and tissue remodelling as well as pathological processes such as cancer invasion and wound healing. Integrin-mediated migration over extracellular matrix (ECM) is accompanied by characteristic morphological changes such as the formation of lamellipodia, which appear as membrane protrusions at the cells leading edge (reviewed by Sheetz et al., 1998; Sanchez-Madrid and del Pozo, 1999). The driving force for cell movement is provided by the dynamic reorganisation of the actin cytoskeleton, directing protrusion at the front end of the cell and retraction at the rear end. Situations where cells are migrating across other cells, such as tumour cell or leukocyte interactions with vascular endothelium, generally involve more labile contact regions and the migration driving force is mediated by transient adhesive cell-cell junctions. In principle this depends on the biomechanics of haptokinetic migration across surfaces, with the difference that cell-cell adhesion receptors, eg. VCAM-1 and $\alpha_4\beta_1$ or $\alpha_4\beta_7$, or LFA-1 and ICAM-1, are involved. It appears that the Rho family of GTPases including RhoA are involved in both cases (Hall, 1998; Laudanna et al., 1996). Rho activity appears to be regulated by a family of Rho-associated serine/threonine kinase enzymes named p160ROCK and ROCK-II, which can induce focal adhesions and stress fibres in adherent cells such as fibroblasts and epithelial cells. A specific inhibitor of p160ROCK named Y-27632 can inhibit the formation of stress fibres and focal adhesions in cultured cells by inhibition of the phosphorylation of myosin (Amano et al., 1996). Recent studies have shown that Y-27632 can inhibit contraction of smooth muscle cells (Seasholtz, 1999), prevent neurite retraction (Hirose et al., 1998), reduce migration of neutrophils (Niggli, 1999), and reduce the invasiveness of tumour cells (Itoh et al., 1999), which suggests that these enzymes are involved both in cell-ECM and cell-cell based migratory mechanisms.

Meltrin γ (ADAM-9) is a widely expressed protein and has been implicated in the shedding of HB-EGF (heparin binding-epidermal growth factor) from epithelial cells (Izumi et al., 1998). Its cytoplasmic tail contains potential SH3 binding motifs and can be phoshorylated by protein kinase C (PKC). The disintegrin domain of meltrin γ contains an ECD (Glu-Cys-Asp) integrin binding motif, which is also present in fertilin β. So far, integrin ligands have identified for only two ADAMs, fertilin β and ADAM-15 (Almeida et al., 1995; Zhang et al., 1998; Nath et al., 1999). Since meltrin γ is expressed on the cell surface (Roghani et al., 1999), it can potentially be involved in cellular interactions.

In this report we show that meltrin γ can mediate cell adhesion to the fibroblast cell lines Wehi 164 and HT1080 by binding specifically to the integrin $\alpha_6\beta_1$ via its disintegrin domain. Furthermore, we examined the ability of fibroblasts to utilise $\alpha_6\beta_1$ in cell migration on laminin and meltrin γ. An in vitro system to quantitatively measure fibroblast migration on purified ligands was used in order to eliminate the contribution of other adhesion mechanisms. We demonstrate that when fibroblasts were plated on meltrin γ there was a dramatic change in their morphology and in the induction (8-10 fold) of their motility compared to plating on laminin. Meltrin γ-enhanced motility of the cells could be reduced by a synthetic inhibitor (Y-27632) of p160 ROCK kinase, the downstream effector of Rho A. To our knowledge this is the first demonstration that meltrin γ binds to an integrin and that this interaction can lead to a marked induction in cell motility.

MATERIALS AND METHODS

Unless otherwise stated all reagents were purchased from Sigma (St Louis, USA).

Cell lines and reagents

The murine cell lines Wehi 164 (fibrosarcoma), EL4 (T cell lymphoma) and L929 (fibroblast) were from the European Tissue Culture Collection. HT1080 k2p (human fibrosarcoma) was obtained from Celltech Therapeutics, UK. Wehi 164 cells were maintained in RPMI containing 10% foetal calf serum (FCS). HT1080 k2p, EL4 and L929 cells were grown 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. Y-27632 was a generous gift from Yoshitomi Pharmaceutical Ind, Koyata, Japan.

Antibodies

The following anti-integrin blocking monoclonal antibodies (mAbs) against human integrins were used. Anti-α_2 integrin clone 5E8 (a kind gift from Dr R. Bankert, Roswell Park Cancer Institute, Buffalo, NY, USA); anti-α_3 clone P1B5 (Chemicon International, Harrow, UK); anti-α_5 clone mAb16 (Becton and Dickinson); anti-α_6 clone GoH3 (Serotech, Oxford, UK); anti-β_1 integrin clone mAb 13 (Becton and Dickinson, UK). The following anti-integrin blocking antibodies against murine integrins were used: anti-α_4 clone CRL1911; anti-α_E clone M290; anti-α_M clone M1/70 and anti-α_L clone TIB217 (gifts from Dr Anthony Shock, Celltech Therapeutics, UK). A blocking antibody against murine β_1 integrin, mAb 9EG7, was a kind gift from Prof. Dietmar Vestweber (Frieburg, Germany) and was also purchased from Serotech Ltd, Oxford, UK. Polyclonal goat anti-human-Fc Ig was obtained from Sigma (St Louis, USA).

Recombinant fusion protein constructs

Construction of Melγ-Fc

A full-length meltrin γ cDNA clone was generated using a combination of colony hybridisation and 5' RACE techniques, from an NS0 cDNA library (Amour et al., 1998). This was used as a PCR template in order to generate a gene fragment encoding the extracellular domain of meltrin γ. The 5' primer introduced a unique *Xba*I site upstream of the initiating methionine codon ATG and the 3' primer introduced a *Sal*I site at nucleotide 2107. The resultant *Xba*I-*Sal*I fragment was cloned into the eukaryotic expression vector pEE12.2 (Nath et al., 1999). This results in the formation of a gene fusion encoding the whole ectodomain sequence, i.e. pre pro

catalytic, disintegrin, cysteine-rich and EGF-like domains of meltrin γ, fused to the hinge and CH2 and CH3 domains of human IgG1 (the Fc portion). The sequence D697G698VD was generated at the junction (numbering according to reference 50; accession no. U41765).

Melγ-Fc protein was generated either transiently from COS-7 cells or stably from NS0 cells, essentially as described previously (Nath et al., 1999; Amour et al., 1998), and both products were essentially the same. Neural cell adhesion molecule-Fc (designated as NCAM-Fc) was a kind gift from Dr Christopher Buckley (University of Birmingham, UK).

Cell adhesion assays

Cell adhesion assays to soluble chimeric fusion proteins were carried out as described previously (Nath et al., 1999), 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 different concentrations. Prior to binding assays, cells were resuspended in HBSS + 0.25% BSA + 1 mM $MnCl_2$ to a concentration of 2×10^6 cell/ml, and 50 µl samples 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 non-adherent 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 10 minutes at room temperature prior to plating and included throughout the binding assay.

Based on the disintegrin loop sequences of meltrin γ, the synthetic peptide Lys-Thr-Ser-Glu-Cys-Asp-Val-Pro (KTSECDVP) as well as a scrambled control peptide Val-Lys-Asp-Thr-Cys-Glu-Ser-Pro (VKDTCESP) were synthesised by Sigma-Genosys. The peptides were dissolved in HBSS and stored at −20°C prior to use. In adhesion assays, cells were preincubated with the peptides at a concentration of 0.1 mM 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 and standard error of three replicates.

Cell migration assays

Cell migration assays were performed in 4-well tissue culture dishes. Wells were coated with laminin at 10 µg/ml and Melγ-Fc was coated onto wells that had already been preadsorbed with anti-human Fc antibody overnight at 4°C. Non-specific sites were blocked with PBS containing 1 mg/ml heat-denatured BSA for 1 hour at room temperature. Melγ-Fc was added at a concentration of 1.5 µg/ml for 3 hours at 37°C. HT1080 k2p and Wehi 164 cells were harvested using PBS/EDTA, washed twice with L15 (air-buffered medium, Sigma) and resuspended in L15 containing 1 mM $MnCl_2$. Approximately 5×10^4 cells were added to wells coated with Melγ-Fc and were allowed to adhere for 1-2 hours at 37°C, after which the non-adherent cells were removed by washing. The dishes were then placed on a microscope stage heated to 37°C. For image analysis, a Nikon eclipse TE300 microscope with phase contrast optics was used. This microscope was connected to a Colour Video Camera (JVC, TK13260) to capture the images. Images were collected for 10 hours at intervals of 2 minutes for cell on Melγ-Fc and 5 minutes for laminin,

and the migration paths were calculated using the Lucia32G/Magic4.11 computer software. Migration paths were plotted and the total distance of migration was calculated using the Lucia G (version 4.11) software.

In some experiments, the tyrosine kinase inhibitor genistein (Calbiochem) at 10 µM (10 mM stock in DMSO) was added to the cells for 30 minutes prior to plating onto either laminin or Melγ-Fc coated wells. Similarly, Y-27632 (1 mM stock in PBS), the p160 ROCK kinase inhibitor was added to the cells at a concentration of 10 µM, 30 minutes prior to plating onto the wells. The inhibitors were present throughout the course of the experiment.

Flow cytometry

Cells were washed with PBS, detached with 2 mM EDTA and resuspended in ice-cold PBS + 0.5% BSA + 5 mM sodium azide (assay buffer). Cells were incubated with anti-integrin antibodies at a concentration of 10 µg/ml for 30 minutes on ice. For Wehi 164, EL4 and L929 cells, the following mAbs were used: anti-β_1 (clone 9EG7, rat anti-mouse), anti-α_6 (clone GoH3, rat anti-mouse/human). For HT1080 cells, anti-β_1 (clone mAb 13, rat anti-human) and anti-α_6 (clone GoH3, rat anti-human/mouse) were used. After incubation with the antibodies, cells were washed with assay buffer and incubated with the FITC-conjugated anti-rat IgG for 30 minutes on ice. Cells were washed once with assay buffer, followed by PBS and then fixed with 2% paraformaldehyde. Analysis was performed using the Coulter EPICS® ELITE cytometer.

Fluorescence staining of cell cultures

8-well chamber slides were coated with either Melγ-Fc or laminin as described in the cell adhesion assay section above. Cells were allowed to adhere for 1 hour at 37°C and non-adherent cells were removed by washing in PBS. For detection of actin filaments, cells were fixed with cold acetone for 10 minutes and incubated with fluorescence isothiocyanate-conjugated phalloidin for 20 minutes at room temperature. After the final wash with PBS, cells were mounted with Citifluor and examined under a Nikon eclipse E800 microscope with epifluorescence.

RESULTS

Production of murine Melγ-Fc fusion proteins

To identify a ligand for meltrin γ we used a recombinant chimeric protein consisting of the entire extracellular domain of meltrin γ fused to the Fc portion of human IgG1 (termed Melγ-Fc). The protein was purified from supernatants of either transiently transfected COS cells or from stably transfected NS0 cell lines using Protein A chromatography. In all our experiments we used NCAM-Fc as a negative control. A schematic representation of the Melγ-Fc construct is shown in Fig. 1A and a Coomassie-stained polyacrylamide gel run under reducing conditions showing the purified form of Melγ-Fc (90 kDa, lane 2) is shown in Fig. 1B. Under non-reducing conditions the recombinant protein migrates at approx. 180 kDa, as expected for a dimeric form of the fusion protein linked through disulphide bonds in the hinge of the Fc region (data not shown). The identity of the protein was confirmed by N-terminal sequencing as being Ala_{206}ValLeuProGln. Ala_{206} is the amino acid immediately after the furin cleavage site (Fig. 1A). Hence, this form corresponds to the metalloprotease domain followed by the disintegrin domain, the cysteine-rich region and the EGF domain of meltrin γ, and the hinge and constant (CH2 and CH3) region (the Fc portion) of human IgG1.
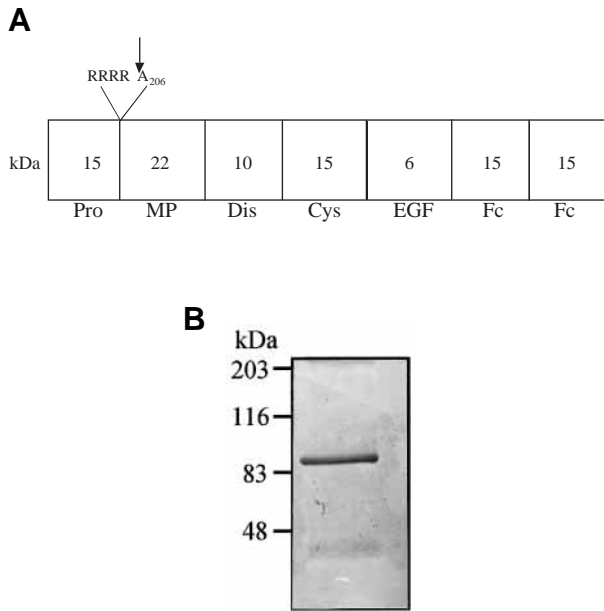
A

B

Fig. 1. Recombinant Melγ-Fc fusion protein. (A) Schematic representation of Melγ-Fc protein. Although drawn as a monomer, the Fc chimeric protein is a dimer as a result of disulphide formation between the Fc regions. The numbers within the boxes are the molecular mass in kDa of each domain. Pro, pre pro catalytic region; MP, metalloprotease domain; Dis, disintegrin domain; Cys, cysteine-rich region; EGF, EGF-like domain; Fc, Fc region og IgG1. (B) 5 μg of affinity purified Melγ-Fc protein was resolved by SDS-polyacrylamide gel electrophoresis under reducing conditions and visualised with Coomassie Blue. The positions of the molecular mass markers are shown at the left.

Melγ-Fc binds to fibroblast cell lines in a divalent cation-dependent manner

In order to identify cellular ligands for meltrin γ we screened a panel of cell lines: Wehi 164 (murine fibrosarcoma), EL4 (murine T cell), L929 (murine fibroblast) and HT1080 k2p (human fibrosarcoma), for their ability to bind to soluble recombinant Melγ-Fc protein immobilised onto plastic via anti-Fc polyclonal antibodies (Fig. 2). Amongst the cell lines screened for binding activity, two lines, Wehi 164 and HT1080 k2p, bound to Melγ-Fc in the presence of Ca^{2+}, Mg^{2+} and Mn^{2+} (Fig. 2A). Binding of the cells to Melγ-Fc was specific since the control protein NCAM-Fc did not bind. Binding to both cell lines occurred in a concentration-dependent manner with maximal adhesion at 1.0 μg/ml Melγ-Fc (Fig. 2B). We included divalent cations in our adhesion buffer because we hypothesised that meltrin γ would bind to an integrin, based on the fact that both fertilin β (ADAM-2) and metargidin (ADAM-15) bind to integrins (Almeida et al., 1995; Zhang et al., 1998; Nath et al., 1999). In addition to Ca^{2+} and Mg^{2+}, we also included Mn^{2+} in our assays because this ion can enhance the adhesive properties of many integrins, although the precise mechanism of this enhancement is not as yet clear. We did not observe significant cell binding in the absence of Mn^{2+} (data not shown), strongly suggesting that Melγ-Fc was binding to an integrin on Wehi 164 and HT1080 k2p cells.

To determine whether meltrin γ might be binding to an integrin we performed cell adhesion assays in the presence of
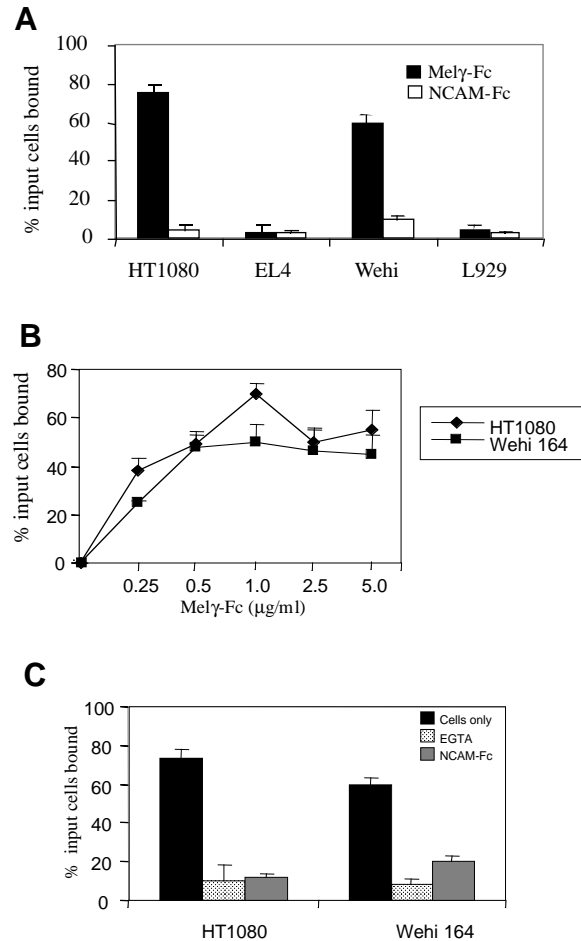
A

B

C

Fig. 2. Solid-phase cell binding assays. The Fc fusions at different concentrations were adsorbed onto microtiter plates precoated with goat anti-human IgG. (A) Melγ-Fc and NCAM-Fc were coated at 1.0 μg/ml concentration. Cell binding assays were performed with four cell lines: HT1080, Wehi 164, EL4 and L929 in HBSS buffer containing 0.5 mM Ca^{2+}, 0.5 mM Mg^{2+} and 1.0 mM Mn^{2+}, as described in Materials and Methods. NCAM-Fc is used as a negative control and represents background cell binding. (B) Dose-dependent binding of HT1080 and Wehi 164 cells to Melγ-Fc. Melγ-Fc protein was adsorbed at varying concentrations and cell binding assays were carried out in the presence of divalent cations as described in A. (C) Divalent cation-dependent cell binding. HT1080 and Wehi 164 cells were incubated in assay buffer alone (0.5 mM Ca^{2+}, 0.5 mM Mg^{2+} and 1.0 mM Mn^{2+}) or washed twice in assay buffer containing 2 mM EGTA. Binding to the control protein NCAM-Fc was done in the presence of all three divalent cations. Cell binding was measured by Methylene Blue staining and the results are expressed as the percentage of total cells bound ± s.d. (n=3).

2 mM EGTA. As shown in Fig. 2C, Wehi 164 and HT1080 k2p did not bind to Melγ-Fc in the presence of EGTA, thus demonstrating strict dependence on divalent cations for cell binding.

Melγ-Fc mediates cell adhesion via its disintegrin domain

It is well established that short synthetic peptides containing integrin binding motifs can be used to define the specificity of integrin-ligand interactions and their functions in biological
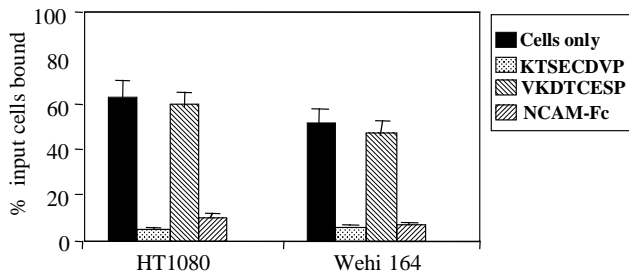
Fig. 3. A peptide (KTSECDVP) containing the putative integrin binding motif of meltrin γ inhibits cell adhesion. HT1080 and Wehi 164 cells in assay buffer (0.5 mM Ca^{2+}, 0.5 mM Mg^{2+} and 1.0 mM Mn^{2+}) were incubated in the presence or absence of 0.1 mM peptides, KTSECDVP and the control peptide, VKDTCESP, for 15 minutes at room temperature, prior to plating onto wells coated with 1 μg/ml Melγ-Fc. Binding to the control protein NCAM-Fc was performed without preincubation with peptides and represents background binding. Results are expressed as the percentage of total input cells bound ± s.d. (n=3).

systems. This approach has been particularly successful using RGD-based peptides to mimic the binding activity of integrin ligands. In addition, these peptides can be used to determine residues involved in binding the ligand. Human ADAM-15 is the only ADAM to date that has the classical RGD integrin binding tripeptide motif. Other ADAMs have a potential XXCD (X is any amino acid) motif within their disintegrin domain. Meltrin γ has an ECD motif, which is also present in ADAMs such as fertilin β and snake venom disintegrins such as atrolysin A. Using synthetic peptides it has been demonstrated that this motif is required for integrin binding by fertilin β and atrolysin A, and that the two acidic residues flanking the cysteine residue are important for binding.

In order to show that the ECD motif in meltrin γ may be involved in integrin binding we tested the ability of a linear peptide KTSECDVP to inhibit cell binding. We found that the binding of HT1080 and Wehi 164 to Melγ-Fc was inhibited when the cells were pretreated with the peptide at a concentration of 0.1 mM prior to the adhesion assays, as shown in Fig. 3. The negative control scrambled peptide, VKDTCESP, at the same concentration had no effect on cell binding (Fig. 3). The control Fc protein, NCAM-Fc, did not bind to the cell lines. These results suggest that the disintegrin domain of meltrin γ is involved in cell binding and may involve the ECD motif.

Adhesion to HT1080 k2p and Wehi 164 cells is inhibited by antibodies to α_6 and β_1 integrins

In order to identify the integrin that binds meltrin γ, we screened a panel of anti-integrin adhesion blocking antibodies for their ability to inhibit the binding of cells to Melγ-Fc. For both HT1080 and Wehi 164, only antibodies to α_6 and β_1 integrin were able to completely inhibit binding to background levels (Fig. 4A,B). Blocking antibodies to other integrin subunits (α_2, α_3 and α_5) did not affect binding of HT1080 cells to Melγ-Fc (Fig. 4A). For Wehi 164 binding, integrin antibodies to α_4, α_E and α_M subunits did not block binding (Fig. 4B). Anti-α_L antibody slightly reduced cell binding, but this was within experimental error. These results demonstrated that $\alpha_6\beta_1$ was the integrin ligand mediating cell binding to

Melγ-Fc. The negative control protein, NCAM-Fc, did not bind to either HT1080 or Wehi 164 cells (Fig. 4A,B).

By FACS analysis, we determined the expression levels of α_6 and β_1 integrin subunits in the cell lines (Fig. 4C). Both HT1080 and Wehi 164 cells expressed higher levels of α_6 integrin compared to the non-binding EL4 and L929 cells (see Fig. 4C legend). The levels of β1 integrin were relatively high in all the cell lines. These results suggest that the ability of HT1080 and Wehi 164 cells to bind to Melγ-Fc may possibly be due to increased expression levels of $\alpha_6\beta_1$ integrin.

Changes in fibroblast cell morphology that accompany adhesion on laminin and meltrin γ

When cells adhere to ECM-coated surfaces they form specialised adhesive structures that link them to the underlying matrix. In addition to establishment of certain actin-based motility structures such as microspikes and membrane ruffles, morphological changes involved in cell spreading also occur. Rho family proteins are known to be involved in regulating cytoskeletal actin organisation. In a recent study it was observed that tumour cells adhering to a recombinant form of the cysteine-rich domain of ADAM-12 also remain rounded and do not spread out (Iba et al., 1999); however, these tumour cells do not migrate significantly on ADAM-12.

We investigated whether there were any morphological differences between cells adhering to laminin versus Melγ-Fc. After 60 minutes adhesion, cells were stained for F-actin using FITC-conjugated phalloidin. Both HT1080 and Wehi 164 cell attached to laminin appeared flattened and spread out and had small, filopodia containing thin actin fibres projecting out from the cell body (Fig. 5A,C). In contrast, both cell types on Melγ-Fc displayed a rounded morphology and lacked thin actin fibres (Fig. 5B,D). Plating cells at a higher density of Melγ-Fc did not appear to enhance cell spreading or motility (data not shown).

Enhanced migration of HT1080 and Wehi 164 cells on Melγ-Fc compared to laminin

Since $\alpha_6\beta_1$ can induce the migration of cells on laminin, we undertook experiments to see whether ligation of the same integrin to meltrin γ could modulate cell motility. The fibroblast lines Wehi 164 and HT1080 were plated on laminin and Melγ-Fc and their migration was monitored for 10 hours by time-lapse video microscopy. The mean rate of migration of HT1080 cells on laminin was 75 μm/hour (Fig. 6A) and of Wehi 164 cells on laminin was 30 μm/hour (Fig. 6B). In marked contrast, their rate of migration was increased eight- to tenfold when they were plated on Melγ-Fc. HT1080 cells migrated at 450 μm/hour (Fig. 6A) and Wehi 164 cells migrated at 280 μm/hour (Fig. 6B). The negative control NCAM-Fc protein did not bind to the cells, thus demonstrating the specificity of the interaction (data not shown).

Migration of cells on Melγ-Fc is inhibited by a specific p160 ROCK kinase inhibitor

It is well established that integrin-ligand interactions can promote cell migration by inducing tyrosine phosphorylation of several proteins that lead to activation of downstream signalling pathways (reviewed by Giancotti, 1997). This is further supported by studies showing that the ability of $\alpha_6\beta_1$ to promote cell migration on laminin is dependent on activation

of the MAP kinase pathway by the α_6 subunit (Wei et al., 1998).

To determine whether tyrosine phosphorylation is important in inducing cell motility on Melγ-Fc, we tested the effects of a general tyrosine kinase inhibitor, genistein, in our migration assays. Cells were treated with genistein (10 μM) for 30 minutes prior to plating them onto either laminin or Melγ-Fc. Pretreatment of cells with genistein did not effect adhesion (data not shown), but markedly reduced cell migration on both substrates (Figs 7, 8). Migration of HT1080 and Wehi 164 cells on laminin was reduced by

approximately 55% and 70% respectively (Fig. 7A,B). Similarly, migration of cells on Melγ-Fc were reduced by approximately 85% for both HT1080 and Wehi 164 cells treated with genistein (Fig. 8A,B).

Many targets of the GTPase Rho have been identified, including Rho-kinase/p160ROCK, myosin-binding subunit (MBS), citron and citron-kinase. p160ROCK is a serine/threonine kinase that directly phosphorylates myosin light chain (MLC). This kinase regulates the formation of stress fibres, focal adhesions, smooth muscle contraction and neurite retraction through the phosphorylation of MLC.
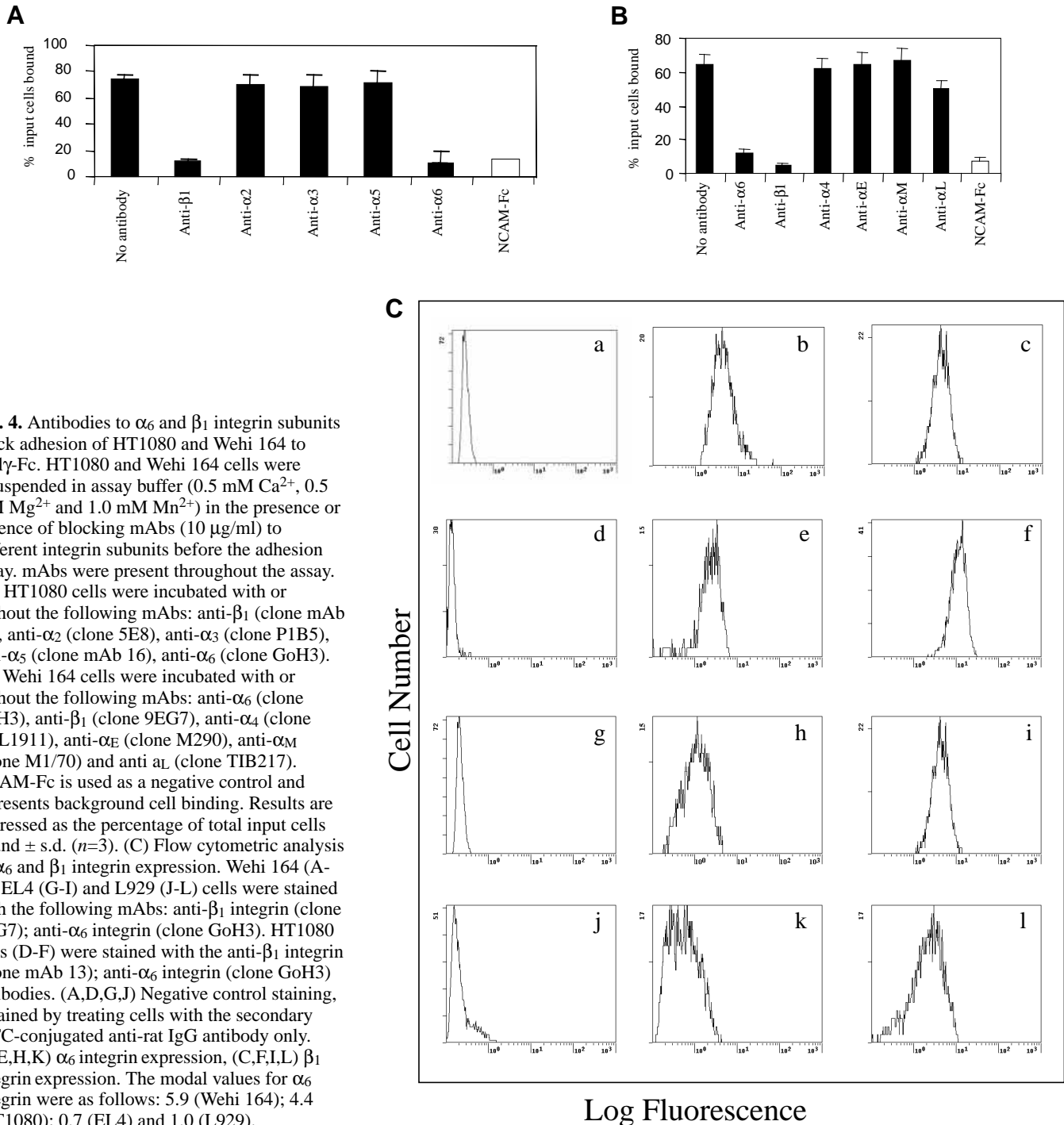

Fig. 4. Antibodies to α_6 and β_1 integrin subunits block adhesion of HT1080 and Wehi 164 to Melγ-Fc. HT1080 and Wehi 164 cells were resuspended in assay buffer (0.5 mM Ca^{2+}, 0.5 mM Mg^{2+} and 1.0 mM Mn^{2+}) in the presence or absence of blocking mAbs (10 μg/ml) to different integrin subunits before the adhesion assay. mAbs were present throughout the assay. (A) HT1080 cells were incubated with or without the following mAbs: anti-β_1 (clone mAb 13), anti-α_2 (clone 5E8), anti-α_3 (clone P1B5), anti-α_5 (clone mAb 16), anti-α_6 (clone GoH3). (B) Wehi 164 cells were incubated with or without the following mAbs: anti-α_6 (clone GoH3), anti-β_1 (clone 9EG7), anti-α_4 (clone CRL1911), anti-α_E (clone M290), anti-α_M (clone M1/70) and anti a_L (clone TIB217). NCAM-Fc is used as a negative control and represents background cell binding. Results are expressed as the percentage of total input cells bound ± s.d. (n=3). (C) Flow cytometric analysis of α_6 and β_1 integrin expression. Wehi 164 (A-C), EL4 (G-I) and L929 (J-L) cells were stained with the following mAbs: anti-β_1 integrin (clone 9EG7); anti-α_6 integrin (clone GoH3). HT1080 cells (D-F) were stained with the anti-β_1 integrin (clone mAb 13); anti-α_6 integrin (clone GoH3) antibodies. (A,D,G,J) Negative control staining, obtained by treating cells with the secondary FITC-conjugated anti-rat IgG antibody only. (B,E,H,K) α_6 integrin expression, (C,F,I,L) β_1 integrin expression. The modal values for α_6 integrin were as follows: 5.9 (Wehi 164); 4.4 (HT1080); 0.7 (EL4) and 1.0 (L929).
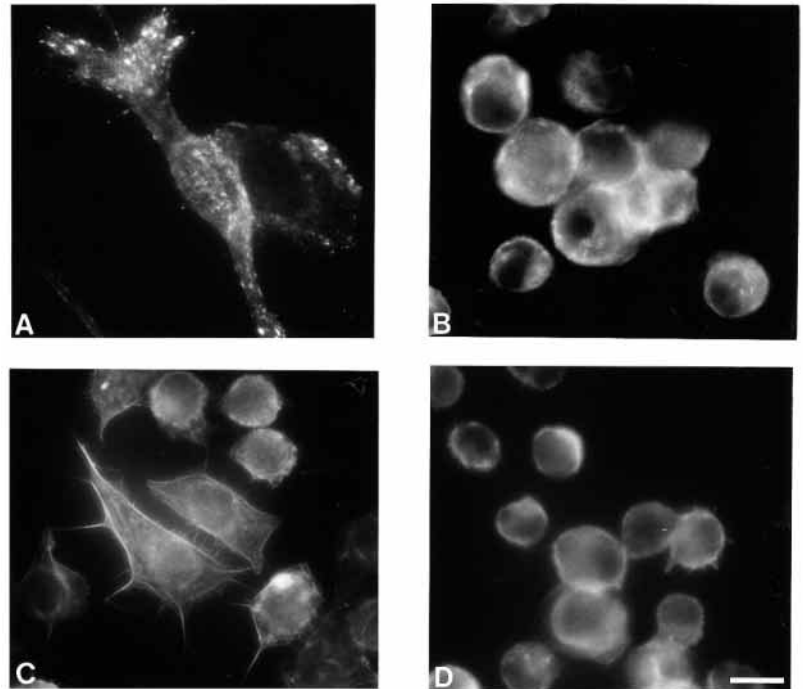
Fig. 5. Morphology of HT1080 (A,B) and Wehi 164 cells (C,D) on laminin (A,C) and Melγ-Fc (B,D). Cells were plated onto wells coated with either laminin (10 µg/ml) or Melγ-Fc (1.5 µg/ml) that was preadsorbed onto anti-human IgG antibody. Cells were fixed and permeabilized and the actin cytoskeleton was visualised by staining with Phalloidin-FITC, 60 minutes after adhesion. Bar, 10 µm.

To determine whether p160ROCK is involved in the migration of HT1080 and Wehi cells on Melγ-Fc, we tested the effects of Y-27632 in our migration assay. Cells were pretreated with 10 µM Y-27632 for 30 minutes prior to plating them onto either laminin or Melγ-Fc. Migration of both HT1080 and Wehi cells on laminin was not effected by Y-27632 (Fig. 7A,B). In marked contrast, migration of HT1080 and Wehi164 cells on Melγ-Fc was decreased by 80% and 90% respectively (Fig. 8A,B) compared to untreated cells. These results together suggest that p160ROCK may be involved in regulating the migration of HT1080 and Wehi 164 cells on Melγ-Fc but not on laminin.
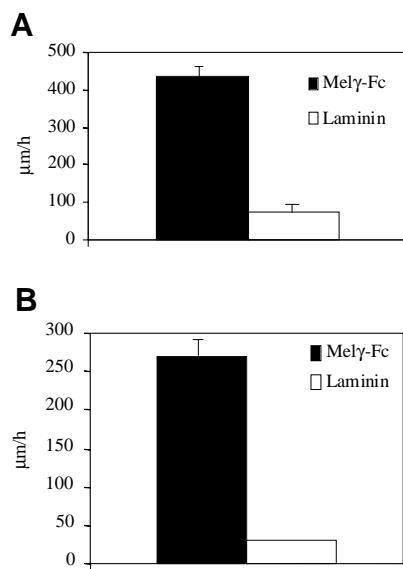

Fig. 6. Time-lapse video microscopy of HT1080 and Wehi 164 cells plated onto laminin and Melγ-Fc. Cells were plated onto dishes that had been coated with either laminin (10 µg/ml) or Melγ-Fc (1.5 µg/ml) preadsorbed onto anti-human IgG antibody (described in Materials and Methods). Cell migration was monitored over 10 hours, and the distance migrated was calculated by tracking the cells individually. (A) Migration of HT1080 cells on laminin and Melγ-Fc. (B) Migration of Wehi 164 cells on laminin and Melγ-Fc. Distance migrated by the cells is denoted in µm/hour and is representative of at least three experiments performed.
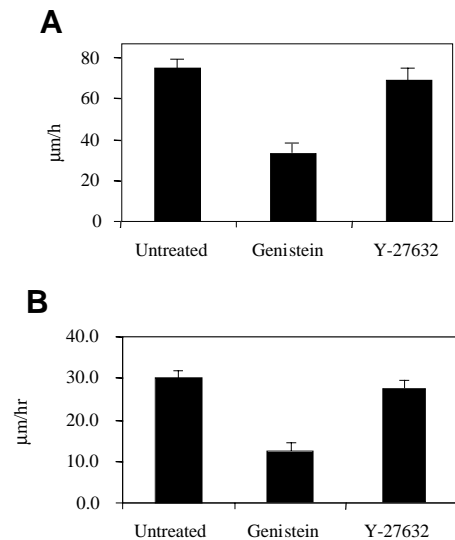

Fig. 7. Effect of genistein and the p160ROCK kinase inhibitor, Y-27632 on cell migration on laminin. HT1080 (A) and Wehi 164 (B) cells were either plated directly onto the matrix or were pre treated with genistein (10 µM) or Y-27632 (10 µM) for 30 minutes prior to plating. Experiments were performed as described in the legend to Fig. 6 and the results are representative of at least three performed. Genistein treatment reduced migration of both HT1080 (A) and Wehi 164 (B) cells on laminin, whereas Y-27632 had no effect on the migration of both cell types.
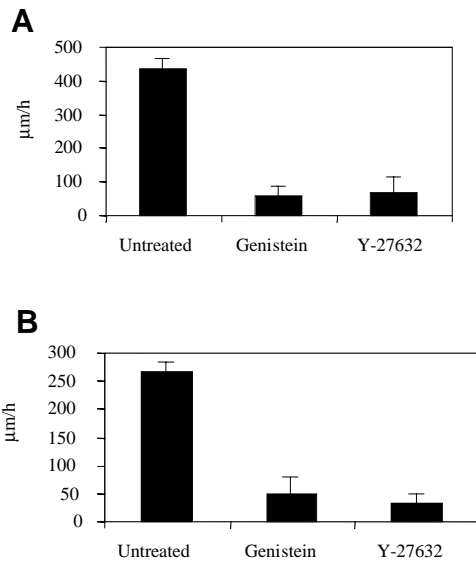
A

B

Fig. 8. Effect of genistein and Y-27632 on migration of HT1080 and Wehi 164 cells plated onto Melγ-Fc. Melγ-Fc (1.5 μg/ml) was coated onto wells that had been preadsorbed with anti-human IgG antibody. HT1080 (A) and Wehi 164 (B) cells were either plated onto Melγ-Fc or were pretreated with genistein (10 μM) and Y-27632 (10 μM) for 30 minutes prior to plating. Distance migrated was calculated as described in the legend to Fig. 6 and the results are representative of at least three experiments. Both genistein and Y-27632 treatment reduced cell migration on Melγ-Fc.

DISCUSSION

Meltrin γ is a member of the ADAM family of proteins and is expressed on the surface of a variety of cells (Weskamp et al., 1996). As the sequence of meltrin γ contains a disintegrin domain, we hypothesised that an integrin on the surface of cells functions as its ligand. In this paper we report three novel findings. Firstly, meltrin γ can function as a cell adhesion molecule by binding to the integrin $\alpha_6\beta_1$. This makes it the second mammalian ADAM after fertilin-β to bind to $\alpha_6\beta_1$ (Almeida et al., 1995). Secondly, the fibrosarcoma lines HT1080 and Wehi 164 can migrate on immobilised meltrin γ and laminin in an $\alpha_6\beta_1$-dependent manner. Thirdly, the rate of cell migration on meltrin γ is increased by a factor of eight- to tenfold compared to migration on laminin. To our knowledge, this is the first demonstration that meltrin γ can bind to an integrin and that this interaction leads to a marked induction in fibroblast motility.

Comparison of the disintegrin loop sequences of several ADAMs reveals that in at least 12 of the ADAMs, including fertilin β and meltrin γ, there is an XECD potential integrin binding motif (reviewed by Wolfsberg and White, 1996). It will interesting in the future to determine whether the other ADAMs that contain the XECD motif also bind to $\alpha_6\beta_1$. Several studies have shown that synthetic peptides based on the ECD motif can efficiently inhibit fertilin β/$\alpha_6\beta_1$ interactions occurring between sperm and egg cells (Myles et al., 1994; Gichuhi et al., 1997; Chen and Sampson, 1999). Our work also suggests that this motif may be important in meltrin γ/$\alpha_6\beta_1$ interactions since a synthetic octomer peptide (KTSECDVP) containing this motif could block HT1080 and Wehi 164 cells

from binding to immobilised Melγ-Fc (Fig. 3A,B). Although short sequence motifs are critical for integrin recognition, they may not account for the entire binding event as synergistic sequences have been identified that collaborate with them. This has been demonstrated for $\alpha_5\beta_1$ integrin binding to fibronectin (Mould et al., 1997). In order to determine whether the octomer (KTSECDVP) meltrin γ disintegrin domain-based peptide was sufficient on its own to mediate cell adhesion we performed solid-phase cell binding assays to immobilised peptide. Even at high peptide concentrations of 1.0 mM, we were unable to observe significant cell binding (data not shown). This suggests that either the integrin binding motif presented in a linear conformation is not sufficient to support cell adhesion, or that additional sequences are required to promote cell adhesion. Since the integrin binding motifs in ADAMs are predicted to be present in a loop structure protruding from the core structure, based on homology with snake venom disintegrins, it is possible that the integrin binding motif has to be presented in a confomationally constrained manner in order to function in integrin binding.

The amino acid sequence of meltrin γ is highly conserved between human and mouse, showing 82% sequence identity (Weskamp et al., 1996). The putative integrin binding motif is identical, suggesting that meltrin γ may bind to the same integrin in both species. Our data supports this idea, because we demonstrate that meltrin γ can bind to $\alpha_6\beta_1$ on both humans and murine cells (Fig. 2). However, conservation of the integrin binding motif does not appear to be a general characteristic of the ADAMs, because in the case of ADAM-15, even though there is 80% amino acid sequence identity between mouse and humans, the disintegrin domain of the human protein contains an RGDC motif, whereas the murine protein has a TDDC sequence at the corresponding position (Lum et al., 1998), which could suggest that ADAM-15 may bind to different integrins in mouse and humans.

Cell migration is essential for several biological and pathological processes. It is well established that adhesive interactions between integrins and their extracellular matrix ligands can provide the traction forces necessary for cell movement. For example, $\alpha_5\beta_1$ and $\alpha_4\beta_1$ mediate migration over fibronectin, the vitronectin receptors $\alpha_v\beta_3$ and $\alpha_v\beta_5$ influence migration of smooth muscle cells, leukocytes, keratinocytes and endothelial cells, and $\alpha_6\beta_1$ is important for the migration of several cell types on laminin (as mentioned in the Introduction). However, less is known about migration of cells influenced by integrin interactions with cell surface ligands. Integrins like $\alpha_4\beta_1$ can also function in cell-cell interactions through binding to the cell surface protein VCAM-1. Leukocytes expressing this integrin can migrate on immobilised VCAM and fibronectin (Chan and Aruffo, 1993; Mould et al., 1994), suggesting that $\alpha_4\beta_1$ may be a locomotive adhesion receptor involved in transendothelial migration of cells. Recently, it has been shown that the integrin $\alpha_9\beta_1$ is important for neutrophil migration on VCAM-1 and chemotaxis across activated endothelium (Taooka et al., 1999). Since $\alpha_6\beta_1$ can confer motility of cells on laminin and can also bind to cell surface ADAMs such as fertilin β and meltrin γ, we wished to examine whether cells can migrate on meltrin γ in an $\alpha_6\beta_1$-dependent manner. We used a system utilising purified proteins to study migration in order to specifically delineate the role of $\alpha_6\beta_1$/laminin and

$\alpha_6\beta_1$/meltrin γ adhesion pairs in this process and also to eliminate the contribution of other adhesion receptor/ligand pairs. Our results demonstrate that $\alpha_6\beta_1$ can induce cell motility on meltrin γ and that the rate of cell migration is at least eight- to tenfold higher on meltrin γ compared to laminin. In a similar study it has been shown that lymphocytes utilising $\alpha_4\beta_1$ can migrate more efficiently on VCAM-Fc than on fibronectin (Chan and Aruffo, 1993). We propose that meltrin γ could function as a locomotive adhesion receptor involved in cell-cell interactions and that $\alpha_6\beta_1$ is another example of an integrin that can mediate both cell-extracellular matrix (ECM) and cell-cell adhesion and migratory processes. The latter are involved in the locomotion of rapidly moving cells such as leukocytes and tumour cells and might involve diffuse, labile cell contacts. Rho family proteins are known to be involved in integrin-ECM interactions and we have confirmed a role for them in our study, by the inhibitory action of the ROCK kinase inhibitor, Y-27632. Since $\alpha_6\beta_1$ is expressed by a wide variety of cells, including lymphocytes and mesenchymal cells, it could be of importance in inflammatory responses. At this stage the relationship between the metalloprotease and disintegrin domains of meltrin γ remains unclear. However, the inclusion of a synthetic hydroxamate inhibitor, BB94, at 1 μM did not modify cell adhesion or motility on meltrin γ study (data not shown).

In summary, we have identified $\alpha_6\beta_1$ as a novel and biologically significant ligand for meltrin γ, and have demonstrated that interaction of this integrin with meltrin γ induces a marked induction of fibroblast motility. In the future, studies examining the distribution of meltrin γ on migrating cells and the nature of its cytoskeletal linkages will allow further elucidation of the role of this ADAM in cell motility.

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