

$\alpha_4\beta_1$ - and $\alpha_6\beta_1$ -integrins are functional receptors for midkine, a heparin-binding growth factor

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Summary

Midkine is a heparin-binding growth factor that promotes the growth, survival, migration and differentiation of various target cells. So far, receptor-type protein tyrosine phosphatase ζ , low-density-lipoprotein-receptor-related protein and anaplastic lymphoma kinase have been identified as receptors for midkine. We found β_1 integrin in midkine-binding proteins from 13-day-old mouse embryos. β_1 -Integrin bound to a midkine-agarose column and was eluted mostly with EDTA. Further study revealed that the α -subunits capable of binding to midkine were α_4 and α_6 . Purified $\alpha_4\beta_1$ - and $\alpha_6\beta_1$ -integrins bound midkine. Anti- α_4 antibody inhibited the midkine-dependent migration of osteoblastic cells, and anti- α_6 antibody inhibited the midkine-dependent neurite outgrowth of embryonic neurons. After midkine treatment, tyrosine

phosphorylation of paxillin, an integrin-associated molecule, was transiently increased in osteoblastic cells. Therefore, we concluded that $\alpha_4\beta_1$ - and $\alpha_6\beta_1$ -integrins are functional receptors for midkine. We observed that the low-density-lipoprotein-receptor-related-protein-6 ectodomain was immunoprecipitated with $\alpha_6\beta_1$ -integrin and $\alpha_4\beta_1$ -integrin. The low-density-lipoprotein-receptor-related-protein-6 ectodomain was also immunoprecipitated with receptor-type protein tyrosine phosphatase ζ . $\alpha_4\beta_1$ - and $\alpha_6\beta_1$ -Integrins are expected to co-operate with other midkine receptors, possibly in a multimolecular complex that contains other midkine receptors.

Key words: Cell migration, Growth factor, Integrin, LDL-receptor related protein, Midkine

Introduction

Integrins are heterodimeric membrane glycoproteins involved in cell-matrix and cell-cell interactions (Giancotti and Ruoslahti, 1999; Hynes, 2002; Kleiman and Mosher, 2002). They play key roles in morphogenesis, inflammation and tumor invasion (Hood and Cheresch, 2002; Watt, 2002; Bokel and Brown, 2002; Brakebusch et al., 2002).

Here, we provide evidence that integrins serve as receptors for the growth factor midkine (MK) (Kadomatsu et al., 1988; Muramatsu, 2002a; Muramatsu, 2002b). MK is a 13 kDa protein with about 50% sequence identity to pleiotrophin (PTN), also called heparin-binding growth-associated molecule (HB-GAM) (Rauvala, 1989; Li et al., 1990), but is not related to other growth factors or cytokines. MK enhances the growth and survival of target cells and activates the transcription of certain genes (Muramatsu and Muramatsu, 1991; Muramatsu et al., 1993; Michikawa et al., 1993; Nurcombe et al., 1993; Owada et al., 1999; Sumi et al., 2002). Furthermore, both MK and PTN/HB-GAM enhance the migration of various cells (Takada et al., 1997; Imai et al., 1998; Maeda and Noda, 1998; Maeda et al., 1999; Horiba et al., 2000; Qi et al., 2001). Of particular importance is the promotion of migration of inflammatory leukocytes (Takada et

al., 1997; Horiba et al., 2000; Sato et al., 2001). We have recently identified MK as a key molecule in the inflammatory response (Horiba et al., 2000; Sato et al., 2001). In MK-deficient mice, leukocyte infiltration into the blood vessels and kidney after ischemic injury is suppressed, leading to the suppression of neointima formation and nephritis after ischemia (Horiba et al., 2000; Sato et al., 2001). In the deficient mice, antibody-induced arthritis and intraperitoneal adhesions after surgery are also suppressed (Inoh et al., 2004; Maruyama et al., 2004).

So far, receptor-type protein tyrosine phosphatase ζ (PTP ζ) (Maeda et al., 1999), anaplastic lymphoma kinase (ALK) (Stoica et al., 2002) and low-density-lipoprotein (LDL) receptor-related protein (LRP) (Muramatsu et al., 2000) have been proposed as the MK receptors. PTP ζ is a chondroitin sulfate proteoglycan with an intracellular tyrosine phosphatase domain, an MK receptor for the migration of embryonic neurons (Maeda et al., 1999) and osteoblast-like cells (Qi et al., 2001), and involved in the survival of embryonic neurons (Sakaguchi et al., 2003). PTP ζ is also a PTN receptor for the migration of embryonic neurons (Maeda et al., 1996; Maeda and Noda, 1998). ALK is a transmembrane tyrosine kinase (Iwahara et al., 1997) and has been reported to serve as a

PTN/HB-GAM and MK receptor primarily in the promotion of cell growth (Stoica et al., 2001; Stoica et al., 2002).

LRP is a member of the LDL-receptor family and is a receptor of MK necessary for the survival of embryonic neurons (Muramatsu et al., 2000). It also plays a role in the internalization of MK (Shibata et al., 2002). Although the LDL-receptor family primarily serves as endocytosis receptors, some members have been found as components of signaling receptors (Herz and Bock, 2002). Their role in the signaling of reelin (Howell et al., 1999; Trommsdorff et al., 1999) is of particular interest. Reelin is an extracellular-matrix (ECM) protein required for proper neuronal migration, and the reelin receptor is composed of three components: a member of the LDL-receptor family (VLDL receptor or apoE2 receptor) (Howell et al., 1999; Trommsdorff et al., 1999); cadherin-related neural receptor (Senzaki et al., 1999); and $\alpha_3\beta_1$ -integrin (Dulabon et al., 2000). In addition, LRP5/6 function as components of the Wnt receptor complex (Tamai et al., 2000; Wehrli et al., 2000). LRP also associates with the platelet-derived growth factor (PDGF) receptor, leading to a downregulation of receptor activity (Boucher et al., 2003).

In this communication, we provide evidence that $\alpha_4\beta_1$ - and $\alpha_6\beta_1$ -integrins bind directly to MK and are involved in MK-dependent cell migration and neurite outgrowth. Furthermore, we propose that integrins and other MK receptors mentioned above co-operate upon MK signaling, as in the case of reelin receptors.

Materials and Methods

Materials

Recombinant human MK was expressed in yeast (*Pichia pastoris* GS115) and purified to homogeneity as described previously (Ikematsu et al., 2000). MK-agarose was prepared by coupling 50 mg human MK to 10 ml CNBr-activated Sepharose 4B (Amersham Biosciences, NJ) as described previously (Muramatsu et al., 2000). Laminin was purchased from BD Bioscience (Bedford, MA, USA) and laminin-agarose was prepared by coupling laminin to CNBr-activated Sepharose 4B. Protein-A/agarose and Protein-G/agarose were from Amersham Biosciences. V-10 peptide (GPEILDVPST) and fibronectin active fragment (GRGDS) were products of Peptide Institute (Osaka, Japan). Protease inhibitor cocktail tablets were from Roche (Mannheim, Germany).

Anti-PTP ζ polyclonal antibody was prepared as described previously (Maeda et al., 1994). Anti-PTP ζ monoclonal antibody (RPTP β) was purchased from BD PharMingen (San Diego, CA, USA), anti-hemagglutinin (anti-HA) was from Roche, anti-FLAG M2 monoclonal antibody, anti- α -tubulin and anti-FLAG M2 affinity gel were from Sigma (St Louis, MO, USA), anti-Myc antibody was from Upstate Biotechnology (Lake Placid, NY, USA) and anti-paxillin antibody was from BD Biosciences. Antibodies for western blotting were as follows: anti- α_3 -integrin (AB1920) and anti- β_1 -integrin (MAB1965) were purchased from Chemicon International (Temecula, CA, USA), anti- α_4 -integrin (sc-6590), anti- α_6 -integrin (sc-6597) anti- β_4 -integrin and anti- β_7 -integrin were from Santa Cruz Biotechnology (Santa Cruz, CA, USA), anti- α_5 -integrin was from BD PharMingen, and anti-phosphotyrosine was from Upstate Biotechnology. Anti- α_4 -integrin antibody (mouse anti-rat CD49d monoclonal antibody) and anti- α_6 -integrin antibody (rat anti-CD49f monoclonal antibody) for functional studies were from Chemicon International. Anti- α_4 -integrin antibody and anti- β_1 -integrin antibody for flow cytometry were from Chemicon International. Horseradish peroxidase (HRP)-conjugated anti-mouse IgG, anti-rat IgG, anti-rabbit IgG and anti-goat IgG were from Jackson Immunoresearch Laboratories (West Grove,

PA, USA). Fluorescein isothiocyanate (FITC)-conjugated anti-rat IgG was from Sigma, and Alexa-Fluor-488/goat-anti-mouse-IgG was from Molecular Probes (Eugene, OR, USA).

Purification of MK-binding proteins and sequence analysis

MK-binding membrane proteins were isolated from about 130 g day-13 mouse embryos as described previously (Muramatsu et al., 2000). They were subjected to sodium-dodecyl-sulfate polyacrylamide-gel electrophoresis (SDS-PAGE) under reducing conditions, and their sequences were analysed using a 494A Protein Sequencer (Applied Biosystems, Foster City, CA) after in-gel trypsin digestion and peptide separation (Muramatsu et al., 2000).

DNA constructs

The coding sequences of human β_1 -integrin (Argraves et al., 1987) and human α_6 -integrin (Tamura et al., 1990) were ligated into an expression vector pcDNA3.1 (Invitrogen Life Technologies, Carlsbad, CA, USA) with the HA tag sequence at the C-terminus. The coding sequence of human α_4 -integrin (Takada et al., 1989) was also ligated into pcDNA3.1 with the FLAG-tag sequence or Myc-tag sequence at the C-terminus. A cDNA encoding the extracellular domain of mouse LRP6 (Brown et al., 1998; Sakaguchi et al., 2003) was ligated into pcDNA3.1 with the FLAG-tag sequence at the C-terminus. The cDNA encoding rat PTP ζ (Maeda et al., 1994) was inserted into pcDNA3.1.

Cell lines and transfection

COS-7 cells and UMR-106 osteoblastic cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). For transfection, 5×10^5 COS-7 cells per 60-mm dish or 1.5×10^6 cells per 100-mm dish were seeded in DMEM supplemented with 10% FCS. After 24 hours, cells were transfected with pcDNA3.1 with the inserts using LipofectAMINE PLUS reagent or LipofectAMINE 2000 reagent (Invitrogen Life Technologies) for 3 hours according to the manufacturer's protocols.

Binding of integrins to MK

The COS-7 cells transfected with HA-tagged β_1 -integrin or α_6 -integrin or FLAG-tagged α_4 -integrin cDNA were cultured in DMEM with 10% FCS for 24 hours and lysed in buffer A (20 mM Tris-HCl, pH 7.5, 0.3% CHAPS {3-[(3-cholamidopropyl)dimethylammonio]propanesulfonic acid}, 0.15 M NaCl, 2 mM CaCl₂, 1 mM MgCl₂, protease inhibitors). The cell lysate was applied to an MK-agarose column (0.2 ml) equilibrated with buffer A. After washing with buffer A, the column was eluted stepwise with 1 ml of buffer B (20 mM Tris-HCl, pH 7.5, 0.3% CHAPS, protease inhibitor) containing 0.2 M, 0.3 M, 0.4 M and 0.5 M NaCl with or without 20 mM EDTA. The eluate was subjected to SDS-PAGE using a 7% gel under reducing conditions, and the proteins were transferred to a PVDF membrane. The membrane was blocked with 5% nonfat milk in PBS and reacted with anti-HA or anti-FLAG antibody. Then, it was reacted with HRP-conjugated anti-rat IgG or anti-mouse IgG, and bands were revealed using an ECL detection kit (Amersham Biotechnology).

COS-7 cells transfected with α_4 -integrin/FLAG cDNA were cultured in DMEM supplemented with 10% FCS overnight. The medium was changed to DMEM without L-methionine, L-cysteine or FCS. After 2 hours, 40 μ l (20 MBq) L-[³⁵S] methionine [PRO-MIX; L-[³⁵S] in vitro cell labeling mix (Amersham Biosciences)] was added and culture was continued for 40 minutes. Then, the cells were lysed in 1 ml buffer A and the lysate was applied to an anti-FLAG-agarose column (0.4 ml). Material eluted with 2 ml of buffer A containing FLAG peptide (100 μ g ml⁻¹) was applied to an MK-agarose column. MK-binding proteins were subjected to SDS-PAGE using a 7% gel under reducing conditions, and analysed using an image analyser

BAS2000 (Fujifilm, Japan). COS-7 cells were also co-transfected with α_4 -integrin/FLAG and β_1 -integrin/HA, and methionine labeled in the same way. The cell lysate was applied to anti-HA/agarose column. Material eluted with buffer A containing 1 mg ml^{-1} HA-peptide was applied to an MK-agarose column and analysed as above.

Immunoprecipitation

After 24–48 hours of culture, the transfected cells were rinsed with PBS. All the following steps were done on ice or at 4°C using ice-cold buffer. The cells on a 60-mm cell culture plate were covered with 0.5 ml buffer A and scraped with a cell scraper. The cell lysate was transferred to a 1.5 ml microcentrifuge tube, incubated for 20 minutes at 4°C and centrifuged at $10,000 \text{ g}$ for 10 minutes to remove cellular debris. The samples were pre-adsorbed with $30 \mu\text{l}$ Protein-A/agarose or Protein-G/agarose by rotating the sample tubes for 2 hours at 4°C . After centrifugation, $1\text{--}10 \mu\text{l}$ ($0.5\text{--}5 \mu\text{g}$) of primary antibody was added to 0.5 ml of the supernatant fluid and the mixture was incubated at 4°C on a rotating device for between 5 hours and overnight. After addition of $30 \mu\text{l}$ of Protein-A/agarose or Protein-G/agarose (for anti-HA antibody), the mixture was incubated for 2 hours. The pellet was collected by centrifugation at 3000 g for 3 minutes, washed three times with buffer A and resuspended in $30 \mu\text{l}$ of $2\times$ electrophoresis sample buffer and subjected to SDS-PAGE and western blotting as mentioned above.

The level of expression of the tagged proteins in the transfected cells were estimated using the [^{35}S]-labeled cells; we determined the percentage of radioactivity immunoprecipitated by an anti-tag antibody to radioactivity precipitated by 10% trichloroacetic acid. The values were 0.03–0.12% for α_4 , α_6 , β_1 and LRP6.

For analysis of paxillin phosphorylation, UMR106 cells were cultured without FCS for 8 hours and then incubated with DMEM containing MK for the indicated periods. Cells were lysed in 0.5 ml buffer A containing 0.5 mM sodium vanadate. The cell lysate was immunoprecipitated with anti-paxillin antibody and subjected to western blotting using anti-phosphotyrosine or anti-paxillin antibody.

Assays for cell migration and neurite outgrowth

The migration of UMR106 cells was assayed using Chemotaxicell (Kurabo, Osaka, Japan) with pores $8 \mu\text{m}$ in diameter, as described previously (Qi et al., 2001). The lower surface of the filter was coated with $20 \mu\text{g ml}^{-1}$ MK or poly-L-lysine in PBS and $600 \mu\text{l}$ 0.3% bovine serum albumin (BSA) in DMEM was placed in the lower chamber. UMR-106 cells (1×10^5) in $100 \mu\text{l}$ 0.3% BSA/DMEM were added to the upper chamber and incubated for 6 hours. For the inhibition assay, UMR-106 cells were preincubated at room temperature for 30 minutes with antibodies, peptides or IgG, and added to the upper chamber with the inhibitors. Statistical analysis was performed with Student's *t* test.

A grid assay of neurite outgrowth was performed as described previously (Kaneda et al., 1996), adopting the method described by Rauvala et al. (Rauvala et al., 1994). Neurite outgrowth on wells coated with $20 \mu\text{g ml}^{-1}$ MK was performed as described previously (Muramatsu et al., 1993).

Flow cytometric analysis

The expression of α_4 and β_1 -integrin on UMR106 cells was analysed by flow cytometry. UMR cells were detached by incubation for 10 minutes at 37°C with non-enzymatic cell dissociation solution (Sigma). The cells (1×10^6) were washed with PBS and suspended in 1 ml PBS containing 1% BSA. The cell suspension was mixed with $10 \mu\text{l}$ rat anti-mouse β_1 subunit or mouse anti-rat CD49d, incubated at 4°C on a rotating device for 45 minutes and, after washing with PBS, stained with FITC-conjugated anti-rat IgG or Alexa-Fluor-488/goat anti-mouse IgG at 4°C for 45 minutes. Background fluorescence intensity was assessed in the absence of primary

antibody. The expression of integrins were quantified using a Beckman Coulter Flow Cytometer (EPICS XL-2)

Results

β_1 -Integrin binds to MK

To identify new components of the MK receptor, membrane proteins of 13-day mouse embryos were solubilized with 0.3% CHAPS and MK-binding glycoproteins were isolated by affinity chromatography on *Ricinus communis* agglutinin-agarose and MK-agarose. These proteins were separated by SDS-PAGE and subjected to trypsin digestion and protein sequence analysis (Muramatsu et al., 2000). In the previous study, a 110-kDa protein that migrated between entactin and heat-shock protein 90- β was not identified (Muramatsu et al., 2000). In the present study, it was identified as β_1 -integrin, because two peptides derived from the protein by trypsin digestion [LSENNIQTIF (amino acids 326–335) and WDTGENPIYK (amino acids 775–784)] were present in β_1 -integrin.

To examine the binding of β_1 -integrin to MK in more detail, we produced HA-tagged β_1 -integrin. The cell lysate of COS-7 cells transfected with HA-tagged β_1 -integrin cDNA was applied to an MK-agarose column. β_1 -Integrin bound to the column at a NaCl concentration of 0.15 M. Under conditions without EDTA, β_1 -integrin was mainly eluted by 0.3 M and 0.4 M NaCl, although a significant portion was also eluted by 0.5 M NaCl and part of it remained in the column and was eluted by adding EDTA (Fig. 1A). This strong binding to an MK-agarose column had been observed in the LRP family (Muramatsu et al., 2000; Sakaguchi et al., 2003) but not in other MK-binding proteins such as PRP-8 (Takahashi et al., 2001) and NCAM (data not shown). The addition of Mn^{2+} did not change the elution profile (data not shown). In the presence of EDTA, a large amount of β_1 -integrin was eluted without changing the NaCl concentration, and the remainder by 0.2 M and 0.3 M (Fig. 1A). Therefore, β_1 -integrin bound specifically to MK and the binding was cation dependent, although the incomplete elution by EDTA indicates that the MK/ β_1 -integrin interaction is somewhat different from the usual interaction of integrins with proteins in ECM.

The α subunits of β_1 -integrin that bind MK are α_4 and α_6 . Each integrin is a non-covalently linked heterodimer containing one member of the α -subunit family and one member of the β -subunit family. The particular combination of α - and β -subunits determines the ligand specificity and function of the integrin (Giancotti and Ruoslahti, 1999; Hynes, 2002; Kleiman and Mosher, 2002). Therefore, we tried to identify an α -subunit of β_1 -integrin capable of binding to MK, using COS-7 cells and mouse 13-day embryos. Western-blot analysis revealed that COS-7 cells used for the present study strongly expressed α_3 -, α_4 -, α_5 -, α_6 - and β_1 -integrin but neither β_4 - nor β_7 -integrin. The lysate of COS-7 cells transfected with HA-tagged β_1 -integrin or 13-day-old mouse embryos was applied to an MK-agarose column and eluted with 0.5 M NaCl containing EDTA. These eluates, which contained β_1 -integrin, were separated by SDS-PAGE and transferred to PVDF membranes, and the membranes were reacted with anti- α_3 -, α_4 -, α_5 - or α_6 -integrin antibody. Consequently, an 80-kDa

fragment from α_4 -integrin (the anti- α_4 -integrin antibody recognizes only the 80-kDa fragment of this integrin) and a 130-kDa band of α_6 -integrin were detected in the transfected COS-7 cells (Fig. 1B), whereas no bands of α_3 - and α_5 -

integrins were detected. In embryos, only an α_6 -integrin band was revealed (H.M. and T.M., unpublished).

Based on these results, we transfected cDNAs of HA-tagged α_6 -integrin and FLAG-tagged α_4 -integrin into COS-7 cells. The lysate of COS-7 cells transfected with FLAG-tagged α_4 -integrin was applied to an MK-agarose column and the adsorbed material was eluted with increasing NaCl concentrations. α_4 -Integrin was mainly eluted with 0.3 M and 0.4 M NaCl, and part of it remained in the column, as in the case of β_1 -integrin (Fig. 1C). When the lysate of COS-7 cells transfected with HA-tagged α_6 -integrin was applied to MK-agarose column and eluted with NaCl, it was also eluted with 0.3 M and 0.4 M NaCl, in a manner similar to β_1 -integrin or α_4 -integrin (Fig. 1D).

We estimated the proportion of integrins bound to the MK column relative to the total amount in the extract by quantifying the amount of HA-tagged or FLAG-tagged integrins by western blotting after SDS-PAGE. In the case of β_1 -integrin, 36% bound to the MK column. The values were 39% and 54% for α_4 - and α_6 -integrins, respectively. Co-transfection of β_1 - and α -subunit cDNAs did not change the result. As an example, when HA-tagged α_6 -subunit and HA-tagged β_1 -subunit cDNAs were transfected, the amount of bound β_1 was 37% of total, and the amount of bound α_6 was 49% of total. The elution profile from the column was also unchanged.

To verify that α_4 -integrin was bound to MK without the help of other molecules, α_4 -integrin/FLAG was purified using an anti-FLAG-monoclonal-antibody/agarose column and eluted with 100 $\mu\text{g ml}^{-1}$ FLAG peptide. We confirmed the purity of the affinity-purified α_4 -integrin by using the [^{35}S]-labeled preparation. Upon SDS-PAGE, the major band was about 130–140 kDa, which corresponds to the bands of both α_4 - and β_1 -subunits (Guan and Hynes, 1990) (Fig. 1E). A minor band of around 70 kDa corresponds to the C-terminal fragment of α_4 -integrin (Hemler et al., 1987). The major band was also detected, when FLAG-tagged α_4 -subunit and HA-tagged β_1 -subunit were co-expressed, and $\alpha_4\beta_1$ -integrin was purified by affinity chromatography on anti-HA-antibody/agarose (Fig. 1E). A slight difference of the size of integrin β_1 -subunit expressed in COS-7 cells was observed between Fig. 1A and Fig. 1E. We interpret this as showing that, upon overexpression for prolonged period as in Fig. 1A, less glycosylated β_1 -subunit is formed. The affinity-purified α_4 -integrin/FLAG also bound to the MK-agarose column (Fig. 1C) in the same manner as the unpurified one (Fig. 1C).

Because one ligand for $\alpha_6\beta_1$ -integrin is laminin, we also purified $\alpha_6\beta_1$ -integrin using a laminin-1/agarose column before application to the MK column. Although $\alpha_6\beta_4$ -integrin also interact with laminin-1, β_4 was not detected in COS-7 cells. The purified integrin bound to the MK column (Fig. 1D) in a manner indistinguishable from the unpurified one (Fig. 1D). Based on all these results, we concluded that MK bound specifically to $\alpha_4\beta_1$ - and $\alpha_6\beta_1$ -integrins.

$\alpha_4\beta_1$ -Integrin is involved in MK-induced migration of osteoblastic cells

MK induces haptotactic migration of UMR-106 rat osteoblastic cells (Qi et al., 2001). Generally speaking, osteoblasts express α_2 -, α_3 -, α_4 -, α_5 -, α_6 - and β_1 -subunits

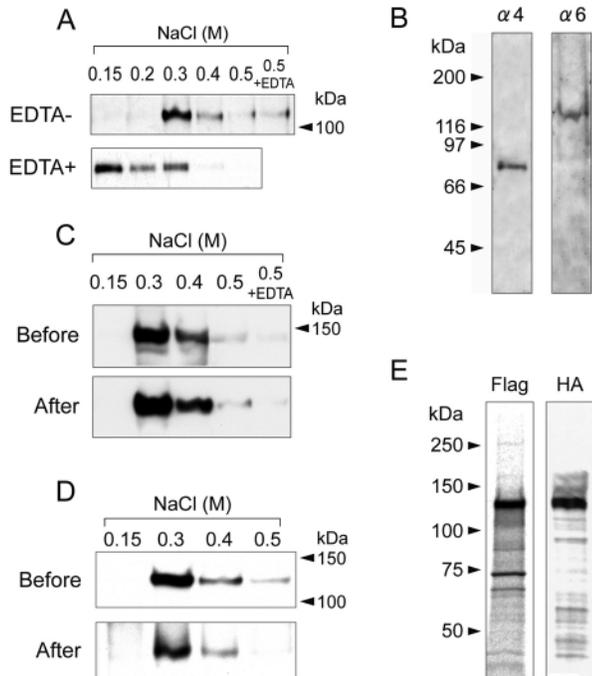


Fig. 1. $\alpha_4\beta_1$ - and $\alpha_6\beta_1$ -integrins bind to MK. (A) The binding of β_1 -integrin to MK. The cell lysate of the COS-7 cells transfected with β_1 -HA-encoding cDNA was applied to an MK-agarose column (0.2 ml) and bound proteins were eluted stepwise with 1 ml of buffer containing 0.15 M, 0.2 M, 0.3 M, 0.4 M and 0.5 M NaCl with or without 20 mM EDTA. A portion of the eluate was subjected to SDS-PAGE and analysed by western blotting using anti-HA antibody to detect β_1 -integrin. (B) Identification of α -subunit of β_1 -integrin capable of binding MK. The lysate of COS-7 cells transfected with β_1 -HA was applied to the MK column and eluted with 0.5 M NaCl containing EDTA. The eluate was analysed by immunoblotting using anti- α_4 -integrin or anti- α_6 -integrin antibodies. (C) Binding of α_4 -integrin to MK. The cell lysate of COS-7 cells transfected with FLAG-tagged α_4 -integrin-encoding cDNA was applied to the MK column before or after affinity purification using anti-FLAG-antibody/agarose, and bound proteins were eluted and analysed as in A, except that anti-FLAG antibody was used. (Before) MK column only. (After) MK column and FLAG column. (D) Binding of α_6 -integrin to MK. The cell lysate of COS-7 cells transfected with HA-tagged α_6 -integrin-encoding cDNA was applied to the MK column before or after affinity purification using laminin-1/agarose, and bound proteins were eluted and analysed as in A. (Before) MK column only. (After) MK column and FLAG column. (E) Binding of metabolically labeled $\alpha_4\beta_1$ -integrin to MK. (Flag) COS-7 cells transfected with FLAG-tagged α_4 -integrin cDNA were labeled with [^{35}S]-methionine. After purification using anti-FLAG-antibody/agarose, the radioactively labeled $\alpha_4\beta_1$ -integrin fraction, which was bound to the MK-agarose column and was eluted by 0.5 M NaCl with 20 mM EDTA, and analysed by SDS-PAGE. (HA) COS-7 cells were transfected with FLAG-tagged α_4 -integrin and HA-tagged β_1 -integrin-encoding cDNAs. After purification using anti-HA-antibody/agarose, the radioactively labeled $\alpha_4\beta_1$ -integrin fraction, which was bound to the MK-agarose column, was analysed as in the case of 'FLAG'.

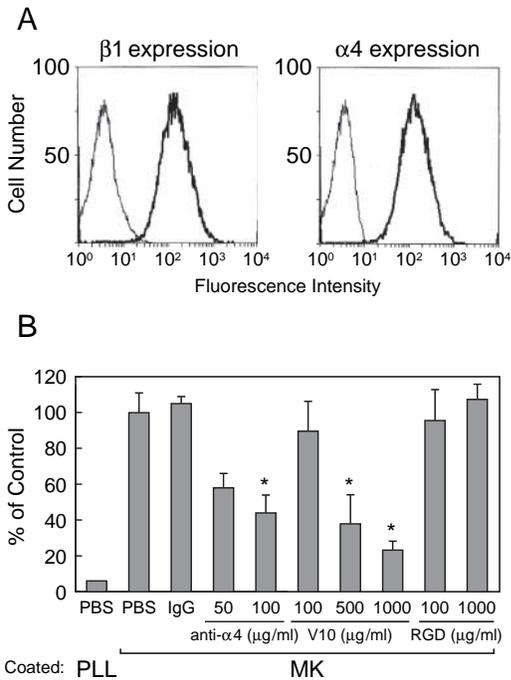


Fig. 2. $\alpha_4\beta_1$ -Integrin is involved in MK-induced migration.

(A) Expression of $\alpha_4\beta_1$ -integrin on the surface of UMR-106 cells as revealed by flow cytometry. UMR cells were incubated with rat anti-mouse- β_1 -integrin or mouse anti-rat- α_4 -integrin antibody, and stained with FITC/anti-rat-IgG or Alexa-Fluor 488-anti-mouse IgG. The negative controls without the primary antibody are indicated with light lines, whereas the signals from integrin staining are shown with a dark line. (B) Involvement of $\alpha_4\beta_1$ -integrin in MK-induced migration. The lower surface of the Chemotaxicell filter was coated with MK or poly-L-lysine (PLL) and a migration assay using UMR-106 cells was performed. Ten fields at 400 \times magnification per filter were counted to determine the number of migrated cells (one field corresponds to 1/160th of the entire surface of the filter). The results are expressed as a ratio to the value obtained without the addition of reagents (PBS). The value is the mean \pm s.e.m. ($n=3$). *, $P<0.001$ versus PBS or IgG. V10, the GPEILDVPST sequence; RGD, the GRGDS sequence.

(Nakayamada et al., 2003). Western-blot analysis indicated that UMR-106 cells strongly expressed $\alpha_4\beta_1$ -integrin. A weaker expression of the α_6 -subunit was also noted. Flow-cytometric analysis confirmed that $\alpha_4\beta_1$ -integrin was expressed on the surfaces of these cells (Fig. 2A). It is known that $\alpha_4\beta_1$ -integrin governs the migration of leukocytes to inflammatory sites (Rose et al., 2001). To evaluate the potential contribution of $\alpha_4\beta_1$ -integrin in MK signaling, we examined whether or not the MK-induced migration of UMR106 cells is mediated by $\alpha_4\beta_1$ -integrin.

MK was coated on the lower surface of a filter at a concentration of 20 $\mu\text{g ml}^{-1}$ and the cells were added to the upper chamber. Function-blocking anti-rat- α_4 -integrin antibody inhibited the cell migration in a dose-dependent manner, whereas mouse IgG did not (Fig. 2B). Anti- α_6 -integrin antibody was not inhibitory (data not shown). Furthermore, the V-10 peptide (GPEILDVPST), which contains the tripeptide of the α_4 -integrin-binding motif [leucine-asparatate-valine (Guan and Hynes, 1990)] also inhibited the migration in a concentration-dependent manner. An RGD peptide used as a

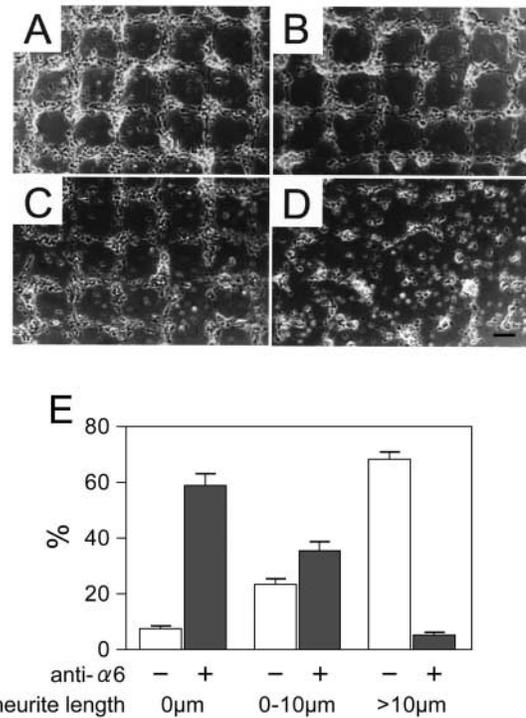


Fig. 3. Inhibition of MK-induced neurite outgrowth by anti- α_6 -integrin antibody. Plastic 24-well culture plates were coated with 10 $\mu\text{g ml}^{-1}$ of MK for 2 hours at room temperature. Then, a grid pattern was made by the ultraviolet-inactivation technique using electron-microscopy grids. After blocking with 10 mg ml^{-1} BSA, brain cells (1×10^6) from mouse embryonic cerebral cortex were cultured without further addition (A) or with 0.0045% NaN_3 (B), 50 $\mu\text{g ml}^{-1}$ anti- α_4 -integrin antibody (C) or anti- α_6 -integrin antibody (D) at 37 $^\circ\text{C}$ under an atmosphere containing 5% CO_2 . (E) Effects of anti- α_6 -integrin antibody on neurite outgrowth on wells coated with 20 $\mu\text{g ml}^{-1}$ MK. The proportion of neurons with a defined neurite length was determined by enumerating cells in ten fields at 400 \times magnification. The average value obtained in three different wells is shown with the s.d. Phase-contrast photomicrographs were taken after culture for 48 hours using a Nikon DIAPHOTO and Olympus CCD camera C5530. Scale bar, 50 μm .

control was not inhibitory. We confirmed that the V-10 peptide at 1 mg ml^{-1} concentration inhibited the binding of $\alpha_4\beta_1$ -integrin to an MK column to 44% compared with the binding in the absence of the inhibitor. These assays were performed by culturing cells for 6 hours. However, in the assay conducted by culturing cells for 3 hours, anti- α_4 -integrin antibody inhibited the migration in an identical manner; the antibody at the concentration of 100 $\mu\text{g ml}^{-1}$ reduced the migration to 41% of the control. The result supports our conclusion that the adhesion is to MK and not to other proteins secreted by the cells. Therefore, we concluded that haptotactic migration of UMR-106 cells induced by MK was mediated by $\alpha_4\beta_1$ -integrin.

Anti- α_6 -integrin antibody inhibits MK-dependent neurite outgrowth

When neurons from rat embryonic brains were cultured on the grid pattern of MK formed on culture plates, they attached and

extended their neurites along the MK tracks (Kaneda et al., 1996) (Fig. 3A). To examine whether α_6 -integrin is involved in neurite outgrowth induced by MK, mouse embryonic brains were cultured with anti- α_6 -integrin antibody. Indeed, anti- α_6 -integrin antibody inhibited the attachment and neurite outgrowth of neurons on the MK-coated substratum (Fig. 3D), whereas anti- α_4 -integrin antibody or 0.0045% NaN₃ (which is present in the anti- α_6 -integrin antibody preparation), or mouse IgG did not have this effect (Fig. 3B,C, H.M. and T.M., unpublished).

We also performed quantitative analysis of the effect of anti- α_6 antibody on neurite outgrowth. In the grid assay mentioned above, individual neurons are difficult to observe. Thus, we just plated brain cells on wells coated with MK. Anti- α_6 antibody dramatically suppressed the number of cells with extended neurites and increased the number of cells without neurites (Fig. 3E).

MK stimulates tyrosine phosphorylation of paxillin

To further examine the physiological significance of MK-integrin interactions, we investigated whether phosphorylation of integrin-associated molecules is changed after stimulation with MK. Consequently, we found that 200–500 ng ml⁻¹ MK transiently increased the tyrosine phosphorylation of paxillin in UMR-106 cells (Fig. 4A). The maximum response was found at 5 minutes after the addition of MK (Fig. 4B). The increase in the phosphorylation was two- to threefold compared with untreated cells. Anti- α_4 antibody suppressed the increased phosphorylation (Fig. 4C). This finding supports the proposal that the binding of MK to $\alpha_4\beta_1$ -integrins in these cells delivers an intracellular signal necessary for the stimulation of cell migration.

$\alpha_6\beta_1$ -Integrin is co-immunoprecipitated with LRP6 and PTP ζ

LRP is an important component of the receptor complex for MK (Muramatsu et al., 2000). Therefore, we were interested in clarifying whether LRP forms a complex with $\alpha_4\beta_1$ - or $\alpha_6\beta_1$ -integrin. Because LRP has molecular mass of 600 kDa, it is hard to express by cDNA transfection. We previously found that LRP6, which is a component of the Wnt receptor complex (Tamai et al., 2000; Wehrli et al., 2000), binds to MK with similar affinity (Sakaguchi et al., 2003). Therefore, we decided to investigate this point by transfection of a cDNA for the LRP6 ectodomain. The ectodomain was used to avoid a possible non-specific association of transmembrane proteins.

COS-7 cells were transfected with cDNAs of HA-tagged β_1 (β_1 -HA) and FLAG-tagged LRP6 ectodomain (LRP6-FLAG) together, LRP6-FLAG alone or β_1 -HA alone. Then, LRP6-FLAG was precipitated with antibody directed against the FLAG epitope, and the immunoprecipitates were probed for the presence of β_1 -HA by immunoblotting. As a result, β_1 -HA could be detected only in the precipitates from co-transfected cells (Fig. 5A). When β_1 -HA was precipitated with anti-HA antibody and the immunoprecipitates were probed for the presence of LRP6-FLAG by immunoblotting, it was detected only in the precipitates from co-transfected cells (Fig. 5B).

Identical sets of experiments were performed for α_6 -

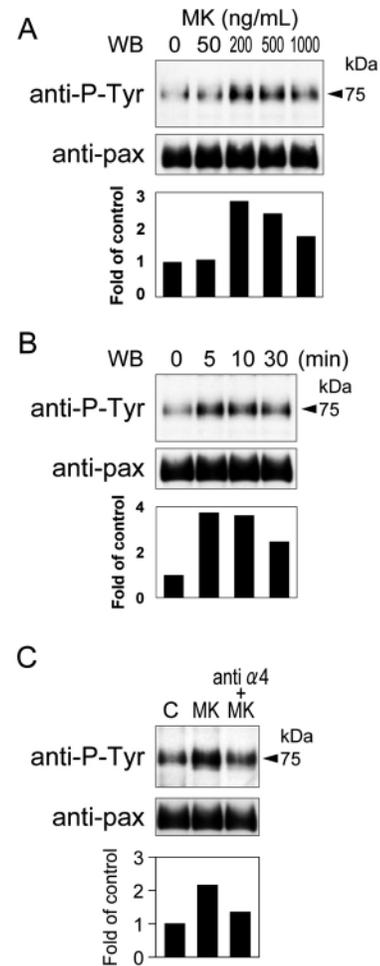


Fig. 4. MK induces paxillin phosphorylation. UMR-106 cells were cultured for 8 hours in DMEM and stimulated with MK (0 ng ml⁻¹, 50 ng ml⁻¹, 200 ng ml⁻¹, 500 ng ml⁻¹ or 1000 ng ml⁻¹) in DMEM for 5 minutes (A) or 200 ng ml⁻¹ of MK for 0 minutes, 5 minutes, 10 minutes or 30 minutes (B). (C) Cells were incubated for 5 minutes without addition (C), with 200 ng ml⁻¹ MK (MK) or 200 ng ml⁻¹ MK plus 100 μ g ml⁻¹ anti- α_4 -integrin antibody (anti- α_4 + MK). Paxillin was immunoprecipitated from cell lysate and analysed by western blotting using anti-phosphotyrosine antibody (Anti-P-Tyr) or anti-paxillin antibody (anti-pax). Relative densitometric densities are shown below the bands obtained by western blotting. In (C), three experiments were performed and a typical result was shown; the increased phosphorylation by MK was suppressed by anti- α_4 -integrin antibody to 36% of the value without the antibody.

integrin. Precipitation of LRP6-FLAG by anti-FLAG antibody co-precipitated α_6 -HA (Fig. 5C) and precipitation of α_6 -HA by anti-HA antibody co-precipitated LRP6-FLAG (Fig. 5D). We also examined the association of LRP6 with α_4 -integrin. COS-7 cells were co-transfected with cDNAs encoding Myc-tagged α_4 (α_4 -Myc) and LRP6-FLAG. After precipitation with anti-Myc antibody, the immunoprecipitates were probed for the presence of LRP6-FLAG by immunoblotting. LRP6-FLAG could be detected only in anti-Myc precipitates from co-transfected COS-7 cells (Fig. 5F). However, precipitation with anti-FLAG antibody and western blotting with anti-Myc antibody revealed no bands (Fig. 5E).

Taken together, these results suggested that the LRP6 ectodomain formed a complex with $\alpha_6\beta_1$ -integrin. The LRP6 ectodomain also appears to form a complex with $\alpha_4\beta_1$ -integrin, but further analysis is required to obtain a definitive conclusion.

We also questioned whether the LRP6 ectodomain is co-immunoprecipitated with PTP ζ . PTP ζ has been identified as a receptor of MK in the MK-dependent migration of UMR-106 osteoblastic cells and embryonic neurons (Maeda et al., 1999; Qi et al., 2001). COS-7 was transfected with cDNAs of PTP ζ and LRP-FLAG. Then, PTP ζ was precipitated with anti-PTP ζ antibody and the immunoprecipitates were probed for the presence of LRP6-FLAG by immunoblotting. A 180-kDa band of LRP6-FLAG could be detected only in anti-PTP ζ precipitates from co-transfected COS-7 cells (Fig. 6A). When LRP6-FLAG was precipitated with anti-FLAG antibody and the immunoprecipitates were probed for PTP ζ by immunoblotting, a broad band of PTP ζ could be detected only in the precipitates from co-transfected cells (Fig. 6B).

As described here, we suggested that the LRP6 ectodomain formed a complex with $\alpha_6\beta_1$ -integrin and also with PTP ζ . It is likely that LRP also forms a complex with $\alpha_6\beta_1$ -integrin. Although the distribution of LRP6 is restricted, LRP is expressed in a variety of cells (Herz and Bock, 2002). Thus, we can infer that $\alpha_6\beta_1$ -integrin and probably $\alpha_4\beta_1$ -integrin form a complex with PTP ζ when they are co-expressed.

To test this possibility, COS-7 cells were transfected with cDNAs of β_1 -HA and PTP ζ , β_1 -HA alone or PTP ζ alone. After precipitation with anti-PTP ζ antibody, the immunoprecipitates were probed for the presence of β_1 -integrin by immunoblotting using anti-HA antibody. Indeed, β_1 -HA could be detected only in the precipitates from co-transfected cells (Fig. 7A). When β_1 -HA was precipitated with anti-HA antibody and the immunoprecipitates were probed for the presence of PTP ζ by immunoblotting using anti-PTP ζ antibody, a broad band of PTP ζ could be detected only in precipitates from co-transfected cells (Fig. 7B). We also examined the possible association of α_6 -integrin or α_4 -integrin with PTP ζ and obtained the same results (Fig. 7C-F). Although a possible non-specific association through the transmembrane domain could not be completely ruled out in this experiment, the result further supports the proposed presence of a large complex consisting of PTP ζ , LRP or LRP6 and $\alpha_4\beta_1$ - or $\alpha_6\beta_1$ -integrin. By transfection with cDNAs of α_6 -HA, LRP6-FLAG and PTP ζ , we also obtained a result supporting the presence of a complex containing all the three molecules. Thus, the immunoprecipitate pulled down with anti-HA antibody contained both LRP6-FLAG and PTP ζ (Fig. 6C).

Finally, we investigated the proportion of a co-immunoprecipitated tagged molecule to the total tagged molecule expressed in the cells; the value was obtained by western blot analysis of a tagged molecule in the immunoprecipitate and in cell extract. α_6 -HA co-precipitated with LRP6-FLAG was 7.9% of total α_6 -HA, and LRP-FLAG co-precipitated with α_6 -HA was 6.9% of total LRP-FLAG.

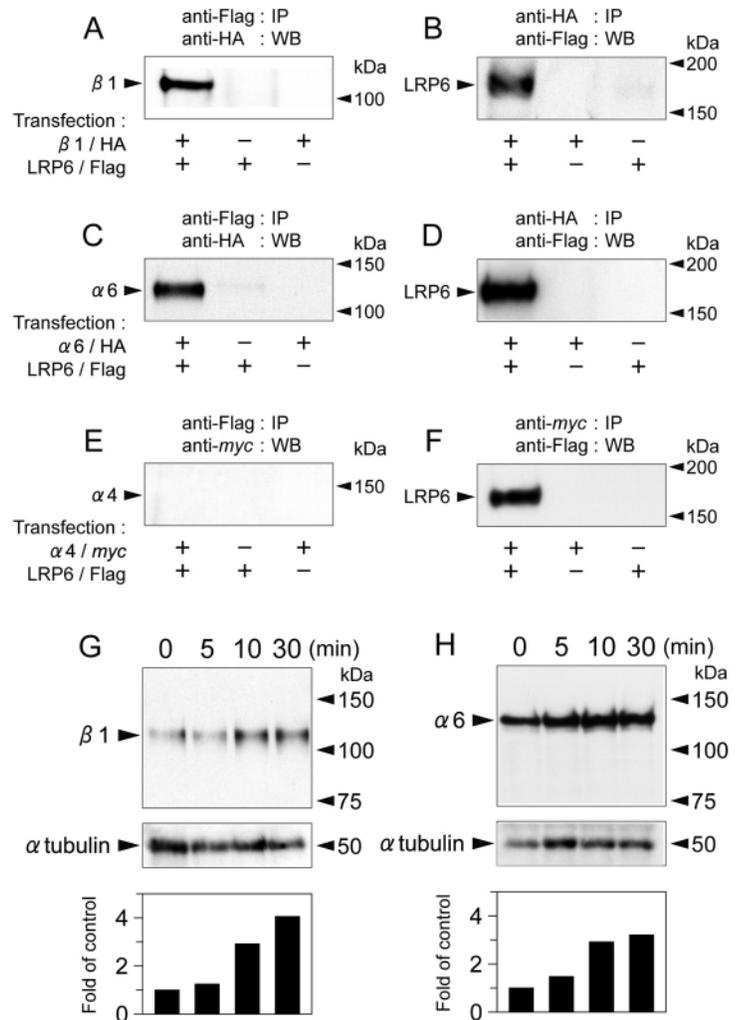


Fig. 5. $\alpha_4\beta_1$ - or $\alpha_6\beta_1$ -Integrin precipitates with LRP6. COS-7 cells were co-transfected with LRP6-FLAG and β_1 -HA (A,B,G), LRP6-FLAG and α_6 -HA (C,D,H) or LRP6-FLAG and α_4 -Myc (E,F). Single transfections were also done. After precipitation with anti-FLAG antibody (A,C,E,G,H), anti-HA antibody (B,D) or anti-Myc antibody (F), the immunoprecipitates were probed for the presence of β_1 -HA (A,G), α_6 -HA (C,H), α_4 -myc (E) or LRP6-FLAG (B,D,F) by immunoblotting. In (G), experiments were performed as in (A) except that cells were incubated with 200 ng ml⁻¹ MK for appropriate period. In (H), experiments were performed as in (C) except that the time of transfection was reduced to 1.5 hours and that cells were incubated with 200 ng ml⁻¹ MK for appropriate period.

PTP ζ co-precipitated with α_6 -HA was 4.2% of total PTP ζ . PTP ζ co-precipitated with LRP6-FLAG was 10% of total PTP ζ . β_1 -HA co-precipitated with LRP6-FLAG was 0.64% of total β_1 -HA, and LRP-FLAG co-precipitated with β_1 -HA was 0.40% of total LRP-FLAG. PTP ζ co-precipitated with β_1 -HA was 0.41% of total PTP ζ . Thus, co-precipitation efficiency of β_1 -HA was low and, in other cases, the value was in the range 4-10%. It is likely that the β_1 -subunit plays an important role in complex formation and the HA tag hinders the process.

When 200 ng ml⁻¹ of MK was added to the culture medium, the proportion of tagged molecules in the co-precipitates of α_6 -HA and LRP6-FLAG did not increase significantly (data not

shown). However, the proportion of β_1 -HA co-precipitated with LRP6-FLAG increased about fourfold (Fig. 5G). When the duration of transfection was shortened to lower the expression of α_6 -HA and LRP6-FLAG, α_6 -HA co-precipitated with LRP6-FLAG was 2.8% of total α_6 -HA. On this occasion, 200 ng ml⁻¹ of MK increased the co-precipitation of α_6 -HA about threefold (Fig. 5H). We concluded that exogenous MK

increased the efficiency of co-precipitation when the degree of co-precipitation was low in the absence of MK.

Discussion

We have provided evidence that MK specifically binds to $\alpha_4\beta_1$ - and $\alpha_6\beta_1$ -integrins. The ligands of $\alpha_4\beta_1$ -integrin are fibronectin, a major component of the ECM, and VCAM, a member of the immunoglobulin superfamily. $\alpha_4\beta_1$ -Integrin recognizes LDV in the alternatively spliced III CS domain of fibronectin (Guan and Hynes, 1990; Kleiman and Mosher, 2002). $\alpha_4\beta_1$ -Integrin plays important roles in cell migration – it governs lymphocyte migration (Rose et al., 2001), is involved in recruitment of neutrophils to inflammatory sites (Burns et al., 2001; Henderson et al., 2001) and is essential for the migration of the heart to form the epicardium (Sengbusch et al., 2002). MK also promotes the migration of neutrophils, macrophages, UMR-106 osteoblastic cells and neurons (Takada et al., 1997; Maeda et al., 1999; Horiba et al., 2000; Qi et al., 2001). Therefore, we postulated that MK-induced migration of UMR-106 cells might be mediated by $\alpha_4\beta_1$ -integrin. Indeed, a function-blocking monoclonal antibody against α_4 -integrin inhibited MK-induced migration of UMR-106 cells in a concentration-dependent manner. Furthermore, we found that an LDV-containing decapeptide of fibronectin (also called CSI), but not an RGD peptide, inhibited the migration of these cells. We also found that, in UMR-106 osteoblastic cells, soluble MK transiently increased the tyrosine phosphorylation of paxillin, which is an adapter molecule that directly binds to the cytoplasmic portion of α_4 -integrins (Turner, 2000; Schaller, 2001; Rose et al., 2002). The phosphorylation of paxillin at a tyrosine residue is known to promote cell migration through downstream systems involving Crk-II (Petit et al., 2000) or by suppressing RhoA (Tsubouchi et al., 2002). It should also be mentioned that substratum-bound MK, but not soluble MK stimulates migration of UMR-106 cells (Qi et al., 2001), whereas both substratum-bound and soluble MK stimulate migration of macrophages (Horiba et al., 2000). It is likely that, in addition to phosphorylation of paxillin, another intracellular signal is required for migration of UMR-106 cells, and that the signal is delivered by substratum-bound MK.

One ligand of $\alpha_6\beta_1$ -integrin is laminin, another component of the ECM. Laminin is one of the most potent promoters of neurite outgrowth of neuronal cells in culture, and $\alpha_6\beta_1$ -integrin is implicated in neuronal adhesion and neurite outgrowth on laminin (DeCurtis and Reichardt, 1993). In embryonic neurons, MK promotes neurite outgrowth and migration, and also has anti-apoptotic activity (Muramatsu et al., 1991; Muramatsu et al., 1993; Michikawa et al., 1993; Owada et al., 1999). These effects on embryonic neurons appear to be at least partially mediated by α_6 -integrin, because MK-

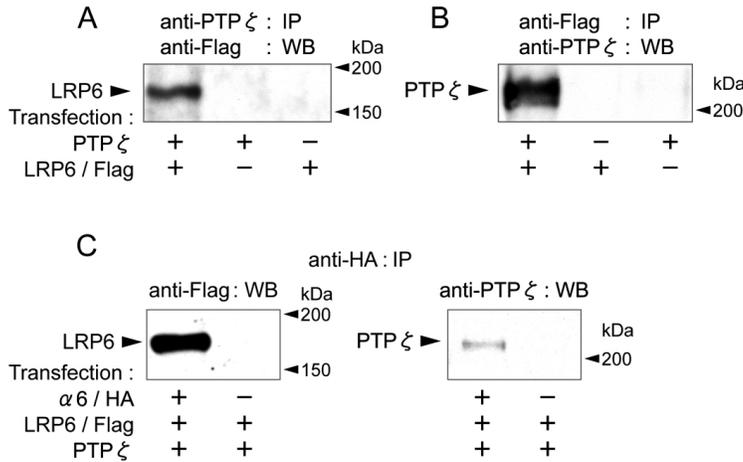


Fig. 6. LRP6 co-precipitates with PTP ζ . (A), (B) COS-7 cells were co-transfected with LRP6-FLAG and PTP ζ , or with each alone. After precipitation with anti-PTP ζ antibody (A) or anti-FLAG antibody (B), the immunoprecipitates were probed for the presence of LRP6-FLAG (A) or PTP ζ (B) by immunoblotting using anti-FLAG antibody (A) or anti-PTP ζ antibody (B). (C) COS-7 cells were co-transfected with LRP6-FLAG, PTP ζ and α_6 -HA. After precipitation with anti-HA antibody, the immunoprecipitate was probed for the presence of LRP6-FLAG or PTP ζ .

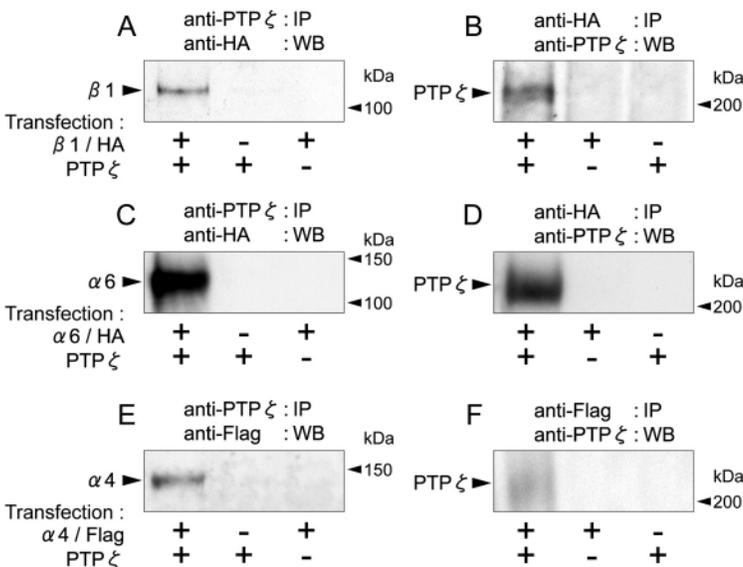


Fig. 7. $\alpha_4\beta_1$ - or $\alpha_6\beta_1$ -Integrin co-precipitates with PTP ζ . COS-7 cells were co-transfected with PTP ζ and β_1 -HA (A,B), PTP ζ and α_6 -HA (C,D) or PTP ζ and α_4 -FLAG (E,F). Single transfections were also done. After precipitation with anti-PTP ζ antibody (A,C,E), anti-HA antibody (B,D) or anti-FLAG antibody (F), the immunoprecipitates were probed for the presence of β_1 -HA (A), α_6 -HA (C), α_4 -FLAG (E) or PTP ζ (B,D,F) by immunoblotting using appropriate antibodies.

induced neurite outgrowth was inhibited by anti- α_6 -integrin antibody.

We conclude that the binding of MK to $\alpha_4\beta_1$ - and $\alpha_6\beta_1$ -integrins is functionally important. We also provide evidence that purified $\alpha_4\beta_1$ - and $\alpha_6\beta_1$ -integrins bound to MK. In the case of $\alpha_4\beta_1$ -integrin, its purity was confirmed by SDS-PAGE. Because MK also binds to proteoglycans (Kojima et al., 1996; Maeda et al., 1999) and LRP (Muramatsu et al., 2000) with high affinity, it was not possible to determine the binding affinity of MK for integrins directly by measuring the amount of MK bound to cells, which were transfected with integrin cDNAs; future experiments should be directed at determining MK binding to purified integrins. However, the profile of elution from the MK column suggests that MK binds to $\alpha_4\beta_1$ - and $\alpha_6\beta_1$ -integrins with an affinity similar to that of LRP (3.5 nM) (Muramatsu et al., 2000). From all these results, we have concluded that $\alpha_4\beta_1$ -integrin and $\alpha_6\beta_1$ -integrin are MK receptors.

An interesting question is whether integrins are used in reception of the migratory signal delivered by PTN/HB-GAM, which is closely related to MK (Rauvala, 1989; Li et al., 1990). It should be mentioned that PTN/HB-GAM also promotes the migration of UMR-106 cells (Imai et al., 1998). More broadly, the role of the thrombospondin-type-1 repeat in interaction with integrins requires systematic study. The repeat is present in many matrix-bound proteins (such as F-spondin) and has weak but significant homology to the β -sheet region of PTN/HB-GAM and MK (Kilpelainen et al., 2000).

There is evidence that PTP ζ (Maeda et al., 1999; Qi et al., 2001; Sakaguchi et al., 2003), LRP family members (Muramatsu et al., 2000; Sakaguchi et al., 2003) and ALK (Stoica et al., 2002) are also MK receptors. Notably, both PTP ζ (Qi et al., 2001) and $\alpha_4\beta_1$ -integrin (this study) serve as functional MK receptors mediating the migration of UMR-106 osteoblastic cells. Therefore, a key issue is whether these receptors function separately or as a molecular complex. We analysed this point using an approach similar to that of Borges et al. (Borges et al., 2000), namely transfection and co-precipitation. To avoid the non-specific association of transmembrane proteins, we used the ectodomain of LRP6. Consequently, we found that $\alpha_4\beta_1$ - or $\alpha_6\beta_1$ -integrin, LRP6 and PTP ζ were co-immunoprecipitated and were likely to form a receptor complex. This proposal is supported by recent findings that integrins can form a cis association with other receptors on the same cell to form receptor complexes (Schneller et al., 1997; Porter and Hogg, 1998; Borges et al., 2000). The $\alpha_v\beta_3$ -integrin associates with PDGF receptor β , allowing a greater proliferative response to PDGF on vitronectin (Schneller et al., 1997). Cell adhesion molecule L1 and its close homolog CHL1 also associate with β_1 integrins and enhance integrin-dependent cell migration (Thelen et al., 2002; Buhusi et al., 2003).

Generally speaking, a multicomponent receptor will not only increase the affinity of the ligand receptor complex but will also increase the specificity of the recognition. In the case of the MK receptor, co-expression of PTP ζ and $\alpha_4\beta_1$ - or $\alpha_6\beta_1$ -integrin will occur much more rarely than expression of a single component of the receptor. However, studies on multicomponent receptors are still at the initial stage, requiring much more work. In case of the putative MK receptor complex, its existence should be confirmed by a method other than co-

immunoprecipitation, especially because the degree of complex formation appeared to be influenced by the density of receptors. The result of the present study suggests that MK stimulates the formation of the receptor complex, when the receptor density is low. Furthermore, it is important to know all molecules constituting the receptor complex.

In this respect, one point that was not explored in the present investigation is the possible role of syndecans in MK signaling. MK binds to syndecans with high affinity (Kojima et al., 1996; Muramatsu, 2002a) and syndecan-3 has been identified as a receptor of PTN/HB-GAM in neurite outgrowth of embryonic neurons (Raulo et al., 1994). Furthermore, Src is associated with the cytoplasmic tail of syndecan-3 and ligand binding appears to alter the activity (Kinnunen et al., 1996). It is still possible that, in certain cases, syndecans might be present in the putative receptor complex and Src is recruited to the complex by association with syndecans.

Whether or not various MK receptors make a molecular complex, we are confident that they co-operate to deliver the MK signal. An increase in tyrosine phosphorylation appears to be a key event after treatment with MK. The requirement of tyrosine phosphorylation for MK-dependent migration of UMR-106 cells was verified in a study using inhibitors (Qi et al., 2001). In contrast to receptor-type tyrosine kinases, PTP ζ is suggested to be inactivated after ligand binding, enhancing the effect of a competing cytoplasmic kinase (Meng et al., 2000). Src is an obvious candidate for such a cytoplasmic kinase, because 1[(4-amino-5-(4-methylphenyl)-7(*t*-butyl)pyrazolo[3,4-D]pyrimidine)] (PP1) inhibits the MK-dependent migration of these cells (Qi et al., 2001). After an extensive examination of the target of increased tyrosine phosphorylation, we found that MK transiently increased tyrosine phosphorylation of paxillin in UMR-106 osteoblastic cells. Because paxillin is associated with the cytoplasmic tail of integrins (Turner, 2000; Rose et al., 2002), the binding of integrins to MK might bring the cytoplasmic portion and paxillin closer to a tyrosine kinase in the receptor complex. Phosphorylated paxillin would stimulate the migration of osteoblastic cells as in the case of bladder tumor cells (Petit et al., 2000) and normal epithelial cells (Tsubouchi et al., 2002).

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