

Midkine and LDL-receptor-related protein 1 contribute to the anchorage-independent cell growth of cancer cells

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Summary

The growth factor midkine (MK) is highly associated with cancer progression. Knockdown of MK expression strikingly suppresses tumor growth in nude mice. Thus, MK is a candidate target for cancer treatment. LDL-receptor-related protein 1 (LRP1) is a receptor for MK. We found that among the four ligand-binding domains of LRP1, the N-terminal half of the second domain (designated as MK-TRAP) had the strongest affinity to MK. MK-TRAP bound to MK, but not to HB-GAM/pleiotrophin, basic fibroblast growth factor or platelet-derived growth factor (PDGF)-BB. Exogenous MK-TRAP inhibited the binding between MK and LRP1. G401 cells that transiently or stably overexpress MK-TRAP showed decreased cell growth in monolayer culture and reduced colony formation in soft agar, which could be rescued by exogenous MK administration. MK-TRAP

collected from conditioned medium also inhibited anchorage-independent growth of G401 cells and CMT-93 cells. Anti-MK antibody also inhibited the anchorage-independent growth. CMT-93 cells stably expressing MK-TRAP formed smaller tumors in a xenograft nude mouse model than control cells. Moreover, GST-RAP, a potent inhibitor of LRP1, inhibited the anchorage-independent growth of control G401 cells but not that of MK-TRAP stable transformants. Collectively, these data demonstrate a crucial role of MK-LRP1 signaling in anchorage-independent cell growth.

Supplementary material available online at
<http://jcs.biologists.org/cgi/content/full/120/22/4009/DC1>

Key words: Midkine, LRP1, Dominant negative

Introduction

Midkine (MK), a heparin-binding growth factor (Kadomatsu and Muramatsu, 2004; Kadomatsu et al., 1988), shows highly increased expression in a number of malignant tumors, including Wilms tumor (Tsutsui et al., 1993), neuroblastoma (Nakagawara et al., 1995), prostate (Konishi et al., 1999), esophageal, gastric (Aridome et al., 1995), colon (Ye et al., 1999), hepatocellular (Koide et al., 1999), breast (Garver, et al., 1994), lung (Garver, et al., 1993), and urinary bladder carcinomas (O'Brien et al., 1996), but restricted expression in normal adult tissues. It is of note that in some tumors, such as neuroblastoma (Nakagawara et al., 1995), glioblastoma (Mishima et al., 1997) and urinary bladder carcinoma (O'Brien et al., 1996), the expression level of MK is significantly correlated with malignant progression and a poor prognosis for patients. This unique characteristic makes MK a candidate target for cancer treatment. Indeed, antisense oligodeoxynucleotide (ODN) targeting MK blocks the growth of mouse colorectal carcinoma cells (CMT-93) in vitro and in vivo (Takei et al., 2002; Takei et al., 2001; Takei et al., 2005). In addition, application of small interfering RNA (siRNA) targeting MK together with atelocollagen significantly suppresses the growth of xenografted PC-3 tumors in nude mice. This effect is

further augmented by the combinational application of the chemotherapeutic drug paclitaxel (PTX) (Takei et al., 2006).

Low-density lipoprotein (LDL)-receptor-related protein 1 (LRP1) is a receptor for MK (Muramatsu et al., 2000). MK enters into the nucleus immediately after rapid LRP1-mediated endocytosis and effects apoptosis suppression (Shibata et al., 2002). Despite an increasing body of evidence that MK is critically involved in cancer development, the significance of MK-LRP1 signaling in cancer has not been explored. Here, we identify a small fragment of LRP1 that exhibits high affinity to MK. This fragment inhibits the binding between MK and LRP1, and could therefore be used as a soluble dominant-negative MK receptor. We report here the new finding that MK-LRP1 is crucial for MK signaling in anchorage-independent cell growth.

Results

The sLRP2N-E fragment possesses the strongest affinity for MK of the four ligand-binding domains of LRP1. LRP1 is a large, multifunctional receptor that contains four putative ligand-binding domains. Each domain consists of EGF precursor repeats and complement-type ligand-binding repeats

and represents a functional unit for the recognition of multiple ligands (Krieger and Herz, 1994; Willnow et al., 1994a). To detect the binding domain of MK, we performed a ligand blot assay using LRP minireceptors that contained the respective ligand-binding domains, as illustrated in Fig. 1A (Obermoeller-McCormick et al., 2001; Obermoeller et al., 1997). Of these four minireceptors, MK displayed substantial binding to the second and fourth ligand-binding domains (Fig. 1B,C). To narrow down further the functional unit for MK binding, we used constructs representing the N-terminal and C-terminal halves of the second and fourth ligand-binding domain. As shown in Fig. 1B,C, the N-terminal portion of the second ligand-binding domain (sLRP2N-E) had the strongest affinity for MK. We renamed this fragment MK-TRAP.

MK-TRAP specifically interacts with MK in vitro

MK-TRAP appeared as a smear migrating with a molecular mass of around 30 kDa on SDS-PAGE gels, with the secreted form shifted above the newly synthesized form, suggesting that MK-TRAP undergoes glycosylation during its synthesis (Fig. 2A). To further confirm the binding between MK-TRAP and MK, we performed an immunoprecipitation experiment using several in vitro systems. First, we directly mixed MK-TRAP-containing medium with MK-containing medium. After pulling down MK with anti-MK antibody, MK-TRAP, which includes an N-terminal hemagglutinin (HA) epitope, was detected with an anti-HA antibody (Fig. 2B). Thus, MK-TRAP could interact with MK in this soluble assay system mixture. We also detected this interaction in the lysate and the medium of M1 cells, which stably overexpress MK-TRAP (Fig. 2C). Second, for the reverse experiment, we generated a version of MK that contained a C-terminal Myc tag. The expression of MK-myc was confirmed by western blot as shown in Fig. 2D. After co-transfection of MK-TRAP and MK-myc, we precipitated MK-TRAP with anti-HA and then detected MK with anti-Myc antibody, and vice versa. We successfully detected MK in MK-TRAP-containing precipitates from both cell lysates and medium, and vice versa (Fig. 2E).

To determine whether this binding is specific, we examined

the binding activity of MK-TRAP with other proteins, such as HB-GAM/pleiotrophin (PTN), basic fibroblast growth factor (bFGF), or platelet-derived growth factor (PDGF)-BB. PTN constitutes a unique family with MK. We added PTN, bFGF or PDGF-BB into MK-TRAP-containing medium. After pulling down PTN, bFGF or PDGF-BB, we checked MK-TRAP in the precipitates. As shown in Fig. 2F, PTN, bFGF or PDGF-BB do not interact with MK-TRAP. The reverse experiments also showed the same result (data not shown). We further confirmed the specificity by a binding-inhibition assay. We added MK or other proteins into a solution containing MK-myc and MK-TRAP to determine whether exogenous protein can inhibit the binding between MK-myc and MK-TRAP. As shown in Fig. 2G, after pulling down MK-TRAP, exogenous MK markedly reduced the co-precipitation of MK-myc, whereas PTN, bFGF or PDGF-BB did not. We also checked the affinity between sLRP3 and MK (Fig. 2H). Human recombinant MK protein was mixed with sLRP3-containing medium, and was then precipitated. There was no detectable binding between sLRP3 and MK (Fig. 2H).

Cells stably overexpressing MK-TRAP show decreased cell growth

To evaluate the effect of MK-TRAP on the growth of cells in monolayer culture, we subjected G401 Wilms tumor cells stably overexpressing MK-TRAP to an MTT assay. As shown in Fig. 3A, clones M1 and M2 showed, over a 4-day culture period, a dramatically decreased growth rate compared with two control cell lines (C1 and C2) that do not express MK-TRAP. To investigate whether this growth suppression was due to trapping of MK by MK-TRAP, we added exogenous human recombinant MK to see whether excess MK can rescue this phenotype. As shown in Fig. 3B, exogenous MK partially abrogated the cell growth inhibition observed in M1 but not control cells (C1).

MK-TRAP inhibits the colony formation ability of G401 and CMT-93 cells

We next investigated the effect of MK-TRAP on anchorage-independent growth of cancer cells, which is known to be a

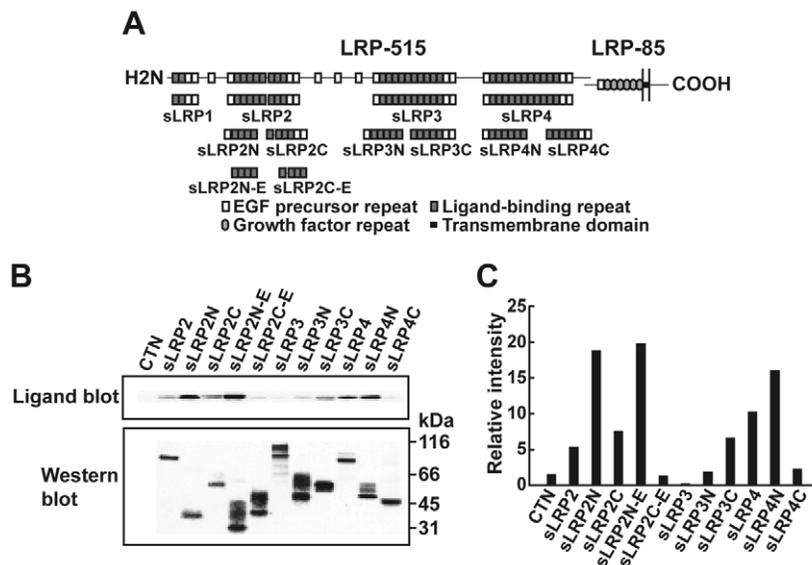


Fig. 1. Ligand blot of MK. (A) Schematic representation of various sLRP fragments. (B) Ligand blot analysis of MK with various fragments. MK was electrophoresed on SDS gels and transferred to a nitrocellulose membrane. Strips of the membranes were then incubated with conditioned medium harvested from sLRP cDNA-transfected COS7 cells. The bound sLRPs were detected using anti-HA antibody. Upper panel, ligand blot result; bottom panel, expression levels of sLRPs. (C) Bar graph of the integrated intensity of each band in (B). The intensities of ligand blot bands were measured and then calculated as the relative intensity (measured intensity/CTN intensity ratio). Experiments were independently performed twice. The results showed the same tendency, and results from one experiment are presented here.

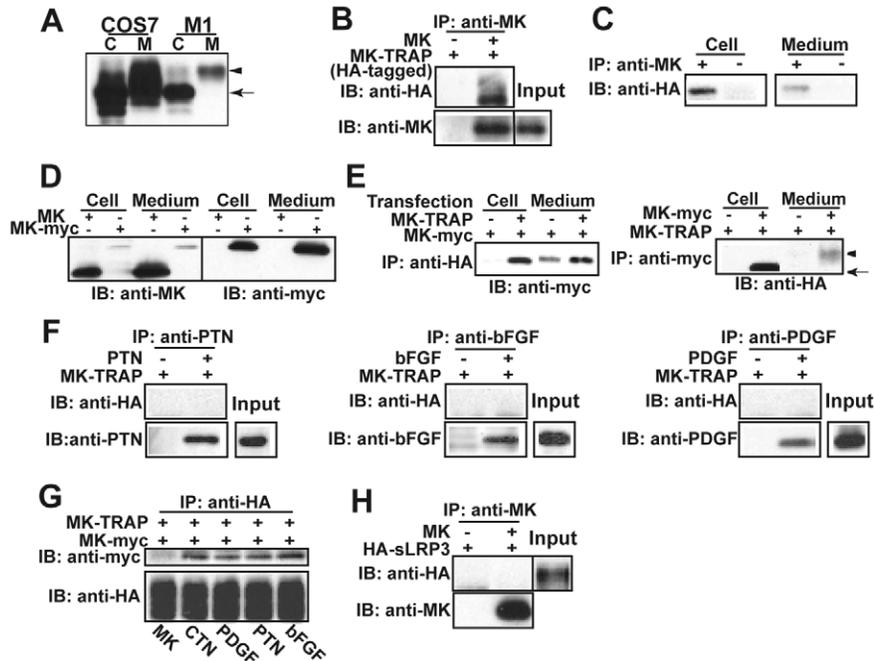


Fig. 2. MK specifically interacts with MK-TRAP in vitro. (A) MK-TRAP expression in COS7 cells transiently overexpressing MK-TRAP or in the G401-derived M1 cell line stably overexpressing MK-TRAP. The arrowhead indicates the secreted form of MK-TRAP; the arrow indicates the pre-secreted form of MK-TRAP; C, cell lysate; M, medium. (B) MK-TRAP-containing medium was mixed with MK-containing medium or control medium, and MK was pulled down with anti-HA antibody. MK-TRAP was detected with anti-HA antibody. (C) MK was pulled down from cellular extracts or medium of the M1 cell line. MK-TRAP was detected in the immunoprecipitates. (D) Expression of MK and MK tagged with a Myc epitope (designated as MK-myc) in COS7 cells transiently transfected with each construct. (E) Cellular extracts or medium from COS7 cells co-transfected with both MK-myc and MK-TRAP were immunoprecipitated with an anti-HA antibody and subsequently detected with anti-Myc antibody, and vice versa. The arrowhead indicates the secreted form of MK-TRAP; the arrow indicates the pre-secreted form of MK-TRAP. (F) MK-TRAP-containing medium was mixed with human recombinant PTN (left panel), bFGF (middle panel) or PDGF-BB (right panel). PTN, bFGF or PDGF-BB was immunoprecipitated with anti-HA antibody. (G) Exogenous proteins (10 μ g of MK, PTN, bFGF or PDGF-BB) were added to a medium containing MK-TRAP and MK-myc to detect their effects on the binding between MK-TRAP and MK-myc. MK-TRAP was pulled down with anti-HA antibody, and MK-myc in the precipitates was detected using anti-Myc antibody. (H) sLRP3-containing medium was mixed with MK. MK was then pulled down with anti-MK antibody, and sLRP3 in the precipitates was detected with anti-HA antibody.

good index of tumorigenicity. First, we determined the colony formation of G401 Wilms tumor cells stably overexpressing MK-TRAP (M1 and M2). We found that colonies of control clones had a well-defined spherical structure; by contrast, those of M1 and M2 were small and diffused (Fig. 4A). The colony numbers of both M1 and M2 clones were markedly reduced compared with those of two control cell clones (Fig. 4B). When we added exogenous MK to M1 cells, this inhibition of colony formation was rescued in a dose-dependent manner (Fig. 4C). We also evaluated the effect of transient overexpression of MK-TRAP on G401 cells. As shown in Fig. 4D, MK-TRAP transfectants showed a significant reduction in colony-forming ability in soft agar, as compared with control cells. Since MK-TRAP is likely to exert its effects at the cell surface, we examined the effect of exogenous MK-TRAP-containing medium on the colony-forming potential of cancer cells. As shown in Fig. 4E, MK-TRAP-containing medium significantly inhibited the colony formation of both G401 cells and CMT-93 cells. Exogenous goat anti-MK antibody also inhibited colony formation (Fig. 4F), supporting the idea that both exogenous MK-TRAP and anti-MK antibody trap extracellular MK, and prevent its binding to its cell surface

receptor, thereby blocking MK signaling and leading to suppression of anchorage-independent cell growth.

MK-TRAP inhibits tumor formation of CMT-93 cells in nude mice

We further inoculated CMT-93 cells stably expressing MK-TRAP or control vector into nude mice. After implantation, the volume of tumors was measured at the indicated intervals. As shown in the left panel of Fig. 4G, MK-TRAP stable transformants formed smaller tumors as compared with control cells. The tumors were removed at day 25 and then weighed; tumors derived from MK-TRAP-expressing cells showed significantly decreased weights (Fig. 4G, right panel).

MK-TRAP is not cytotoxic

To rule out the possibility that all the phenotypes were due to the toxicity of MK-TRAP, we investigated the toxicity of MK-TRAP-containing medium using mouse fibroblast NIH3T3 and L cells, which do not express MK. After replacing the culture medium with MK-TRAP-containing medium or control medium, we monitored cell growth in a monolayer culture. There was no significant difference between the two groups

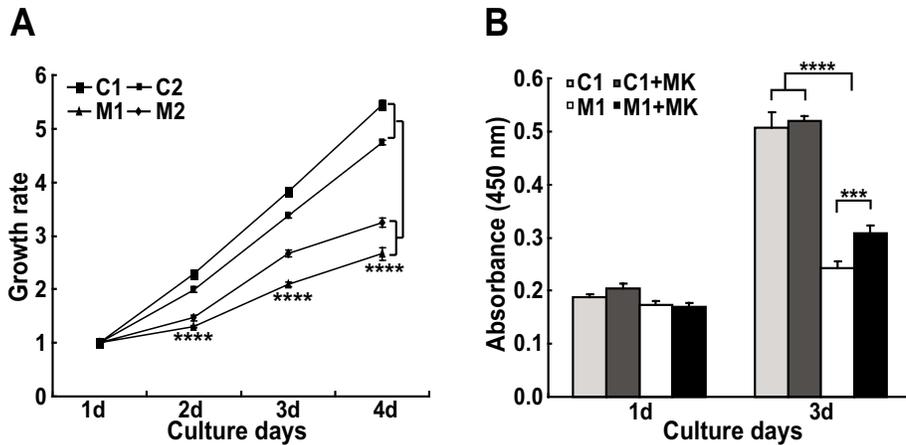


Fig. 3. The effect of MK-TRAP on the proliferation of G401-derived cell lines in monolayer culture. (A) G401-derived cell lines M1 and M2 that stably overexpress MK-TRAP, and control cell lines C1 and C2 were seeded into 96-well dishes and then counted with a cell counting kit over 4 consecutive days. The growth rate was calculated as the ratio of the measured OD on each day to the OD on day one. $n=8$; error bars, \pm s.e.m.; **** $P<0.001$ (MK-TRAP cells versus control cells). (B) Exogenous MK (100 ng/ml) was added to assess its effect on the growth of M1 cells versus control (C1) cells. $n=8$; error bars, \pm s.e.m.; *** $P<0.005$, **** $P<0.001$.

(see supplementary material Fig. S1A,B). We also evaluated apoptosis of NIH3T3 cells. We used serum-free medium (SFM) as a negative control and 30 μ M of the anticancer drug cisplatin (CDDP) as a positive control. As shown in supplementary material Fig. S1C-O, MK-TRAP did not induce apoptosis of NIH3T3 cells.

MK-TRAP acts by blocking the MK-LRP1 pathway

To investigate whether MK-TRAP really blocks the binding between MK and LRP1, we performed the experiments shown

in Fig. 5. We used CHO-derived LRP-null cells (CHO-LRP⁻) and mLRP4-expressing cells (CHO-mLRP4). CHO-mLRP4 cells were generated by stably overexpressing mLRP4, a minireceptor that represents the fourth ligand-binding domain of LRP1 (Bu and Rennke, 1996; Obermoeller et al., 1998). Cells were exposed to ¹²⁵I-MK and treated with the water-soluble crosslinker BS³. As shown in Fig. 5A, externally added MK-TRAP almost completely abolished the ¹²⁵I-MK-crosslinked band observed on SDS-PAGE, whereas sLRP3 did not. Furthermore, we evaluated the effect of GST-RAP, a potent

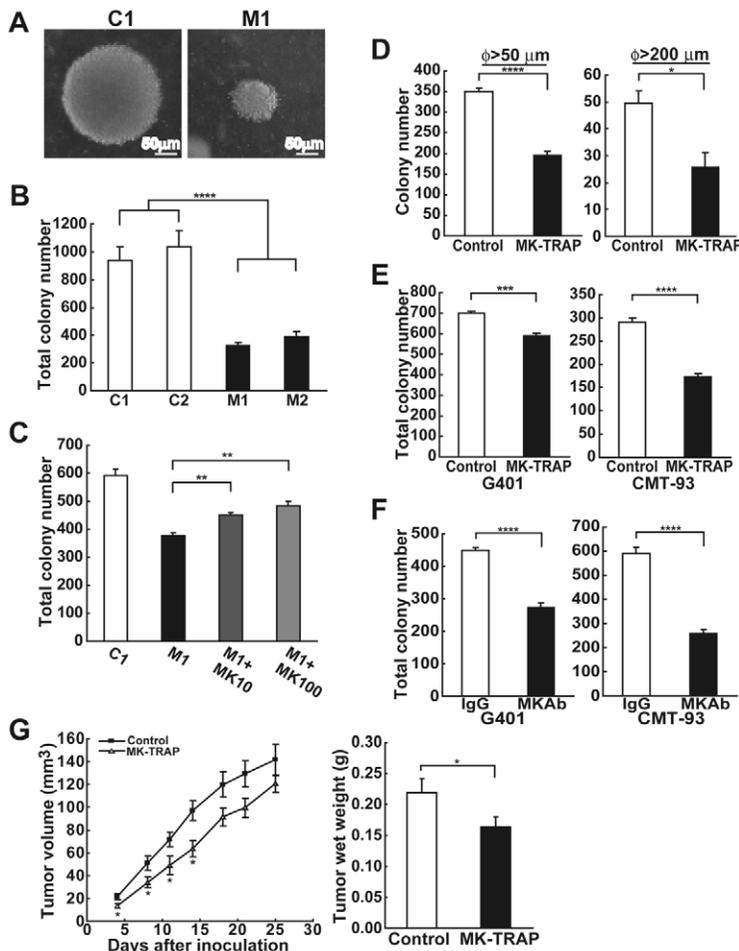


Fig. 4. The effect of MK-TRAP on anchorage-independent growth of G401 and CMT-93 cells. (A) M1 and C1 cells (3000 cells per dish) were plated in 35-mm dishes in soft agar. Two weeks later, representative colonies were photographed. (B) The total numbers of colonies of M1 cells versus C1 cells were counted and calculated as the mean of three independent experiments ($n=3$); error bars indicate \pm s.e.m.; **** $P<0.001$. (C) Exogenous MK (10 ng/ml or 100 ng/ml) was added to both upper and bottom gels to rescue decreased anchorage-independent growth of M1 cells. The results are presented as means ($n=3$); error bars, \pm s.e.m.; * $P<0.01$. (D) G401 cells transiently transfected with MK-TRAP or empty vector (control) were subjected to soft agar colony-formation assay, and the numbers of colonies with a diameter (ϕ) >50 μ m or >200 μ m in each group were counted and calculated as means. $n=3$; error bars, \pm s.e.m.; * $P<0.05$; **** $P<0.001$. (E) MK-TRAP-containing medium was produced by transient overexpression in COS7 cells and then added to both upper and bottom gels, in order to evaluate effects on anchorage-independent growth of G401 cells and CMT-93 cells. Left, G401 cell line; right, CMT-93 cell line. $n=3$; error bars, \pm s.e.m.; *** $P<0.005$; **** $P<0.001$. (F) Goat anti-MK antibody (15 μ g/ml) was added to upper gels to evaluate its effects on anchorage-independent growth of G401 cells and CMT-93 cells. Left, G401 cell line; right, CMT-93 cell line. $n=5$; error bars, \pm s.e.m.; **** $P<0.001$. (G) CMT-93 bulk stable transformants were generated as described in the Materials and Methods section. Cells were inoculated subcutaneously into nude mice. The volume of the tumors was measured at the indicated intervals (left panel). The data are shown as the means \pm s.e.m. ($n=8$). Twenty-five days later, the tumors were isolated and weighed immediately (right panel). Data are given as the means \pm s.e.m. ($n=8$); * $P<0.05$.

inhibitor for LRP1 (Herz et al., 1991; Willnow et al., 1994b), on the colony formation of G401 cells (control: C1; MK-TRAP-expressing: M1 cells). Although GST-RAP dramatically inhibited the colony formation in control cells, it had little effect on M1 cells (Fig. 5B), which supports the idea that LRP1 is involved in the anchorage-independent growth of G401 cells.

Discussion

Although it has been established that MK plays an important role in cancer development, its mechanisms of action are poorly understood. Several MK-binding cell-surface proteins have been identified, including syndecans, integrins, anaplastic lymphoma kinase, protein tyrosine phosphatase ζ and LRP1 (Kadomatsu and Muramatsu, 2004; Muramatsu et al., 2004). For example, protein tyrosine phosphatase ζ has chondroitin sulfate chains on its ectodomain, which are important for MK-binding (Maeda et al., 1999). The interaction between MK and protein tyrosine phosphatase ζ plays a role in cell migration (Qi et al., 2001). After we identified LRP1 as an MK receptor (Muramatsu et al., 2000; Shibata et al., 2002), we showed that LRP1 is involved in MK-mediated neural cell survival (Muramatsu et al., 2000). LRP1 mediates MK endocytosis, and endocytosed MK enters the nucleus where it exerts anti-apoptotic activity (Shibata et al., 2002). On the basis of these findings, it is conceivable that some MK-binding proteins form a receptor complex with MK. To understand the mechanism of action of MK in cancer development, it is important to determine which component of the receptor complex is essential for the function of MK in cancer. In this paper, we show that LRP1 is a functional receptor for MK-mediated anchorage-independent cell growth. This finding also provides a basis for the development of a therapeutic strategy targeting MK in cancer treatment.

MK expression is associated with the premalignant stages of several kinds of tumors, such as colorectal carcinomas (Ye et al., 1999) and prostate carcinomas (Konishi et al., 1999). MK expression increases with the progression of cancer, and is closely associated with poor prognosis (Kadomatsu and Muramatsu, 2004). Similarly, increased LRP1 expression has been reported in malignant astrocytomas (Yamamoto et al., 1997), an effect that was shown to be due to the amplification of the LRP1 gene (Baum et al., 1998). LRP1 also promotes the invasiveness of breast cancer cells in vitro (Li et al., 1998). The phosphorylated cytoplasmic tail of LRP1 associates with Shc, a cellular docking protein, and LRP1 modulates JNK, a member of the MAP kinase family (Barnes et al., 2001; Lutz et al., 2002). Finally, both MK and LRP1 are induced by hypoxia, which is often manifested in carcinoma tissues (Horiba et al., 2006; Koong et al., 2000; Reynolds et al., 2004). These data collectively suggest overlapping functions of MK and LRP1 in cancer development, and are consistent with the findings presented in this paper.

Tumorigenesis, inflammation and some other diseases are always accompanied by overexpression of growth factors and cytokines. Therefore, inhibition of these activities by dominant-negative soluble receptors represents a potential strategy for clinical treatment of these diseases. In support of this idea, trapping of VEGF or TNF α has been successful in some diseases. A VEGF trap is effective in the treatment of

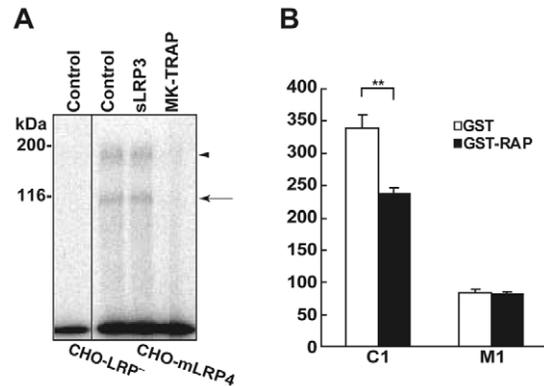


Fig. 5. MK-TRAP acts by blocking MK-LRP1 interaction. (A) CHO-derived cells were exposed to ^{125}I -MK and treated with the chemical crosslinker BS 3 . The ^{125}I -MK-crosslinked band appeared in CHO-mLRP4 but not CHO-LRP $^-$ cells. MK-TRAP- or sLRP3-containing medium was added to CHO-mLRP4 cells to assess their effects on crosslinking. CHO-LRP cells are deficient in LRP1 expression; CHO-mLRP4 is a stable transfectant expressing LRP1-binding domain IV in the background of CHO-LRP $^-$ cells. The arrowhead indicates the unprocessed form of the protein; the arrow indicates the mature form of the protein. (B) GST-RAP, an inhibitor of LRP1, and GST control were administered to M1 and control C1 cells, and the total numbers of colonies in each group were counted and compared by Student's *t*-test. $n=3$; error bars, \pm s.e.m.; **, $P<0.01$.

angiogenesis-related diseases such as tumor- and age-related macular degeneration (Holash et al., 2002; Nguyen et al., 2006), and Etanercept, which traps TNF α , is used to treat rheumatoid arthritis (Goldenberg, 1999; Mohler et al., 1993). The soluble ectodomains of receptors of many other growth factors, such as IGF-I (Hongo et al., 2003), GDNF (Cerchia et al., 2003), PDGF (Borkham-Kamphorst et al., 2004; Zwerner and May, 2002) and Eph (Brantley et al., 2002; Dobrzanski et al., 2004), have also been reported to be effective in suppressing the function of their cognate growth factors by competing for binding to soluble growth factors. The MK-TRAP fragment presented in this study may represent a clinically applicable trapping reagent for cancer treatment.

Materials and Methods

Proteins and antibodies

GST and GST-RAP proteins were produced and purified according to the protocol reported previously (Muramatsu et al., 2000). Recombinant human MK was prepared in yeast and purified as described previously (Muramatsu et al., 2000). Recombinant human PTN, bFGF and PDGF-BB were purchased from R&D Systems, Inc. (Minneapolis, MN). Rabbit antibodies against MK were prepared as described previously (Kadomatsu et al., 1997). Affinity-purified goat anti-human MK antibody was produced as described previously (Maehara et al., 2007). This antibody recognizes both mouse and human MK. Anti-HA rat monoclonal antibodies were purchased from Roche Diagnostics Corporation (Mannheim, Germany). Anti-Myc mouse monoclonal antibodies were from Upstate Biotechnology (Lake Placid, NY). Goat polyclonal antibodies against PTN, bFGF and PDGF-BB were from R&D Systems, Inc. Peroxidase-conjugated secondary antibodies against rabbit, rat or mouse immunoglobulins were from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA).

Construction of cDNAs for sLRPs and mLRP4

Plasmid constructs representing sLRP1 6-171, sLRP2 787-1244, sLRP2N 787-994, sLRP2N-E 826-994, sLRP2C 995-1244, sLRP2C-E 995-1164, sLRP3 2462-3004, sLRP3N 2462-2712, sLRP3C 2713-3004, sLRP4 3274-3843, sLRP4N 3274-3553, sLRP4C 3554-3843 and mLRP4 were generated as reported previously (Obermoeller et al., 1997). The regions of each sLRP are illustrated in Fig. 1. An HA epitope was inserted after the signal cleavage site in each construct.

Cell culture, transient and stable transfection

The G401 Wilms tumor cell line (the Japanese Cancer Research Resource Bank, Tokyo), the CMT-93 cell line derived from mouse rectal carcinoma, the COS7 cell line (American Type Culture Collection, Rockville, MD), the mouse fibroblast L cell line and CHO-derived cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS) at 37°C in humidified air with 5% CO₂. CHO-mLRP4 cells were generated as described previously (Bu and Rennke, 1996; Obermoeller et al., 1998). Mouse fibroblast NIH3T3 cells were cultured in DMEM supplemented with 10% heat-inactivated bovine serum. For transient transfection, cells were transfected with various plasmids at 60–80% confluence using LipofectAMINE PLUS reagent (Life Technologies, Inc., Grand Island, NY) according to the manufacturer's instructions. To generate MK-TRAP-expressing cell lines, G401 cells transfected with MK-TRAP or control vector were selected by culture in 800 µg/ml G418 (Sigma, St Louis, MO) for 2 weeks, and then neomycin-resistant colonies were isolated and screened for MK-TRAP expression. For CMT-93 cells, transfected cells were selected by culturing in 600 µg/ml G418 for 2 weeks; then all cells were reseeded into a 10-cm dish for further culturing without picking colonies (designated as bulk stable transformant).

Protein preparation, immunoprecipitation and western blotting

Cells were washed with ice-cold phosphate-buffered saline (PBS), lysed with lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2 µg/ml aprotinin, and 2 µg/ml leupeptin). Cell lysates or medium were subjected to a 10-minute centrifugation at 13,000 g at 4°C. To harvest secreted proteins, COS7 cells were transfected with MK-TRAP or empty vector (control). Twenty-four hours later, medium was replaced with serum-free DMEM and collected after another 24 hours. Immunoprecipitation with primary antibody was performed at 4°C for 1–2 hours, followed by pulldown assay using protein G (for anti-HA) or protein A (for anti-MK) beads for an additional 2 hours. The immunoprecipitates were extensively washed with lysis buffer and then resuspended in Laemmli sample buffer. For western blot, cell preparations were denatured by boiling for 5 minutes and then loaded (~20 µg) onto a 10% (for sLRPs) or 15% (for MK) polyacrylamide gel. Electrophoresis was performed on a Bio-Rad (Hercules, CA) apparatus. Proteins were transferred to nitrocellulose membranes and blocked for 1 hour with PBS containing 5% nonfat dry milk and 0.1% Tween-20. Membranes were then washed and probed with primary antibodies, followed by horseradish-peroxidase-conjugated secondary antibodies. Blots were visualized using an enhanced chemiluminescence detection reagent (Amersham, Buckinghamshire, UK) and autoradiography film. The intensity of each band was measured using the public domain NIH Image program.

Ligand blot assays

Ligand blot assays were performed as described previously (Obermoeller et al., 1997). Briefly, purified human recombinant MK was subjected to SDS-PAGE and then transferred to nitrocellulose membranes. Membranes were blocked with 10% FCS-containing DMEM, and then incubated with conditioned medium from COS7 cells that transiently overexpress various sLRP fragments or control empty vector; bound proteins were detected using anti-HA antibody.

Binding-inhibition assay

MK-TRAP-containing and MK-myc-containing medium harvested from transiently transfected COS7 cells was mixed, and then 10 µg of MK or other proteins was added to the reaction solution, respectively. After 1 hour of incubation at 4°C, MK-TRAP was immunoprecipitated with anti-HA antibody, and co-precipitated MK-myc was detected with anti-myc antibody.

Monolayer culture cell proliferation assay

G401-derived cells were seeded into 96-well culture dishes at a density of 4×10³ cells (2×10³ cells for NIH3T3 and L cells) per well in DMEM supplemented with 10% serum. Sixteen hours later, the medium was replaced with serum-free DMEM (1% CS/DMEM for NIH3T3 cells and 1% FCS/DMEM for L cells), and cells were cultured for approximately 4 days. Cell proliferation was detected using a cell counting kit (Dojin; Kumamoto, Japan). Human recombinant MK protein was added to some cultures as indicated in Fig. 3B.

Soft agar colony formation assay

Cells were suspended in 2 ml of top agar (0.35% agar in DMEM containing 20% FCS) and then plated on 3 ml of bottom agar (0.5% agar in DMEM containing 20% FCS) in a 35-mm dish. Colonies were counted after 14-day incubation. Soluble reagents tested for growth inhibition were added into both the top and bottom culture layers.

Chemical crosslinking

Radioiodination of MK was performed as described previously (Shibata et al., 2002). The specific activity obtained was approximately 7×10⁷ cpm/µg. For chemical crosslinking, cells at 70–80% confluence were washed with ice-cold DMEM supplemented with 10% FCS, and then incubated with ¹²⁵I-labelled MK at

4°C for 2 hours while gently shaking. After washing with ice-cold PBS containing 0.9 mM Ca²⁺, 0.33 mM Mg²⁺ (PBS-B) and 0.1% bovine serum albumin (PBS-B-BSA) three times, and with PBS-B once, the water-soluble crosslinker BS³ (Pierce) was added at a final concentration of 1 mM in PBS-B. After crosslinking for 15 minutes at 4°C, the reaction was stopped with detaching buffer [10 mM Tris-HCl pH 7.5, 10% glycerol, 1 mM CaCl₂, 1 mM MgCl₂ and 1 mM phenylmethylsulfonyl fluoride (PMSF)]. Cells were scraped into 500 µl detaching buffer, collected by centrifugation and lysed with 200 µl of lysis buffer [20 mM Tris-HCl pH 7.5, 0.6% CHAPS, 150 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂ and 1 mM PMSF].

Apoptosis assay

NIH3T3 cells were plated into 8-well slide chambers (Nunc, n=4). Sixteen hours later, the medium was replaced with MK-TRAP- or control vector-containing medium. SFM was used as a negative control, and CDDP (30 µM) as a positive control. Twenty four hours and 48 hours later, the apoptosis of cells was measured by the TUNEL method using a MEBSTAIN Apoptosis kit (Medical & Biological Laboratories Co., Nagoya, Japan) according to the manufacturer's manual. 4',6-diamidino-2-phenylindole (DAPI) was used to counterstain the cell nuclei. After mounting with mounting medium (90% glycerin, 10% PBS), slides were viewed by fluorescent microscopy (Olympus), and the pictures of eight fields per sample were acquired and counted with MetaMorph software (Molecular Devices, Downington, PA).

Tumor growth in a nude mouse xenograft model

3×10⁶ CMT-93 bulk stable transformants were injected subcutaneously into the abdomen of nude mice (6 weeks old, male). The length and width of the tumors were measured at the indicated intervals, and then the volumes were calculated as 1/2 (length×width×width). Twenty-five days after inoculation, all the tumors were isolated and weighed immediately. Animal experiments in the present study were performed in compliance with the guidelines of the Institute for Laboratory Animal Research, Nagoya University Graduate School of Medicine.

Statistical analysis

The data were compared using two-sided Student's *t*-test. *P*-values of *P*<0.05 were considered significant.

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