Fibroblasts plated on a type I collagen gel can reduce the size of the gel in a way that mimics the reorganization of the collagen matrix that accompanies the wound healing process. We demonstrated previously that lactoferrin (Lf) specifically binds to WI-38 human fibroblasts and enhances their collagen gel contractile activity. The effect of Lf correlated with the phosphorylation of myosin light chain (MLC), suggesting that Lf promotes fibroblast contractile activity by regulating MLC phosphorylation. We found here that the binding of Lf to WI-38 cells was inhibited by recombinant receptor-associated protein (RAP), a universal competitor for ligand binding to LRP (LDL receptor-related protein), and RAP can also promote the collagen gel contractile activity. These observations suggest that LRP is a receptor that mediates the Lf-induced enhancement of collagen gel contractile activity in WI-38 fibroblasts. To confirm the hypothesis, we utilized LRP antisense oligonucleotide, which was modified by morpholino linkage. Suppression of LRP expression abrogated the Lf-induced enhancement of the contractile activity in fibroblasts. Treatment of fibroblasts with Lf enhanced the phosphorylation of ERK1/2 and the activation of MLC kinase (MLCK). These effects were attenuated by suppression of LRP expression. These findings suggest that LRP is involved in the Lf-enhanced collagen gel contractile activity of WI-38 fibroblasts by converting the Lf binding signal into the activation of ERK1/2 and MLCK.

Fibroblasts cultured in a three-dimensional type I collagen gel are able to reorganize the surrounding collagen gel matrix into a more dense and compact structure. This phenomenon is called collagen gel contraction and is considered to mimic the reorganization of the collagen matrix that accompanies wound healing and pathological tissue contracture (1). Collagen gel contraction is due to the tractive force exerted by fibroblasts. It involves numerous actions, including cell adhesion to the collagen fibers, cell migration through the collagen matrix. The extent of collagen gel contraction appears to reflect the motility of the cells in the collagen gel. Certain cytokines (e.g. lysophosphatidic acid) and growth factors such as transforming growth factor (TGF-β), platelet-derived growth factor (PDGF), and fibroblast growth factor (FGF), are known to promote the collagen gel contraction mediated by fibroblasts (2). In addition, it has been found that collagen gel contractile activity and fibroblast motility is regulated by myosin light chain (MLC) phosphorylation (3, 4). We have reported that lactoferrin (Lf) is another factor that is able to promote the collagen gel contractile activity and MLC phosphorylation in WI-38 human fibroblasts (5).

Lf is an iron-binding glycoprotein belonging to the transferrin family. It is synthesized by neutrophils and glandular epithelial cells and is found in many mammalian secretions, including milk, colostrum, semen, tears, and saliva. It is believed that Lf acts as a defense in host animals against microbes and viruses, since it has a broad spectrum of antimicrobial and antiviral activities (6). It also appears to be involved in the inflammatory and immune response against tumors (7). Several studies indicate that Lf is able to modulate various biological functions of mammalian cells. For example, it promotes the activation of natural killer (NK) cells (8) and neutrophils (9), the proliferation of epithelial cells (10), and the maturation of T and B lymphocytes (11, 12). It also inhibits osteoclast-resorbing activity (13) and platelet aggregation (14). Each of these Lf-mediated activities seems to be due to the ability of this molecule to modulate cellular signaling events by interacting with target cells. The cell surface receptors for Lf on various types of mammalian cells have been isolated and characterized, including LRP (LDL receptor-related protein) (15), glycosylphosphatidylinositol-anchored Lf-binding protein (16), and CD14 (17). However, the molecular mechanisms that mediate the cellular responses to Lf ligand binding are still poorly understood.

The purpose of this study was to elucidate the Lf receptor that participates in the Lf-induced enhancement of the collagen gel contractile activity in fibroblasts. We hypothesized that LRP mediates the signal transduction pathway that converts the Lf binding signal into the collagen gel contractile activity in the fibroblasts. It has been reported that Lf can interact with the extracellular domain of LRP (15) and that the removal of Lf

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The abbreviations used are: PDGF, platelet-derived growth factor; Lf, lactoferrin; bLf, bovine lactoferrin; LRP, low density lipoprotein receptor-related protein; DMEM, Dulbecco’s minimal essential medium; HRP, horseradish peroxidase; FBS, fetal bovine serum; MLC, myosin light chain; TMB, tetramethylbenzidine.
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From plasma (18) and translocation of Lf across the blood brain barrier (19) is mediated by LRP.

LRP is a membrane glycoprotein that is a member of the low density lipoprotein (LDL) receptor family. LRP is abundantly expressed on hepatocytes, neurons, smooth muscle cells, and fibroblasts and consists of an extracellular 515-kDa heavy chain and 85-kDa light chain that span the membrane. The extracellular domain of LRP contains four ligand-binding clusters, denoted I to IV. Similar to other members in the LDL receptor family, LRP is known as an endocytic receptor and participates in the uptake of lipoproteins containing triglyceride and cholesterol by hepatocytes. However, the broad range of its ligand diversity and lethality of LRP conventional knockout mice suggests that LRP is involved in diverse physiological and pathological processes other than just lipid protein metabolism. These processes may include cell migration, fibronolysis, thrombosis, and atherosclerosis, in addition to lipoprotein metabolism (20). It has been reported that the binding of various ligands (e.g., urokinase plasminogen activator [uPA], apolipoprotein E [apoE], and α-defensin) to LRP regulates the migration in cultured cells and the contraction of smooth muscle cell (21–26). However, the underlying biochemical signaling pathways elicited by LRP-ligand interaction remain to be elucidated.

To address the above hypothesis, we investigated the effect of the LRP antisense oligonucleotide on the collagen gel contractile activity in WI-38 human fibroblasts. We found that suppression of LRP expression in this way abrogated the Lf-enhanced augmentation of the collagen gel contractile activity and that the Lf-enhanced phosphorylation of ERK1/2 and MLC plays a critical role in the process.

EXPERIMENTAL PROCEDURES

Cell Culture—WI-38 human fetal fibroblasts were obtained from the RIKEN Cell Bank (Tsukuba, Japan). Cells were cultured in Dulbecco’s minimal essential medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) at 37 °C in 5% CO₂ and 95% air atmosphere.

Materials—Materials and chemicals were obtained as follows: purified bovine Lf (bLf) from Nippon Protein (Tokyo, Japan); type I collagen from Nitta Gelatin (Osaka, Japan); anti-MLC monoclonal antibody, anti-MLC kinase monochlonal antibody, MLC substrate peptide, and calmodulin from Sigma; anti-ERK1 monoclonal antibody from Transduction Laboratories (Lexington, KY); anti-phospho-p44/42 MAP kinase monoclonal antibody, anti-p38 MAP kinase polyclonal antibody, and anti-phospho-p38 MAP kinase polyclonal antibody from Cell Signaling (Beverly, MA); anti-human urokinase-type plasminogen activator receptor (uPAR) monoclonal antibody from R&D Systems (Minneapolis, MN); Pansorbin cells from Calbiochem (La Jolla, CA); anti-LRP (615 kDa) monoclonal antibody, and recombinant receptor-associated protein (RAP) from Progen (Heidelberg, Germany); PD98059 from Wako (Osaka, Japan); HRP (horseradish peroxidase)-conjugated streptavidin, and tetramethylbenzidine (TMB) substrate reagent from BD Pharmingen (San Jose, CA); Morpholinoderivatized LRP antisense and control oligonucleotides from Gene Tools (Philomath, OR); LRP PCR primer from Hokkaido System Science (Sapporo, Japan); Taq DNA polymerase (Ampli Taq) from Applied Biosystems (Foster City, CA); human β-actin control primer sets from Stratagene Cloning Systems (La Jolla, CA); ISOGEN total RNA extraction reagent from Nippon Gene (Tokyo, Japan); 1st strand cDNA synthesis kit from Roche Applied Science. A monoclonal antibody against the phosphorylated serine 19 of MLC was a gift from Dr. Minoru Seto (Asahi Chemical Industry, Shizuoka, Japan); Polyclonal rabbit antisera against the phosphorylated serine 19 of MLC was a gift from Dr. Minoru Seto (Asahi Chemical Industry, Shizuoka, Japan); Polyclonal rabbit antisera against the cytoplasmic domain of LRP was a gift from Prof. Joachim Herz (University of Texas, Dallas, TX).

Biotinylation of Lf and Cell Surface Binding Assay—Biotinylation of bLf and the detection of Lf binding to WI-38 fibroblasts was performed according to the method described by Rejman et al. (27). Briefly, WI-38 fibroblasts were plated into collagen-coated 96-well tissue culture plates (2 × 10⁴ cells/well) and incubated with biotinylated Lf for 4 h at 4 °C in the presence or absence of a 100-fold molar excess of unlabelled Lf. The cells were washed and incubated with HRP-conjugated avidin for 1 h at room temperature. The biotin-avidin complex was detected with the TMB substrate reagent. The reaction was terminated with 18 μL of 4 M H₂SO₄, and absorbance at 450 nm was measured using an automated plate reader.

LRP Antisense Oligonucleotide Transfection—The antisense oligonucleotide 5′-CAGCATGTTGCGGCTGATGACC-3′, which corresponds to nucleotides 458–572 of human LRP, was utilized to repress LRP expression in WI-38 fibroblasts. The oligonucleotide was modified by a morpholino linkage to increase its solubility and to provide highly specific antisense activity in transfected cells (28). The oligonucleotide corresponding to the inverted sequence of the LRP antisense oligonucleotide was used as a negative control. WI-38 fibroblasts were transfected with the oligonucleotides by a 3-h incubation in DMEM containing 1.5 μg morpholino oligonucleotide and an equal volume of precipitated polyethylenimine (EPEI) transfection reagent. The cells were subsequently cultured for 5 days in DMEM containing 0.5% FBS and harvested by treatment with phosphate-buffered saline containing 0.25% trypsin and EDTA.

Reverse Transcription and PCR of LrP mRNA—Total RNA was isolated from WI-38 fibroblasts by the guanidine thiocyanate/phenolchloroform extraction method with the ISOGEN total RNA extraction reagent. Total RNA (1 μg) was reverse-transcribed by a 1st strand cDNA synthesis kit according to the manufacturer’s instructions. The PCR primers were designed based on the published human LRP gene sequence: LRP forward primer, 5′-GTAATCCAGGGCTGCGGTG-3′; LRP reverse primer 5′-TCGACCAGCTATCCTGCTU-3′. The PCR reaction was performed with the cDNA with 1.5 μg of initial denaturation step at 94 °C for 4 min, followed by 34 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min. The size and the amount of the PCR products were analyzed by electrophoresis on a 1.5% agarose gel.

Collagen Gel Contraction Assay—A type I collagen solution (3 mg/ml) was gently mixed with 5-fold concentrated cold DMEM on ice. The pH of the mixture was adjusted to 7.4 with 200 mM HEPES containing 2.2% Na₂HCO₃. The final concentration of collagen was adjusted to 1.5 mg/ml by chilling distilled water. A collagen gel was prepared by pouring 0.4 ml of the mixture into each well of a 24-well culture plate and allowing the mixture to set for 2 h at 37 °C. The fibroblasts suspended in 0.4 ml of serum-free DMEM were plated on the top of each collagen gel at an initial cell density of 8 × 10⁴ cells/ml. Gel contraction was initiated by detaching the edge of the collagen gel from the well. The morphological changes of each collagen gel were observed by a charge-coupled device (CCD) camera, and the area of each collagen gel was measured by NIH Image software.

Western Blotting—WI-38 cells were washed twice with chilled phosphate-buffered saline and homogenized with TNE buffer (20 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 1% Nonidet P-40, 1 mM EDTA, 5 μg/ml mercaptoethanol, 1 mM phenylmethylsulfonylfluoride, and 10 μg/ml aprotinin). The cell homogenates were centrifuged at 15,000 × g for 15 min, and aliquots of the supernatants containing 10 μg of protein were resolved on a 7.5% SDS-PAGE in Laemmli system from Pharmacia, transferred onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). In the case of the detection of phosphorylated and total MLc, the proteins were transferred onto an Immobilon-P membrane (Millipore, Bedford, MA). Subsequently, the membrane was treated with a blocking reagent (Tris-buffered saline containing 0.1% Tween-20 and 1% bovine serum albumin (BSA)) for 2 h at room temperature. The blocked membrane was probed with primary antibodies and further incubated with a secondary antibody conjugated with HRP. The immunoreactivity was detected by an enhanced chemiluminescence system (Amersham Biosciences).

Immunoprecipitation of MLc—The cell lysates were incubated with 20 μl of a 10% Pansorbin suspension for 30 min at 4 °C. The Pansorbin cells were removed by centrifugation, and the supernatant was incubated with the anti-MLc antibody for 1 h at 4 °C. Subsequently, 0.1 μg of anti-mouse IgG was added, and the complexes were further incubated for 15 min at 4 °C. The immunocomplexes were precipitated from the lysates by incubation for 30 min at 4 °C with 20 μl of the L5 antisera, followed by washing twice with TNE buffer containing 1 μg sucrose and washing twice with TNE buffer. The immunocomplexes obtained were resuspended in 20 μl of TNE buffer.

In Vitro Kinase Assay—The kinase activity of MLc was determined by quantifying the amount of radiolabeled phosphate incorporated into the MLc substrate peptides (ASKP, ATQPSYK). L5c was immunoprecipitated from the WI-38 cell lysate (containing 500 μg of protein) as described above, and an aliquot of the immunocomplexes was mixed with 18 μl of an ice-cold reaction mixture (20 mM HEPES [pH 7.5], 10 mM MgCl₂, 2 mM dithiothreitol, 0.5 mM CaCl₂, 1 μM calmodulin, and 2.4 μM
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FIG. 1. RAP competes with bLf for binding to WI-38 fibroblasts. WI-38 fibroblasts were plated onto collagen-coated 96-well culture plates and incubated for 3 h at 4 °C with biotin-labeled bLf (1 μM) in the presence of the indicated concentrations of RAP (open circle) or BSA (closed circle). The binding of biotin-labeled bLf to WI-38 cells was detected by incubation with HRP-conjugated avidin, followed by adding the TMB substrate reagent and measuring the absorbance at 450 nm. Binding is expressed as mean values of three parallel measurements. The error bars indicate S.D.

results

RAP Competes with bLf for Binding to WI-38 Fibroblasts—We previously demonstrated that bLf specifically binds to WI-38 fibroblasts (5). To determine whether RAP is involved in the specific binding of bLf to WI-38 fibroblasts, we investigated whether RAP could compete with Lf for binding to WI-38 cells. RAP is a high affinity ligand for LRP that is utilized as a universal competitor for LRP ligands (29). WI-38 fibroblasts were incubated with 1 μM biotin-labeled bLf in the presence of increasing concentrations of the RAP (0–4 μM). As shown in Fig. 1, RAP inhibited the binding of biotin-labeled bLf to WI-38 cells, whereas BSA did not affect the binding of bLf to WI-38 fibroblasts. These observations suggest that RAP is involved in the specific binding of bLf to WI-38 fibroblasts.

RAP Promotes the Collagen Gel Contractile Activity of Fibroblasts—It has been shown that Lf and RAP both interact with the same extracellular domain of LRP (30), and that either ligand can compete with the other for binding to LRP (31). These observations suggest that RAP, as well as Lf, could promote the collagen gel contractile activity of WI-38 fibroblasts. We tested this by performing the collagen gel contraction assay and asessing the effect of RAP in 3, 6, 12, and 24 h after initiating gel contraction by observing the morphological changes of the gel by a CCD camera. Recombinant RAP (final concentration 0.5 μM) enhanced the collagen gel contractile activity of the fibroblasts (Fig. 2A) in a dose-dependent manner that was identical to that of bLf (Fig. 2B). These observations reinforce the possibility that LRP participates in the Lf-enhanced collagen gel contractile activity of fibroblasts.

Inhibition of LRP Expression by Antisense Oligonucleotide Against LRP—We next assessed whether blocking LRP expression would affect Lf binding to WI-38 fibroblasts. To block LRP expression, WI-38 cells were incubated for 3 h with 1.5 μM LRP antisense oligonucleotide against LRP modified by a morpholino linkage (28). WI-38 fibroblasts were also incubated with 1.5 μM oligonucleotide corresponding to the inverted sequence of the LRP antisense oligonucleotide (control). 5 days later, the expression level of LRP mRNA was assessed by RT-PCR analysis, which revealed that the antisense oligonucleotide reduced the LRP mRNA expression to undetectable levels (Fig. 3A). In contrast, the control oligonucleotide did not affect LRP mRNA expression. Western blotting using antibodies against either the LRP heavy chain (515 kDa) or the LRP light chain (85 kDa) confirmed that LRP protein expression levels in the LRP oligonucleotide-treated cells were significantly suppressed (Fig. 3B). However, neither of the oligonucleotides affected the protein levels of another receptor, uPAR (Fig. 3B).
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We examined the effect of the LRP antisense oligonucleotide treatment on the specific binding of Lf to WI-38 fibroblasts (Fig. 4). Unexpectedly, the suppression of LRP expression did not inhibit the binding of biotin-labeled Lf to WI-38 fibroblasts. This suggests that LRP is not the initial binding site for Lf on WI-38 fibroblasts, and an alternative Lf-binding molecule is expressed on the surface of WI-38 fibroblasts.

**LRP Antisense Oligonucleotide Inhibits the Lf-enhanced Collagen Gel Contractile Activity**—We investigated the effect of the LRP antisense oligonucleotide on the Lf-enhanced collagen gel contractile activity of WI-38 fibroblasts. As shown in Fig. 5, it neutralized the collagen gel contraction promoting activity of Lf. In contrast, the control oligonucleotide did not affect the activity. This observation indicates that LRP is required for Lf-enhanced collagen gel contraction. In the absence of Lf, suppression of LRP expression did not affect the collagen gel contractile activity of the fibroblasts. This suggests that the role of LRP in Lf-enhanced collagen gel contraction is related to the ability of Lf to facilitate the intracellular events that is required for the gel contractile processes.

**Lf Enhances the Phosphorylation and Activation of ERK1/2 and MLCK in WI-38 Cells**—The extracellular-regulated kinase (ERK) participates in cell migration and the wound healing process (32, 33). Furthermore, it was reported that the collagen gel contraction mediated by fibroblasts is associated with the activation and phosphorylation of ERK1/2 (34). We investigated the effects of bLf on the phosphorylation of ERK and p38 MAPK (mitogen-activated protein kinase) in WI-38 fibroblasts. The phosphorylation levels of ERK and p38 MAPK were estimated by Western blotting using antibodies against the phosphorylated residues of these proteins. Administration of bLf (final concentration, 1 µM) enhanced the phosphorylation of ERK1/2 within 10 min. The phosphorylation level of ERK1/2 was decreased to baseline levels by 60 min after stimulation. In contrast, the phosphorylation level of p38 MAPK was not affected by bLf treatment (Fig. 6).

We next investigated the effect of bLf on the activity of MLC contraction mediated by fibroblasts is associated with the activation and phosphorylation of ERK1/2 (34). We investigated the effects of bLf on the phosphorylation of ERK and p38 MAPK (mitogen-activated protein kinase) in WI-38 fibroblasts. The phosphorylation levels of ERK and p38 MAPK were estimated by Western blotting using antibodies against the phosphorylated residues of these proteins. Administration of bLf (final concentration, 1 µM) enhanced the phosphorylation of ERK1/2 within 10 min. The phosphorylation level of ERK1/2 was decreased to baseline levels by 60 min after stimulation. In contrast, the phosphorylation level of p38 MAPK was not affected by bLf treatment (Fig. 6).
kinase (MLCK). MLC phosphorylation is a critical step in the formation of actin stress fibers and the modulation of the migratory and collagen gel contractile activities of fibroblasts (32, 35). MLCK is known to be a critical regulator of MLC phosphorylation in fibroblasts and myofibroblasts as well as in smooth muscle cells (36). We have previously demonstrated that administration of bLf enhanced MLC phosphorylation in WI-38 fibroblasts (5). Supporting this observation, we found here that the MLCK activity of WI-38 cells was increased by more than 6-fold within 10 min after 1 μM bLf treatment (Fig. 7A). This MLCK activation was sustained in the 60 min after the administration of bLf.

It has been reported that the activation of MLCK by mitogen is associated with the increased phosphorylation of MAPK, and that ERK/MAPK is able to phosphorylate (activate) MLCK (32). In addition, it was found that the activation of MLCK by ERK increases MLC phosphorylation, and enhances the COS-7 cell migration or leukocyte phagocytic activity (32, 37). To assess the link between ERK and MLCK activation in Lf-enhanced fibroblast collagen gel contraction, we investigated the effect of the MEK inhibitor (PD98059) on the Lf-enhanced activation of MLCK in WI-38 cells. We exposed WI-38 cells to bLf (1 μM) in the presence or absence of PD98059 and measured the kinase activity of MLCK 10 min later. PD98059 (final concentration 50 μM) prevented the Lf-enhanced activation of MLCK (Fig. 7B), which indicates that ERK participates in the Lf signal transduction pathway in fibroblasts by activating MLCK.

We found previously that the MLCK inhibitor (ML-7) and PD98059 both prevented the Lf-enhanced collagen gel contractile activity of human fibroblasts (5). The observation in this study is consistent with the hypothesis that Lf modulates the collagen gel contractile activity of WI-38 fibroblasts by regulating the ERK- and MLCK-dependent signaling pathway.

**LRP Antisense Oligonucleotide Inhibits the Lf-enhanced Phosphorylation of ERK1/2 and MLC—**To further confirm the involvement of LRP in Lf-enhanced WI-38 fibroblast collagen gel contractile activity, we investigated the effect of the LRP antisense oligonucleotide on the Lf-enhanced phosphorylation (activation) of ERK1/2 and MLC. WI-38 fibroblasts were harvested 5 days after treatment with 1.5 μM LRP antisense oligonucleotide, seeded in collagen-coated culture grade dishes and treated with 1 μM bLf. The phosphorylation of ERK1/2 and MLC was examined 10 and 30 min later, respectively. The LRP antisense oligonucleotide abrogated the Lf-enhanced phosphorylation of ERK1/2 and MLC (Fig. 8).

Our data in this study strongly suggest that ERK1/2 and MLCK phosphorylation (activation) are involved in the Lf-enhanced collagen gel contractile activity of fibroblasts, and LRP converts the Lf binding signal into the gel contractile activity by activating the ERK1/2 and MLCK signaling pathway.

**DISCUSSION**

The traditional role ascribed to LRP (and other members of the LDL receptor family) is that it is involved in the receptor-mediated endocytosis by hepatocytes of lipoproteins containing triglyceride and cholesterol. However, LRP contains multiple different ligand-binding clusters in its extracellular domain, and over 30 different ligands can bind to this domain, including apoE, α2-macroglobulin, uPA, RAP, β-amyloid precursor protein (APP), α-defensin, and lipoprotein lipase (20). This ligand...
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Fig. 8. LRP antisense oligonucleotides abrogate the Lf-enhanced phosphorylation of ERK1/2 and MLC. WI-38 fibroblasts were incubated with the LRP antisense oligonucleotide (1.5 μM) or the control oligonucleotide (1.5 μM) for 5 days. Cells were harvested by trypsin treatment, plated in collagen-coated dishes, and cultured in serum-free DMEM for 12 h. Subsequently, bLf (1 μM) was added to the culture media, after which the cells were harvested by TNE buffer at the indicated times. The total and phosphorylated forms of ERK1/2 and MLC were detected by Western blotting, using antibodies recognizing the phosphorylated forms of the proteins (ERK1, Thr-202/Tyr-204; MLC, Ser-19).

The purpose of this study was to elucidate the functional Lf receptor responsible for the Lf-enhanced collagen gel contractile activity of WI-38 fibroblasts. Our experiments showed that LRP is required for this activity since suppressing LRP expression with an antisense oligonucleotide blocked the ability of Lf to augment the collagen gel contractile activity of the fibroblasts (Fig. 5). Furthermore, we found that the Lf-enhanced contractile activity of the fibroblasts was associated with an increased phosphorylation of ERK and MLC and that these Lf-enhanced phosphorylation levels were reduced by suppressing LRP expression (Fig. 8). Thus, LRP may be an Lf receptor on fibroblasts. Supporting this notion is that it has been reported that Lf can interact with the extracellular domain of LRP (15) and that LRP participates in Lf metabolism by mediating the endocytotic clearance of Lf from the circulation by hepatocytes (18). Our study also shows that LRP may act as an Lf-signaling mediator to elicit the phosphorylation (activation) of ERK and MLCK (Figs. 6 and 7) that promotes the collagen gel contractile activity of WI-38 fibroblasts.

It has been suggested previously that LRP is involved in regulating cell motility or contractility (21–26). For example, Okada et al. (21) have reported that inhibiting LRP function prevents uPA-induced smooth muscle cell migration. Nassar et al. (23) have also shown that uPA promotes the contraction of smooth muscle cells by an LRP-dependent mechanism. In contrast, Webb et al. (24) reported that inhibition of LRP function enhanced ERK phosphorylation in fibrosarcoma cells and up-regulated their migratory properties. In addition, Swertfeger et al. (26) showed that the apoE-mediated inhibition of PDGF-enhanced cell migration is mediated by LRP. Notably, these inhibitory effects of LRP on cell migration were observed only with cells cultured on vitronectin and were not observed when type I collagen matrices were used. Thus, the opposite effects of LRP in cell migration that have been reported may result from substratum dependence of the cell surface receptors or signaling molecules that mediate ERK phosphorylation (activation). In any case, these reports clearly indicate that LRP can modulate the migratory or contractile activity of cells by regulating the ERK/MAPK signaling pathway.

uPA binds not only to LRP, but it also interacts with an alternative signaling receptor, uPAR. The binding of uPA to uPAR activates diverse signaling cascades, including ERK and MLCK, and promotes the motility of target cells (22, 28, 38). It has been suggested that LRP may regulate cell migration by clearing cell surface-bound uPA by receptor-mediated endocytosis (24). However, there is also increasing evidence that suggests that LRP participates in signal transduction pathways originating from the binding of non-lipoprotein ligands to LRP. Our observations in the study presented here show that Lf (and RAP) directly activates the collagen gel contractile activity of the fibroblasts by an LRP-dependent mechanism. Additional studies will be required to identify the molecules involved in transducing the Lf signal from LRP to the ERK/MAPK pathway.

Recently, it was shown that PDGF treatment of fibroblasts and smooth muscle cells causes the cytoplasmic domain of LRP to be phosphorylated (39, 40). The tyrosine phosphorylation of LRP generates a binding site for Shc. Shc is an adapter protein that contains a Src homology 2 (SH2) domain and a PTB domain and is involved in the signal transduction mediated by a protein tyrosine kinase. Thus, LRP may be able to act as a co-receptor that modulates the signaling pathway transduced by the PDGF receptor. Furthermore, a recent study using the yeast two-hybrid system and immunoprecipitation revealed that the cytoplasmic tail of LRP interacts with a variety of cytoplasmic adaptor or scaffold proteins, such as FE65, PSD-95, JIP-1/2, and mDab (41). Most of the LRP-interacting proteins contain a PTB or PDZ domain. These proteins are believed to be involved in regulating MAPK activity, cytoskeletal reorganization, and cell adhesion (42). Additional experiments with in vitro cultured cells and genetically manipulated animals are required to confirm the physiological significance of these protein interactions in cellular signaling and modulation of cellular function.

We found that RAP, as well as Lf, is also able to promote the collagen gel contractile activity of WI-38 cells (Fig. 2). RAP is a high affinity ligand for LRP and is known as a universal competitor for ligands that bind to LRP (29–31). It has been reported that LRP binding to either RAP or apoE inhibits PDGF-induced smooth muscle cell migration (21, 26). It has also been reported that apoE4-induced apoptosis of F11 neuronal cells and uPA-induced contraction or migration of vascular smooth muscle cells, are antagonized by RAP (23, 43). These observations suggest that LRP differentially mediates the signal transduction pathway against diverse ligands. A ligand blotting study revealed that the ligand-binding site of LRP for most ligands is restricted to clusters II and IV (30). Only RAP and apoE are capable of binding to cluster III. RAP prevents the binding of all known ligands for LRP, whereas in contrast most LRP ligands apart from Lf and lipoprotein lipase are not able to compete with RAP (30, 31, 44). These differences in ligand-LRP interactions could explain why RAP and other LRP ligands modulate the cellular response to ligand-receptor interaction in different ways. Additional studies are required to confirm this hypothesis.

Another significant finding of our study was that we demon-
stated that Lf can activate MLCK by an ERK/MAPK-dependent mechanism. To investigate how Lf affects mammalian cells, several Lf receptors have been identified in various mammalian cell types, including lymphocytes, hepatocytes, and intestinal cells. However, the cellular events that are induced by Lf remained to be elucidated. We demonstrate here that LRP is required for Lf-enhanced ERK activation. However, the involvement of LRP is not sufficient for explaining all the functions of Lf in mammalian cells. We found that suppressing LRP expression did not affect the specific binding of bLf to WI-38 fibroblasts (Fig. 4) and that RAP only partially inhibited the Lf binding to WI-38 fibroblasts (Fig. 1). It has been reported that cell surface heparin and chondoritin sulfate proteoglycan may play an important role in the interaction between Lf and mammalian cells. However, the cellular events that are induced by Lf may not be the primary recognition site for Lf on WI-38 fibroblasts. In conclusion, LRP might act as a co-receptor to mediate the Lf-triggered signaling pathway, as well as the PDGF-initiated signaling pathway.

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Low Density Lipoprotein Receptor-related Protein (LRP) Is Required for Lactoferrin-enhanced Collagen Gel Contractile Activity of Human Fibroblasts
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