The Low Density Lipoprotein Receptor-related Protein/α2-Macroglobulin Receptor Is a Receptor for Connective Tissue Growth Factor*

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The Low Density Lipoprotein Receptor-related Protein (LRP)/α2-macroglobulin receptor (LRP), the identification of LRP as a receptor for CTGF was validated by several studies: 1) binding competition with many ligands that bind to LRP, including receptor-associated protein; 2) immunoprecipitation of CTGF-receptor complex with LRP antibodies; and 3) cells that are genetically deficient for LRP were unable to bind CTGF. Last, CTGF is rapidly internalized and degraded and this process is LRP-dependent. In summary, our data indicate that LRP is a receptor for CTGF, and may play an important role in mediating CTGF biology.

Connective tissue growth factor (CTGF) (M₉ = 38,000 Da), is a member of the CCN family of growth factors, which is characterized by the presence and conserved spacing of some 38 cysteine residues. CTGF was initially identified in and subsequently purified from human umbilical vein endothelial cells conditioned media (1). Early studies demonstrated the strong induction of CTGF expression by transforming growth factor-β (TGF-β), and the promoter region of the CTGF gene contains a unique TGF-β response element not shared by other members of the CCN family (2). In an anchorage-independent growth assay of TGF-β, neutralization of CTGF activity with antibodies or inhibition of CTGF expression with antisense oligonucleotides reduced the ability of the cells to form colonies (3), suggesting that CTGF is a necessary part of the cascade for induction of anchorage-independent growth. More recently, CTGF was shown to be positively regulated by vascular endothelial growth factor (4, 5), epidermal growth factor, fibroblast growth factor (4), plasma clotting factor VIIa (6, 7), thrombin (7, 8), and by lysophosphatidic acid and serotonin activation of heptahedral receptors (9), but negatively regulated by tumor necrosis factor-α (10) and the Wilms tumor suppressor WT1 (11).

The initial discovery of CTGF in vascular endothelium (1), and the subsequent demonstration that CTGF is involved in the proliferation and migration of vascular endothelial cells (12), suggests that CTGF is also an angiogenic factor. The isolation of CTGF from uterine fluid and localization in embryonic and placental tissues suggests a role for CTGF in embryo implantation (13, 14).

CTGF is expressed at high levels during granulation tissue deposition in normal healing wounds (15, 16). Expression of the extracellular matrix proteins fibronectin, α5-integrin, and type I collagen is regulated by CTGF (16–18). Co-administration of CTGF with TGF-β produced a persistent fibrotic reaction that lasted for 14 days, but had resolved and was absent by 7 days in animals treated with TGF-β alone (19). The overexpression of TGF-β in fibrotic lesions is well documented (reviewed in Refs. 20 and 21), and now many reports indicate that CTGF, too, is overexpressed in many fibrotic lesions (Refs. 22–28 and reviewed in Refs. 29). The emerging understanding that CTGF is actively involved in the induction and/or maintenance of persistent fibrosis has provided a target for the modulation of matrix overproduction in fibrotic disease.

The low density lipoprotein receptor-related protein (LRP)/α2-macroglobulin receptor (LRP) (heretofore referred to as LRP), is a member of the family of low density lipoprotein (LDL) receptors (30). The LDL receptor family includes two subfamilies: one containing “small” receptor members of ~120 kDa, including the LDL receptor (LDLR), apoE receptor-2 (apoER2), and very low density lipoprotein receptor; and one containing “large” receptor members of ~600 kDa, including LRP,
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epithelial glycoprotein 330/megalin, and a related protein, sorLA. LDL family receptors bind multiple ligands. At least two of these protein ligands, apolipoprotein E (apoE) and a 39-kDa receptor-associated protein (RAP), bind to all LDL receptor family members (30). A number of functionally and structurally distinct ligands bind LRP and the diversity of these ligands suggests that it may function in a variety of distinct physiological processes, such as lipoprotein metabolism, protease regulation, tissue repair and remodeling, and embryonic development (reviewed in Ref. 30).

Here we have further characterized the major CTGF-binding protein. This protein was affinity purified and identified as LRP. Competitive inhibition of CTGF binding using other LRP ligands and immunoprecipitation of CTGF receptor complexes with LRP antibodies has confirmed that LRP is a receptor for CTGF. Additionally, this report shows that CTGF is rapidly internalized and degraded by cells through an LRP-dependent pathway. This is the first identification of direct interaction of a growth factor with LRP. Our findings suggest that LRP has a regulatory role in the biology of CTGF.

EXPERIMENTAL PROCEDURES

Materials—A baculovirus expression system was used for the preparation of recombinant human CTGF (rhCTGF) and the protein was purified as described. Apolipoprotein E (apoE), LDL, lactoferrin, sM, and chloroquine were purchased from Sigma. Membrane grade Triton X-100 was purchased from Roche Molecular Biochemicals Corp. (Indianapolis, IN). Heparin was purchased from Life Technologies, Inc. (Bethesda, MD). A panel of receptor-grade detergents, including Nonidet P-40 (Nonidet P-40), Triton X-100, Tween 20, digitonin, dodecyl-β-maltoside, octylglucoside, deoxycholic acid, and CHAPS was purchased from Roche Molecular Biochemicals.

Cell Lines—The cell lines MG63, MEF, PEA10, and PEA13 were purchased from the American Type Culture Collection (ATCC). Cells were maintained as recommended by the ATCC. The murine bone marrow stromal cell line BMS 2 was graciously provided by Dr. Jeff Nations. Alternatively, 0.1 mM ethylene glycolbis(sulfosuccinimidyl) carbonate (Sulfo-EGS, Pierce Chemical Co., Rockville, IL). 50 mM HEPES buffer, pH 7.4, 100 mM NaCl, and 3 mM MgCl2. The nuclei and whole cells were removed by centrifugation for 5 min at 800 × g, and the supernatant was collected. The supernatant was centrifuged over a cushion of 45% sucrose in Dulbecco’s PBS, pH 7.2, for 1 h at 24,000 × g. The membrane fraction located at the sucrose/PBS interface was carefully collected, diluted, and concentrated by centrifugation at 100,000 × g for 15 min. The membrane pellet was resuspended in Dulbecco’s PBS, and protein content estimated with the Pierce BCA reagent against an albumin standard (Pierce Chemical Co.). From a preparation of an estimated 109 cells, 106–107 of rhCTGF was iodinated with chloramine-T (Sigma) by the procedures described. For binding analysis, cells were plated at 2 × 104 cells/cm2 in 24-well dishes, 16–24 h prior to a binding experiment. Cells were washed twice with binding buffer (Dulbecco’s phosphate-buffered saline (PBS) (Life Technologies, Inc.) containing 0.2% bovine serum albumin and 0.2% sodium azide) at 4 °C. Binding experiments were performed by incubating monolayers of cells with various concentrations of 125I-rhCTGF in binding buffer for 4 h at 4 °C with gentle rocking. Duplicate wells were incubated with at least 100-fold excess of unlabelled rhCTGF for the determination of nonspecific binding.

We determined the kinetics of binding of 125I-rhCTGF to cells as follows. Supernatants were collected from the cells and directly counted for cross-linking. Cross-linking proceeded at room temperature for 15 min (BS) or at 4 °C for 30 min (Sulfo-EGS) and was terminated by removing the medium and washing the cells three times with buffer containing 250 mM sucrose, 10 mM Tris, pH 7.4, and 10 mM EDTA at 4 °C. The complexes were collected in the soluble fraction following cell lysis with 1% Triton X-100 (unless indicated) in PBS with a mixture of protease inhibitors (Calbiochem, San Diego, CA). In some experiments, 0.5% Nonidet P-40 was used for cell lysis.

Preparation of Crude Cell Membranes—Membranes were prepared from BMS2 cells by a modification of the procedure described by Atkin et al. (31). The cells were grown to confluence in roller bottles, and then dissociated with 5 mM EDTA in Dulbecco’s PBS lacking calcium and magnesium. Cell pellets were then collected by centrifugation, washed, and lysed with 1% Triton X-100 in PBS with protease inhibitors (Calbiochem, San Diego, CA).

In some experiments, 0.5% Nonidet P-40 was used for cell lysis.

Affinity Chromatography—One milligram of rhCTGF prepared from baculovirus was immobilized on Reacti-Gel(TM) GF-2000 (Pierce Chemical Co.) according to the manufacturer’s specifications. The membranes (10–15 mg) were solubilized in 0.2% Triton X-100, 20% glycerol, in Dulbecco’s PBS (Buffer A) containing a mixture of protease inhibitors, centrifuged (14,000 × g) for 10 min at 4 °C to remove insoluble material, and applied to the CTGF affinity matrix. The sample was re-circulated over the column 5–10 times. The flow through was collected and washed with 20 column volumes of Buffer A. The bound sample was eluted with a gradient of 0.135–2 M NaCl in buffer containing 0.2% Triton X-100, 20% glycerol, and PBS. The CTGF receptor-containing fractions were identified using a solution binding assay (described below). Further purification was achieved by electrophoresis of the fractions in 5% SDS-PAGE under nonreducing conditions.

Solution Binding Assay—Preparations of membrane proteins (5 μg each) were solubilized in 0.2% Triton X-100, 20% glycerol in Dulbecco’s PBS (Buffer A) and the insoluble material was removed by centrifugation (14,000 × g) for 10 min at 4 °C. Fractions from the affinity matrix were used directly in the solution binding assay (10 μl fraction). The samples were incubated with 0.2 mM 125I-rhCTGF for 3–4 h. The cross-linker, BS3, was added to a final concentration of 0.5 mM, and the reaction proceeded at room temperature for 15 min. Gel sample buffer was added to each sample, the samples were then heated for 2 min at 100 °C, and applied to 5% SDS-PAGE. Following electrophoresis, the gels were dried and analyzed by autoradiography.

Spectroscopy—The band of interest (migrating above the 220-kDa marker) was excised with a fresh razor band, destained, and subjected to trypsin digestion (32). The recovered peptide fragments were analyzed by liquid chromatography-mass spectrometry. Microelectrospray columns of 360 μm outer diameter × 100 μm inner diameter fused silica capillary were packed with 10–12 cm PORGOS 10R2, a reversed phase packing material (PerSeptive Biosystems, Framingham, MA) (33). The flow from the high performance liquid chromatography pump (typically 150 μl/min) was split pre-column to achieve a flow rate of 500 n/min. The mobile phase for the gradient elution consisted of (A) 0.5% acetic acid and (B) acetonitrile/water 80:20 (v/v) containing 0.5% acetic acid. The gradient was linear from 0 to 60% B in 30 min. Mass spectra were recorded on an LCQ ion trap mass spectrometer (Finnigan MAT, San Jose, CA) equipped with a microelectrospray ionization source (33). Tandem mass spectra were acquired during the entire gradient automatically as previously described (34). Protein sequence data bases were searched with the tandem mass spectra using the computer program SEQUEST (35). SEQUEST correlates tandem mass spectra of peptides with amino acid sequences from protein and nucleotide data bases (15 public data bases available). The NCBI Nonredundant protein sequence database was used to search an ASCII file in the FASTA format from Frederick Biomedical Supercomputing Center (ncbi.nlm.nih.gov in/pub/nr/db) by anonymous ftp. Each sequence produced by SEQUEST was verified by manually inspecting the fit of the amino acid sequence to the corresponding tandem mass spectrum.

Purification of RAP—Plasmid DNA containing the human RAP pro-
tein sequence was purchased from the ATCC as the I.M.A.G.E. Consortium Clone ID 511113 (36). The RAP cDNA sequence was excised from the vector with the restriction enzymes \( \text{Bam} \)HI and \( \text{Xho} \)I, and the 1-kilobase fragment was gel purified. The \( \text{pGEX-4T-1} \) vector (Amer-
sham Pharmacia Biotech) was cut with \( \text{Bam} \)HI and \( \text{Xho} \)I, into which the RAP cDNA was ligated. Expression of RAP from this expression
construct results in the formation of a fusion protein of RAP and
\( \text{glutathione S-transferase} \). Correct RAP cDNA insertion was verified by
sequence analysis. The RAP-GST fusion protein and the GST protein
alone (control vector) were expressed in \( \text{Escherichia coli} \) and purified as
described (37).

**Antibodies and Immunoprecipitation**—A mouse monoclonal antibody
reactive to the COOH terminus of LRP was prepared from IgG-11H4
hybridoma supernatant (ATCC) and purified by Protein G-Sepharose
(Amersham Pharmacia Biotech). Monoclonal antibodies reactive with
the extracellular domains of the \( \alpha \) chain and \( \beta \) chain of LRP were
purchased from American Diagnostica (catalog numbers 3402 and
3501, respectively).

Affinity labeled cells were extracted with 1% TritonTM X-100 or 0.5%
Nonidet P-40 in PBS containing a mixture of protease inhibitors. The
extracts were incubated for 2.5 h at 4 °C with 1 μg of antibody to LRP.
Protein G-Sepharose was added to the sample and the incubation
continued at 4 °C for 1 h. The sample that bound to the suspension was
collected by centrifugation, washed four times with PBS, then eluted by
boiling with Laemmli gel buffer. The eluted proteins were applied to 5%
SDS-PAGE, and analyzed by autoradiography.

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**Fig. 1. Purification of a CTGF receptor.** A, the solubilized receptor binds CTGF. Crude membranes were prepared from BMS2 cells and
solubilized in 1% Triton in PBS with 10% glycerol, or 20% glycerol, and a mixture of protease inhibitors. Intact membranes were used as a binding
control (far left panel). The membranes were affinity labeled with 0.2 nM \(^{125}\text{I}-\text{rhCTGF} \) and cross-linked. Half the samples also contained a 200-fold
excess of unlabeled CTGF. The cross-linked samples were separated on 5% SDS-PAGE under nonreducing conditions. The gels were dried and
visualized by autoradiography. Molecular size markers are indicated at the left in kDa, and an arrow indicates the CTGF-receptor complex. The
far right panel demonstrates \(^{125}\text{I}-\text{rhCTGF} \) cross-linked to BMS2 cells in monolayer culture. B, binding assay of solubilized membrane proteins
separated by CTGF affinity chromatography. Crude membrane preparations were solubilized in 1% Triton X-100 in PBS with 20% glycerol and
protease inhibitors. The sample was applied to a CTGF affinity matrix and washed through with Buffer A. The bound material was eluted with a
NaCl gradient. An aliquot of the loaded sample, the flow through, and each fraction collected during wash and elution were analyzed by affinity
labeling, cross-linking, and separation on nonreducing 5% SDS-PAGE. Peak fractions are indicated with a bracket. An arrowhead indicates the
CTGF-receptor complex. C, Coomassie staining of affinity purified receptor. The peak fractions were pooled and separated on nonreducing 5%
SDS-PAGE. The upper band (long arrow) migrates at the expected position for the previously identified complex. Short arrows indicate 2 other
proteins co-purifying on the column. The three indicated bands were excised for further analysis.
Internalization Experiments—To determine the kinetics of internalization of CTGF following binding to the cell surface, MG63 cells grown in 6-well dishes were affinity labeled at 4 °C with 0.2 nM 125I-rhCTGF for 3 h in binding buffer prepared with minimal essential medium containing 0.2% bovine serum albumin (MEM/BSA). Nonspecific binding, internalization, and degradation were determined by the addition of 50 nM unlabeled CTGF and subtracted from experimental values. In some samples, 3 μg/ml heparin or 250 nM lactoferrin was added to the cells during binding. After the binding period, the binding medium was removed and the cells were carefully rinsed twice with fresh, cold MEM/BSA. Pre-warmed MEM/BSA was then added to the cells and the cells were incubated at 37 °C in a CO2 humidified incubator. At 0, 15, 30, 60, 120, and 180 min after transfer to 37 °C, the cells were harvested. The medium was collected, treated with 10% trichloroacetic acid, and the precipitable fraction was separated by centrifugation. In these experiments, the supernatant resulting from trichloroacetic acid precipitation contained the degradation products of 125I-rhCTGF that had been internalized, degraded, and returned to the extracellular space (i.e. to the conditioned media). The internalized fraction was determined following trypsinization of the cell layer for 10 min. The resulting cell pellet was collected by centrifugation. The radioactivity associated with the trypsin-resistant pellet represented the internalized fraction of 125I-rhCTGF. To demonstrate LRP-mediated endocytosis of CTGF, MG63 cells were affinity labeled with 0.2 nM 125I-rhCTGF in MEM/BSA in the presence or absence of 50 nM RAP-GST. Nonspecific binding, internalization, and degradation of 125I-rhCTGF were determined by the addition of 50 nM unlabeled CTGF and subtracted from experimental values. To inhibit lysosomal activity, 200 μM chloroquine was added to some of the cultures. After a 2-h incubation period at 37 °C, the degraded and internalized fractions were determined as described above.

RESULTS

Solubilized Receptor Assay—The bone marrow stromal cell (BMS2) line was chosen as a source for purification of the receptor due to their high level of CTGF-binding to a low affinity receptor. The ease of growth and subculture combined with the high level of CTGF binding were factors involved in choosing these cells for receptor purification. Prior to affinity purification, we optimized the binding and solubilization conditions that would allow for solubilization of cell membrane proteins as well as retain the capacity to bind CTGF. Crude membranes were prepared from BMS2 cells by homogenization and differential centrifugation. A panel of receptor-grade detergents was examined individually in order to solubilize the membrane proteins. Solubilized membranes were incubated with 0.2 nM 125I-rhCTGF in the presence or absence of 200-fold excess unlabeled CTGF. The samples were cross-linked with BS3 and separated by 5% SDS-PAGE. Competitive cross-linking and binding was achieved using 1% Triton X-100 containing glycerol to help stabilize the membrane proteins (Fig. 1A). The left panel demonstrates CTGF binding and cross-linking to intact membrane fragments; the addition of 10 and 20% glycerol to the solubilized membranes gave similar binding results, with the inclusion of 20% glycerol providing more favorable results (middle and right panels, Fig. 1A). These conditions were used for the affinity purification of the receptor.

Affinity Purification—Membranes prepared from BMS2 cells were solubilized and applied to a column of CTGF coupled to Sepharose, washed with the same solvent, and bound proteins were eluted with a salt gradient as described above. Fractions were collected and incubated with 125I-rhCTGF to assay for CTGF binding activity (Fig. 1B). The peak CTGF binding fractions eluted between 50 and 75% buffer B (1.15–1.65 M NaCl). Peak fractions were pooled and separated by SDS-PAGE (Fig. 1C). A band migrating above the highest Mr standard (220,000) and estimated as >400,000 was observed in the Coomassie-stained gel. We occasionally observed additional proteins migrating at Mr = 200,000 and 150,000 (as shown in Fig. 1C). These bands were excised and analyzed by mass spectroscopy.

Table I

| Peptide Sequence | M(r) | Kd (nM) |
|------------------|------|--------|
| RAAALSGLVALNTEK | 100,000 | 200,000 |
| KNAVQQLEPGHLLVHPLR | 150,000 | 300,000 |
| KRSRPFFFEIR | 200,000 | 400,000 |
| KTVLWPNGLSLIDIPAGR | 250,000 | 500,000 |
| KTVLWPNGLSLIDIP | 300,000 | 600,000 |
| KTVLWPNGLSLID | 350,000 | 700,000 |

The >400-kDa band contained the peptides listed in Table I and data base analysis identified the protein as LRP. Repeat purification and mass spectroscopy analysis confirmed the result. Identification of the M(r) 200,000 and 150,000 proteins was not achieved.

LRP Ligands Compete for CTGF Binding—That LRP was a binding protein for CTGF was further confirmed. A number of commercially available LRP ligands were tested for their ability to compete with CTGF for binding to cells (Fig. 2). All ligands tested inhibited 125I-rhCTGF binding to cells (Fig. 2A) albeit with 5–10-fold lower affinity than that of CTGF. Additionally, RAP which is able to displace all LRP ligands, prevented CTGF binding (Fig. 2B). Cross-linking analyses confirmed that the inhibition was due to lack of binding to the high M(r) receptor protein (Fig. 2C). These data support the identity of LRP as a CTGF-binding protein.

Binding of CTGF to LRP-deficient Cells—The availability of cells isolated from LRP gene deletion mouse embryos provided an opportunity to study CTGF binding on cells genetically lacking LRP. The cell lines examined include a homozygous LRP-deficient mouse embryo fibroblast cell line, PEA13 (−/−), a heterozygous LRP-deficient cell line, PEA 10 (+/−), and a wild type mouse embryo fibroblast, MEF1 (+/+).

Each of these cell lines was examined for CTGF binding in a CTGF-binding assay. The binding parameters obtained (determined by nonlinear regression analysis) are summarized in Table II. MEF1 (+/+) and PEA10 (+/−) cells bound 125I-rhCTGF with single site binding kinetics, while the LRP-deficient cells, PEA13, did not bind 125I-rhCTGF in this assay. The heterozygous cell line, PEA10, appeared to have approximately one-fifth the number of CTGF-binding sites as observed in wild type MEF1 cells, yet had the same Kd for binding. These data are suggestive of a gene dosage affect on LRP protein expression levels, but not on the kinetics of ligand association/dissociation.

Cross-linking analysis was performed using these LRP-deficient cells to confirm that the observed binding of CTGF to these cells involved the high Mr protein (Fig. 3). Both MEF (+/+) and PEA10 (+/−) cells, but not PEA 13 (−/−) cells, bound and could be cross-linked to CTGF to the high Mr protein. The binding of CTGF to the LRP-expressing cells, but not to the LRP-deficient cells, supports the identification that LRP is a CTGF-binding protein.
Immunoprecipitation of CTGF/High Mr Complexes—Further confirmation that LRP is the high Mr CTGF-binding protein was demonstrated using LRP antibodies. Detergent lysates were prepared from 2 cell types, BMS2 and MG63, that had been affinity labeled with $^{125}$I-rhCTGF, cross-linked, and immunoprecipitated with antibodies directed to LRP (Fig. 4). Antibodies specific to the extracellular epitopes in both the $\alpha$/H9251 and $\alpha$/H9252 subunit chains of LRP, as well as an antibody that recognizes a cytoplasmic domain of LRP, immunoprecipitated the CTGF-containing complex, while control normal murine IgG did not. CTGF, migrating at the front of the gel, was poorly immunoprecipitated with these LRP antibodies. Notably, the antibody recognizing the LRP $\alpha$/H9251 chain was most active for MG63 (human origin) cells while the antibody recognizing the LRP $\alpha$/H9252 chain was most active for BMS2 (murine origin) cells. The LRP $\alpha$/H9251 chain antibody is immunoreactive with the human protein only. While the $\alpha$/H9252 chain antibody is reactive with both human and rodent protein, the difference in recognition could be due to different detergent extraction conditions.

Internalization of CTGF—The internalization kinetics of CTGF following binding was examined. For these experiments, the MG63 cell line was utilized because the conditions often promoted lifting of BMS2 cells from monolayers whereas the MG63 cultures remained intact. Monolayer cultures of MG63 cells were incubated at 4 °C with 0.2 nM $^{125}$I-rhCTGF in medium (Fig. 2). Competition of LRP/$\alpha$/H9251 2MR ligands with CTGF. A, monolayers of BMS2 cells were incubated with 100 pM $^{125}$I-rhCTGF in the presence of indicated concentrations of unlabeled CTGF, apoE-4, LpL, or LDL. The bound material was collected and determined by counting. Bound is represented as a fraction of total (non-competing) CTGF bound. Each point is the average of duplicate samples and error bars indicate the standard deviation. B, monolayers of BMS2 cells were incubated with 50 pM $^{125}$I-rhCTGF in the presence of indicated concentrations of unlabeled CTGF, RAP, or RAP-GST. The bound material was collected and determined by counting. Bound is represented as a fraction of total (non-competing) CTGF bound. Each point is the average of duplicate samples and error bars indicate the standard deviation. C, monolayers of BMS2 cells were incubated with 50 pM $^{125}$I-rhCTGF in the presence of unlabeled competitor: 21.5 nM CTGF, 250 nM lactoferrin (LF), 574 nM apoE-4, 400 nM LpL, or 100 nM methylamine-activated $\alpha$/M (a2M-Me). The bound material was cross-linked to the cells. Lysates were separated by SDS-PAGE; the gel was dried and visualized by autoradiography. An arrow indicates the position of the CTGF-receptor complex.

**Table II**

| Cell line | Phenotype | $K_d$ (pM) | $B_{max}$ (fmol/10$^6$ cells) | No. receptors/cell |
|-----------|-----------|------------|-----------------------------|-------------------|
| MEF1      | +/+       | 489        | 40                           | 24,008            |
| PEA 10    | +/−       | 456        | 9                            | 5,227             |
| PEA 13    | −/−       | 0          | <0                          |                   |

**Fig. 3**. Affinity labeling and cross-linking of $^{125}$I-rhCTGF to LRP/$\alpha$/MR-deficient cells. MEF1 (+/+), PEA 10 (+/−), and PEA 13 (−/−) cells were grown in monolayer culture; affinity labeling with $^{125}$I-rhCTGF and cross-linking were performed. The Triton X-100 soluble fraction was run on SDS-PAGE; the gel was dried and visualized by autoradiography. An arrow indicates the position of the CTGF-receptor complex.

Immunoprecipitation of CTGF/High Mr Complexes—Further confirmation that LRP is the high Mr CTGF-binding protein was demonstrated using LRP antibodies. Detergent lysates were prepared from 2 cell types, BMS2 and MG63, that had been affinity labeled with $^{125}$I-rhCTGF, cross-linked, and immunoprecipitated with antibodies directed to LRP (Fig. 4). Antibodies specific to the extracellular epitopes in both the α and β subunit chains of LRP, as well as an antibody that recognizes a cytoplasmic domain of LRP, immunoprecipitated the CTGF-containing complex, while control normal murine IgG did not. CTGF, migrating at the front of the gel, was poorly immunoprecipitated with these LRP antibodies. Notably, the antibody recognizing the LRP α chain was most active for MG63 (human origin) cells while the antibody recognizing the LRP β chain was most active for BMS2 (murine origin) cells. The LRP α chain antibody is immunoreactive with the human protein only. While the β chain antibody is reactive with both human and rodent protein, the difference in recognition could be due to different detergent extraction conditions.

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Fig. 4. Immunoprecipitation of CTGF complexes with anti-LRP/α-MR antibodies. A, immunoprecipitation from MG63 cells. MG63 cells were affinity labeled and cross-linked with 125I-rhCTGF. The complexes were extracted with Triton X-100 and immunoprecipitated with anti-LRP-α, anti-LRP-β, or nonimmune murine IgG. A portion of the extract was saved and applied to the gel without immunoprecipitation. An arrow indicates the CTGF-receptor complex. B, immunoprecipitation from BMS2 cells. BMS2 cells were affinity labeled and cross-linked with 125I-rhCTGF. The complexes were extracted with Nonidet P-40 and immunoprecipitated with anti-LRP-α, anti-LRP-β, or mAb11H4. An arrow indicates the CTGF-receptor complex.

Diagrams and figures are not transcribed directly, but describe immunoprecipitation experiments showing the binding of CTGF to LRP under various conditions.

Fig. 5. Kinetics of the internalization and degradation of CTGF. MG63 cells were affinity labeled with 125I-rhCTGF alone (solid circles), with 3 μg/ml heparin (open squares), or with 250 nM lactoferrin (open circles), at 4 °C. After 3 h, the cells were shifted to 37 °C and at the indicated time points, the internalized (A) or degraded (B) fractions were determined. Internalized CTGF was measured as the trypsin-resistant cell pellet. Degraded CTGF was measured in the supernatant of trichloroacetic acid-treated cell medium. Each point is the average of triplicate samples and error bars indicate the standard deviation.

Pathway for the LRP-mediated internalization of CTGF, with ligand dissociation required for its degradation. RAP addition to the cultures eliminated CTGF internalization and degradation. These results provide evidence that CTGF uptake and degradation by cells are LRP dependent.

Discussion

In this study, we have presented evidence that the major cell membrane protein to which CTGF binds is a high Mr, protein, identified previously as LRP. This study provides the first report of a growth factor that binds directly to LRP, although recently the Wnt family of secreted molecules has been demonstrated to bind to other members of the LRP family (39). Previous suggestion of growth factor interaction with LRP has been through complexes with αM as a clearance molecule (40).

We were unable to obtain sequence information for two lower Mr proteins that were occasionally affinity co-purified with LRP (estimated Mr of 150,000 and 200,000). These proteins were not detected in the cross-linking/binding assay. CTGF, and the structurally related protein, Cyr61, bind to a number of integrins, including αvβ3, αmβ2, and αvβ1 (41–44). Recently it has been demonstrated that CTGF-dependent cell adhesion induces cell signaling through integrin-mediated pathways (42). While the identity of the co-purifying bands is unclear, the Mr estimates are suggestive for some of the integrin subunits. The possibility exists that these bands represented breakdown products of the large LRP protein. Further purification and analysis will be necessary for unambiguous sequence identity of these proteins.

Our data indicate that at least part of the CTGF-binding site on LRP is similar or common to the binding site utilized for
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FIG. 6. Internalization and degradation of CTGF is LRP/α2MR dependent. MG63 cells were incubated with 125I-rhCTGF alone, with RAP-GST, or with chloroquine for 2 h at 37 °C. After 2 h, the internalized (A) and degraded (B) fractions were measured as described in the legend to Fig. 5 and as described under “Experimental Procedures.” Each point is the average of triplicate samples and error bars indicate the standard deviation.

many of the LRP ligands, as these ligands competed for CTGF binding, although with lower affinity (Fig. 2). LRP contains multiple copies of cysteine-rich repeats known as LDLR class A repeats (complement-type repeats) arranged in 4 clusters, forming ligand-binding sites (45–49). The binding site for many LRP ligands has been sublocalized (50), and NMR solution structure of the complement-like repeat CR3 from LRP has recently been determined (51). It will be of interest to sublocalize the binding site for CTGF within the extracellular domain of LRP.

With the exception of RAP, which binds to LRP with very high affinity (Kd = 3 nM (52)), most LRP ligands bind LRP with a moderate affinity. For example, hepatic lyase binds LRP with a Kd of 52 nM (53); PEA binds LRP with a Kd of 14 nM (54); and, coagulation factor VIII binds LRP with a Kd of ~50 nM (55). Urokinase-type plasminogen activator (uPA) is synthesized as a single chain zymogen, pro-urokinase, and binds LRP with a Kd of 45 nM; the active two-chain enzyme (tc-uPA) binds LRP with a Kd of 60 nM. Complex formation with plasminogen activator inhibitor type I increases the affinity of uPA to LRP, such that the uPA/plasminogen activator inhibitor type I complex binds LRP with a Kd of 1–3 nM (56, 57).

Many LRP ligands are polyanionic proteins that bind heparin, suggesting that their binding to cell surface heparin sulfate proteoglycan may be required for interaction with LRP (58). Heparin sulfate proteoglycan may serve to concentrate the ligands at the cell surface, promoting binding with the relatively low affinity interaction with LRP. The observed Kd for CTGF binding to LRP is 0.5–1 nM, which is a much higher binding affinity than reported for other LRP ligands. In Neshytt et al., we showed that, while CTGF is a heparin-binding protein, CTGF is capable of binding to the surface of cells depleted of GAGs. Perhaps the higher affinity of CTGF for LRP obviates concentration of CTGF on the cell surface by heparan sulfate proteoglycans. Nevertheless, the significance of the high affinity binding of CTGF to LRP is unclear at this time, but suggests an important role for LRP in CTGF biology.

Experiments aimed at creating mice lacking LRP by genetic deletion of the LRP allele failed to produce viable embryos (59), suggesting an important role for LRP during early development. CTGF is highly expressed in uterine epithelium and in decidualizing endometrial stromal cells during early pregnancy, as well as in embryonic ectoderm, endoderm, and at the ectoplacental cone after implantation (13). LRP is localized to invading trophoblastic cells, in decidualizing tissue, and at the ectoplacental cone after implantation (61, 62). The similar developmental localization patterns support possible interaction between CTGF and LRP during embryonic development.

We have demonstrated that CTGF is rapidly internalized and degraded by cells, and that this represents an LRP-mediated process. We have experienced a rapid disappearance of CTGF from culture medium after addition of CTGF to cell cultures. A rapid internalization and degradation pathway would account for the inability to detect CTGF in conditioned medium. We suggest that one function of LRP in CTGF biology is to modulate the concentration of free CTGF in the extracellular space or at the cell surface.

Whether or not LRP serves as a signaling receptor for CTGF bioactivity remains to be determined. LRP binds and internalizes numerous ligands that would necessitate separate ligand-specific signaling mechanisms; yet, signaling involving LRP has been suggested in at least some systems. Most recently, signaling by the Wnt family of proteins requires the LRP-5 or LRP-6 members of the LRP family to function as co-receptors with the Frizzled family of receptors (39, 63, 64). In a different system, LRP mediates long-term potentiation in hippocampal neurons (65) and activation with activated α2M promotes calcium influx in neuronal cells (66). Other members of the LDLR family have recently been demonstrated to transduce extracellular signals. For instance, very low density lipoprotein receptor and apoE-R2 function as receptors in the Reelin/disabled-mediated neuronal migration pathway (67–70). The short cytoplasmic domain of LRP contains multiple potential endocytosis motifs including 2 NPXY motifs; but, a recent study demonstrated that a YXXIL motif serves as the dominant signal for LRP endocytosis (71), leaving the NPXY motifs available for interaction with other signaling or adaptor proteins. Disabled-1 (dab-1) interacts with the NPXY motifs of LRP in neuronal cells (69, 70, 72, 73). When tyrosine phosphorylated, mDab1 binds nonreceptor tyrosine kinases, such as src, fyn, and abl (74). Another member of this family, mDab2/p96/DOC-2, has ~50% sequence conservation with the amino-terminal sequence of mDab1, but is expressed in a wider variety of cells (75). Dab2/p96/DOC-2 has been shown to compete with SOS to bind Grb2, suggesting a negative regulatory role in the Ras signaling cascade (76) and may negatively regulate mitogenesis (77). Whether Dab2/p96/DOC-2 or an unidentified member of the Dab family binds to LRP remains unknown and is of much interest to investigate.

We have been unable to detect tyrosine phosphorylation of LRP in cells treated with CTGF (data not shown). In addition, we have examined LRP purified by CTGF affinity chromatography in kinase assays. Although an associated kinase activity toward casein co-purified with LRP, this kinase activity was found not from LRP itself, but rather from an associating protein. Most importantly, the kinase activity did not respond to CTGF stimulation. Therefore it remains unclear whether LRP phosphorylation plays any role in mediating biological functions of CTGF.

LRP and many of its ligands have been localized to the senile

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plagues of Alzheimer's disease (78–80), in plaque-associated activated astrocytes, but not in resting astrocytes (80). Recent biochemical data support a role for LR.P in the pathogenesis of Alzheimer's disease (81, 82). There is an increase in LR.P expression in monocytes from patients with coronary heart disease (83), and LR.P is abundantly expressed by smooth muscle cells and macrophages in human atherosclerotic lesions (84). In the renal ablation model of experimental kidney fibrosis, LR.P expression is significantly increased in the glomeruli and interstitium with preferential localization to the glomerular lesions (60). While to date CTFG has not been documented in Alzheimer's lesions, it is highly expressed in fibrotic kidney disease (22) and atherosclerotic plaques (24). With co-localization of CTFG and LR.P in these diseases, it will be important to understand the functional role of the high affinity interaction of these proteins.

It will be of interest to examine whether altered LR.P expression is a general marker of disease characterized by impaired healing or fibroproliferative disorders. Future work examining the role of LR.P in CTGF signaling by itself or as a co-receptor to a yet unidentified CTGF receptor, and the effect of the modulation of LR.P on CTGF activity will be important studies to probe the biology of CTGF and for the design of therapies for intervention in fibrotic disease.

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