A Connective Tissue Growth Factor Signaling Receptor in Corneal Fibroblasts

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Purpose. To biochemically characterize the receptor for connective tissue growth factor (CTGF) of human corneal fibroblasts (HCF).

Methods. Radiolabeled recombinant human CTGF was used to determine the specificity and time course of binding to low-passage cultures of HCF. The affinity and number of receptors present were calculated by Scatchard and best-fit analyses. In vitro immunoprecipitation assays with radiolabeled CTGF and soluble mannose 6-phosphate/insulin-like growth factor 2 receptor (M6P/IGF-2-R) alone, or with CTGF-related growth factors were conducted. Additionally, ^{125}I-CTGF-binding and CTGF-stimulated proliferation were measured in cultures of M6P/IGF-2-R knockout fibroblasts.

Results. Binding of ^{125}I-CTGF to fibroblast cultures was significantly displaced by CTGF, but not by related growth factors. Scatchard plot analysis indicated the presence of both a high-affinity, low-abundance binding site, and a low-affinity, high-abundance binding site; whereas, the best-fit analysis suggests a single high-affinity, low-abundance binding site. A 280 kDa complex containing cross-linked ^{125}I-CTGF was immunoprecipitated by antibodies to CTGF or M6P/IGF-2-R. M6P/IGF-2-R knockout cells have a reduced proliferative response to TGF-β, and don't proliferate at all in response to CTGF.

Conclusions. CTGF binds to the M6P/IGF-2-R with high affinity, and the M6P/IGF-2-R is required for CTGF-stimulated proliferation in fibroblasts. These observations suggest that the M6P/IGF-2-R may be a new antifibrotic target. (*Invest Ophthalmol Vis Sci.* 2012;53:3387–3394) DOI:10.1167/iovs.12-9425

Connective tissue growth factor (CTGF) is a 38 kDa secreted, cysteine-rich protein that was first identified in conditioned media from cultures of human umbilical vein endothelial cells.[1,2] CTGF belongs to the CCN (CTGF, Cyr61/Cef10, Nov) family of proteins, which all possess growth regulatory functions and are involved in cell differentiation.[3–5] CTGF stimulates proliferation of fibroblasts, induces contraction of fibroblast-populated collagen matrix, and increases synthesis of components of the extracellular matrix (ECM) components, including collagen and fibronectin.[6] Transforming growth factor beta (TGF-β) stimulates synthesis of CTGF, and CTGF mediates many of TGF-β's effects on proliferation, contraction, and ECM synthesis.[7–9] Expression of TGF-β and CTGF mRNA are significantly increased in many fibrotic diseases, including biliary fibrosis, sclerosis, corneal scarring, atherosclerotic blood vessels, and types of inflammatory bowel disease, leading to the hypothesis that TGF-β and CTGF play key roles in regulating scar formation.[10–14]

A complete understanding of the biological effects of CTGF on target cells depends on establishing the identity of the CTGF receptors and signal transduction pathways. Currently, there is limited information on CTGF receptors. The initial report of CTGF binding to cells indicated ^{125}I-CTGF binding to human chondrosarcoma cells (HCS-2/8) reached a plateau after 60 minutes, and was displaced by unlabeled CTGF, but not by unlabeled platelet-derived growth factor BB (PDGF-BB) or basic fibroblast growth factor (bFGF).[15] Scatchard analysis of specific binding suggested two classes of binding sites: a high-affinity class with low-capacity, and a low-affinity class with high capacity. Cross-linking of ^{125}I-CTGF to the HCS-2/8 labeled a protein, of approximately 250 kDa, that was displaced by unlabeled CTGF. CTGF has been primarily detected immunohistologically by the authors, and others, in a perinuclear cellular location, and it has been previously argued that this location represents newly endogenously synthesized CTGF in the Golgi.[16,17] Exogenous CTGF, however, also tracks to this perinuclear location,[18] suggesting that the CTGF-positive perinuclear vesicles may be endosomes. One known receptor, of approximately 280 kDa, that translocates from the cell surface to the endosomes is the cation-independent mannose 6-phosphate/insulin-like growth factor 2 receptor (M6P/IGF-2-R). This hypothetical endosomal connection makes the M6P/IGF-2-R an ideal candidate for a cell surface receptor for CTGF binding and uptake.

Another study utilized a murine bone marrow stromal cell line (BMS2) for characterization and purification of the CTGF-binding protein because the cells expressed a high level of relatively low-affinity CTGF binding.[19] Affinity purification of membrane proteins from BMS2 cells with CTGF identified three proteins with molecular weights (MWts) of 620 kDa, 200 kDa, and 150 kDa. Mass spectrometric analysis indicated the largest protein was the low-density lipoprotein receptor-related protein/α_2-macroglobulin receptor (LRP). Several LRP ligands, including apolipoprotein E4, lipoprotein lipase, and receptor-associated protein (RAP), inhibited ^{125}I-CTGF binding to the 640 kDa protein, albeit with a 5- to 10-fold lower affinity than that of unlabeled CTGF. Additional experiments by this group

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demonstrated that mouse embryo cell lines, which lack LRP, did not bind ^{125}I-CTGF, while those that were heterozygous for LRP, or were from wild-type embryos, bound ^{125}I-CTGF with a single-site binding kinetics. Immunoprecipitation with anti-LRP antibodies of solubilized membrane proteins cross-linked with ^{125}I-CTGF produced a complex of approximately 620 kDa. These data demonstrated that LRP is able to bind CTGF at a site that is utilized for many of the LRP ligands, but it is not clear whether the LRP is a signaling receptor for CTGF.

To further define the CTGF receptor system, the authors biochemically characterized CTGF binding to low-passage cultures of human corneal fibroblasts (HCF), and found CTGF associated with a 280 kDa receptor complex in these fibroblasts. Since molecular size and cellular location suggested the putative CTGF receptor could be the M6P/IGF-2-R,[20] the authors assessed CTGF binding to soluble M6P/IGF-2-R, as well as binding to, and stimulation of, proliferation in M6P/IGF-2-R knockout mouse fibroblast cultures.

Materials and Methods

Iodination of Recombinant Human CTGF

Recombinant human CTGF produced by using a baculovirus expression system[6] was labeled with iodine-125 (^{125}I; Amersham, Plc., Amersham, UK) using a low Chloramine-T method, per the manufacturer's kit instructions (formerly IODO-BEADS; Pierce Chemical Co., Rockford, MA). A PD-10 G-25 Sephadex column (Pharmacia, Stockholm, Sweden) was pre-equilibrated with reaction buffer containing 0.1% BSA and 0.05% Tween-20 (Thermo Fisher Scientific Inc., Waltham, MA) to reduce nonspecific binding to the column. The reaction solution was applied to a desalting column from which 1-mL fractions were collected, and the radioactivity profile was determined by γ-scintillation counting. Because CTGF can be cleaved into lower MWt fragments, column fractions containing ^{125}I-CTGF were further characterized by electrophoresis on SDS-15% polyacrylamide gels (Bio-Rad Laboratories, Inc., Hercules, CA), followed by autoradiography and Western blotting. The fractions containing intact 38 kDa CTGF with the highest specific activity were pooled and used for binding experiments. The pooled specific activity was approximately 33 μCi/μg.

Establishment of Fibroblast Cultures

Cultures of normal HCF were established from corneal explants (Lions Eye Bank, Jacksonville, FL). Briefly, epithelial and endothelial cells were removed from corneas that were unsuitable for corneal transplantation; the stroma was cut into cubes of approximately 1 mm^3, placed in culture medium consisting of equal parts Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Grand Island, NY), medium 199 (Gibco BRL, Grand Island, NY), and Ham's F12 nutrient mixture (Gibco BRL) containing 1 mM $NaHCO_3$ and buffered with 25 mM HEPES at pH 7.4. The medium was supplemented with 10% heat-inactivated normal calf serum and 1× antibiotic-antimycotic (Gibco BRL).

The M6P/IGF-2-R is necessary for postpartum viability, and mutant, or knockout, mice die within 6 to 12 hours after birth.[20] A cell line was established via outgrowth from lung tissue harvested from newborn, knockout mouse pups using methods similar to the establishment of the corneal fibroblasts, described above, and were provided by Mark W. K. Ferguson from the University of Manchester, UK. Lung fibroblasts from normal tissue in common laboratory (C57BL/6) mice were used as controls.

For all cultures used, the cells were harvested by trypsin/EDTA (Gibco BRL) when the outgrowth of fibroblasts reached approximately 50% confluence, and were passaged at a four-to-one split. All experiments were performed using fibroblasts between the fourth and seventh passages.

Time Course, Specificity, and Scatchard Analysis of ^{125}I-CTGF Binding to HCF Cells

To measure the time course of CTGF binding to HCF, cells were grown to confluence in 48-well plates. Cells were washed and incubated with chilled binding buffer (serum-free culture medium plus 1 mg/mL BSA), or binding buffer containing 2 μg/mL unlabeled CTGF at 4°C for 1 hour. ^{125}I-CTGF (25,000 cpm) was then added to the wells, and incubated at either 4°C or 37°C for the indicated times (0 minutes, 15 minutes, 30 minutes, 1 hour, 2 hours, and 4 hours). Cell layers were washed five times with chilled PBS containing 1 mg/mL BSA, solubilized in 1 mL of 1 N NaOH, and γ-radioactivity was counted as before. Specific binding of ^{125}I-CTGF was calculated by subtracting nonspecific binding (wells containing unlabeled CTGF) from total binding (wells without unlabeled CTGF). Each time point was performed in triplicate using the same cell line.

To measure the specificity of CTGF binding to cell surface receptors, HCFs were grown to confluence in a 48-well plate. Cells were washed as above, and incubated with 1 μg/mL of various unlabeled competitors (TGF-β_1, TGF-α, PDGF, epidermal growth factor [EGF], FGF, IGF-1, IGF-2 [all R&D Systems, Minneapolis, MN], insulin [Gibco BRL], CTGF, or mannose 6-phosphate [Sigma-Aldrich, St. Louis, MO]) for 1 hour at 4°C, then ^{125}I-CTGF (25,000 cpm) was added to each well and incubated for 1 hour at 37°C. Cells were washed, solubilized, and radioactivity was measured as before. Six technical replicate wells were used for each experimental condition, and mean binding values were compared for statistical significance using ANOVA and Tukey's HSD post hoc test.

A binding and uptake assay was performed on HCF cultures grown to confluence in a 48-well plate.[21] Cells were washed and incubated with increasing concentrations of unlabeled CTGF (1 pM to 1.0 μM) for 1 hour at 4°C, then ^{125}I-CTGF (50,000 cpm) was added to each well and incubated at 37°C for better physiological relevance for 1 hour, since the initial incubation experiments indicated that ^{125}I-CTGF binding and uptake plateaued at 1 hour (Fig. 1A). Cells were washed, solubilized, and γ-radioactivity was measured. Six replicate wells populated with the same cell line were used for each condition. The dissociation constants and receptor number values were calculated using both Scatchard plots and nonlinear fit analysis with either one or two specific binding sites using GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA).

M6P/IGF-II-R Western Blot Analysis

Fibroblasts, both basal and those stimulated with 5 ng/mL of TGF-β_1 for 24 hours, were extracted after cross-linking with dithiobis-succinimidyl propionate (Sigma Chemical, St. Louis, MO). Western blots of fibroblast cellular extracts and soluble bovine M6P/IGF-2-R was performed, essentially as described previously,[22] though the samples here were resolved on a 6% gel, and the lower MWts were allowed to run off of the gel.

SDS-PAGE of ^{125}I-CTGF Cross-Linked to HCF

HCFs were grown to confluence in two 75 cm^2 flasks. To maximize the yield of cross-linked CTGF-receptor complexes, cells were stimulated for 24 hours with 5 ng/mL TGF-β_1.[23] One flask was incubated with 2 μg/mL CTGF for 1 hour at 4°C. Then 4,000,000 cpm of ^{125}I-CTGF was added to the culture medium in each flask, and incubated for 2 hours at 4°C. The cells were washed, cross-linked, solubilized, and then the cleared supernatants were resolved by SDS-PAGE on a 3–8% gel (Bio-Rad Laboratories, Inc.) and an autoradiograph was generated using X-ray film.

Immunoprecipitation of ^{125}I-CTGF- M6P/IGF-2-R Binding Reactions

To assess the interaction between CTGF and the M6P/IGF-2-R, in vitro binding reactions were performed, followed by immunoprecipitation of complexes using a simple polyethylene glycol precipitation method,

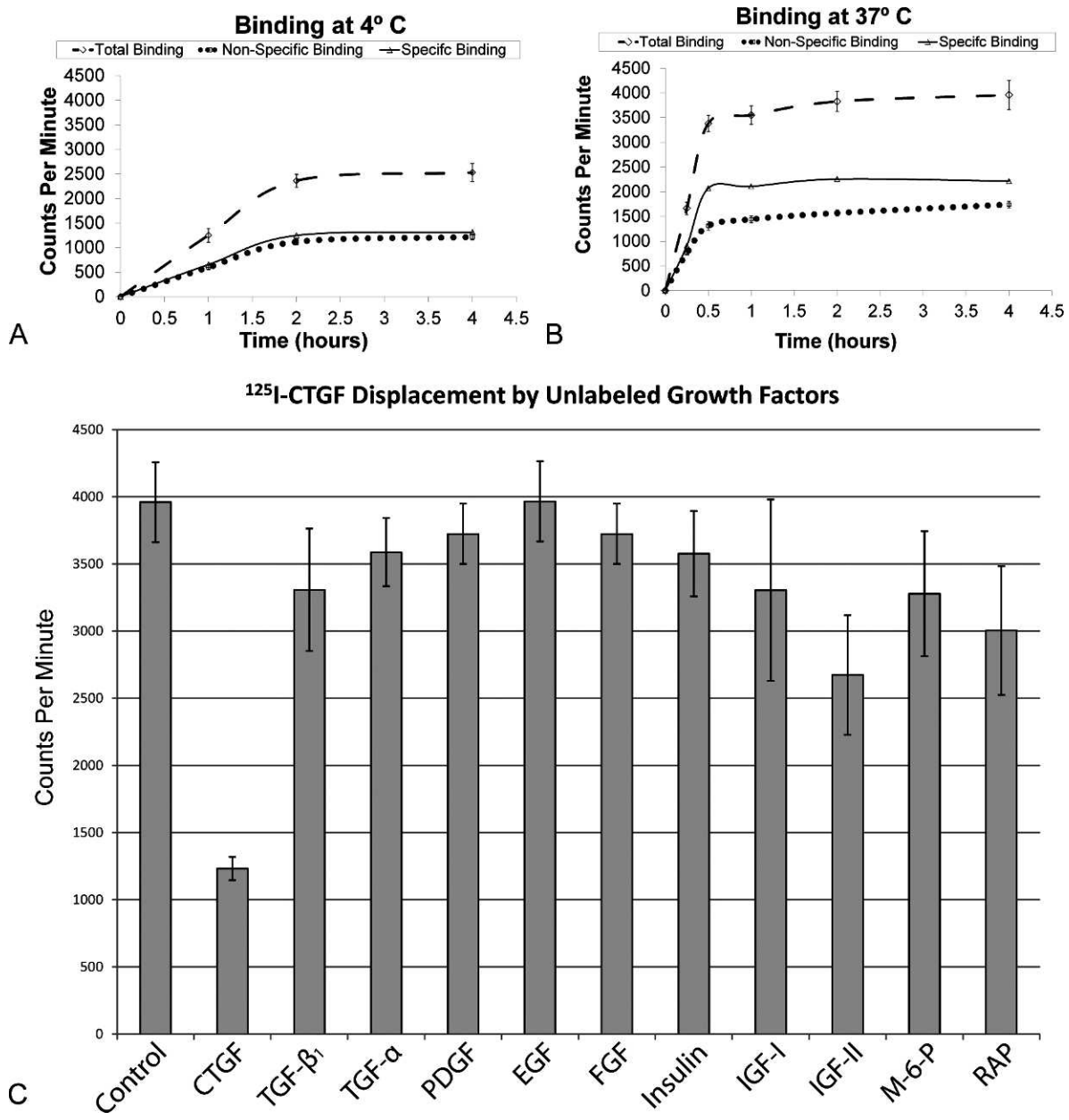


Figure 1. (**A**) and (**B**) Specific binding of ^{125}I-CTGF plateaus after 2 hours at 4°C or after 1 hour at 37°C ($n = 3$). (**C**) Radiolabeled CTGF is strongly displaced by unlabeled CTGF and slightly displaced by IGF-2 ($n = 4$).

which was found to give comparable results to Protein A/G precipitation. Binding studies were performed using a soluble form of the cation-independent M6P/IGF-2-R isolated from fetal bovine serum, supplied by Peter Lobel, of the University of Medicine and Dentistry of New Jersey, Piscataway, NJ.[24]

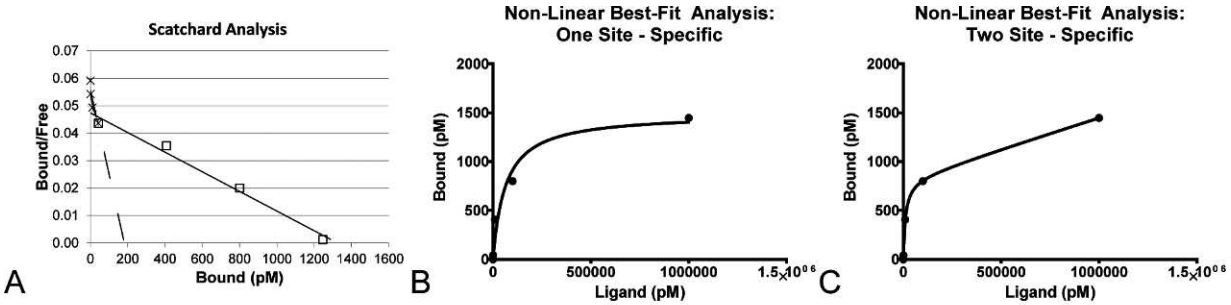
Precipitating antibodies were either an affinity-purified polyclonal goat anti-human CTGF,[22] or an affinity-purified polyclonal goat anti-bovine soluble cation-independent M6P/IGF-2-R, prepared similarly to the anti-CTGF antibody. Reactions containing the components in the combinations presented in Figure 4A were assembled in 1.5 mL microcentrifuge tubes and were incubated overnight at 4°C. Reaction complexes were then precipitated by adding 150 μL of 10 mg/mL gamma globulins (Sigma-Aldrich) and 150 μL of 20% polyethylene glycol (PEG) (MWt = 8000). Reactions were centrifuged at 14,000*g* for 15 minutes at 4°C, the pellets were then washed with 20% ethanol, and γ-radioactivity was counted. Each binding reaction was performed in triplicate using the same cell line, and ANOVA and Tukey's HSD post hoc test were used to assess statistical significance between groups.

Immunoprecipitation of Cross-Linked HCF Receptors

Reactions were assembled in the presence of 10 μL of 1 mg/mL BSA, 1000 cpm of ^{125}I-CTGF cross-linked HCF extracts (from above), and either affinity-purified polyclonal goat anti-human CTGF, or polyclonal goat anti-bovine soluble cation-independent M6P/IGF-2-R, and incubated overnight at 4°C. The reactions were precipitated and measured as explained above.

CTGF-Stimulated Cell Proliferation Assay in Wild-Type or M6P/IGF-2-R Knockout Mouse Fibroblasts

Wild-type control and M6P/IGF-2-R knockout lung fibroblasts were separately seeded into 48-well plates (10,000 cells per well), and



D

	Classical Scatchard	Non-linear: Single Site - Specific	Non-linear: Two Site - Specific
$K_{d,High}$ (nM)	3.3	63.6	10.9
$Sites_{High}$	18,000	1,493	820
$K_{d,Low}$ (nM)	133.3	n/a	1.8×10^{13}
$Sites_{Low}$	83,000	n/a	1.1×10^{13}
R^2_{High}	0.82	0.97	0.99
R^2_{Low}	0.98	n/a	

Figure 2. (**A**) A Scatchard plot was initially used to ascertain the binding kinetics and receptor numbers for two classes of binding to corneal fibroblasts. (**B**) Nonlinear best-fit analysis with one specifically binding site, and (**C**) with two specific binding sites. (**D**) The tabulated data obtained from the three different analyses of the same data.

cultured for 48 hours in serum-supplemented medium. The cultures were then washed and maintained in serum-free medium for 48 hours. The medium was removed and replaced by 1 of 10 test media: (1) 10% normal calf serum (Gibco BRL), (2) 5 ng/mL TGF-β_1 (R&D Systems), (3) 25 ng/mL CTGF, (4) 25 ng/mL CTGF + 6.5 μg/mL RAP (EMD Biosciences, Rockland, MA), (6) 6.5 μg/mL RAP (EMD Biosciences), (7) 1 ng/mL IGF-1 (R&D Systems), (8) 1 ng/mL IGF-2 (R&D Systems), (9) 5 ng/mL TGF-β_1 with 10 μM CTGF antisense oligonucleotide (ISIS Pharmaceuticals, Carlsbad, CA), (10) 5 ng/mL TGF-β_1 with 10 μM scrambled oligonucleotide (ISIS Pharmaceuticals). After 48 hours of additional culturing, cell proliferation was measured using a nonradioactive MTS cell proliferation assay (Promega, Madison, WI). Absorbance readings were measured from six biological replicate samples for each condition, and ANOVA and Tukey's HSD post hoc test were used to assess statistical significance between groups.

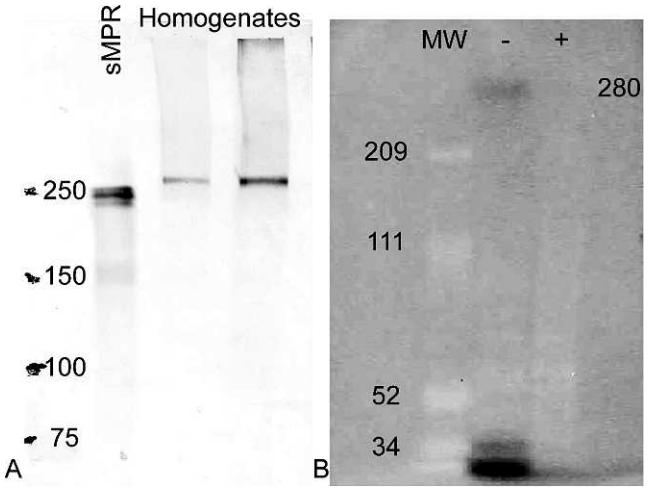


Figure 3. (**A**) M6P/IGF-2-R Western blot. Lane 1 is purified bovine soluble M6PR/IGF-2-R standard, while lanes 2 and 3 are cross-linked homogenates. Lane 2 represents basal receptor levels, and lane 3 shows an increase in cross-linked receptor levels following TGF-β_1 stimulation. (**B**) Autoradiograph of ^{125}I-CTGF cross-linked fibroblast extracts. In the absence of unlabeled CTGF (–), ^{125}I-CTGF could bind to fibroblasts. A cross-linked complex with a MWt of approximately 280 kDa was present in cell lysates without unlabeled CTGF (–). Nearly no radiolabeled CTGF was bound to fibroblasts in the presence of unlabeled CTGF (+).

Results

CTGF Binds Specifically to Two Distinct Cell Surface Receptors

As shown in Figure 1A, total binding of ^{125}I-CTGF to HCF at 4°C, increases steadily for 2 hours then reaches a maximum plateau of binding that is sustained between 2 and 4 hours. The observed profile of specific binding of ^{125}I-CTGF at 4°C, which minimizes internalization of plasma membrane receptors and degradation of ligands, is consistent with the presence of a binding protein located in the plasma membrane of HCF. The specific binding of ^{125}I-CTGF at 37°C reached a maximum at, or slightly before, 1 hour, and remained essentially constant during the next 3 hours. The level of specific binding measured at 37°C is approximately twice that of specific binding measured at 4°C in a similar number of HCFs, which is likely due to uptake of CTGF.

The specificity of ^{125}I-CTGF binding to HCF at 4°C is demonstrated by the lack of significant inhibition of ^{125}I-CTGF binding by the addition of other closely related growth factors

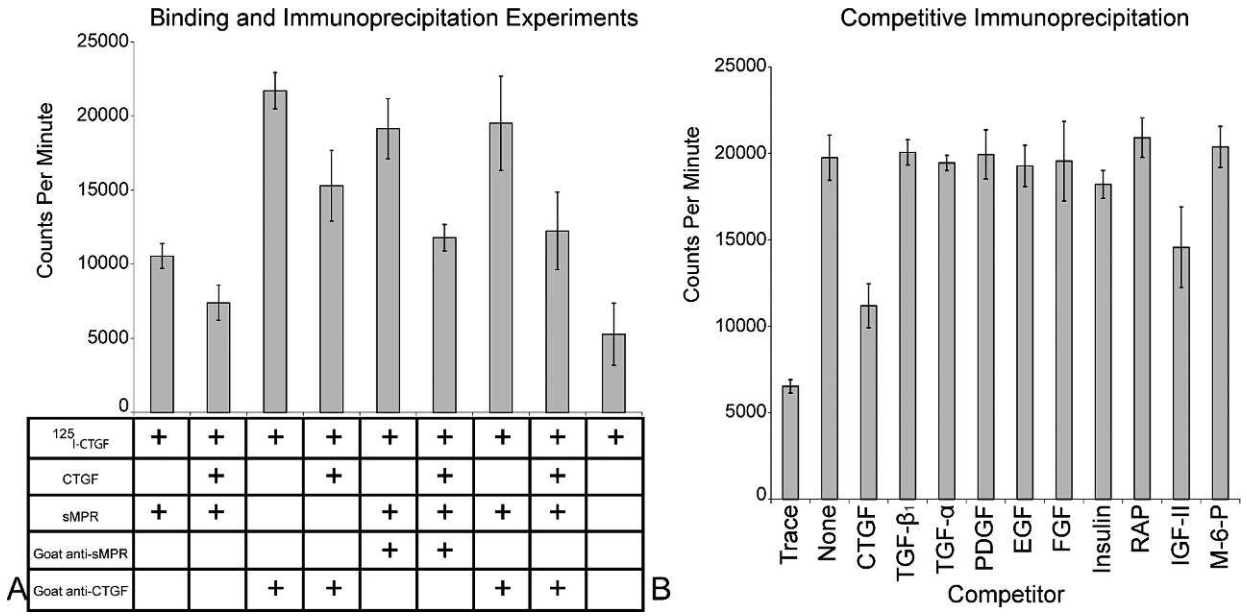


Figure 4. **(A)** Radiolabeled CTGF is co-immunoprecipitated with antibodies to sM6PR and the interaction can be inhibited by unlabeled CTGF ($n = 3$). **(B)** The interaction between radiolabeled CTGF and the sM6PR can be strongly inhibited by unlabeled CTGF, and slightly inhibited by IGF-2, but not other related competitors ($n = 3$).

($P > 0.05$, Fig. 1C). This suggests that CTGF binding to receptors on the surface of the cells is specific. Scatchard analysis of CTGF binding to HCF at 4°C reveals two classes of cell-surface receptors (Fig. 2A). One high-affinity, low-abundance class was observed ($K_d = 3.3$ nM, 18,000 receptors/cell). A second class of receptors, with lower affinity and higher abundance, was also detected ($K_d = 133.3$ nM, 83,000 receptors/cell). Using nonlinear curve fitting analysis for one specific binding site (Fig. 2B, $R^2 = 0.97$) revealed a K_d of 63.6 nM (11.3–115.7 nM, 95% Confidence Interval [CI]) with 1493 binding sites (1198–1787 sites, 95% CI); while nonlinear analysis for two specific binding sites (Fig. 2C, $R^2 = 0.99$) gave more ambiguous results of a $K_{d,high}$ between 0.0 and 54.9 nM with 753 to 867 receptors per cell (95% CI), and a $K_{d,low}$ in the kilo-molar range (10^{16} pM). The kinetics data obtained from these three different analyses are summarized in Figure 2D.

^{125}I-CTGF Binds to HCF and Forms a Macromolecular Complex with a Mass Consistent with M6P/IGF-2-R Ligand Complexes

Western blot studies of cross-linked basal, or TGF-β_1–stimulated, fibroblast homogenates revealed an anti–M6P/IGF-2-R immunoreactive band of approximately 280 kDa that was slightly larger than the soluble M6P/IGF-2-R standard (Fig. 3A). Moreover, TGF-β_1 stimulation increased the detectable amount of the M6P/IGF-2-R; thus, our autoradiographic analysis was performed on TGF-β_1–stimulated corneal fibroblasts, and revealed that ^{125}I-CTGF formed a cross-linked receptor complex of approximately 280 kDa, which was not observed in the presence of excess unlabeled ligand (Fig. 3B).

CTGF Binds Specifically to the Type-2 Insulin-Like Growth Factor Receptor

To confirm the authors' hypothesis that the M6P/IGF-2-R was the 280 kDa receptor identified on their autoradiograph, they first performed in vitro binding studies with ^{125}I-CTGF and soluble bovine M6P/IGF-2-R. The interaction of ^{125}I-CTGF with soluble M6P/IGF-2-R (sM6PR) is inhibited in the presence of unlabeled CTGF (Fig. 4A, bars 1 and 2). An affinity-purified antibody to CTGF precipitates ^{125}I-CTGF, and is inhibited by the addition of unlabeled CTGF (Fig. 4A, bars 3 and 4). The ^{125}I-CTGF with sM6PR binding complexes are precipitated by affinity-purified antibodies, either to human sM6PR (Fig. 4A, bars 5 and 6) or to CTGF (Fig. 4A, bars 7 and 8). The amount of trace ^{125}I-CTGF precipitated by PEG alone is much lower than the binding reactions ($P < 0.01$, Fig. 4A, bar 9). Figure 4B shows that other closely related growth factors and IGF family members do not exhibit strong inhibition of ^{125}I-CTGF binding to sM6PR, IGF-2 somewhat diminishes CTGF's binding ($P = 0.09$), but not to the degree of unlabeled CTGF ($P < 0.01$). These results demonstrate that CTGF binds to the M6P/IGF-2-R, and that the binding is not strongly inhibited by any of the other related growth factors tested.

To correlate the specificity of in vitro immunoprecipitations with cellular receptors, the authors demonstrated that HCF cell membrane extracts, which are covalently cross-linked with ^{125}I-CTGF, are immunoprecipitated by affinity-purified antibodies to either sM6PR or to CTGF (Fig. 5). The amount of precipitated counts is significantly higher ($P < 0.01$) when compared to the control nonspecific antibody, or the absence of the primary antibody. This result shows that CTGF binds, not only to the M6P/IGF-2-R in vitro, but also those present on fibroblast cell membranes in cell culture.

Type-2 Insulin-Like Growth Factor Receptor Knockout Cells are Insensitive to CTGF Mediated Proliferation

Binding and cell proliferation experiments were performed in knockout cells lacking the putative receptor to confirm that CTGF binds to and signals through the type- 2 IGF receptor in cell cultures. Figure 6A shows that there is no significant binding to cells lacking the type-2 IGF receptor when compared to normal mouse lung fibroblasts ($n = 6$, $P < 0.01$). The same two cell cultures were used to measure cell proliferation in response to exogenously added growth factors and agents (Fig. 6B). Both cell types showed high levels of cell

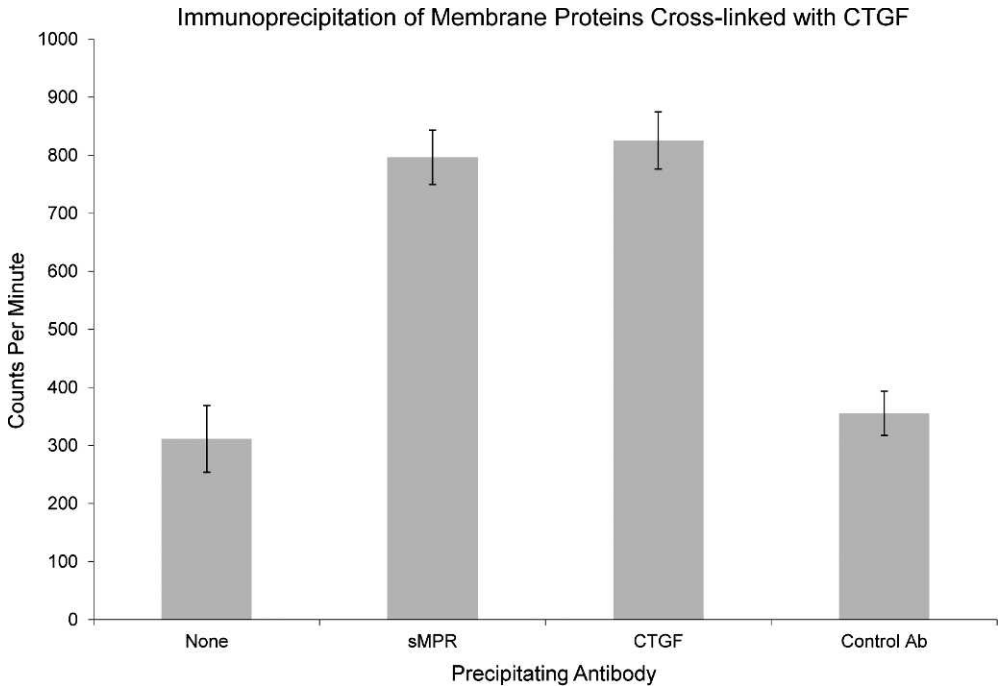


Figure 5. Radiolabeled CTGF cross-linked to fibroblasts can be immunoprecipitated with an antibody to CTGF or co-immunoprecipitated with an antibody to soluble sM6PR, but not by irrelevant goat IgG ($n = 3$).

proliferation in response to serum, though the knockout cells seemed to have a reduced capacity to proliferate. Wild-type fibroblasts increased proliferation when exposed to TGF-β_1 or CTGF, as expected. In marked contrast, the M6P/IGF-2-R knockout fibroblasts showed an attenuated proliferation in response to TGF-β_1, and did not proliferate at all when exposed to CTGF. These results show that the M6P/IGF-2-R is necessary for CTGF-stimulated proliferation of corneal fibroblasts. The presence of RAP appeared to have no effect on cell proliferation, suggesting that the low-density LRP is not responsible for CTGF-mediated TGF-β_1 signaling in this cell type. A marked difference between IGF-2–induced and IGF-1–induced cell proliferation is evident between the knockout and wild- type cells ($P < 0.01$). The result for IGF-2 is expected, due to absence of the type-2 receptor, while the result for IGF-1 is more puzzling. The decrease in proliferation in response to IGF-1 suggests that there may be a role for the M6P/IGF-2-R in the regulation of the type-1 receptor, or its activity in a more indirect, nonbinding, manner. Finally, CTGF antisense oligonucleotides reduced TGF-β_1–induced cell proliferation in both cell types, suggesting that CTGF synthesis is required, and acts as a mediator of TGF-β_1's effects on the cells ($P = 0.17$). This blockage is not seen in the presence of scrambled control oligonucleotides.

Discussion

The results of these studies provided evidence for the presence of up to two CTGF-specific receptors on the surface of HCF. The presence of a high-affinity, low-abundance receptor was consistent among the analysis methods chosen. The Scatchard plot data roughly agreed with the findings of Nishida et al.[15]

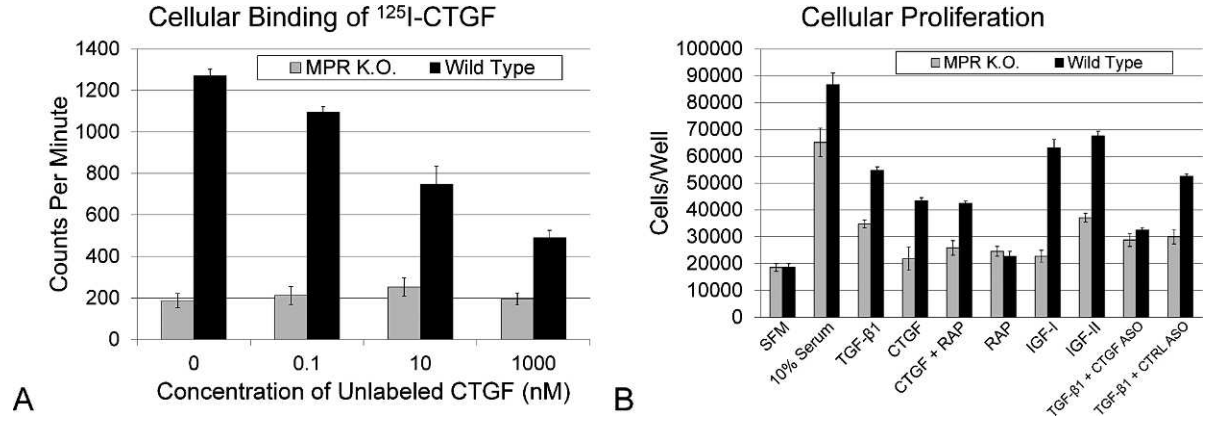


Figure 6. (**A**) Radiolabeled CTGF binds to wild-type fibroblasts, but not fibroblasts derived from a M6P/IGF-2-R knockout mouse ($n = 6$). (**B**) M6P/IGF-2-R knockout fibroblasts do not proliferate in response to CTGF, and have a diminished proliferative response to other mitogenic agents ($n = 3$). Also, a CTGF antisense oligonucleotide reduced TGF-β_1–induced proliferation in wild-type cells to a level comparable with that of the knockout cells.

and indicated that CTGF may possess two distinct binding interactions on the cell surface of cultured HCF. The authors did not measure any interaction with kinetics equivalent to those measured by Segarini et al.,[19] for CTGF binding to LRP, which may be due to differences in the cell types used, and the authors' choice of incubation temperature. Additionally, while the 280 kDa complex (Fig. 3) was observed, there were not any higher MWt complexes observed, nor did the LRP binding antagonist, RAP, block CTGF binding to our cells (Fig. 1B, 6B). These data suggest that the LRP binding CTGF receptor is not present in the cell types studied.

The in vitro experiments demonstrated that radiolabeled CTGF can co-immunoprecipitated with an antibody to the sM6PR (Fig. 4A). Furthermore, the co-immunoprecipitation of ^{125}I-CTGF with the sM6PR is blocked by CTGF, it is only slightly inhibited by the addition of IGF-2, but not any of the other growth factors tested, including the LRP binding protein RAP (Fig. 4B); thus, mirroring the findings of the cell surface binding studies (Fig. 1C). The M6P/IGF-2-R is known to have at least three defined ligand binding domains, one that binds IGF-2 and the remaining two bind M-6-P.[25] The data now suggests that there is a fourth distinct domain that binds CTGF since neither M-6-P nor IGF-2 caused significant displacement of ^{125}I-CTGF. The marginal inhibition of CTGF binding with the addition of IGF-2 could be consistent with a change in K_d due to allosteric changes upon binding of either CTGF or IGF-2. Finally, the authors have demonstrated that mouse lung fibroblasts from mice lacking the M6P/IGF-2-R have decreased binding capacity for CTGF, and are insensitive to TGF-β_1–induced, CTGF-mediated proliferation. These combined data indicate that CTGF binds to the M6P/IGF-2-R, and suggests this interaction is necessary for CTGF-induced proliferation of fibroblasts.

The data presented herein demonstrated that CTGF may bind in two distinct interactions on the cell surface of HCF. An explanation for this observation is that CTGF, like FGF and hepatocyte growth factor, can bind to both low-affinity, nonsignaling, cell surface proteoglycans, and to high-affinity, signal-transducing, transmembrane receptors.[26,27] The C-terminal domain of CTGF (domains 3 and 4) is known to bind heparin,[28] and heparin was previously demonstrated to be necessary for CTGF binding.[29] This known interaction with heparin may account for the observed ambiguous low-affinity, high-abundance receptor class, while the authors' novel identification of the M6P/IGF-2-R most likely accounts for the observed high-affinity, low-abundance binding receptor class. Additional Scatchard analysis with the knockout fibroblasts could provide more solid evidence of the identities and binding characteristics of the two classes of observed binding interactions.

The identification of the M6P/IGF-2-R as the signaling receptor for CTGF-induced proliferation opens a substantial body of work, which contains facts that provide further insight into many of the disparate observations from those studying CTGF. For instance, it has been demonstrated that treatment of cells with cAMP can inhibit CTGF-mediated collagen synthesis.[22] When this fact is integrated with the fact that the M6P/IGF-2-R possesses a cytosolic domain, which is a known cAMP-dependent kinase substrate,[25,30] new testable hypotheses emerge about components of CTGF signaling.

TGF-β_1 levels, and levels of key extracellular matrix proteins, have been correlated with incidence of corneal haze after excimer laser ablation[31] It has also been demonstrated that TGF-β_1 can induce the expression of CTGF,[32] and increasing evidence shows that CTGF is an essential factor in mediating TGF-β_1–induced fibroblast proliferation and myofibroblast transdifferentiation during fibrosis.[33–35] The fact that the authors were able to mitigate TGF-β_1–induced proliferation with an antisense oligonucleotide to CTGF, but not with a scrambled single stranded DNA oligonucleotide, adds to this body of evidence implicating CTGF as the mediator of TGF-β_1's pathological fibrotic activities. Additional experiments observing other known fibrotic activities are necessary to affirm the theoretical role of CTGF mediation.

In total, the evidence presented here demonstrates that the M6P/IGF-2-R is necessary for TGF-β_1–induced, CTGF-mediated proliferation in fibroblast cell cultures, and, as such, is a cell surface signaling receptor for CTGF.

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