Connective Tissue Growth Factor CCN2 Interacts with and Activates the Tyrosine Kinase Receptor TrkA

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Connective tissue growth factor (CTGF) is implicated as a factor promoting tissue fibrosis in several disorders, including diabetic nephropathy. However, the molecular mechanism(s) by which it functions is not known. CTGF rapidly activates several intracellular signaling molecules in human mesangial cells (HMC), including extracellular signal–related kinase 1/2, Jun NH2-terminal kinase, protein kinase B, CaMK II, protein kinase Cα, and protein kinase Cδ, suggesting that it functions via a signaling receptor. Treating HMC with CTGF stimulated tyrosine phosphorylation of proteins 75 to 180 kD within 10 min, and Western blot analysis of anti-phosphotyrosine immunoprecipitates identified the neurotrophin receptor TrkA (molecular weight approximately 140 kD). Cross-linking rCTGF to cell surface proteins with 3,3′-dithiobis(sulfosuccinimidylpropionate) revealed that complexes formed with TrkA and with the general neurotrophin co-receptor p75NTR. rCTGF stimulated phosphorylation of TrkA (tyr 490, 674/675). K252a, a known selective inhibitor of Trk, blocked this phosphorylation, CTGF-induced activation of signaling proteins, and CTGF-dependent induction of the transcription factor TGF-β-inducible early gene in HMC. It is concluded that TrkA serves as a tyrosine kinase receptor for CTGF.

Recent studies in the past few years indicate that CTGF is strongly implicated in the pathogenesis of a variety of fibrotic disorders, including diabetic nephropathy (2). Functionally, the growth factor has been shown to regulate many aspects of cell behavior, including cell adhesion, migration, growth, chemotaxis, and the synthesis and accumulation of matrix proteins (3). However, the molecular mechanism(s) by which it functions is not clear. The presence of multiple domains in CTGF suggests a role for the growth factor as an integrator of several other signaling molecules such as growth factors, integrins, and extracellular matrix proteins. CTGF has been reported to bind directly bone morphogenic protein–4 and TGF-β through its von Willebrand type C domain, leading to inhibition of bone morphogenic protein and enhancement of TGF-β signaling (4). CTGF also has been shown to bind to integrins (5–10), and this interaction may mediate some of the cellular phenomena mentioned above.

Recently, we proposed that CTGF directly enhances the TGF-β/Smad signaling pathway. The mechanism underlying this seems to be via the rapid induction of the transcription factor TGF-β-inducible early gene (TIEG) (11). TIEG has been shown to bind to the promoter of the Smad7 gene and represses its transcription (12). Because Smad7 is a potent inhibitory Smad, it seems that CTGF simply blocks the negative feedback loop of the TGF-β signaling pathway, allowing its continued activation. The rapid activation of several intracellular signaling pathways by CTGF, together with its ability to induce the expression of several genes in mesangial cells, including the early gene TIEG, suggested that a specific signaling receptor must exist. Previous cross-linking studies revealed CTGF-receptor complexes with an apparent molecular weight of 280 kD, present in chondrocytes, osteoblasts, and endothelial cells (13). CTGF was also found to bind to LDL receptor–related protein. However, this may facilitate clearance rather than signal transduction (14).

In the present report, we provide for the first time evidence that CTGF interacts with TrkA and p75NTR, a dual-receptor system that is known to transduce neurotrophin signals. There are three Trk receptor tyrosine kinase genes (TrkA, TrkB, and TrkC) and a single gene encoding the neurotrophin receptor, p75NTR. On ligand binding, Trk dimerize and autophosphorylate, leading to the activation of several small G proteins, including Ras, Rap-1, and the Cdc 42-Rac-Rho family, as well as of pathways regulated by mitogen activated protein kinase (MAPK), phosphatidylinositol 3-kinase, and phospholipase C-γ (15). On activation, Trk also promote a rapid increase in cytoplasmic calcium level. This seems to arise from both release of intracellular stores and uptake of extracellular calcium (16,17). Moreover, activated Trk interact, directly or indirectly, with...
many cytoplasmic adaptor proteins, leading to a variety of biologic responses, including, cell proliferation and survival; axonal and dendritic growth and remodeling; assembly and remodeling of cytoskeleton; membrane trafficking and fusion; and synapse formation, function, and plasticity (18).

The pan-neurotrophin receptor p75NTR is a member of a superfamily that includes the TNF, B cell antigen CD40, and Fas receptors (19). p75NTR signals via pathways involved with activation of sphingomyelinase and ceramide production (20), J Am Soc Nephrol 16: 340–351, 2005 CTGF Activates the TrkA Receptor 341

seems that Trk play the central role in signaling. However, the both receptors mediate the biologic effects of neurotrophins, it seems that Trk play the central role in signaling. However, the contribution of p75NTR is not fully understood. It has been proposed that p75NTR can act as a co-receptor with Trk to create high-affinity receptors for different neurotrophins (23) either by presenting the ligand to the Trk receptor, or by altering the conformation of the Trk receptors through allosteric interaction (24). It can also function on its own (25). Yet another interesting finding is that p75NTR can function as a receptor for the immature unprocessed forms of neurotrophins, whereas Trk functions as a receptor for the mature forms (26). Cross-talk between the Trk and other membrane receptors, such as G protein-coupled receptor, vanniloid receptor, and c-Ret, have also been reported (18).

Ligand engagement stimulates the endocytosis of the ligand–Trk complex into vesicles via a clathrin-dependent mechanism (27). The receptors remain catalytically active within the sorting vesicles, which are called “signaling endosomes” (28). Inhibiting endocytosis inhibits extracellular signal–related kinase (ERK) 1/2 activation in response to neurotrophin stimulation (29).

As Trk receptors have been found to bind a large number of adaptor proteins and multipleintracellular signaling pathways are activated by them, as well as modulated by p75NTR, this may explain the multifunctional properties of CTGF. The results presented in this study show that Trk tyrosine kinase activity is required for the CTGF-dependent induction of the transcription factor TIEG.

Materials and Methods
Cell Cultures, Antibodies, and Reagents
Primary normal adult human mesangial cells (HMC; CC-2259, lot 3F1510) were purchased from BioWhittaker (Wokingham, Berkshire, UK), maintained in culture as described previously (2), and used at passages 9 to 10. Phospho-Akt (P-Ser473) antibody was from Cell Signaling Technology, Beverly, MA. Phospho-Akt (P-Thr308) and ERK5 antibodies were from New England BioLabs (Hitchin, Herts, UK). Phospho-CaMKII (P-Thr286) antibody was from Cell Signaling Technology, Beverly, MA. Anti–CTGF-C terminal domain–sepharose column (Pierce). When rCTGF was used, CTGF cross-linked proteins were either immunoprecipitated with rabbit anti-CTGF antibody or captured on a Pull-Down PolyHis column (Pierce). When rCTGF was used, CTGF cross-linked proteins were captured on a goat anti–CTGF-C terminal domain–sepharose immunoaffinity column, using an IgG-sepharose column as a control (Biogenex Inc.). After extensive washing of the columns with solubilizing buffer, bound proteins were solubilized in reducing SDS-PAGE loading buffer, boiled for 5 min, and resolved on a 12% gradient gel by SDS-PAGE. Gels were either stained with Coomassie blue or used for Western blotting.

RNA Extraction and Reverse Transcriptase–PCR Analysis
Total RNA was extracted from 6 × 10⁶ mesangial cells using the RNAzol B method (AMS Biotechnology [UK] Ltd., Oxfordshire, UK). Equal amounts of total RNA (2 μg) from each sample were reverse-transcribed into cDNA using SuperScript II RNase H⁻ reverse transcriptase (Life Technologies BRL, Paisley, Scotland, UK) and random primers. Equal amounts (0.5 μl) of the reverse transcription reaction (20 μl) were subjected to PCR amplification in a 100-μl volume that contained 10 μl of 10X PCR buffer, 16 μl of dNTP (1.25 mM each), 2 mM MgCl₂, 5 M betaine (Sigma), 0.5 μM of each specific primer, and 1.25 U of Amplitaq DNA polymerase (Life Technologies BRL). Amplification was started with 5 min of denaturation at 94°C followed by 30 PCR cycles for all genes. Each cycle consisted of 60 s at 94°C, 60 s at 55°C, and 60 s at 72°C. The final extension was for 10 min at 72°C. A control omitting the reverse transcriptase step was also performed. The sequences of primers were as described by Anderson et al. (30).

CTGF Activates the TrkA Receptor
CTGF (CTGF/V5 fusion protein) was expressed in transformed HMC and purified from the medium using Talon metal affinity resin, as reported previously (2). Alternatively, r-CTGF (nonfusion protein) was expressed in the baculovirus system and was a gift from FibroGen Inc. (South San Francisco, CA). Rabbit anti-CTGF (pAb2) and chicken anti-CTGF (plgY3) were also supplied by FibroGen Inc. Phosphothioate antisense and control oligonucleotides directed to TrkA (GTAAGAT-GAAGCTTGT; ACTACTAGTACACTAC) and p75NTR (TTCTGCT-TGTCCGT; GCTCTATGACTCCCAG) were designed and manufactured by Biognostik GmbH (Göttingen, Germany), who own the intellectual property rights to the sequences.

Cross-Linking and Membrane Preparation
Cell layers were washed twice with cold binding buffer (PBS and 0.5% glucose) and incubated with CTGF in binding buffer for 2 h at 4°C. After incubation, the cell layers were washed five times with cold binding buffer and incubated with 1 mM 3,3′-dithiobis(sulfosuccinimidylpropionate) (DTSSP) or disuccinimidyl suberate (Pierce Biotechnology, Tattenhall, Cheshire, UK) in PBS for 30 min at room temperature. The reaction was quenched for 15 min at room temperature by the addition of 50 mM Tris buffer (pH 7.5). Cell layers were washed with wash buffer (10 mM Tris buffer [pH 7.5], 5 mM MgCl₂, and 150 mM NaCl), scraped in homogenizing buffer (100 mM Tris buffer [pH 7.5], 250 mM sucrose, 1 mM EDTA, 5 mM MgCl₂, 150 mM NaCl, and 1× protease inhibitor cocktail; Roche Applied Science, Mannheim, Germany), passed through a 25-G needle, and homogenized on ice with 30 to 40 cycles in a Dounce homogenizer. The homogenate was centrifuged for 10 min at 2500 g at 4°C. The supernatant was centrifuged for 90 min at 45,000 g at 4°C. The membrane-enriched pellet was solubilized for 1 h in solubilizing buffer (10 mM Tris buffer [pH 7.5], 5 mM MgCl₂, 150 mM NaCl, 1% Triton-X100, and 1× protease inhibitor cocktail). Soluble membrane proteins were collected after further centrifugation for 1 h at 45,000 g at 4°C. When rCTGF/V5 fusion protein was used, CTGF cross-linked proteins were either immunoprecipitated with rabbit anti-CTGF antibody or captured on a Pull-Down PolyHis column (Pierce). When rCTGF was used, CTGF cross-linked proteins were captured on a goat anti–CTGF-C terminal domain–sepharose immunoaffinity column, using an IgG-sepharose column as a control (FibroGen Inc.). After extensive washing of the columns with solubilizing buffer, bound proteins were solubilized in reducing SDS-PAGE loading buffer, boiled for 5 min, and resolved on 4 to 12% gradient gels by SDS-PAGE. Gels were either stained with Coomassie blue or used for Western blotting.
Western Blotting

Cells were lysed in reducing SDS-PAGE loading buffer and immediately scraped off the plate. Cell lysates were sonicated for 10 s to shear DNA. Samples were boiled for 5 min and resolved on 4 to 12% gradient gels by SDS-PAGE. Proteins were transferred onto a polyvinylidene difluoride membrane filter (Immobilon-P, Millipore, Bedford, UK) using a BioRad transfer apparatus. Blots were incubated in blocking buffer that contained 1X TBS and 0.1% Tween-20 with 5% (wt/vol) nonfat dry milk for 1 h. Immunodetection was performed by incubating the blots in primary antibody at the appropriate dilution in antibody dilution buffer (1X TBS and 0.1% Tween-20 with 5% BSA) overnight at 4°C. Blots then were washed three times with washing buffer (1X TBS and 0.1% Tween-20) and incubated with secondary horseradish peroxidase-conjugated antibodies for 1 h at room temperature. Bound antibodies were visualized using the enhanced chemiluminescence reagent Luminol (Autogen Bioclear UK Ltd, Wiltshire, UK). Prestained molecular weight standards (Amersham International PLC, Amersham, UK) were used to monitor protein migration.

Immunofluorescence Staining

Cells were fixed with 3.7% paraformaldehyde and permeabilized with 0.5% Triton X-100 in PBS for 10 min at room temperature. Coverslips then were incubated overnight at 4°C with serum (5% in PBS) from the same species as that in which the secondary antibody was raised. After this, they were incubated with primary antibodies (at optimum dilution in PBS that contained 3% BSA) for 1 h at 37°C. Coverslips then were washed and incubated in the dark for 1 h with fluorescein-conjugated secondary antibody (Sigma Aldrich, Dorset, UK). After staining, the coverslips were mounted on glass slides with antifade mounting media (Vector Labs, Peterborough, UK). Prestained molecular weight standards (Amersham International PLC, Amersham, UK) were used to monitor protein migration.

Results

CTGF Activates Several Intracellular Signaling Pathways

To understand the molecular mechanisms by which CTGF functions, we used purified rCTGF-V5 fusion protein to identify the intracellular signal pathways that are activated in response to the growth factor in HMC. We found that CTGF rapidly triggers the activation of the classical MAPK (ERK1/2) and JNK pathways (Figure 1, A and B) but not the p38 MAPK (data not shown). The figure shows maximal activation of these kinases after 15 min of CTGF stimulation. CTGF stimulation also led to the activation of Akt, also known as protein kinase B (PKB), at both the known phosphorylation sites: thr-308 and ser-473 (Figure 1C). It is interesting that the activation of thr-308 (by a phosphoinositide-dependent kinase 1 whose activity is strictly dependent on 3-phosphorylated inositol lipids [31]) seems to be rapid and sustained in comparison with the activation of ser-473. Phosphorylation of the latter (by integrin-linked kinase [32]) seems to be transient with a maximal level at 15 min and return to a level close to the basal one within 30 min of CTGF exposure. CTGF stimulation also led to the transient activation of CaMK II (Figure 1C). Other kinases that seem to be activated in response to CTGF are PKCα and PKCδ (Figure 1D). These results indicate that CTGF signals through a receptor, most probably a receptor tyrosine kinase (RTK), because RTKs are commonly known to activate these kinases. To test this hypothesis, we exposed HMC to CTGF for various periods of time, prepared cell lysates, and performed Western Blot analysis using an anti-phosphotyrosine antibody. Short-term exposure of the blot (<5 s) showed that CTGF fusion protein (40 ng/ml) stimulates the tyrosine phosphorylation within 10 min of at least two major proteins with apparent molecular weight of approximately 75 to 80 and 140 to 150 kD in HMC (Figure 2). Another phosphotyrosine protein (molecular weight 45 kD) was detected in control cell lysate and seems to be reduced in response to the CTGF treatment.

CTGF Interacts with HMC Surface Proteins

To investigate whether CTGF interacts with HMC surface proteins, we allowed CTGF to bind to the cell surface, performed a subsequent cross-linking procedure, and isolated a membrane-enriched fraction from the cells. After solubilization, this was immunoprecipitated with a rabbit anti-CTGF antibody. Covalently linked CTGF complexes then were analyzed by PAGE and Western blotting with a chicken anti-CTGF antibody. As shown in Figure 3, lane 2, CTGF seems to be cross-linked with membrane proteins to form complexes of apparent molecular weight 180 kD and >220 kD, the latter being a large diffuse band. These complexes were not immunoprecipitated from the membrane-enriched fraction when the cross-linking step was eliminated (lane 1).

To ascertain whether CTGF activates an RTK, we incubated serum-starved HMC in the presence or absence of CTGF for 15 min, lysed the cells, and immunoprecipitated phosphotyrosine proteins. The immunoprecipitated proteins were analyzed by Western blotting using antibodies against TrkA (Figure 4A, B, C, and E) and EGF receptor (EGFR; data not shown), known tyrosine kinase receptors. Only the anti-TrkA antibody cross-reacted strongly with a band of approximately 140 kD (Figure 4A). The intensity of this band was stronger when cells were incubated with CTGF (lane 2), indicating its activation by the growth factor. The interaction of CTGF with the TrkA receptor was confirmed by different experiments in which either His-tagged CTGF/V5 fusion protein or rCTGF expressed in the baculovirus system was allowed to bind to the cell surface and then cross-linked to its ligand(s) using the reversible cross-linker DTSSP. The latter is cleaved by reducing agents. Subsequently, a membrane fraction was prepared and cross-linked CTGF complexes were captured on affinity metal beads or on anti–C-terminus CTGF antibody affinity beads. The captured complexes were subjected to SDS-PAGE under reducing conditions and analyzed by Western blots. The results in Figure 4, B through D, clearly indicate that CTGF interacts with the TrkA receptor. As Trk receptors have been shown to interact with the pan neurotrophin receptor p75NTR, plots were stripped and reprobed using an anti-p75NTR antibody. As shown in Figure 4E, the antibody cross-reacted with a protein of the correct molecular weight for p75NTR. Thus, our results indicate that CTGF interacts with TrkA and p75NTR, two receptors that are known to be activated by the neurotrophin nerve growth factor (NGF). To confirm this interaction, MC were depleted of TrkA or p75NTR receptors using antisense oligonucleotides (2 μM for 3 d), as described previously by Wahab et al. (2). Equal amount of r-CTGF were allowed to bind to the cell surface and then cross-linked with DTSSP as above. Membrane preparations
Figure 1. Connective tissue growth factor (CTGF) activates intracellular signaling pathways. Serum-starved human mesangial cells (HMC) were incubated in the presence of CTGF/V5 fusion protein for the periods of time indicated, after which the cells were lysed. Equal amounts of lysate protein were subjected to SDS-PAGE and analyzed by Western blotting using phospho-specific antibodies against the constituent proteins of the mitogen-activated protein kinase (MAPK) pathway including the MEK1/2 and their downstream targets extracellular signal–related kinase 1 and 2 (ERK1/2) and p90-ribosomal-S6 kinase (p90RSK; A) Jun NH2-terminal kinase (JNK; B), and protein kinase B (PKB) and CamKII (C). β-Actin is shown as a marker for equal protein loading.

(D) Cells were grown on coverslips and serum-starved for 48 h before incubation in medium in the absence (a and c) or presence of 40 ng/ml CTGF-fusion protein (b and d) for 30 min. Cells were fixed, permeabilized, and probed with anti-phospho PKCδ (a and b) and PKCα (c and d) primary antibodies and then with fluorescein-conjugated secondary antibody, as described in the Materials and Methods section. White arrows in b and d point to plasma membrane translocation of PKCδ and PKCα, characteristic of PKC enzyme activation. Results are representative of three separate experiments.
were made, and equal amounts of solubilized protein were incubated with anti–C-terminus CTGF antibody affinity beads. Bound complexes were subjected to SDS-PAGE under reducing conditions and analyzed by Western blots. Figure 5 shows that reducing the expression level of the TrkA receptor by treatment with antisense oligonucleotide (lane 2) resulted in a reduction in the amount of TrkA receptors that bound to CTGF (Figure 2B), but it had no significant effect on the amount of p75NTR bound to CTGF (Figure 2C). In contrast, reducing the expression level of p75NTR (lane 4) reduced the amount of both receptors interacting with CTGF (Figure 4, B and C). Both TrkA and p75NTR control antisense oligonucleotides did not effect the amount of the interacting receptors (compare lanes 3 and 5). Attempts to confirm chemically the identity of these receptors using MALDI-TOF-MS analysis were unsuccessful because of their relatively low abundance and, probably, high level of glycosylation of these receptors.

**HMC Express Trk Receptors**

To investigate whether HMC express any members of the Trk, we extracted total RNA from HMC and performed reverse transcriptase–PCR analysis. The results (Figure 6) show that HMC express the three members of the neurotrophin receptor family—TrkA, TrkB, and TrkC—as well as the pan receptor p75NTR. It is interesting that HMC also express the neurotrophin factors NGF and brain-derived growth factor.

**CTGF Activates TrkA in HMC**

On binding its ligand, TrkA autophosphorylates on several tyrosine residues, leading to the association and activation of multiple effectors. Phosphorylation at Tyr490 is required for Shc association and activation of the Ras-MAPK cascade. Phosphorylations at Tyr674/675 lie within the catalytic domain and reflect Trk kinase activity. Therefore, we tested whether stimulating cells with CTGF leads to the phosphorylation of TrkA at these residues. The results in Figure 7 show that CTGF induces an increase in the phosphorylation of the receptor at the Tyr490 and Tyr674/675 residues by 1.8-fold and 2.5-fold, respectively.

**Inhibition of CTGF-Induced Signaling Pathways by K252a**

K252a is an alkaloid-like kinase inhibitor that is known to selectively inhibit Trk (33). K252a blocked the rapid phosphorylation of ERK1/2/JNK/ERK5 and PKB in HMC stimulated with CTGF (Figure 8), indicating that the phosphorylation of these kinases is indeed induced by ligand activation of the Trk receptor. Furthermore, treatment with K252a led to the accumulation of TrkA at the plasma membrane of HMC that were exposed to CTGF (Figure 9). This indicates that internalization and sorting of receptor–CTGF complexes was blocked as a result of inhibition of the receptor tyrosine kinase activity by K252a. This is consistent with the hypothesis that TrkA activation is an obligatory step in the recruitment of activated Trk receptors to clathrin-coated pits and entry into the endocytic pathway (34). The results in Figure 9 also indicate that K252a completely blocks TrkA phosphorylation at Tyr490 but is less effective in blocking phosphorylation at Tyr674/675. Tyr674/675 are within the catalytic domain of TrkA but are not associated with signaling. Phosphorylation of these tyrosines might turn over at a slower pace than those such as tyr 490, which are associated with effector binding (35). Thus, tyr 674/675 may be more readily detected in the presence of K252a than tyr 490.

It is interesting that similar results were obtained with HMC that were treated with 50 ng/ml NGF (Figure 10). The activation of TrkA by both CTGF and NGF were confirmed by Western blot analysis, as shown in Figure 11.

For further evaluating the effect of K252a on TrkA autophosphorylation and signaling, HMC were serum starved for 24 h,
pretreated with increasing concentrations of the inhibitor for 30 min, and then stimulated with 40 ng/ml CTGF/V5 for 15 min. Cells were lysed and analyzed for phosphorylation of TrkA and the downstream effector MAPK. Figure 12 shows that CTGF-induced total tyrosine phosphorylation of TrkA, phosphorylation at TrkA tyr-490 residue, and p-MAPK all are significantly inhibited by K252a at 10-nM concentration. This result is in good agreement with the published IC50 for Trk (approximately 3 nM) (33,36), whereas inhibitory effects on other kinases require much higher concentrations (36).

TrkA Tyrosine Kinase Activity is Required for CTGF-Dependent Induction of TIEG

To determine whether Trk tyrosine kinase activity is required for CTGF-dependent induction of the transcription factor TIEG-1, we treated HMC with K252a 30 min before incubation with CTGF for 2 h. As shown in Figure 13, CTGF enhanced the expression of TIEG-1 by 3.4-fold ($P < 0.0001$). K252a was a potent inhibitor for TIEG induction at a concentration that is known specifically to inhibit Trk kinase activity in other cells (33,36).

Discussion

It is becoming clear that CTGF is implicated in the pathogenesis of diabetic nephropathy and possibly of other fibrotic disorders. Thus, therapeutic approaches that could selectively block CTGF activity would be beneficial. However, it is important first to understand fully the precise role and the molecular effector mechanisms of this growth factor.

In this report, we provide evidence that CTGF triggers the rapid simultaneous activation of the signaling pathways MAPK (ERK 1/2), JNK, ERK5, phosphatidylinositol 3-K, CaM-KII, PKCα, and PKCδ in HMC. By subsequent binding, cross-linking, and immunoprecipitation studies, we identified the tyrosine kinase receptor TrkA and p75NTR as two receptors that interact with CTGF. TrkA and p75NTR are known to be activated by the neurotrophin factor NGF. Neurotrophins are survival and differentiation factors in the nervous system, and although both receptors mediate the biologic effects of neurotrophins, it seems that TrkA plays the central role in signaling, whereas the contribution of p75NTR is not fully understood. Activation of TrkA in HMC in response to CTGF treatment was further supported by the ability of the Trk selective inhibitor K252a to block the rapid phosphorylation of ERK1/2, JNK, PKB, and ERK5.

On binding and activation of Trk by neurotrophins, the ligand–receptor complex is endocytosed into vesicles via a clathrin-dependent mechanism (27,29), where receptors remain catalytically active within these vesicles (28). Inhibiting endocytosis with specific inhibitors, or chemical inhibition of Trk kinase, inhibits ERK1/2 activation in response to neurotrophin stimulation (29).

Previously, we demonstrated that CTGF is endocytosed from the cell surface in endosomes, from which the growth factor translo-
cates into the nucleus (37). In the present study, we showed that the addition of CTGF to HMC induces the activation of TrkA and its accumulation in vesicles (Figure 9), which were similar to those observed when the cells were treated with NGF. Activation of the ERK5 pathway in HMC that were treated with CTGF is consistent with its preferential activation within vesicles (38). Treatment of HMC with CTGF or NGF in the presence of K252a led to the accumulation of TrkA at the plasma membrane. However, this phenomenon was seen only when probing with the phospho-Trk

Figure 5. Effect of TrkA and p75NTR antisense oligonucleotide treatment on receptor interaction with CTGF in HMC. HMC were treated or not treated with phosphothioate antisense or control oligonucleotides directed to TrkA or p75NTR for 3 d, after which the cells were used to perform cross-linking experiments with r-CTGF, as described in Figure 4C. Equal amounts of solubilized membrane proteins were incubated with anti-C-terminus-CTGF antibody affinity beads. Bound proteins were analyzed by Western blotting using anti-TrkA and p75NTR antibodies (A). (B) Densitometric quantification of the TrkA blots. (C) Densitometric quantification of the p75NTR blots. Results (mean ± SEM) represent three separate experiments (n = 6). *P < 0.003; **P < 0.0001; ++P < 0.007.

Figure 6. HMC expresses Trk receptors. Total RNA was extracted from HMC and used for reverse transcriptase–PCR as described in the Materials and Methods section. After amplification, 10 µl of each PCR reaction product was electrophoresed through a 1.2% (wt/vol) agarose gel that contained ethidium bromide (0.5 µg/ml). Results are representative of three separate experiments.

Figure 7. CTGF activates TrkA in HMC. Serum-starved HMC were incubated in the absence (lane 1) or the presence (lane 2) of CTGF/V5 (40 ng/ml) for 15 min, after which the cells were lysed. Equal amounts of lysate protein were subjected to SDS-PAGE and analyzed by Western blotting. Blot A was probed with anti-TrkA antibody. Blot B was probed with anti-phospho-TrkA (Tyr490) antibody, whereas blot C was probed with anti-phospho-TrkA (Tyr674/675). Results are representative of three separate experiments.

Figure 8. Inhibition of CTGF-induced signaling pathways by K252a. Serum-starved HMC were incubated with CTGF/V5 (40 ng/ml) in the presence or absence of K252a (100 nM) for the period of time indicated, after which the cells were lysed. Equal amounts of lysate protein were subjected to SDS-PAGE and analyzed by Western blotting using phospho-specific antibodies against the indicated protein kinases. β-Actin is shown as a marker for equal protein loading.
(Tyr674/675) antibody, which is specific for phosphorylated tyrosine residues that lie within the catalytic domain. In contrast, the phospho-Trk (Tyr490) antibody, which is specific for the tyrosine residue required for Shc association and activation of the Ras-MAPK cascade, did not show accumulation of phospho-TrkA at the plasma membrane. This suggests that K252a is more potent toward selective residues and indicates that internalization and sorting of the receptor–ligand complex is blocked as a result of the overall inhibition of the receptor tyrosine kinase activity by K252a.

This is consistent with the hypothesis that TrkA activation is an obligatory step in the recruitment to clathrin-coated pits and entry into the endocytic pathway (34). K252a also blocks the CTGF-dependent induction of the transcription factor TIEG (Figure 11), indicating that Trk tyrosine kinase activity is required for CTGF to induce expression of this gene.

The principal domains in Trk that determine affinity and specificity of binding of neurotrophins are the two IgC2 domains (39), whereas p75NTR binding is facilitated through four negatively charged cysteine-rich repeats (19). It is likely that localization of the CTGF binding sites on these receptors will ultimately lead to the generation of selective small molecule antagonists.
It is of interest that NGF receptors have been detected in many nonneuronal cells under normal and pathologic conditions. Increased TrkA expression was reported in human skin during diabetes (40). Low expression of p75NTR was reported in normal glomeruli and in a subpopulation of renal interstitial cells. However, the level of this receptor was found to be increased during inflammatory kidney diseases and in diabetic nephropathy (41,42). It was concluded that p75NTR is a marker of mesangial cell injury or activation and a potential molecule for signaling mechanisms to recruit or activate cells at sites of tubulointerstitial injury. NGF receptors were also detected on activated murine CD4 T cells (but not on resting T cells) (43), human B lymphocytes (44), monocytes (45), skin and lung fibroblasts (46), keratinocytes (40), melanocytes (47), periarticular connective tissue cells (40), and human osteoarthritic chondrocytes (48).

The finding that CTGF acts via TrkA/p75NTR is intriguing, as there are some similarities between CTGF and neurotrophins. All of the neurotrophins are initially synthesized and secreted as 30- to 35-kD precursor proteins. These are proteolytically cleaved in the middle to release the biologically active 12- to 14-kD C-terminal mature forms (49). It is proposed that the N-terminal domain allows for correct protein folding and secretion (49,50), and it may also possess some biologic activities (51). However, secreted unprocessed immature or proneurotrophins are often present in abundance (52).

CTGF is also synthesized and secreted as a 36- to 38-kD peptide, but different forms between 10 and 20 kD are also detected and are believed to be proteolytic products of the 38-kD form (53). The C-terminal 10- to 12-kD peptide is biologically active.

Mature neurotrophins dimerize and adopt a novel tertiary fold as a result of a cystine knot motif present in each monomer (54). This cystine knot is present in the C-terminal of CTGF (55). Moreover, CTGF contains a characteristic heparin-binding domain in its C-terminus. Similarly, two novel NGF-like neurotrophins, NT-6/7, that contain an additional 15 to 22 amino acid residues with heparin-binding ability in their mature forms have been identified (56,57). They were found to bind p75NTR and activate TrkA but not TrkB or TrkC (56,57).

It is interesting that it has been shown that mature neurotrophins dimerize and preferentially activate the Trk, whereas the unprocessed precursor forms preferentially activate p75NTR to induce a proapoptotic response (26,58). Thus, it has been proposed that the survival or death of neurones that coexpress the N-terminal domain and a Trk receptor could depend on pro-
cessing of the neurotrophin ligands (59). Activation of p75NTR by proneurotrophins may also promote other functions through pathways involving increased NF-κB or c-Jun kinase activities.

We used recombinant full-length CTGF in our experiments. However, our preparations always contain the 10- to 20-kD C-terminal form. Currently, we do not know whether there is preferential binding of the different forms of CTGF toward each receptor. If this proved to be the case, then it would mean that postsecretory proteolytic processing of CTGF is essential for efficient Trk receptor binding and activation. At present, the differential ability of precursor and mature neurotrophins to bind selective receptors to mediate distinctive biologic actions is thought to be unique. However, if CTGF has similar properties, then the phenomenon is more common than first thought.

Our finding that CTGF activates the Trk receptor that is known to associate with numerous cytoplasmic adaptors and signaling proteins such as the nonreceptor tyrosine kinase c-abl, an ankyrin repeat-rich membrane spanning protein, ion channels, GRK-2, B-Arrestin I, Sh2B, rAPS, p62 dok, and atypical PKC enzymes (15,18), may explain the multifunctional properties of CTGF. Cells may respond differently to CTGF depending on their expression level of the Trk and p75 receptors and the availability of different forms of CTGF in their environment. Different cells may also express different subsets of adaptor proteins that may compete with each other for binding to activated receptors, leading to differential repertoire of signaling proteins, which exhibit differences in signal transmission.

It is also tempting to speculate that CTGF may act as a fine-tuner for the TGF-β signaling pathway by regulating the level of Smad7. Thus, under certain pathologic conditions, it may activate the Trk receptor and induce TIEG expression to enhance TGF-β signaling, whereas under other conditions, it may induce the expression of Smad7 through the activation of p75NTR and NF-κB. This would lead to diminishing the effects of TGF-β. Further experiments to address these issues should provide insight into the mechanisms by which CTGF regulates cell function under normal and pathologic conditions.

Acknowledgments

We thank the Medical Research Council (UK) for the financial support. We are grateful to FibroGen Inc. for recombinant CTGF, CTGF antibodies, and anti-CTGF-C-terminal domain–sepharose.

References

19. Smith CA, Farrah T, Goodwin RG: The TNF receptor su-
50. Suter U, Heymach JV Jr, Shooter EM: Two conserved domains in the NGF propeptide are necessary and sufficient for the biosynthesis of correctly processed and biologically active NGF. EMBO J 10: 2395–2400, 1991


Correction


The authors regretfully report an error in quoting the findings of the Irbesartan in Patients with Type 2 Diabetes and Microalbuminuria 2 (IRMA 2) Study at the bottom of the left column on page 3043. The second sentence of the paragraph beginning, “Various clinical studies support…” should read as follows:

The largest trial in patients with type 2 diabetes and microalbuminuria, the Irbesartan in Patients with Type 2 Diabetes and Microalbuminuria 2 (IRMA 2) Study, found that progression of diabetic nephropathy occurred in approximately 5% of patients who had diabetes and received a high dose of ARB (irbesartan 300 mg/day): In 10% of those who received a low dose of ARB (irbesartan 150 mg/day) and in 15% of the control group who received placebo on top of other antihypertensive medication (32).

Correction


In this article, Figure 5 is in fact a duplicate of Figure 12. Please see the correct Figure 5 below. The legend for Figure 5 is correct as published. The publisher regrets this error.

Also in this article, the authors would like to correct a reference to Figure 13. On page 347, in the second line from the top of the right column, the textual reference to Figure 11 should refer instead to Figure 13. The authors regret this error.