Uptake and intracellular transport of the connective tissue growth factor: a potential mode of action

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Connective tissue growth factor (CTGF) is a secreted cysteine-rich protein now considered as an important effector molecule in both physiological and pathological processes. An increasing amount of evidence indicates that CTGF plays a key role in the pathogenesis of different fibrotic disorders including diabetic nephropathy. However, the molecular mechanisms by which CTGF exerts its effects are not known. Here we provide the first evidence for the existence of an intracellular transport pathway for the growth factor in human mesangial cells. Our results demonstrate that CTGF is internalized from the cell surface in endosomes and accumulates in a juxtanuclear organelle from which the growth factor is then translocated into the cytosol. In the cytosol CTGF is phosphorylated by protein kinase C and PMA treatment can enhance this phosphorylation. Phosphorylated CTGF may have an important role in the cytosol, but it is also translocated into the nucleus where it may directly affect transcription.

Key words: CTGF, endocytosis, mesangial cells, protein kinase C, fibrosis.

INTRODUCTION

Connective tissue growth factor (CTGF) is an immediate early gene encoding a cysteine-rich protein belonging to the CTGF, Cyr61/Cef10 and nephroblastoma overexpressed gene (CCN) family of growth regulators. Members of the CCN family are distinguished by their organization into four conserved domains; an insulin-like growth factor (IGF)-binding protein domain, a von Willebrand factor type C domain, a thrombospondin type I repeat domain and a heparin-binding domain at the C-terminus [1]. The precise biological function of each domain remains to be established. CTGF is a secreted monomeric protein having a molecular mass of 36–38 kDa, depending on its degree of glycosylation [2]. However, other forms also exist, such as the 10-, 12-, 16-, 18-, 19-, 20- and 24-kDa fragments, which have been found in different cell types, tissues and body fluids [2–7].

The physiological role of CTGF is not yet fully understood. However, *in vitro* it has mitogenic activity [8], mediates cell adhesion [9], induces angiogenesis [10], stimulates cell migration [11] and cell survival [12], and regulates gene expression [8,13,14]. Moreover, present evidence indicates that CTGF plays a role in the pathogenesis of several different fibrotic disorders. It has been reported to be involved in inflammatory bowel disease [15], skin lesions in scleroderma and systemic sclerosis [16,17], renal fibrosis [14,18], liver fibrosis [19], heart fibrosis [20], idiopathic pulmonary fibrosis and pulmonary sarcoidosis [21]. The molecular mechanisms by which CTGF exerts these effects are not clear. However, the multimodular structure of the growth factor suggests that its effect may depend on interactions with other proteins and glycosaminoglycans. It may act through the IGF-signalling pathway, as it has been reported that CTGF binds IGF-I and IGF-II with low affinity [22]. It could also act through an integrin-mediated signalling pathway [12,23,24], or by binding to a specific CTGF receptor, or a more general cell surface receptor [5].

Using a suppression subtraction hybridization technique we identified upregulated expression of CTGF in human glomerular mesangial cells exposed long term to high concentrations of glucose *in vitro* [25]. These conditions mimic those found in diabetics with hyperglycaemia, and subsequently we detected high levels of CTGF immunostaining in glomeruli of non-obese diabetic mouse kidneys and in renal biopsies from humans with diabetic nephropathy [14]. Moreover, the growth factor mediates both high glucose- and transforming growth factor β-induced fibronectin expression in human mesangial cells (HMCs) [14], suggesting that it may play a key role in promoting excessive extracellular matrix deposition in diabetic nephropathy.

Immunostaining of HMCs grown under different glucose conditions shows CTGF protein in the nuclear compartment of HMCs maintained in medium containing 30 mM D-glucose. This observation, together with the report by Perbal [26] showing that nephroblastoma overexpressed gene-H ('NOVH'), another member of the CCN family, is localized in the nucleus of some cell lines, prompted us to investigate whether CTGF protein is transported to the nuclear compartment. In this study, we present evidence for the first time demonstrating that the secreted growth factor is internalized, phosphorylated and transported into the nucleus of HMCs where it appears to act directly on gene transcription.

MATERIALS AND METHODS

Cell cultures, antibodies and reagents

Primary normal adult HMCs (CC-2259, lot 3F1510) were purchased from BioWhittaker (Wokingham, Berks., U.K.) and maintained in culture as described previously [27]. A rabbit polyclonal anti-human CTGF antibody (pAb2) was a kind gift from FibroGen (South San Francisco, CA, U.S.A.). We have

Abbreviations used: aFGF, acidic fibroblast growth factor; CREB, cAMP-response element binding protein; CTGF, connective tissue growth factor; CCN family, CTGF, Cyr61/Cef10 and nephroblastoma overexpressed gene family; HMC, human mesangial cell; IGF, insulin-like growth factor; MBP, myelin basic protein; PAI-1, plasminogen activator inhibitor-1; PKC, protein kinase C; REP-1, Rab escort protein 1; rCTGF, recombinant CTGF.

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reported the characterization of this antibody [14]. Antibodies against calnexin, cAMP-response element binding protein (CREB) and phosphoserine were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). An anti-V5 epitope antibody was purchased from ClonTech (Basingstoke, Hampshire, U.K.). An antibody against Rab escort protein 1 (REP-1) was a kind gift from Professor M. Seabra (Cell and Molecular Biology Section, Division of Biomedical Science, Imperial College, London, U.K.). Alexa 488 was from Molecular Probes (Eugene, OR, U.S.A.). Myelin basic protein (MBP) was from Sigma–Aldrich (Poole, Dorset, U.K.).

Fractionation of cells

Cells were scraped in ice-cold PBS, recovered by centrifugation at 500 ***g*** for 10 min and resuspended in 500 μl of buffer A {10 mM Hepes, pH 7.4, 1.5 mM $MgCl_2$, 10 mM NaCl, 1 × protease inhibitors cocktail [1 mM EDTA, 1 mM EGTA, 0.2 mM tosyl-lysylchloromethane ('TLCK'), 1 mM *N*-ethylmaleimide, 0.1 mM tosylphenylalanylchloromethane ('TPCK') and 2 mM PMSF; Sigma], 1 mM NaF and 1 mM Na_3VO_4}. After incubation on ice for 20 min, Nonidet P40 was added to a final concentration of 0.6 % (v/v) and vigorously vortex-mixed for 10 s. The nuclei were pelleted at 4 °C by centrifugation for 5 min at 12000 ***g***. The supernatant, which represents the crude cytoplasmic fraction and subcellular organelles other than nuclei, was carefully transferred to a fresh vial and centrifuged once more to eliminate remaining debris. The nuclear pellet was washed once with buffer A, collected by centrifugation, resuspended in 500 μl of buffer B [10 mM Hepes, pH 7.4, 1.5 mM $MgCl_2$, 450 mM NaCl, 1 × protease inhibitors cocktail, 1 mM NaF, 1 mM Na_3VO_4 and 20 % (v/v) glycerol] and vortex mixed for 15 min at 4 °C. The lysate was centrifuged at 12000 ***g*** for 5 min at 4 °C and the supernatant containing the nuclear proteins transferred to a fresh vial. Protein concentration was measured by Bradford assay. Extracts were stored at −70 °C until further use. The purity of each fraction was determined by Western blotting for calnexin, REP-1 and CREB.

Immunoprecipitation and immunoblotting

Equal amounts (500 μg) of cytoplasmic and nuclear protein fractions were diluted in 1.5 ml of ice-cold RIPA buffer (1 × PBS, 1 % Nonidet P40, 0.5 % sodium deoxycholate, 0.1 % SDS, 1 × protease inhibitors cocktail, 1 mM NaF and 1 mM Na_3VO_4). Lysates were precleared by adding 1 μg of appropriate control IgG (corresponding to the host species of the primary antibody) together with 20 μl of Protein A/G-coupled agarose beads (Autogen Bioclear, Calne, Wiltshire, U.K.). Samples were centrifuged and supernatants transferred to fresh tubes. Primary antibody (2–4 μg) was added and incubated overnight at 4 °C on an orbital shaker. The immunocomplex was captured by adding 20 μl of Protein A/G-coupled agarose beads. After a further 2 h incubation at 4 °C, the immunocomplex was centrifuged, washed 4 times with 1 ml of PBS, solubilized in 40 μl of reducing Laemmli sample buffer [28] containing the cocktail of protease inhibitors, and analysed by SDS/PAGE. Proteins were transferred on to PVDF membrane (Immobilin-P; Millipore, Bedford, MA, U.S.A.) using a BioRad transfer apparatus. Immunodetection was performed essentially as described previously [14]. Bound antibodies were visualized using the enhanced chemiluminescence system (ECL®) (Autogen Bioclear, Wiltshire, U.K.). Pre-stained molecular-mass standards (Amersham International) were used to monitor protein migration.

In vivo phosphorylation and phospho-amino-acid analysis

HMCs were serum-starved for 24 h and incubated with phosphate-free media for 6 h, after which 100 μCi/ml $^{32}PO_4^{3-}$ and 80 ng/ml CTGF were added and incubation continued for 12 h. The cells were washed with PBS and fractionated in the presence of phosphate and protease inhibitors. Cytoplasmic and nuclear protein fractions were prepared, immunoprecipitated with anti-CTGF antibody, and subjected to SDS/PAGE. Proteins were transferred onto PVDF membrane filters and analysed by autoradiography. ^{32}P-labelled bands were cut out from the membrane and hydrolysed in 200 μl of 6 M HCl for 90 min at 110 °C. Samples were then lyophilized and Cerenkov counts determined. The hydrolysate was resuspended in 10 μl of buffer (50 ml of 88 % formic acid, 156 ml of glacial acetic acid and 1794 ml of deionized water, pH 1.9) containing 5 μg each of unlabelled phospho-amino-acid standard (phosphoserine, phosphothreonine and phosphotyrosine). Phosphate of approx. 100 c.p.m. was loaded as a spot onto a 20 cm × 20 cm thin-layer cellulose plate. Electrophoresis was carried out in the first dimension using a pH 1.9 buffer and with a pH 3.5 buffer (100 ml of glacial acetic acid, 10 ml of pyridine, 1890 ml of deionized water) for the second dimension. Plates were air-dried, baked in an oven at 65 °C for 10 min and then sprayed with 0.25 % ninhydrin in acetone to visualize the phospho-amino-acid standards. The plates were then exposed to X-ray film (Kodak).

In vitro phosphorylation

For protein kinase C (PKC) phosphorylation reactions, 2 μg of recombinant CTGF (rCTGF) protein or MBP was suspended in 200 μl of kinase buffer (20 mM Hepes, pH 7.5, 10 mM NaCl, 0.1 % Nonidet P40, 10 % glycerol, 10 mM dithiothreitol and 0.1 mM PMSF). This was then mixed with 10 μl of 40 mM $CaCl_2$, 1 μl of 400 mM $MnCl_2$ and 1 μl of [γ-^{32}P]ATP. The reactions were incubated at 30 °C for 2 min, after which 150 ng of PKC (PKM, the catalytically active fragment of PKC; Biomol, Mamhead, Exeter, U.K.) was added, mixed, and the incubation continued for a further 30 min. Tubes were heated at 95 °C for 5 min to stop the reaction. MBP was precipitated from reactions by 20 % ammonium sulphate and resuspended in Laemmli sample buffer. CTGF protein was captured on heparin beads (Sigma), washed 4 times with kinase buffer, then resuspended in Laemmli sample buffer and analysed by SDS/PAGE and autoradiography.

Transcription and translation *in vitro*

The PROTEINscript II system (Ambion, AMS Biotechnology, Abindon, Oxon, U.K.) was used for coupled transcription/translation *in vitro* of CTGF. Briefly, 0.5 μg of CTGF TOPO and CTGF–V5 TOPO plasmid DNA [14] were transcribed with sp6 and T7 polymerase enzymes respectively. Transcripts were translated for 1 h at 30 °C in a rabbit reticulocyte lysate in the presence of 33 μM [^{35}S]methionine (1200 Ci/mmol; Amersham International). After translation the lysate was dialysed against dialysis buffer (140 mM NaCl, 20 mM Hepes and 2 mM $CaCl_2$, pH 7.4) to remove free [^{35}S]methionine. The lysate was then analysed by SDS/PAGE and autoradiography.

Transcription in isolated nuclei and analysis of RNA

Nuclei were purified according to Marzluff and Huang [29] and stored in liquid nitrogen. Nuclear run-off transcription assay was

performed by incubating 200 μl of thawed nuclei (2×10^6), with 200 μl of 2 × reaction buffer (10 mM Tris/HCl, pH 8, 5 mM $MgCl_2$, 0.3 M KCl and 0.2 mM dithiothreitol), 2 μl each of ATP, CTP, GTP (100 mM each), CTGF (0–20 ng) and 10 μl of [α-^{32}P]UTP (800 Ci/mmol and 10 mCi/ml; Amersham International). Fibronectin (20 ng) was included in some incubations as a control protein. The reaction was incubated at 30 °C for 45 min with shaking. Total RNA was extracted using the RNAWIZ reagent (Ambion). Unincorporated label was removed by repeated suspension and precipitation of RNA (three times) using RNase-free water and 1/10 volume of 2.5 M ammonium acetate and 3 volumes of cold ethanol. Samples were finally suspended in 20 μl of water, of which aliquots of 2 μl were assayed for radioactivity in a Beckman LS 6000SC liquid scintillation counter. Labelled total RNA was also used to probe an array of cDNAs spotted in doublet onto a Zeta-Probe membrane (Bio-Rad, Hemel Hempsted, Herts., U.K.). Each spot represented 500 ng of approx. 500 bp of PCR-amplified fragments of collagen type I and III, fibronectin, CTGF, plasminogen activator inhibitor-1 (PAI-1) and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase. Hybridization was carried out using Rapid-hyb buffer (Amersham Pharmacia Biotech, Little Chalfont, Bucks., U.K.).

Immunofluorescence staining

CTGF was labelled using the Alexa 488 protein labelling kit according to the manufacturer's instructions (Molecular Probes Europe BV, Leiden, The Netherlands). Cells were grown on glass coverslips, incubated with Alexa-CTGF labelled for various periods of time and fixed with 3.7 % (w/v) paraformaldehyde fixed in PBS for 20 min at 4 °C. For double-staining experiments, paraformaldehyde-treated cells were first permeabilized with 0.5 % Triton X-100 in PBS for 10 min at room temperature. Coverslips were then 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 containing 5 % BSA) for 1 h at 37 °C. Coverslips were then washed and incubated in the dark for 1 h with fluorescein-conjugated secondary antibody (Sigma–Aldrich). After staining the coverslips were mounted on glass slides with anti-fade mounting media (Vector, Peterborough, UK) and examined using a fluorescence microscope (Olympus AX70).

RESULTS

Subcellular localization of CTGF

We investigated the subcellular distribution of CTGF in HMCs maintained for up to 21 days in either normal (4 mM D-glucose), or high glucose conditions (30 mM) to mimic chronic hyperglycaemia in diabetes *in vivo*. At steady state, CTGF is located predominantly in the juxtanuclear area of HMCs maintained in medium containing either normal or high glucose concentration at all times (Figure 1). CTGF can be also seen clustered on the cell surface (Figures 1D and 1E, open arrowhead), in vesicles throughout the cytoplasm (Figures 1B–1E, filled arrow) and, possibly, in the nuclei of cells. This distribution was essentially unchanged over 21 days culture under the different glucose conditions.

To confirm the steady state subcellular localization of CTGF, proteins from mesangial cells maintained in high glucose for 3 days were fractionated into cytosolic/subcellular membrane and nuclear fractions. Both fractions were then analysed by Western blotting for the presence of REP-1, calnexin, CREB and CTGF proteins. The results in Figure 2 show that the transcription factor CREB (43 kDa) was only detected in the nuclear fraction (Figure 2C) whereas, as expected, the marker of the endoplasmic reticulum, calnexin (90 kDa), and the cytosolic REP-1 (98 kDa), were detected only in the cytosolic/subcellular membrane fraction (Figures 2A and 2B). The nature of the bands which cross react with the anti-(REP-1) antibody (approx. 46 kDa and 70 kDa) and with the anti-CREB antibody (approx. 66 kDa) in the cytosolic fraction is not known. The results rule out the possibility that CTGF protein detected in the nuclear fraction (Figure 2D) is an artifact due to contamination with the cytosolic fraction. Figure 2D also shows that only about 15 % of the total CTGF protein is present in the nuclear fraction. The same results were also obtained for CTGF when the cytosolic/membrane and nuclear fractions were added to heparin–Sepharose beads to extract the growth factor before analysis by Western blotting (results not shown).

Endocytosis and intracellular transport of exogenous CTGF added to HMCs

To investigate uptake and intracellular transport of CTGF in HMCs, we labelled the growth factor with Alexa and followed its subcellular distribution after adding it to cells and leaving them at 4 °C for 2 h, followed by washing with cold PBS and then incubation at 37 °C for various periods of time. Figure 3(A) shows that when the cells were incubated with the growth factor at 0 °C, it was bound fairly uniformly to the cell surface. When the cells were incubated subsequently for 8 min at 37 °C, the labelled growth factor appeared as intracellular spots, indicating uptake in vesicles (Figure 3B). These vesicles accumulated in the juxtanuclear area with increasing time (Figures 3C–3E). After 4 h a small amount of labelled CTGF remained in the juxtanuclear area (Figure 3F). A second experiment gave the same results. The identity of the compartment where the growth factor accumulates is not clear. However, our preliminary experiments using markers for different intracellular organelles suggest that it could be the recycling endosome compartment (results not shown) which consists of a juxtanuclear pericentriolar collection of membranous tubular elements [30]. The accumulation of endocytosed CTGF appears to be similar to that of endocytosed transferrin which accumulates in the recycling endosome compartment. Under steady-state conditions, about 80 % of the endocytosed transferrin accumulates in this compartment [31].

Phosphorylation of CTGF

When recombinant CTGF was incubated with mesangial cells in the presence of $Na_3{}^{32}PO_4$ and the growth factor subsequently immunoprecipitated from cytosolic and nuclear fractions it was found to be phosphorylated (Figure 4A). The major band has a higher apparent molecular mass (approx. 44 kDa) than the original rCTGF (approx. 36–38 kDa), attributable to its phosphorylation. Phospho-amino-acid analysis showed that the phosphorylation occurs on serine residues (Figure 4B). The deduced amino acid sequence of CTGF contains four putative Ser-X-(Arg-Lys) motifs, potential sites for phosphorylation of Ser-119, -218, -227 and -275 by PKC. To investigate whether endogenous PKC can phosphorylate CTGF, we incubated HMCs without or with 0.1 μM and 0.3 μM PMA in the presence of 40 ng/ml rCTGF for 30 min. Cells were lysed in RIPA buffer and equal amounts of lysate protein incubated with phosphoserine-agarose affinity beads. Bound proteins were analysed by Western blotting, using anti-CTGF antibody. The

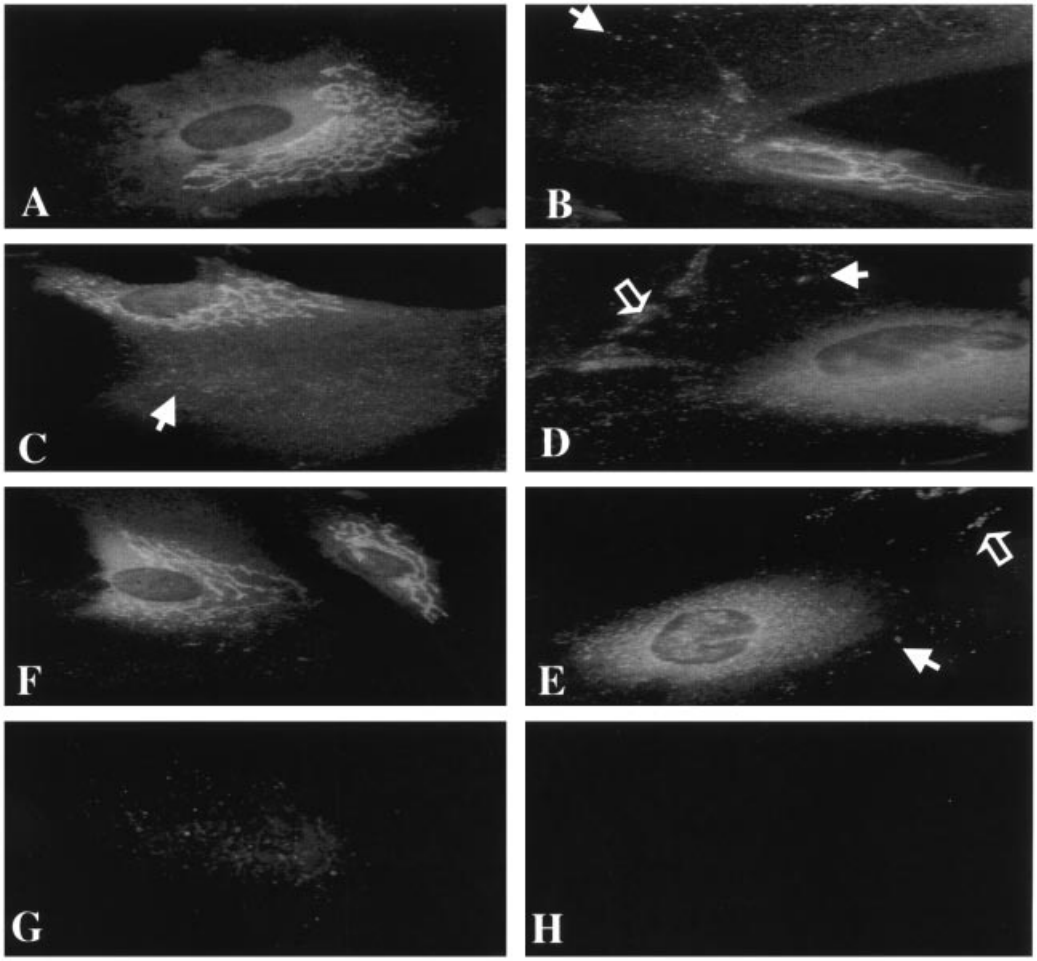

Figure 1 Subcellular localization of CTGF protein in HMCs by indirect immunofluorescence

Cells were maintained under normal glucose (**A**, **C** and **F**) or high glucose (**B**, **D** and **E**) conditions for periods of 1 day (**A** and **B**), 14 days (**C** and **D**) and 21 days (**F** and **E**). Cells were fixed and stained for CTGF as described in the Materials and methods section. Specificity was checked by using CTGF antibody which has been adsorbed with rCTGF (**G**) or by omission of the primary antibody (**H**). Magnification, $\times 760$.

results confirm that endocytosed rCTGF undergoes phosphorylation in non-PMA-treated cells since it is captured by the phosphoserine affinity beads (Figure 5, lane 1). PMA treatment enhanced CTGF phosphorylation in a dose-dependent manner (Figure 5, lanes 2 and 3), indicating that this was due to the activity of endogenous PKC. Phosphorylated endogenous CTGF was also detected in the absence of PMA stimulation (Figure 5, lane 4).

Following PMA stimulation of the cells we also detected some faint bands of higher and lower molecular mass than the prominent rCTGF monomer band (Figure 5, lane 3). The faint bands must be derived from phosphorylated rCTGF since they were captured by the phosphoserine affinity beads and bound anti-CTGF antibody on the Western blot. Similar bands were seen in anti-CTGF antibody immunoprecipitates of the cytosol of cells labelled with $Na_2[^{32}PO_4]$ (Figure 4A, lane 1). These are likely to be due to cleavage products of rCTGF (< 44 kDa) and to SDS-stable complexes formed between CTGF and its cleavage products (e.g. 56 kDa), as described previously [14], or possibly to CTGF dimerization (> 90 kDa). Figure 6(A) shows that PKC phosphorylates rCTGF directly *in vitro* (Figure 6A, lane 1) and that several bands of higher molecular mass than the recombinant protein were also detected which were not present in either the control MBP incubation (Figure 6A, lane 2), or in the original recombinant protein preparation (Figure 6B). CTGF and the associated bands were recovered from the phosphorylation reaction by heparin-affinity beads. This result provides further evidence suggesting that phosphorylated rCTGF can undergo dimerization with itself (approx. 75–80 kDa bands; Figure 6A, lane 1) or with spontaneously generated cleavage products. However, although these higher molecular mass forms of CTGF are prominent in the autoradiogram (Figure 6A), Western-blot analysis of the products of *in vitro* PKC phosphorylation reactions carried out with non-radioactive ATP (results not shown) were similar to Western blots of *in vivo* phosphorylations (Figure 5, lane 3). Thus the higher molecular mass bands are quantitatively a minor component relative to the main monomer CTGF bands.

Uptake of exogenous CTGF and phosphorylation during its translocation to the nucleus

To investigate further the uptake of exogenous CTGF and its translocation via the cytosol to the nucleus, cells were serum

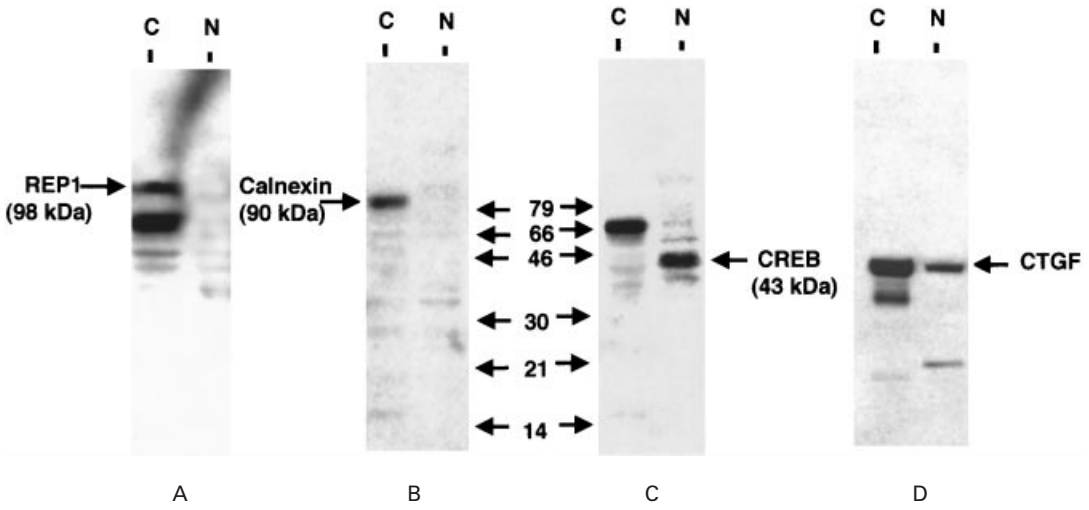

Figure 2 Subcellular localization of CTGF protein in HMCs detected by immunoblotting

Cells were maintained under high glucose conditions for 3 days, and then fractionated into cytosolic and nuclear fractions as described in the Materials and methods section. Equal amounts of protein were resolved by SDS/PAGE (4–12% gel) under reducing conditions and Western blotted with anti-(REP-1) (**A**), anti-calnexin (**B**), anti-CREB (**C**), and anti-CTGF (**D**) antibodies. C, cytosolic fraction; N, nuclear fraction.

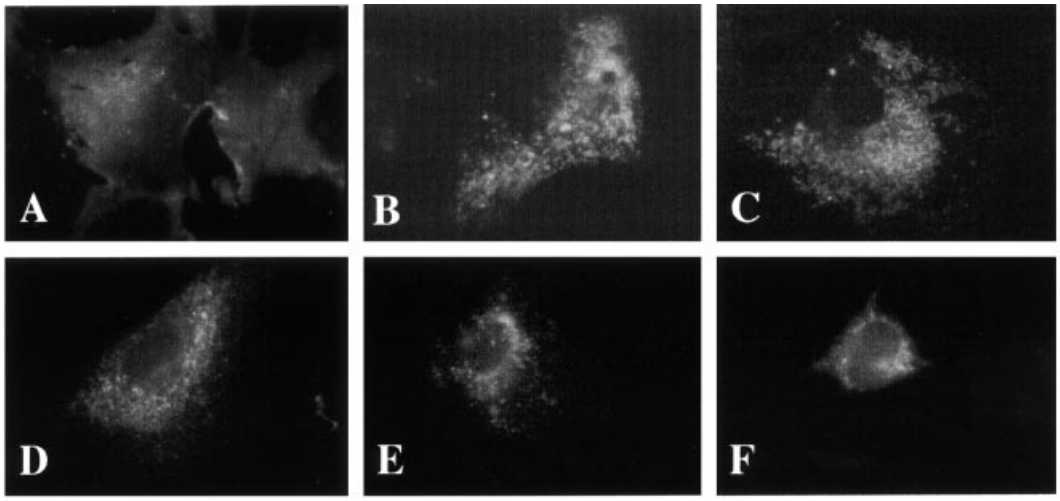

Figure 3 Uptake and intracellular transport of fluorescently labelled CTGF in HMCs

Cells were incubated with Alexa 488-labelled CTGF at 4 °C for 2 h to allow binding of the growth factor. They were then washed with cold PBS and either fixed immediately, 0 min (**A**), or incubated at 37 °C for 8 min (**B**), 15 min (**C**), 30 min (**D**), 45 min (**E**) and 4 h (**F**), before fixation. A second experiment gave the same results. Magnification, × 760.

starved for 24 h and then incubated with [^{35}S]methionine-labelled CTGF–V5 fusion protein and unlabelled methionine (1 mM) in the presence or absence of unlabelled CTGF (200 ng/ml) at 37 °C for various periods of time. Any ^{35}S-labelled CTGF–V5 bound at the cell surface was washed off with an acid-salt buffer and the cells were then fractionated into cytosolic and nuclear fractions. CTGF–V5 fusion protein was immunoprecipitated from each fraction using affinity purified anti-V5 antibody and analysed by SDS/PAGE and autoradiography. As shown in Figure 7(A), no radiolabelled CTGF–V5 protein was detected in the cytosolic or nuclear fractions at time zero of the incubation (Figure 7A, lanes 1 and 2), while after 10 min, the labelled growth factor was detected in only the cytosolic fraction (Figure 7A, lane 3). Between 10 and 30 min of incubation, the labelled CTGF–V5 was detected in both the cytosolic and the nuclear fractions (Figure 7A, lanes 5 and 6). The amount of labelled growth factor increased in both fractions after 1 h of incubation (Figure 7A, lanes 7 and 8). An excess of unlabelled rCTGF was able to compete out the binding of the [^{35}S]methionine-labelled CTGF–V5 (Figure 7A, lanes 9 and 10), indicating the binding specificity. The same results were obtained if the cytosolic and nuclear fractions were incubated with heparin–Sepharose affinity beads and the bound proteins analysed by SDS/PAGE and autoradiography (Figure 7B).

Interestingly, the results in Figure 7(A) show that there is a difference in the molecular mass of the added labelled fusion

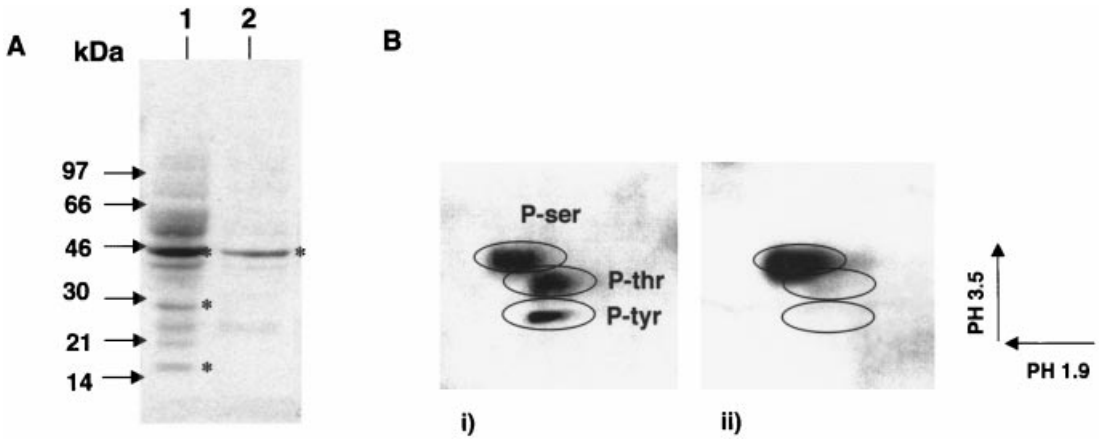

Figure 4 ***In vivo*** **phosphorylation of CTGF**

Cells were incubated with rCTGF in 4 mM D-glucose conditions in the presence of Na3[$^{32}PO_4$]. The growth factor was then immunoprecipitated from the cytosolic and nuclear fractions with anti-CTGF antibody and resolved by SDS/PAGE (4–12% gel) and autoradiography. (**A**) Lane 1, cytosolic fraction. Lane 2, nuclear fraction. *, bands which were excised from the PVDF membrane for phospho-amino-acid analysis. (**B**) Two-dimensional phospho-amino-acid analysis of the 46 kDa band of CTGF from (**A**), lane 1. Samples were prepared and subjected to electrophoresis in two-dimensions as described in the Materials and methods section. Plates were sprayed with ninhydrin to visualize the phospho-amino-acid standards (i) and then exposed to X-ray film (ii). All bands indicated by * in (**A**) gave the same result.

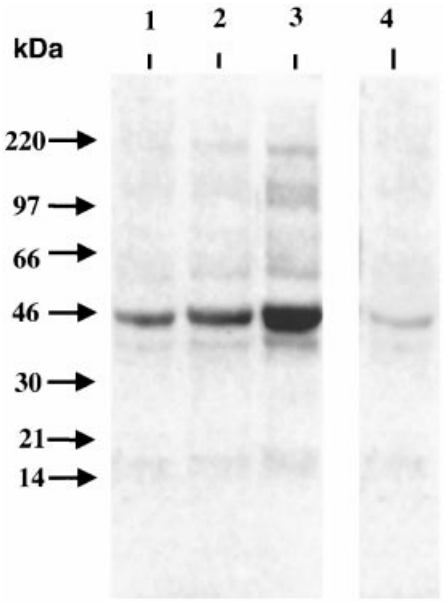

Figure 5 Stimulation of CTGF phosphorylation by PMA

HMCs were incubated without (lane 1) or with 0.1 μM PMA (lane 2), or 0.3 μM PMA (lane 3), in the presence of 40 ng/ml rCTGF for 30 min. HMCs were also incubated without either exogenous rCTGF or PMA as a control (lane 4). Cells were lysed and equal amounts of protein were incubated with phosphoserine-affinity beads. Bound proteins were analysed by Western blotting using anti-CTGF antibody.

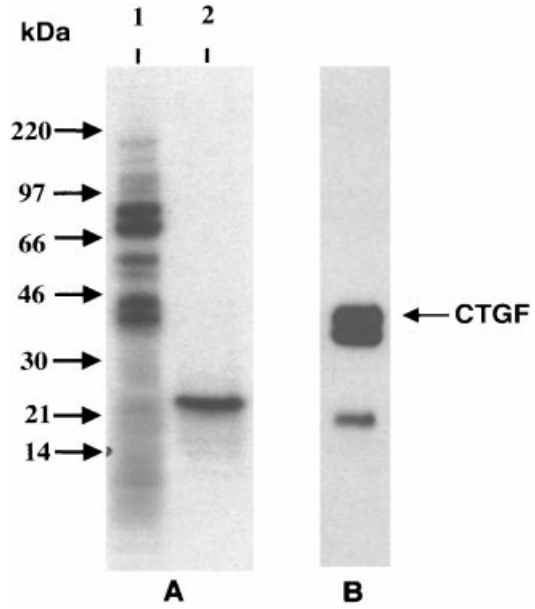

Figure 6 ***In vitro*** **phosphorylation of CTGF by PKC**

(**A**) Autoradiogram of phosphorylation products separated by SDS/PAGE (4–12% gel) under reducing conditions. Phosphorylation reactions were performed as described in the Materials and methods section in the presence of [γ-^{32}P]ATP and either CTGF (lane 1) or MBP protein as control (lane 2). (**B**) A Western blot represents the same amount of CTGF protein used in the phosphorylation reaction.

protein (lane 11) and that transported intracellularly (lanes 5–8). This change could be due to phosphorylation of the growth factor during its translocation. To investigate this we incubated the cytosolic and nuclear fractions with phosphoserine-affinity beads and analysed the bound proteins for the presence of [^{35}S]methionine-labelled CTGF–V5 fusion protein. Both the cytosolic and nuclear [^{35}S]CTGF–V5 bound to the phosphoserine-affinity beads (Figure 7C). However, the beads did not bind dialysed [^{35}S]methionine-labelled CTGF–V5 before adding it to the cells (results not shown). These results confirm the uptake of CTGF by HMCs and demonstrate that it is phosphorylated en route to the nuclear compartment of the cells.

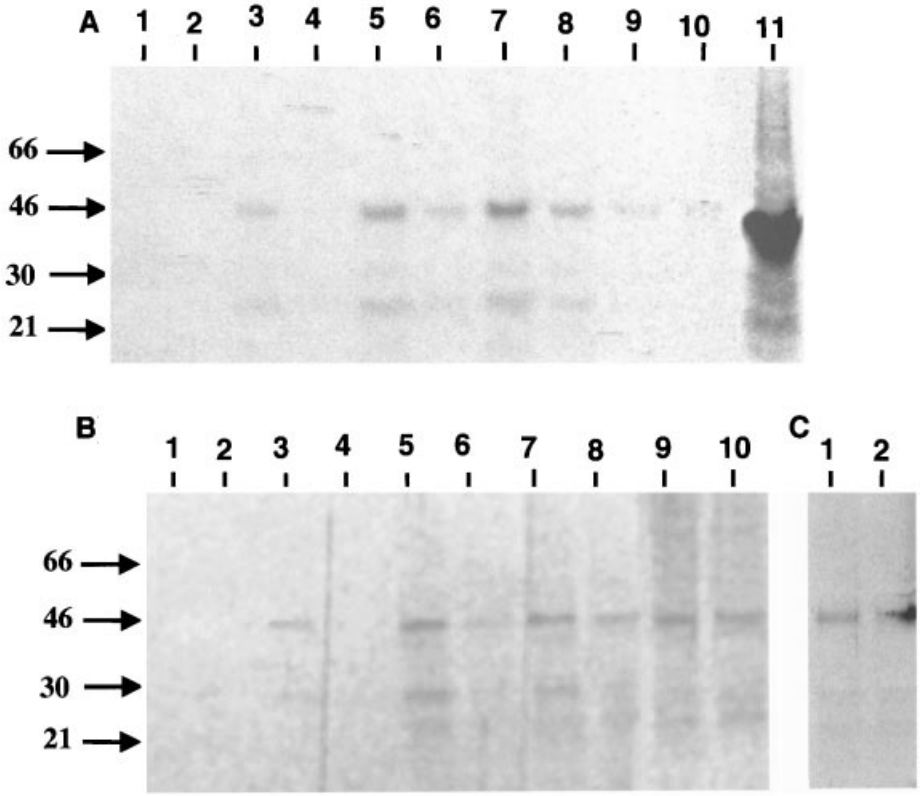

Figure 7 Transport of externally added CTGF to the cytosol and nucleus

(**A**) Serum starved cells were incubated with [^{35}S]methionine-labelled CTGF–V5 at 37 °C for 0 min (lanes 1 and 2), 10 min (lanes 3 and 4), 30 min (lanes 3, 5 and 6) or 60 min (lanes 7–10) before being washed and fractionated into cytosolic (lanes 1, 3, 5, 7 and 9) and nuclear fractions (lanes 2, 4, 6, 8 and 10). Equal amounts of proteins from each fraction were immunoprecipitated with anti-V5 antibody and analysed by SDS/PAGE and autoradiography. Lanes 9 and 10 are from 60 min incubations with [^{35}S]CTGF–V5 in the presence of excess of unlabelled rCTGF. Lane 11, dialysed [^{35}S]methionine-labelled CTGF–V5 before adding to the cells. (**B**) Equal amounts of protein from cytosolic and nuclear fractions were incubated with heparin–Sepharose affinity beads and bound proteins analysed by SDS/PAGE and autoradiography. Lanes 1–8, as for (**A**). Lanes 9 and 10, cytosolic and nuclear fractions from cells incubated with [^{35}S]CTGF–V5 for 120 min. (**C**) Lanes 1 and 2 are cytosolic and nuclear fractions from cells incubated with [^{35}S]CTGF–V5 for 60 min. The subcellular fractions were then incubated with phosphoserine-affinity beads and bound proteins were analysed by SDS/PAGE and autoradiography.

Effect of CTGF on the transcription of RNA in isolated nuclei from HMCs

To determine whether CTGF can act directly on the transcriptional machinery of the cell, studies *in vitro* were carried out with isolated nuclei from HMCs. Nuclei were incubated in a reaction mixture containing [α-^{32}P]UTP with or without CTGF, or with fibronectin as a control protein. RNA was extracted and newly transcribed RNA assayed for radioactivity. In the absence of CTGF, 19841 ± 1402 c.p.m. were incorporated into newly-transcribed total RNA per 10^5 nuclei ($n = 3$). Incorporation was unchanged in the presence of 20 ng/ml fibronectin (20008 ± 1630 c.p.m.). However, there was a greater than 2-fold (44775 ± 4516 c.p.m.) and greater than 3-fold (66071 ± 3726 c.p.m.) increase in incorporation of radioactivity into total RNA in the presence of 5 ng and 20 ng/ml CTGF respectively. A second experiment gave very similar results. To determine whether the addition of rCTGF to isolated nuclei directly affected the transcription of known target genes for the growth factor [15] we analysed the mRNA pools for collagen I, collagen III, fibronectin, CTGF, PAI-1 and glyceraldehyde-3-phosphate dehydrogenase. The mRNA pool sizes of each were not affected by addition of CTGF to the isolated nuclei. This suggests that the increase in newly-transcribed total RNA in the presence of CTGF is largely, if not exclusively, due to increased transcription of ribosomal RNA.

DISCUSSION

CTGF is now considered as an important effector molecule in both physiological and pathological processes. Our major goal is to understand the molecular mechanisms by which this growth factor is involved in the pathogenesis of diabetic nephropathy. The mode of action of CTGF has not yet been established. However, its secretion and intrinsic multimodular structure suggest that its function may depend on interactions with several other proteins, including cell surface receptors, for its function. However, no specific cell surface receptor has yet been identified.

The presence of CTGF in the juxtanuclear area of HMCs under steady state conditions could represent molecules either waiting for release by exocytosis or accumulated endocytosed molecules. In the present study we demonstrated endocytic uptake of CTGF. The internalized growth factor appears first in endosomes and then in a juxtanuclear organelle. The nature of this organelle requires further investigation.

We have shown, *in vitro*, that CTGF contains functional site(s) for phosphorylation by PKC. However, the precise number and

location of these is still under investigation. CTGF is phosphorylated on serine residues *in vivo* and PMA treatment of HMCs enhances this phosphorylation. Thus, the growth factor can be phosphorylated by PKC *in vivo*.

The cellular phosphorylation of externally added CTGF by HMC is an indication of its translocation to the cytosol and nucleus, since these are the only places where PKC is known to exist [32,33]. PKC is not found inside vesicular compartments. The deduced amino acid sequence of CTGF contains a putative nuclear localization sequence, PFPRRVK (residues 149–155), in the von Willebrand factor type C domain which could potentially target the growth factor to the nucleus. The present work shows that ^{35}S-labelled CTGF–V5 added externally is found in the cytosol within 10 min and in the nucleus within 30 min. This time course, together with the increase in its molecular mass, indicates that the cytosol is the site of CTGF phosphorylation.

Protein kinase activity has been implicated in the regulation of transport of proteins to the nucleus [34–36]. However, although externally added acidic fibroblast growth factor (aFGF) is phosphorylated at Ser-130 by PKC [37,38], its nuclear translocation is not regulated by its phosphorylation status [39].

An increasing number of cytokines and growth factors have now been found to be transported to the nucleus including platelet-derived growth factor [40], aFGF [41], basic FGF [42], interleukin 1 [43], angiogenin [44], Schwannoma-derived growth factor [45] and insulin [46]. The transport pathway for aFGF is the most studied of these, and there is increasing evidence that the growth factor acts via both receptors at the cell surface and inside the cell. After binding to its tyrosine kinase receptors on the plasma membrane, aFGF activates several intracellular signalling cascades, including phospholipase Cγ and mitogen-activated protein kinase [47]. However, the growth factor is also endocytosed upon binding to its receptor and is targeted for lysosomal degradation, resulting in down-regulation of the receptors. Despite this, a considerable amount of the growth factor accumulates in a juxtanuclear organelle, from which it leaves the endocytic pathway and translocates to the cytosol and the nucleus [37,41,48–51]. The mechanism of this translocation is not yet clear but it appears to require an acidic environment and phosphatidylinositol 3-kinase activity [39,52]. Nuclear translocation of aFGF has been correlated with its increased mitogenic activity [41,52]. In this respect, we have shown that CTGF acts directly on the transcriptional machinery of isolated nuclei from HMCs, although this action may be modified *in vivo* by its phosphorylation. However, the transcription of known target genes (collagens, fibronectin, CTGF and PAI-1, see [15]) was unaffected and it seems more likely that the large increases in total RNA transcription are due to CTGF triggering release of nuclei from G0-phase cells to enter G1 phase with accompanying increases in rRNA. Further experiments are in progress to study the potential role of CTGF transported into HMCs and the significance of its phosphorylation. Interestingly, bioinformatic analysis using the PSI-BLAST method [53] detected sequences in the C-terminal domain of CTGF that share significant alignments with the R3H motif. This motif is predicted to bind to single-stranded nucleic acids [54].

In conclusion, the results presented here demonstrate for the first time a transport pathway for the growth factor CTGF from the cell surface into the cytosol, where its phosphorylation occurs, and then to the nucleus, where it may affect gene transcription. It is not clear whether this intracellular route represents the main effector pathway for CTGF signalling, or even whether it is the only such pathway since to date, 10 years after the discovery of the growth factor, no specific receptor has been identified for it. Here HMCs are able to both synthesize and endocytose CTGF. This occurs predominantly when the growth factor is present at a high extracellular concentration under abnormal conditions (high glucose). The identification of such a pathway suggests autocrine and putative paracrine functions for the growth factor towards other target cells. Interestingly, Ito et al. [18] have shown that renal tubule cells do not express CTGF mRNA using the *in situ* hybridization technique. However, during immunostaining for CTGF protein in mouse or human renal biopsy specimens, we found that a specific subset of distal tubule cells contain a high level of CTGF protein (N. A. Wahab, H. Brinkman and R. M. Mason, unpublished work).

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