Establishment of a recombinant expression system for connective tissue growth factor (CTGF) that models CTGF processing *in utero*

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Connective tissue growth factor (CTGF) stimulates cell proliferation, migration, adhesion and extracellular matrix production, and functions in processes such as development, differentiation, angiogenesis, implantation, wound healing and fibrosis. CTGF is a 38 kDa protein that comprises four discrete structural modules (modules 1–4) but is susceptible to limited proteolysis *in utero* yielding bioactive isoforms that comprise either modules 3 and 4 (16–20 kDa) or module 4 (10 kDa). Here we report the development of a stable cell line, termed DB1, that was generated by transfecting cDNA encoding full-length human CTGF into Chinese hamster ovary cells that were mutant for heparin sulphate and chondroitin sulphate. DB1 cells produced 38 kDa CTGF and low molecular mass CTGFs that had N-termini between modules 2 and 3 at Ala¹⁸¹ (20 kDa), Leu¹⁸⁴ (18 kDa) or Ala¹⁹⁷ (16 kDa)

or between modules 3 and 4 at Gly²⁵³ (10 kDa). CTGF was exported from DB1 cells as early as 5 min after synthesis and all isoforms were readily purified from conditioned medium by sequential steps of heparin affinity, cation exchange, and reverse-phase chromatography. The 38 kDa CTGF was faithfully glycosylated and underwent limited proteolysis in the presence of thrombin, kallikrein or uterine fluids, the last of which was antagonized by anti-thrombin III. All CTGF isoforms promoted cell adhesion, mitosis and epithelial transdifferentiation *in vitro* as well as subcutaneous fibrosis *in vivo*. The establishment of this recombinant expression system allows for mass-scale production of all previously reported uterine CTGF isoforms, demonstrates that module 4 contains functional domains involved in a broad range of biological activities, and will facilitate studies of CTGF processing *in vitro*.

Introduction

Connective tissue growth factor (CTGF) is a multifunctional polypeptide that regulates several aspects of cell function including differentiation, proliferation, survival, migration, adhesion and stimulation of extracellular matrix production (Grotendorst, 1997; Brigstock, 1999; Lau and Lam, 1999; Moussad and Brigstock, 2000; Perbal, 2001). CTGF is a 38 kDa protein comprising four discrete structural modules that are found in a variety of unrelated extracellular proteins (Bork, 1993; Brigstock, 1999). These modules contain motifs that predict potential roles in insulin-like growth factor (IGF) binding (module 1), oligomerization (module 2), cell attachment (module 3), and dimerization or receptor binding (module 4). It is likely that the modular

configuration of CTGF allows it to interact with soluble, cellular or extracellular proteins associated with the matrix. These interactions may regulate the activity and bioavailability of CTGF and its binding partners within the pericellular environment (Brigstock, 1999; Moussad and Brigstock, 2000; Perbal, 2001).

Many different cell types produce and respond to CTGF and it is likely that autocrine and paracrine circuits are responsible for its actions (Moussad and Brigstock, 2000). Although CTGF overproduction and action has been linked to various fibrotic and desmoplastic pathologies (Grotendorst, 1997; Brigstock, 1999; Clarkson *et al.*, 1999; Goldschmeding *et al.*, 2000; Gupta *et al.*, 2000; Moussad and Brigstock, 2000; Denton and Abraham, 2001), several studies have highlighted the likely importance of this molecule in normal biological processes such as wound repair (Igarashi *et al.*, 1993; Pawar *et al.*, 1995; Schwab *et al.*, 2000; Ujike *et al.*, 2000; Wunderlich *et al.*, 2000; Blom *et al.*, 2001), endochondral ossification (Nakanishi *et al.*, 1997, 2000, 2001; Nishida *et al.*, 2000) and angiogenesis (Shimo

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et al., 1999). Within the context of the reproductive tract, both the uterus and ovary of the adult have been identified as sites of CTGF production and action. Ovarian CTGF expression is regulated by transforming growth factor β (TGF- β) and gonadotrophins (Harlow *et al.*, 2002; Liu *et al.*, 2002), whereas uterine CTGF expression is regulated by TGF- β , oestrogen and progesterone (Rageh *et al.*, 2001). Cysteine-rich 61 (CYR61), which is 80% homologous to CTGF, is also oestrogen- and progesterone-dependent (Sampath *et al.*, 2001a, 2002; Xie *et al.*, 2001a), and both CYR61 and CTGF are expressed in steroid-dependent tumours of the breast or uterus (Frazier and Grotendorst, 1997; Tsai *et al.*, 2000, 2002; Uzumcu *et al.*, 2000; Sampath *et al.*, 2001a,b, 2002; Xie *et al.*, 2001a,b). Thus CTGF and CYR61 may mediate the action of hormones and growth factors in the reproductive tract. In the ovary, CTGF has been implicated in theca cell recruitment and mitogenesis during follicular development and maintenance of the corpus luteum after ovulation (Wandji *et al.*, 2000; Slee *et al.*, 2001; Harlow and Hillier, 2002; Harlow *et al.*, 2002; Liu *et al.*, 2002). In the uterus, luminal and glandular epithelial cells are a major source of CTGF during the oestrous cycle and preimplantation (Surveyor *et al.*, 1998; Moussad and Brigstock, 2000; Moussad *et al.*, 2002). In species exhibiting non-invasive placentation, there is a transient decrease in CTGF production by these cells during the attachment phase, which is temporally and spatially associated with extensive stromal remodelling and neovascularization (Moussad *et al.*, 2002). In species with invasive placentation, CTGF is downregulated in uterine luminal epithelial cells before implantation and is then markedly upregulated in decidualizing stromal cells (Surveyor *et al.*, 1998; Uzumcu *et al.*, 2000). Irrespective of the mode of placentation, CTGF is also produced by endothelial cells of the uterine and extra-embryonic vasculature (Surveyor *et al.*, 1998; Moussad and Brigstock, 2000; Uzumcu *et al.*, 2000; Moussad *et al.*, 2002). Collectively, these results indicate that CTGF is involved in driving proliferation, differentiation, angiogenesis and extracellular matrix remodelling during placentation and decidualization. CTGF has also been detected in the uterine luminal fluid (ULF) in which its concentration changes as a function of the oestrous cycle (Brigstock *et al.*, 1997; Ball *et al.*, 1998; Surveyor *et al.*, 1998). In pigs, CTGF in ULF is detectable sooner in pregnancy than during the oestrous cycle (day 12 versus day 14) and exhibits overall lower concentrations and earlier peak production in pregnant animals compared with cyclic animals (day 12–14 versus day 16–18) (Ball *et al.*, 1998), supporting a modified role for uterine CTGF during the pre- and peri-attachment periods.

ULF was found to contain stable C-terminal isoforms of CTGF, which comprise essentially modules 3 and 4 (16–20 kDa) or module 4 alone (10–12 kDa) (Brigstock *et al.*, 1997; Ball *et al.*, 1998). These proteins were

detected in ULF of pigs and mice and arise via limited proteolysis of the 38 kDa uterine CTGF protein (Brigstock *et al.*, 1997; Ball *et al.*, 1998; Surveyor *et al.*, 1998). Low molecular mass CTGF isoforms possess some of the biological and physicochemical properties as full-length CTGF showing that functional domains reside within the C-terminal region of CTGF. Similar, but non-identical low molecular mass CTGF isoforms have been reported in amniotic, follicular, peritoneal, cerebrospinal fluids, serum and in conditioned medium of various mammalian cell lines maintained *in vitro* (Steffen *et al.*, 1998; Yang *et al.*, 1998; Boes *et al.*, 1999; Williams *et al.*, 2000). Although not yet proven, it is expected that the presence of modules 3 or 4, but not modules 1 or 2, will affect the repertoire of binding interactions of low molecular mass isoforms compared with the intact molecule.

Although the early studies of uterine secretory CTGF provided an important lead with respect to CTGF structure and function (Brigstock *et al.*, 1997; Ball *et al.*, 1998), detailed studies of low molecular mass CTGFs have been hampered by the difficulty of obtaining and processing large volumes of ULF and the relatively low yields of each isoform. Moreover, no other convenient source of these proteins is available for preparative purposes. As progress in this area is important so that the intrinsic biological properties of low molecular mass CTGFs can be studied in depth, we have developed a mammalian recombinant expression system in which 38 kDa CTGF undergoes faithful post-translational processing (glycosylation, proteolysis) and from which all CTGF isoforms can be readily purified and characterized.

Materials and Methods

Establishment of a stable CTGF-producing cell line

Full-length human CTGF cDNA was isolated by RT-PCR of human foreskin fibroblast RNA as described by Steffen *et al.* (1998). The amplified product was verified using Sequenase Version 2 (USB, Cleveland, OH) and ligated into pcDNA3.1 (Invitrogen, San Diego, CA). The ligation product, termed pcDNA3.1/CTGF, was transfected into DH5 α competent *Escherichia coli* and isolated by large-scale alkaline lysis. The protocol for lipofectamine (Gibco/BRL, Grand Island, NY) was used and 2 μ g pcDNA3.1/CTGF was used to transfect pgsA mutant Chinese hamster ovary (CHO) 745 cells (deficient in xylosyltransferase; negative for heparan sulphate and chondroitin sulphate (Esko *et al.*, 1985), kindly provided by J. Esko, University of California, San Diego, CA). After 2 days, the transfected cells were placed in Dulbecco's modified Eagle's medium (DMEM)–10% fetal bovine serum (FBS) containing 500 μ g gentamycin (G418) ml⁻¹ for selection. After 10 days, the cells were seeded in the same medium at 0.5 cells per well in six 96-well plates. Medium from wells containing single colonies

was screened for the presence of CTGF by western blot analysis and one clone, termed DB1, was selected for further processing.

DB1 cells were expanded into ten T-175 culture flasks in Ham's F12 (CellGro, Fisher Scientific, Pittsburgh PA) containing 10% FBS and 500 μg G418 ml^{-1} (30 ml per flask). Once the cells had reached confluency, they were trypsinized and placed in 15 Nunclon 500 cm^2 triple flasks (Nalge Nunc International Corp., Naperville, IL) in fresh Ham's F12–10% FBS–G418 medium (95 ml per flask). After 2–3 days, the medium was replaced with 85 ml per flask of serum-free CHO-SFMII (Gibco/BRL) containing 500 μg G418 ml^{-1} . After 3–4 days, conditioned medium was collected (approximately 1.2 l total volume), centrifuged (10 000 g for 20 min), and the supernatant clarified using a 0.2 μm filter.

Heparin affinity chromatography

A first step of heparin affinity chromatography was performed either by loading conditioned medium on to an Econo-Pac heparin column (3.6 cm $\times$ 0.7 cm; Bio-Rad, Hercules, CA) at a flow rate of 1 ml min^{-1} at 4°C (Brigstock *et al.*, 1997) or on to a Poros HE2/P heparin column (5.0 cm $\times$ 0.46 cm; Applied Biosystems, Foster City, CA) at a flow rate of 5 ml min^{-1} at room temperature. A gradient of 40 ml of 0.2–2.0 mol NaCl l^{-1} in 10 mmol Tris–HCl l^{-1} (pH 7.4) was used to develop each column and bound proteins were collected into fractions of 1 ml. Selected fractions from four individual first-step purifications were pooled, diluted 1:3 with 10 mmol Tris–HCl l^{-1} (pH 7.4), and subjected to a second step of heparin affinity chromatography using a TSK® heparin column (7.5 cm $\times$ 0.8 cm; TosoHaas, Philadelphia, PA), essentially as described by Brigstock *et al.* (1997) and Ball *et al.* (1998).

Cation exchange chromatography

Selected fractions from the second step of heparin affinity chromatography were diluted 1:3 in 10 mmol Tris–HCl l^{-1} (pH 7.4) and loaded on to an Econo-Pac carboxymethyl (CM) cation exchange column (1 cm $\times$ 5 cm; Bio-Rad, Hercules, CA). After washing the column with 10 ml of 10 mmol Tris–HCl l^{-1} (pH 7.4)–0.2 mol NaCl l^{-1} , the bound proteins were eluted using a gradient of 40 ml of 0.2–2.0 mol NaCl l^{-1} at a flow rate of 1 ml min^{-1} and collected into fractions of 1 ml.

Reverse-phase HPLC (RP-HPLC)

RP-HPLC was performed on pooled CM fractions as described by Brigstock *et al.* (1997) except that a semi-preparative C_8 column (25 cm $\times$ 1 cm, 10 μm ; Vydac, Hesperia, CA) was used. The column was equilibrated with water containing 5% (v/v) acetonitrile and 0.1%

(v/v) trifluoroacetic acid (TFA). Selected fractions from the CM cation exchange step were pooled, adjusted to 5% acetonitrile–0.1% TFA, and clarified using a 0.2 μm filter before injection. After sample injection, the column was washed with 5% acetonitrile–0.1% TFA for 30 min and then developed using a multi-linear acetonitrile gradient at a flow rate of 1 ml min^{-1} . Fractions were collected into tubes containing 50 μl 0.25 mol NaOH l^{-1} to neutralize the TFA.

Gel filtration chromatography

Gel filtration HPLC was performed on a Biosep S-2000 column (30 cm $\times$ 0.78 cm; separation range 827–300 kDa; Phenomenex, Torrance, CA). A peak fraction (100 μl) from a second step of heparin affinity chromatography was injected on to the column and eluted with 0.5 mol NaCl l^{-1} in PBS at a flow rate of 0.5 ml min^{-1} .

DNA synthesis

Aliquots of column fractions were tested for their ability to stimulate [^3H]thymidine incorporation into DNA of Balb/c 3T3 cells as described by Brigstock *et al.* (1997).

Cell adhesion

PBS (100 μl) containing fixed dilutions of sequential column fractions or individually quantified CTGF isoforms were incubated overnight at 4°C in Costar 96-well round-bottom medium binding polystyrene ELISA plates (Corning Inc, Reynoldsburg, OH). Wells were blocked with 200 μl PBS containing 3% (w/v) BSA and then incubated for 1 h at 37°C with 100 μl PBS containing Balb/c/3T3 cells at a concentration of 5×10^5 cells ml^{-1} . Adherent cells were then fixed for 15 min with 5% (w/v) formaldehyde and non-adherent cells were removed by washing each well three times with PBS. The remaining cells were measured by fluorescent emission from the wells at 520 nm following addition of 100 μl Cytoquant reagent (Molecular Probes, Eugene, OR) in lysis buffer.

Protein analysis

Aliquots of column fractions were subjected to SDS-PAGE, silver staining, and western blot analysis as described by Brigstock *et al.* (1997). N-terminal amino acid sequencing was performed on purified CTGF isoforms that had been transferred to polyvinylidene difluoride (PVDF) blots as described by Brigstock *et al.* (1997) and Ball *et al.* (1998). CTGF protein quantification was carried out using the Micro BCA Protein assay Kit (Pierce, Rockford, IL) with BSA as a standard.

Fig. 1. For legend see facing page.

Animals

All protocols involving animals were approved by the Institutional Animal Care and Use Committee of the Children's Research Institute, Columbus, OH.

Transdifferentiation

Techniques published by Ross *et al.* (1993) were adapted to isolate corneal epithelial cells from the eyes of adult Swiss Webster mice by enzymatic digestion and differential centrifugation. Cells were cultured for 7 days in DMEM–10% FBS and then grown for 2 days in serum-free DMEM containing insulin, transferrin and sodium selenite. This medium was then replenished and supplemented with 0–400 ng CTGF ml⁻¹ for 24 h, after which cells were stained for alpha smooth muscle actin (α SMA) using 1.7 μ g mouse monoclonal antibody ml⁻¹ (clone 1A4; Dako, Denmark).

Fibrosis in vivo

A model of persistent subcutaneous fibrosis in neonatal mice was established as described by Mori *et al.* (1999) except that prior or co-administration of TGF- β was omitted. Briefly, 340 ng of each CTGF isoform was administered in 20 μ l PBS into the subcutaneous region of the neck of Swiss Webster mice of 3 days of age consecutively for 7 days ($n=3$ per group). Control mice ($n=3$) received subcutaneous injections of 20 μ l PBS consecutively for 7 days. Mice were killed 14 days after the last injection and the treated areas were resected, fixed, sectioned and stained for α SMA as described above.

CTGF antibody production

Western blots were probed with a peptide antiserum raised against residues 247–260 of human CTGF as described by Brigstock *et al.* (1997) and Ball *et al.* (1998). In addition, two New Zealand White rabbits (rabbits 38A and 38B), from which blood was taken to collect preimmune serum, were injected subcutaneously with 100 μ g pure 38 kDa CTGF in Freund's complete

adjuvant, followed 2 weeks later by an intramuscular injection of 200 μ g pure 38 kDa CTGF in Freund's incomplete adjuvant. Blood samples were collected from animals 10 days later and twice a month thereafter for collection of CTGF antiserum, which was used for radioimmunoprecipitation. The CTGF used for immunization was purified from DB1 conditioned medium by sequential two-cycle heparin affinity chromatography, cation exchange chromatography, and reverse-phase HPLC as described above.

Radioimmunoprecipitation assay (RIPA)

Control non-transfected CHO cells or DB1 cells were seeded in 6- or 12-well plates and allowed to grow for 1–3 days. They were transferred to serum-free cysteine and methionine-deficient DMEM for 1 h and labelled with 100 μ Ci [³⁵S]cysteine and [³⁵S]methionine ml⁻¹ (Trans³⁵S-label; ICN Biomedical, Costa Mesa, CA). After incubation for up to 24 h, the labelling medium was removed and the cells were lysed in 1 ml cold RIPA buffer (50 mmol Tris-HCl l⁻¹ (pH 8.0) containing 150 mmol NaCl l⁻¹, 1% (v/v) Nonidet P-40, 0.5% (w/v) deoxycholic acid, and 0.1% (w/v) SDS). Media and cell lysate were subjected to RIPA by addition of 10 μ l rabbit 38B antiserum ml⁻¹ for 1 h at room temperature, followed by 12.5 μ l protein A beads (Pierce Chemical Co., Rockford, IL) for 1 h at 4°C to precipitate the immune complexes. Beads were washed three times with 1 ml RIPA buffer after which bound proteins were extracted with 20 μ l 2 $\times$ SDS-PAGE sample buffer and subjected to SDS-PAGE and autoradiography. Specific experimental conditions are given in the figure legends.

Enzymatic digestion of CTGF

DB1-derived 38 kDa CTGF, purified by HPLC, was incubated overnight at 37°C with ULF essentially as described by Ball *et al.* (1998) except that the reaction was performed on ULF samples that had been preincubated for 1 h at 37°C in the presence or absence of 1 U anti-thrombin III (Calbiochem, San Diego, CA).

Fig. 1. Purification of recombinant connective tissue growth factor (CTGF) isoforms from DB1 conditioned medium. (a) DB1 conditioned medium (1.2 l) was subjected to a first step of heparin affinity chromatography. This step was repeated on three additional samples of conditioned medium and fractions containing CTGF from the four experiments were pooled, diluted and applied to a TSK[®] heparin column that was developed with a gradient of 40 ml of 0.2–2.0 mol NaCl l⁻¹ in 10 mmol Tris-HCl l⁻¹ (pH 7.4) at a flow rate of 1 ml min⁻¹. Aliquots (3 μ l) of selected fractions were tested by western blot analysis using anti-CTGF(247–260) peptide antiserum (inset). (b) Heparin-purified CTGFs were diluted and applied to an Econo-Pac CM cation exchange column. The column was developed at a flow rate of 1 ml min⁻¹ with a gradient of 40 ml of 0.2–2.0 mol NaCl l⁻¹ and fractions of 1 ml were collected. The inset shows CTGF-immunoreactive proteins in aliquots of 3 μ l of selected fractions as assessed by western blot analysis using anti-CTGF(247–260). (c) Pooled CM fractions were adjusted to have 10% acetonitrile–0.1% trifluoroacetic acid (TFA) and injected on to a semi-preparative C₈ column (25 cm $\times$ 1 cm) which was then eluted with a multilinear acetonitrile gradient in water–0.1% TFA at a flow rate of 1 ml min⁻¹. Fractions (1 ml) that were collected 35–60 min after sample injection were tested at 1 μ l per well for their ability to promote 3T3 cell adhesion. The inset shows the CTGF proteins in aliquots of 1 μ l of fractions 37–59 assessed by western blot analysis using anti-CTGF(247–260).

Fig. 2. Structural features of recombinant connective tissue growth factor (CTGF) isoforms. N-termini of recombinant 10 kDa (Gly²⁵³), 16 kDa (Ala¹⁹⁷), 18 kDa (Leu¹⁸⁴) and 20 kDa (Ala¹⁸¹) CTGFs purified from DB1 conditioned medium and their relationship to the N-termini of native 10 kDa (Glu²⁴⁷, Glu²⁴⁸), 16 kDa (Ala¹⁹⁷, Cys¹⁹⁹) or 18–20 kDa (Asp¹⁸⁶) CTGFs in pig uterine secretions (Brigstock *et al.*, 1997; Ball *et al.*, 1998) and 19 kDa (Ala¹⁸¹) CTGF in bovine endothelial cell conditioned medium (Boes *et al.*, 1999). The western blots show that although the lower molecular mass forms of CTGF are readily detectable in both uterine secretions (upper blot) and DB1 medium (lower blot), the 38 kDa CTGF protein is only appreciably present in the recombinant system; sequence analysis of this latter protein was unsuccessful and was probably due to a blocked N-terminus. The recombinant CTGF isoform denoted with its first residue in parentheses (Asn¹⁹⁸) was produced by thrombin digestion of the 38 kDa CTGF protein.

In addition, the various CTGF isoforms were treated for 2.5 h at 37°C with 0.2 or 2.0 U kallikrein (Sigma Chemical Co., St Louis MO) or overnight at 37°C with 20 U thrombin (Roche Molecular Biochemicals, Indianapolis, IN). Samples were assessed by western blot analysis. Specific experimental details are given in the figure legends.

Results

CTGF purification, isolation and biological activity

In preliminary experiments, it was found that export of recombinant CTGF by stably transfected wild-type CHO K1 cells into the medium was significantly enhanced in the presence of 100 µg heparin ml⁻¹ (data not shown), an observation that is consistent with the previously reported ability of CTGF and CYR61 to associate with cell surfaces via heparin sulphate proteoglycans (Yang and Lau, 1991; Kireeva *et al.*, 1997). Since heparin introduced undefined contaminants into the preparation, we next stably transfected CTGF cDNA into the CHO 745 mutant cell line that lacks xylosyl transferase and exhibits heparin sulphate and chondroitin sulphate deficiency (Esko *et al.*, 1985). This was expected to facilitate deposition of CTGF into the conditioned medium without the need for addition of heparin. Indeed, western blot analysis of the conditioned medium from 52 colonies of transfected CHO 745 cells revealed that 13 colonies were positive

for 38 kDa CTGF in the absence of added heparin and that the amount of CTGF released was substantially greater than that seen in transfected parental CHO cells (data not shown). In addition, the conditioned media from eight of the CTGF-positive colonies also contained lower molecular mass (10–20 kDa) forms of CTGF (data not shown). As the antibody used in this experiment was directed towards the C-terminus of CTGF (residues 247–260), these findings indicate that the recombinant 38 kDa CTGF was subject to limited proteolysis as observed *in vivo* (Brigstock *et al.*, 1997; Ball *et al.*, 1998) and that the proteins were probably C-terminal fragments of CTGF. One colony, termed DB1, which exhibited the highest CTGF production was selected for mass-scale culture to characterize and purify all CTGF isoforms.

Heparin affinity chromatography of DB1 conditioned medium demonstrated that all CTGF isoforms would bind heparin and required approximately 0.8 mol NaCl l⁻¹ for elution from heparin beads (Fig. 1a), as shown for native CTGF isoforms by Brigstock *et al.* (1997) and Ball *et al.* (1998). The relative abundance of the purified CTGF proteins was 16–20 kDa CTGF > 30 kDa CTGF > 10 kDa CTGF and did not change in subsequent purification steps. Since the various CTGF isoforms did not demonstrate differential heparin-binding properties and could not therefore be individually studied, they were co-eluted and subjected to cation exchange chromatography (Fig. 1b). In this step, salt gradient elution of the column allowed for the successive elution of 38 kDa CTGF, 16–20 kDa CTGF and 10 kDa CTGF demonstrating that the net negative charge of 10 kDa CTGF > 16–20 kDa CTGF > 38 kDa CTGF. C₈ reverse-phase HPLC resulted in the sequential elution of 10 kDa CTGF at 37–38.5 min, 16–20 kDa CTGF at 39–47 min, and 38 kDa CTGF at 50–59 min (Fig. 1c), allowing the isolation of each isoform and showing that the net hydrophobicity of 38 kDa CTGF > 16–20 kDa CTGF > 10 kDa CTGF. When non-tissue culture ELISA plates were precoated with fixed aliquots of each fraction, each CTGF isoform promoted the adhesion of Balb/c 3T3 cells to the plastic wells (Fig. 1c). These data are consistent with previous reports showing that 38 kDa CTGF can promote cell adhesion (Babic *et al.*, 1999; Jedsadayanmata *et al.*, 1999; Lau and Lam, 1999; C. C. Chen *et al.*, 2001; Schober *et al.*, 2002) and further showed that the lower molecular mass CTGF isoforms contain sufficient structural information to support this process. Yields of the HPLC-purified proteins per litre of conditioned medium were approximately 1 mg 16–20 kDa CTGF, 0.5 mg 38 kDa CTGF, and 0.1 mg 10 kDa CTGF. CTGF constituted 2.1% of the total protein in the conditioned medium, with > 90% recovery after heparin affinity and cation exchange chromatographies and approximately 62% recovery after reverse-phase HPLC (data not shown). No high molecular mass CTGF proteins (for example, > 38 kDa) were detected in the conditioned medium before or after purification.

Fig. 3. Connective tissue growth factor (CTGF) glycosylation, kinetics of release and stability. (a) Control non-transfected Chinese hamster ovary (CHO) 745 cells (lanes 1, 2) or DB1 cells (lanes 3–6) were seeded at 1.6×10^5 cells per well in a six-well plate for 24 h in 2 ml Dulbecco's modified Eagle's medium (DMEM)–10% fetal bovine serum (FBS) after which the medium was changed to 1 ml cysteine and methionine-free DMEM containing 100 μ Ci [35 S]cysteine and [35 S]methionine ml^{-1} . Wells received either no treatment (lanes 1, 3, 5, 6) or 5 μ g tunicamycin ml^{-1} (lanes 2, 4) for the next 24 h at 37°C, after which medium was collected (lanes 1–4) or the cells were lysed with radioimmunoprecipitation assay buffer (lanes 5, 6). Samples were incubated with 10 μ l preimmune serum (lane 5) or 10 μ l anti-38 kDa CTGF antiserum (rabbit 38B) (lanes 1–4, 6) and immunoprecipitates were analysed on 18% SDS-PAGE gels under reducing conditions, dried and exposed to X-ray film for 2 h. (b) DB1 cells were seeded at 8×10^4 cells per well in 1 ml DMEM–10% FBS in a 12-well plate for 3 days. The medium in each well was then replaced for 1 h with 1 ml cysteine and methionine-free DMEM, which was then replaced with 0.5 ml of the same medium containing 100 μ Ci [35 S]cysteine and [35 S]methionine ml^{-1} for 15 min. The labelling medium was then removed; the cells were washed twice with PBS, and 0.5 ml DMEM–10% FBS was then

Structural characterization of CTGF isoforms

No amino acid sequence was obtained for 38 kDa CTGF indicating that the protein had a blocked N-terminus. N-terminal analysis of the lower molecular mass CTGF isoforms revealed that they commenced either between modules 2 and 3 with N-termini at Ala¹⁸¹ (20 kDa CTGF), Leu¹⁸⁴ (18 kDa CTGF) or Ala¹⁹⁷ (16 kDa CTGF) or between modules 3 and 4 with an N-terminus at Gly²⁵³ (10 kDa CTGF). As shown (Fig. 2), the 10–20 kDa CTGFs were structurally identical or very similar to the various CTGF isoforms identified in pig uterine fluids (Brigstock *et al.*, 1997; Ball *et al.*, 1998) or cultured bovine endothelial cells (Boes *et al.*, 1999).

Kinetics of CTGF release and CTGF glycosylation

Antiserum raised against purified 38 kDa CTGF was able to immunoprecipitate the 16–20 kDa and 38 kDa isoforms of CTGF produced by DB1 cells whereas preimmune serum was ineffective (Fig. 3a). No immunoreactive CTGF proteins were apparent in non-transfected CHO 745 cells, showing production of endogenous CTGF was not detectable under normal circumstances (Fig. 3a). DB1-derived 38 kDa CTGF occurred as a protein triplet, the upper band of which was absent after incubation of the cells with tunicamycin, an inhibitor of N-linked glycosylation (Fig. 3a). However, tunicamycin had no effect on the electrophoretic mobility of 16–20 kDa CTGFs (Fig. 3a).

Pulse chase analysis of metabolically labelled CTGF showed very high concentration of 38 kDa CTGF in cell lysates for the first 60 min after 15 min of labelling, with progressively diminishing concentration up to 6 h (Fig. 3b). The 38 kDa CTGF was detectable in the medium as early as 5 min after synthesis although secreted amounts remained relatively low for the first 20 min, increased appreciably between 45 and 60 min, and reached a plateau between 90 min and 6 h. Collectively these data show that 38 kDa CTGF was secreted very efficiently from the cells and that > 90% of the protein had been exported from the cells within 3 h of synthesis. The 20 kDa CTGF was observed in both cell lysates and conditioned medium 45 min after the labelling period and its concentration was correlated with those of 38 kDa CTGF (Fig. 3b). At 37°C in cell-free culture medium, both

added to the wells for 0, 5, 10, 20, 45, 60, 90, 120, 180, 240, 300 or 360 min (lanes 1–12, respectively). Cell lysates (upper panel) and media (lower panel) were harvested from each well and subjected to immunoprecipitation using rabbit 38B antiserum. Samples were run under reducing conditions on 18% SDS-PAGE gels, which were then dried and exposed to X-ray film for 16 h. (c) Purified 38 kDa CTGF (lanes 1–3) or 16–20 kDa CTGF (lanes 4–6) were incubated in culture medium at 37°C for 1 day (lanes 1, 4), 2 days (lanes 2, 5) or 3 days (lanes 3, 6) and then subjected to SDS-PAGE and assessed by western blot analysis using anti-CTGF(247–260).

Fig. 4. Biological activities of recombinant connective tissue growth factor (CTGF) proteins. (a) Heparin-purified CTGFs (100 μ l) were injected on to a Biosep S-2000 column that was then eluted with PBS containing 0.5 mol NaCl l^{-1} at a flow rate 0.5 ml min^{-1} . Fractions (100 μ l) were collected and tested at 5 μ l per well for their ability to stimulate [^3H]thymidine incorporation (●) in 3T3 cells and at 9 μ l per lane for detection of CTGF proteins by SDS-PAGE and western blot analysis using anti-CTGF(247–260). (b) Dose-dependent stimulation of 3T3 cell adhesion

purified 38 kDa CTGF and 16–20 kDa CTGFs were stable for at least 72 h (Fig. 3c). These data support the notion that the cell-associated proteases were responsible for CTGF processing and that various CTGF isoforms in conditioned medium at 3 days (Fig. 1) were not generated by an intrinsic instability of 38 kDa CTGF over the culture period of 3 days.

Biological characterization of CTGF

Previously, 38 kDa CTGF has been shown to regulate various cell functions including stimulation of cell adhesion (Yang and Lau, 1991; Kireeva *et al.*, 1997), α SMA production (Frazier *et al.*, 1996) and mitosis (Bradham *et al.*, 1991; Frazier *et al.*, 1996), the last of which is also stimulated by C-terminal 10 kDa fragments of CTGF (Brigstock *et al.*, 1997). Each isoform was assessed in several biological assays to verify that these properties were preserved in DB1-derived 38 kDa CTGF and to investigate the bioactivity of 10–20 kDa CTGFs.

When subjected to size-exclusion chromatography, three peaks of Balb/c 3T3 cell mitogenic activity were eluted from the column that were correlated with the successive elution of 38 kDa, 16–20 kDa, and 10 kDa CTGF as determined by western blot analysis (Fig. 4a). As similar data were obtained with respect to the ability of the individual isoforms to stimulate Balb/c 3T3 cell adhesion after purification by reverse-phase HPLC (Fig. 1c), specific activity curves were generated to assess the relative ability of each isoform to promote cell adhesion. All isoforms exhibited comparable activity, with dose-dependent stimulation of cell adhesion at CTGF coating doses of 10–100 ng per well (Fig. 4b). Higher maximal cell adhesion was achieved by 100–1000 ng 10 kDa CTGF per well compared with the same doses of the other CTGF isoforms.

α SMA production in response to each CTGF isoform was assessed *in vitro* using immunocytochemical staining of primary cultures of rat corneal epithelial cells. After 24 h exposure to 100–400 ng each isoform ml^{-1} , there was profound induction of α SMA in the cells, showing that the various CTGF proteins were similarly effective at inducing transdifferentiation of epithelial cells into myofibroblastic-like cells (Fig. 4c). As subcutaneous injection of 38 kDa CTGF stimulates persistent fibrosis as assessed by increased frequency of α SMA-positive myofibroblastic cells (Mori *et al.*, 1999), each purified CTGF isoform was tested for its

effect on α SMA production *in vivo* using the same approach. Neonatal mice received daily subcutaneous administration of 340 ng CTGF consecutively for 7 days and were killed 14 days after the last injection. Compared with control animals, which demonstrated approximately 25% α SMA-positive myofibroblasts, animals receiving 10 kDa, 16–20 kDa or 38 kDa CTGF demonstrated, respectively, 76%, 68% or 45% myofibroblasts (data not shown). These data showed that the recombinant CTGF was active *in vivo* in a well-established model of persistent fibrosis and that the C-terminus of CTGF contained sufficient information to elicit this effect.

CTGF processing

It was shown previously that pig uterine secretions contain CTGF proteases that rapidly degrade 38 kDa CTGF into lower molecular mass forms (Ball *et al.*, 1998). DB1-derived 38 kDa CTGF was incubated with uterine fluids for up to 6 h to verify that it was similarly susceptible. Partial degradation of 38 kDa to 20 kDa CTGF was observed in the presence of uterine fluids after exposure for 30 min, and complete disappearance of the 38 kDa protein was observed after 6 h of treatment (Fig. 5a). In contrast, no degradation was observed over this period when 38 kDa CTGF was incubated in PBS (Fig. 5a). The 20 kDa C-terminal product of this reaction was completely stable (Fig. 5a), as reported by Ball *et al.* (1998). ULF-mediated degradation of CTGF was inhibited by anti-thrombin III (Fig. 5b) whereas thrombin was effective in proteolysing both 38 kDa CTGF to 16–28 kDa CTGF and purified 18–20 kDa CTGFs to 16 kDa CTGF (Fig. 5c). When the incubation of 38 kDa CTGF with thrombin was repeated on a semi-preparative scale, the resulting 16 kDa product was found to have an N-terminus at Asp¹⁹⁸ corresponding to one residue fewer than the 16 kDa isoform present in DB1-conditioned medium or pig uterine fluids (see Fig. 2). In addition, the effect of kallikrein on CTGF degradation was tested in view of the current interest in this enzyme in pig reproductive tract function (Vonnahme *et al.*, 1999; Geisert *et al.*, 2001). At 0.02 U, kallikrein degraded 18 kDa CTGF and 38 kDa CTGF with the concomitant production of a 12 kDa isoform, whereas at 2 U kallikrein all of the CTGF isoforms were degraded yielding hitherto unreported immunoreactive proteins of 9, 11 and 15 kDa (Fig. 5c).

by different CTGF isoforms; ●: 10 kDa CTGF; ○: 16 kDa CTGF; ■: 16–20 kDa CTGF; □: 38 kDa CTGF. Values are mean $\pm$ SD of triplicate determinations. (c) Corneal epithelial cells were isolated from adult mice and cultured for 7 days in Dulbecco's modified Eagle's medium (DMEM)–10% fetal bovine serum (FBS). The cells were then grown for 2 days in serum-free DMEM containing insulin, transferrin and sodium selenite. This medium was then replenished for 24 h with either no further addition or with addition of 400 ng each CTGF isoform ml^{-1} . Alpha smooth muscle actin (α SMA) was detected by immunohistochemistry and nuclei were counterstained with haematoxylin. α SMA fibres are clearly apparent in CTGF-treated cells. Comparable data were obtained with 100 ng CTGFs ml^{-1} (data not shown).

Fig. 5. Enzymatic degradation of uterine luminal fluid (ULF). (a) The 38 kDa connective tissue growth factor (CTGF) was incubated in 20 μ l PBS (lanes 1, 2) or 19 μ l PBS plus 1 μ l ULF (lanes 3, 4) for 30 min (lanes 1, 3) or 6 h (lanes 2, 4) at 37°C. Samples were assessed by western blot analysis using anti-CTGF(247–260) antiserum. (b) The 38 kDa CTGF was incubated overnight at 37°C in a final reaction volume of 20 μ l in PBS (lanes 1, 2) or PBS with pig uterine fluid (lanes 3, 4), after 1 h pre-incubation alone (lanes 1, 3) or with 1U anti-thrombin III (lanes 2, 4). Samples were assessed by western blot analysis using anti-CTGF(247–260) antiserum. (c) Purified 38 kDa CTGF (lanes 1, 2), 16–20 kDa CTGF (lanes 3, 4), or all CTGF isoforms (lanes 5–7) were incubated overnight at 37°C alone (lanes 1, 3) or in the presence of 20 U thrombin (lanes 2, 4), or for 2.5 h at 37°C alone (lane 5) or in the presence 0.2 U kallikrein (lane 6) or 2.0 U kallikrein (lane 7). The products of the reaction were assessed by SDS-PAGE and western blot analysis using anti-CTGF(247–260). The arrow shows a CTGF isoform that was obtained from a comparable preparative-scale digestion and found to commence at Asn¹⁹⁸ (see Fig. 2). The proteins marked with a 'T' are thrombin that was detected non-specifically in the reaction mixture.

Discussion

Although CTGF was discovered more than a decade ago, detailed structural and functional studies of this protein have not been performed. Although the modular structure of CTGF has been indicative of potentially important domains within the molecule, the biological role of these domains has not been determined. CTGF interacts with IGF-I (Kim *et al.*, 1997) and promotes cell adhesion (Babic *et al.*, 1999; Jedsadayanmata *et al.*, 1999; Lau and Lam, 1999; C. C. Chen *et al.*, 2001; Schober *et al.*, 2002) and these properties are consistent with the proposed presence of IGF binding and cell attachment motifs in modules 1 and 3 respectively (Bork, 1993). The serendipitous discovery of heparin-binding biologically active C-terminal fragments of CTGF in uterine fluids provided a new lead for CTGF structure–function studies in that putative domains that are heparin-

binding and receptor-binding were mapped to the C-terminal 102 residues (Brigstock *et al.*, 1997). Unfortunately, the difficulty of isolating these proteins from their native sources has severely hampered their further study. In addition, there are virtually no convenient sources of the native 38 kDa CTGF protein that are amenable for large-scale purification. For example, although CTGF is produced by many cell types in culture (Moussad and Brigstock, 2000), scale-up of these *in vitro* systems has not been reported and would be unlikely readily to generate acceptable yields without considerable time and expense.

CTGF contains 38 cysteine residues (10% by mass), which are presumed to form intrachain disulphide bridges necessary for its modular structure (Bork, 1993). It is critical that this very complex protein structure be faithfully reproduced in recombinant expression systems. Although post-translational modifications of CTGF have

not been widely studied, human CTGF appears to be N-linked glycosylated (Yang, *et al.*, 1998) although this modification does not appear to occur in CTGF of other species. In addition, 38 kDa CTGF is highly susceptible to limited proteolysis, yielding 10–20 kDa CTGF isoforms comprising modules 3 and 4 or module 4 alone (Brigstock *et al.*, 1997; Ball *et al.*, 1998; Steffen *et al.*, 1998; Yang *et al.*, 1998; Williams *et al.*, 2000). It is unclear whether these proteins can be faithfully generated by direct cloning and expression of the corresponding cDNA or whether their cleavage from the 38 kDa CTGF protein is a prerequisite for their structural and functional integrity.

In the present study, we have developed a recombinant CTGF expression system in a mutant CHO cell line. After transfection of a full-length human CTGF cDNA into the cells, 38 kDa CTGF was produced that underwent limited proteolysis yielding 10–20 kDa C-terminal isoforms that were virtually indistinguishable from those found *in utero* and which exhibit a comparable biological dose–response and a repertoire of biological actions as full-length CTGF. As the mass of 38 kDa CTGF was decreased in the presence of tunicamycin, it was concluded that the recombinant protein was glycosylated as is predicted from the presence of potential N-glycosylation sites at Asp²⁸ and Asp²²⁵. However, the decrease in mass of 38 kDa CTGF was modest indicating that the N-glycan groups in 38 kDa CTGF are relatively small. As tunicamycin had no effect on 16–20 kDa CTGFs that contained Asp²²⁵ but not Asp²⁸, these data collectively indicate that Asp²⁸ is preferentially utilized for glycosylation or that Asp²²⁵ was subsequently deglycosylated. As some 38 kDa CTGF moieties were not affected by tunicamycin, it may be concluded that they were either slightly truncated at the N-terminus and lacked Asp²⁸ or represented authentic non-glycosylated forms of the protein.

Previously, an attempt to express 38 kDa CTGF in *E. coli* was apparently successful in producing an immunoreactive protein but no biological activity was associated with the recombinant protein (Bradham *et al.*, 1991). A more successful strategy has been to produce recombinant baculoviruses allowing for the expression in infected insect cells of human CTGF (Frazier *et al.*, 1996; Y. Chen *et al.*, 2001), mouse CTGF (Kireeva *et al.*, 1997) or FLAG-tagged CTGF (Yang *et al.*, 1998; Fan and Karnovsky, 2000). The 38 kDa CTGF produced in this manner has been purified from serum-free conditioned medium using single steps of Sepharose S chromatography (Kireeva *et al.*, 1997), heparin-affinity chromatography (Frazier *et al.*, 1996) or anti-FLAG antibody (Yang *et al.*, 1998), or by sequential steps of HS20 sulphopropyl and CM-20 cation exchange chromatography (Y. Chen *et al.*, 2001) or Q Sepharose chromatography followed by anti-FLAG affinity chromatography (Fan and Karnovsky, 2000). In some instances, the purity, structure, secondary modifications, and

specific activity of the end-product have often not been rigorously analysed and, importantly, proteolytic processing of CTGF by insect cells has yet to be reported. An alternative approach has been to use a CMV-driven mammalian expression plasmid to stably overexpress CTGF cDNA in HeLa cells. CTGF produced in this manner was isolated from serum-free conditioned medium by ammonium sulphate precipitation, heparin affinity chromatography, and anti-CTGF affinity chromatography (Nishida *et al.*, 1998). Other mammalian cell CTGF expression systems have involved production of FLAG-tagged CTGF in 293T cells (Inoki *et al.*, 2002) or a CTGF-V5 fusion protein in mesangial cells (Wahab *et al.*, 2001) from which CTGF was purified using anti-FLAG or anti-V5 affinity chromatography, respectively. In all cases, the 38 kDa CTGF molecule appears to be the sole form of the protein produced.

The results of the present study show that the DB1 expression system is a valuable tool for preparative-scale production and isolation of all CTGF isoforms identified to date. Purification of recombinant CTGFs was facilitated by the lack of expression of heparan sulphate proteoglycans by DB1 cells, which allowed for highly efficient export of CTGF into defined serum-free medium. Once conditioned medium was collected from DB1 cells, the relative proportion of the CTGF isoforms remained unchanged through the chromatographic steps showing that they were intrinsically very stable. This stability was also apparent when purified 18–20 kDa or 38 kDa CTGFs were incubated at 37°C for 3 days. However, 18–20 kDa or 38 kDa CTGFs were rapidly processed to lower molecular mass isoforms by DB1 cells or after exposure to uterine fluids, kallikrein or thrombin, the last of which converted 38 kDa CTGF to a 16 kDa isoform that contained a single residue fewer than 16 kDa CTGF produced by the pig uterus (Ball *et al.*, 1998) or DB1 cells.

It was shown previously that CTGF protease concentrations in pig ULF were highly correlated with the concentrations of 10–20 kDa CTGF and were detectable at relatively earlier stages of pregnancy than during the oestrous cycle (day 11 versus day 14, respectively) (Ball *et al.*, 1998). The present study shows that the CTGF-degrading activity in ULF is anti-thrombin III-sensitive, which implicates involvement of one or more serine proteases of the blood coagulation system such as thrombin, plasmin, kallikrein or protease factors IXa, Xa, Xia and XIIa. When thrombin and kallikrein were tested directly in this regard, each enzyme was able to catalyse CTGF proteolysis, indicating that they are possible candidates *in utero* for this role. Aside from their role in blood coagulation *in utero*, thrombin regulates endometrial stromal cell proliferation and myometrial contraction (Arena *et al.*, 1996; Asselin and Fortier, 1996; Elovitz *et al.*, 2000), whereas kallikrein regulates embryo implantation and maintenance of utero–placental blood flow (Valdes *et al.*, 1998, 2001;

Vonnahme *et al.*, 1999). More recently, kallikrein has been implicated in proteolysis of IGF binding proteins during the oestrous cycle and early pregnancy in pigs (Geisert *et al.*, 2001), an observation that may be relevant to our current studies since CTGF binds IGF-I and IGF-II, albeit weakly (Kim *et al.*, 1997). Although the potential importance of kallikrein and thrombin in CTGF processing has not been documented previously, it is likely that these or similar serine proteases account for the production of low molecular mass CTGFs *in utero* and in the DB1 cell culture system. Thrombin is also a direct stimulator of CTGF expression in several systems (Chambers *et al.*, 2000; Pendurthi *et al.*, 2000; Howell *et al.*, 2001) indicating that it exerts control over CTGF production during transcription and after translation. As the CTGF isoforms produced by DB1 cells are readily detectable by purification or immunoprecipitation, this culture system will greatly facilitate studies designed to identify the specific enzymes responsible for CTGF processing.

Although the degree of CTGF processing is likely to affect the repertoire of interactions of CTGF with other binding moieties, our data indicate that important functional domains reside in module 4 since isoforms containing only this module appear to exhibit at least some of the biological activities as full-length CTGF. As such, CTGF isoforms that comprise essentially module 4 will be extremely useful ligands with which to study CTGF binding interactions with target cells since other binding domains are absent. However, it should be emphasized that this does not rule out the possibility that modules 1, 2 or 3, either individually or collectively, also contain functional domains that contribute to the overall activity of a given CTGF isoform. A case in point is that while 10 kDa CTGF is effective at promoting cell adhesion, it is also possible that the putative cell attachment motif in module 3 also contributes to the cell adhesion properties exhibited by 16–20 kDa or 38 kDa CTGFs. Nonetheless it appears that module 4 contains one or more functional domains that account for its ability to bind to heparin and to stimulate diverse cell functions including adhesion, mitosis and α SMA production. These data support the conclusions of previous studies that indicate that native 10 kDa CTGF is mitogenic and heparin-binding and that synthetic peptides corresponding to residues 247–260, 274–286 and 305–328 are strongly heparin-binding (Brigstock, *et al.*, 1997). These data indicate that module 4 is fundamentally important for CTGF function and are supported by the finding that the related protein, CYR61, lost its ability to bind to heparin and did not promote cell adhesion after mutagenesis of two putative heparin-binding domains in module 4 (Chen *et al.*, 2000). On the other hand, Cop1, a CTGF-related protein that comprises modules 1–3 but lacks module 4 entirely, exhibits a broad range of activities including stimulation of integrin-mediated osteoblast cell adhesion (Kumar *et al.*,

1999), inhibition of osteocalcin production by osteoblasts (Kumar *et al.*, 1999), and growth suppression of vascular smooth muscle cells or transformed fibroblasts (Zhang *et al.*, 1998; Delmolino *et al.*, 2001). Thus the net activity of a given CTGF family member probably reflects the unique structural properties of each isoform and, possibly, functional overlap or redundancy between discrete regions of the protein.

In conclusion, the development of the DB1 cell line represents an important step in providing preparative quantities of the various CTGF isoforms that were identified *in utero* several years ago but which previously have not been produced recombinantly. In addition, this cell line will serve as a useful model for studying limited proteolysis of the CTGF protein. Whereas the individual modules of CTGF appear very stable, the regions between either modules 2 and 3 or modules 3 and 4 are highly susceptible to proteolytic cleavage, a phenomenon that is probably attributable, at least in uterine fluids, to serine proteases such as thrombin or kallikrein. Moreover, the resulting C-terminal fragments exhibit comparable activities to the intact CTGF protein. Future studies will be focused on mapping the domains in the C-terminus of CTGF that are critical for its functionality, establishing the potential co-operativity of these domains with other regions of the CTGF molecule, and understanding the relationship between CTGF proteolysis, bioavailability, and mode of action.

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