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The Connective Tissue Growth Factor/Cysteine-Rich 61/Nephroblastoma Overexpressed (CCN) Family*

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I. Introduction

THE LAST 5–6 yr have seen the emergence of a new gene family that currently comprises connective tissue growth factor (CTGF; also termed fisp-12), cysteine-rich 61 (cyr61), nephroblastoma overexpressed (nov), expressed low in metastasis 1 (elm1; also termed WISP-1), heparin-inducible CTGF/cyr61/nov (CCN)-like protein (HICP; also termed rCop-1, CTGF-3 or WISP-2), and WISP-3 (Fig. 1). Family members have been characterized from human, mouse, rat, pig, cow, chicken, quail, and frog and are predicted to have arisen from a common ancestral gene more than 40 million years ago (Fig. 2). Although these proteins were initially classified as immediate early gene products or growth factors, this concept has had to be modified in light of more detailed studies of their activities as well as the discovery of unique family members that exhibit quite different biological properties. Since CTGF, cyr61, and nov were the prototype members of this family, this article adopts the term "CCN family" as introduced by Bork in 1993 (1). Implicit in this usage is that reference to the CCN family applies to all paralogs, and that CTGF, cyr61, and nov are not necessarily representative of the full range of gene or protein structures and biological properties.

The primary translational products of most CCN family members contain 343–381 residues and generate secreted proteins of 35–40 kDa that contain 38 conserved cysteine residues that are organized into four distinct structural modules. Exceptions are rCop-1 and its orthologs, which lack the fourth structural module and contain only 28 conserved cysteine residues, and WISP-3, which lacks 4 of the cysteine residues that are usually present in module 2. The biological properties of CTGF and cyr61 include stimulation of cell proliferation, chemotaxis, adhesion, and extracellular matrix (ECM) formation. CTGF and cyr61, but not nov, elm1, or HICP/rCop-1, are encoded by growth factor-inducible immediate early genes whereas nov, elm1, and HICP/rCop-1 are expressed in cells demonstrating growth arrest or quiescence. The CCN family appears to be involved in normal processes such as implantation, placentation, embryogenesis, differentiation, and development as well as processes related to tissue pathology, including wound healing and fibrotic disorders.

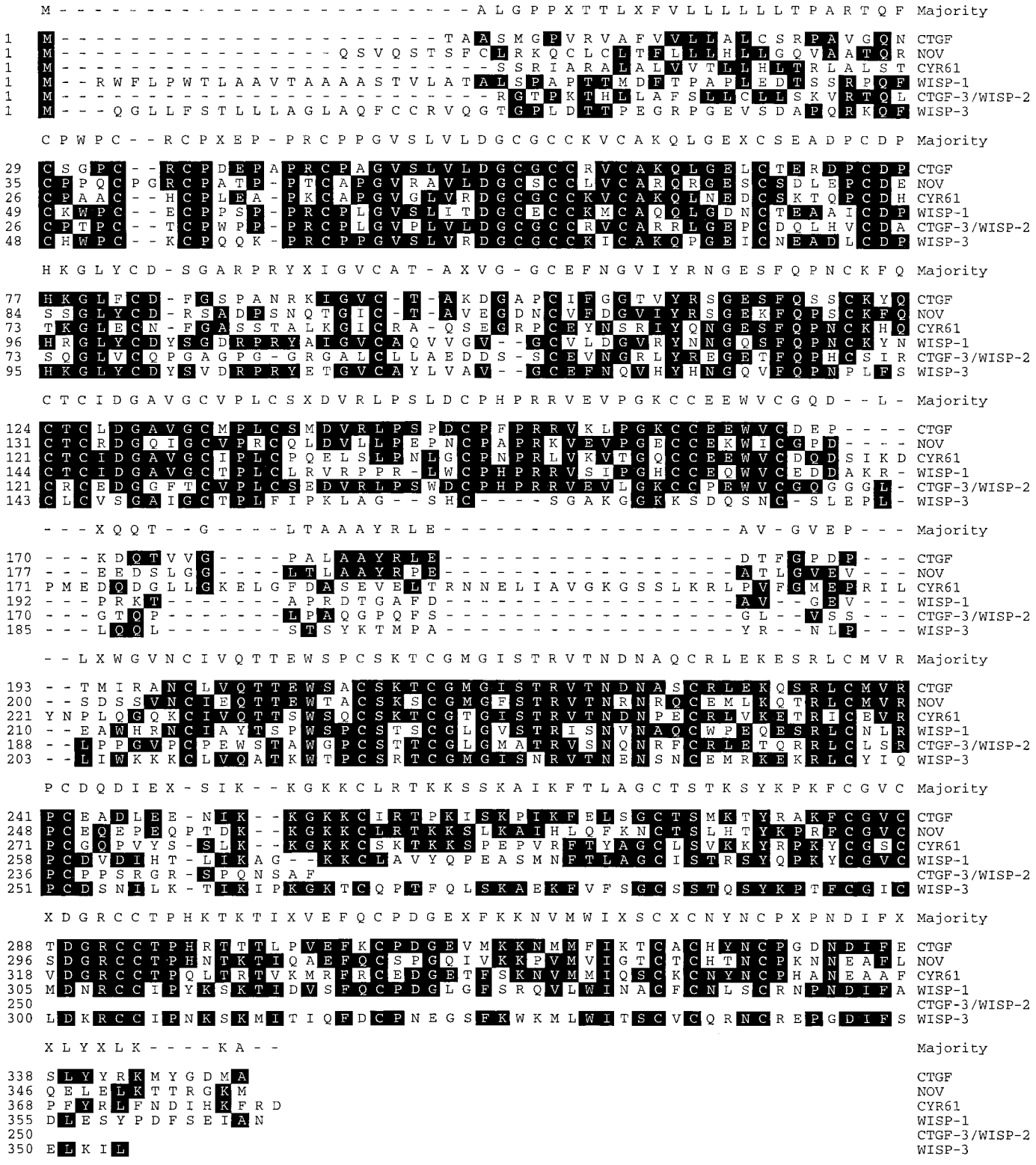

FIG. 1. Amino acid sequence alignment of human CCN paralogs. Sequences were aligned using the J. Hein method. *Shading* represents residues that are identical to the consensus sequence.

II. The Modular Structure of Connective Tissue Growth Factor/Cysteine Rich 61/Nephroblastoma Overexpressed (CCN) Family Members

Through evolution, exons with specific biological functions ("modules") have been shuffled, forming genes that encode mosaic proteins which exhibit new biological properties (2–4). CTGF, cyr61, and nov were shown to contain four distinct structural modules that exhibit homology to conserved regions in a variety of extracellular mosaic proteins (1). All four modules are represented in WISP-1 and WISP-3, and three of the modules are present in rCop-1 and its orthologs (Fig. 3). Each module is involved in protein binding and contains conserved cysteine, hydrophobic, and polar residues. Except for module 2 in WISP-3, the modules in the various CCN proteins are 38–98% conserved with the corresponding module in human CTGF (hCTGF) (Table 1). While complex intrachain disulfide bridging is likely, this is predicted to occur within, rather than between, the modules (1).

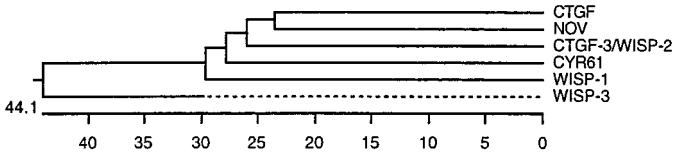

FIG. 2. Dendrogram of human CCN paralogs. The x-axis is millions of years.

The proposed modular configuration of CCN family members has become a provoking model for those engaged in determining their biological functions. Introns occur between the modules in the CTGF, cyr61, and nov genes (5–8), a feature that is typical in genes of many other modular proteins (9). The presence of the modules is further supported by the susceptibility of CTGF to proteolysis at sites between, rather than within, the modules (10, 11) and by

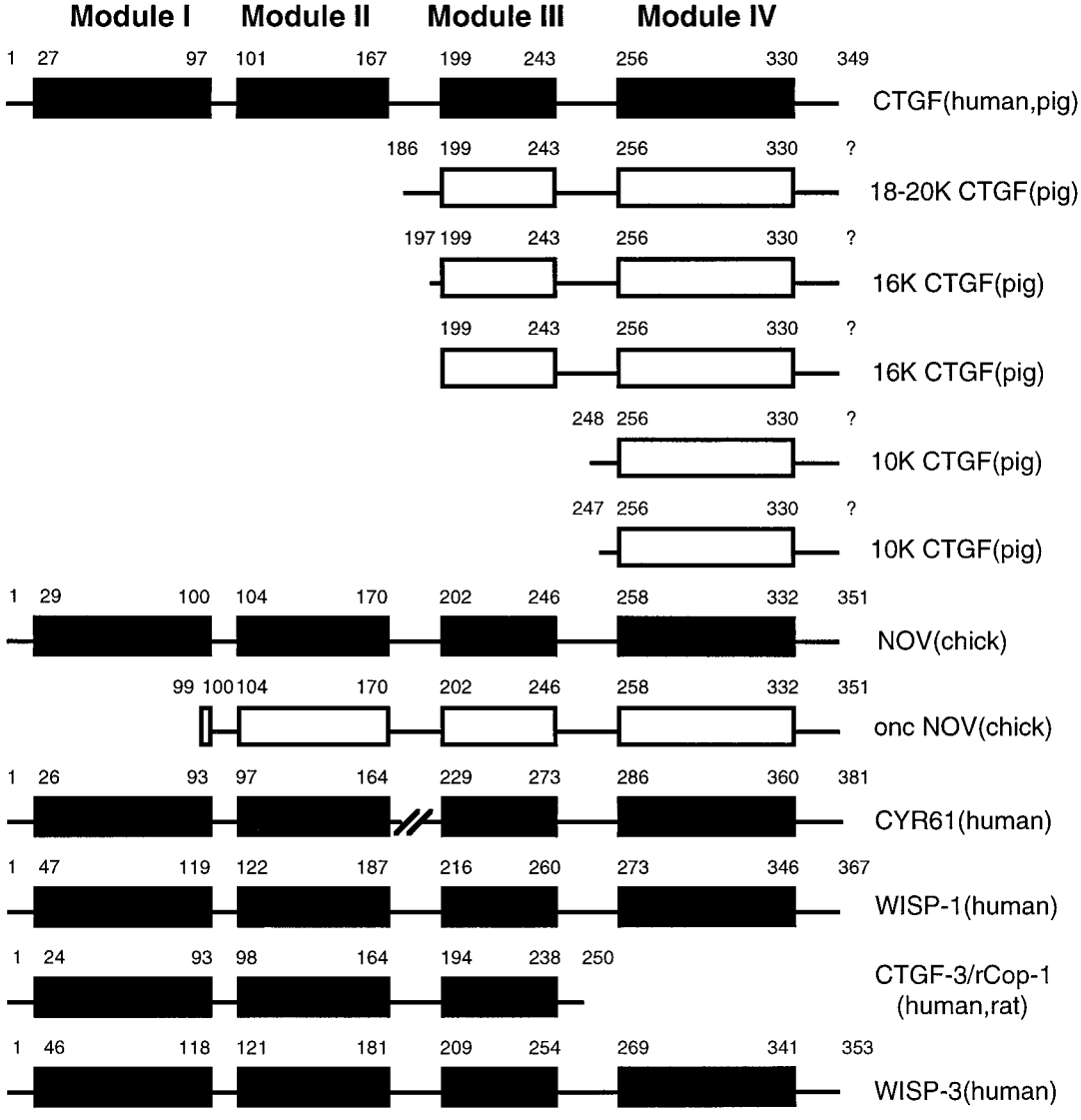

FIG. 3. Modular structure of individual CCN family members. Proteins shown with *black boxes* are the predicted primary translational products. Proteins shown with *open boxes* are biologically active derivatives arising by proteolysis (CTGF) or viral DNA integration (Nov). *Question marks* indicate that the C termini of N-terminally truncated CTGF proteins have not been experimentally determined.

TABLE 1. Amino acid sequence homologies (%) of individual modules and complete sequences of CCN proteins as compared to those of hCTGF

CCN protein	Module I	Module II	Module III	Module IV	Entire sequence
hCTGF	100.0	100.0	100.0	100.0	100.0
mCTGF/FISP12	88.7	94.0	95.5	94.6	91.1
pCTGF	84.5	97.0	97.7	96.0	92.3
bCTGF	87.3	89.5	93.3	96.0	90.2
xCTGF	73.2	88.0	95.5	93.3	80.2
hCYR61	52.1	61.2	73.3	52.0	43.6
mCYR61	50.7	61.2	71.1	52.0	45.1
cCYR61/CEF10	50.7	61.2	71.1	50.6	44.3
hNOV	47.8	61.2	73.3	54.6	49.0
mNOV	46.5	56.7	73.3	56.0	48.6
cNOV	50.6	61.2	73.3	57.3	51.9
qNOV	50.6	59.7	71.1	58.6	51.0
xNOV	47.9	67.1	75.5	57.3	50.7
hWISP-1	54.1	52.9	53.3	37.8	39.0
mELM1	56.3	50.7	57.7	40.0	39.8
hCTGF-3/hWISP-2	53.5	56.7	57.8		29.8
rHICP/rCop-1	52.1	55.2	55.5		30.4
mWISP-2	53.4	54.4	55.6		25.8
hWISP-3	56.0	26.5	57.8	37.8	35.8

functional properties such as the binding of CTGF to insulin-like growth factors (IGF) or putative cell surface receptors (10–14), and the promotion of cell adhesion by CTGF or cyr61 (15, 16). However, it is unclear whether the biological properties of CCN proteins reflect the individual properties of each module or the overall combination of the modules and other sequences within each protein.

Module 1 is approximately 32% identical with the N-terminal cysteine-rich regions of the six "classic" IGF-binding proteins, IGFBP-1 to -6 (1), and contains a motif (GCGCCXXC) that is involved in binding IGF (17). ^{125}I-labeled IGF-I or -II specifically binds recombinant hCTGF (rhCTGF) (12), although with much lower affinity than classic IGFBPs and more comparable to mac25, a low-affinity IGFBP, also termed IGFBP-7 or IGFBP-related protein (IGFBP-rP)-1 (18). Nov was also reported to bind IGFs (19), but this result has not proven reproducible (20). Recently, the terms IGFBP-8, -9, and -10 were introduced as synonyms for CTGF, nov, and cyr61, respectively (12), although it has since been proposed to use the names IGFBP-rP-2, -3, and -4 until their relationship to the classic IGFBPs has been determined (21). Indeed, a case against classifying the CCN family as IGFBPs has recently been made (22). Whatever the terminology, it remains unclear whether the interaction of IGFs with CCN family members occurs physiologically, whether sequences within module 1 are actually responsible for the observed binding of IGF, and what consequences this interaction has on the respective half-life, bioavailability, or activity of each of the molecules involved. However, there is general agreement that direct, IGF-independent effects of CTGF on the cell cycle are likely to be more significant than its low affinity binding of IGF (12, 22).

Module 2 comprises a Von Willebrand type C domain (VWC) that occurs in Von Willebrand factor as well as various mucins, thrombospondins, and collagens (1). Many proteins that contain VWC modules participate in oligomerization, which may be preceded by a dimerization event (23). Since module 4 of CTGF is a putative dimerization domain, module 2 may mediate the formation of complexes from CTGF dimers. Unlike all other CCN proteins, module 2 in WISP-3 contains only 6 of the 10 cysteines, the functional significance of which has yet to be established (24). Module 3 is a thrombospondin type 1 (TSP1) that contains the local motif WSXCSXXCG (1) and appears to be a cell attachment motif that binds sulfated glycoconjugates (1, 25–27). While cyr61 and CTGF promote cell adhesion (15, 16), the role of module 3 in this process remains unexplored.

Module 4 is a C-terminal (CT) module that also occurs in the C termini of a variety of unrelated extracellular mosaic proteins (1). Six of the 10 cysteine residues in the CT module appear to adopt the cystine knot motif that also occurs in nerve growth factor (NGF), transforming growth factor-β (TGF-β), and platelet-derived growth factor (PDGF) (1). This complex structure comprises two 2-stranded β-sheets that lie face-to-face and are linked by three interlocking disulfide bridges and has defined a new superfamily of growth regulators (28, 29) to which members of the CCN family (except orthologs of rCop-1, which lack module 4; see below) may also belong. Since some of the receptor binding properties of NGF, TGF-β, and PDGF reside in variable regions within the cystine knot (28), the CT module likely contains both dimerization and receptor-binding domains (1). Although dimerization of CCN proteins has not been reported, bioactive forms of 10-kDa porcine CTGF (pCTGF) comprise the C-terminal 102 or 103 residues of the primary translational product (Fig. 3) (10) and thus support the proposed role of module 4 in binding cell surface receptors (1). Module 4 is absent from rCop and its orthologs (Figs. 1 and 3), suggesting that they are functionally distinct from other CCN proteins.

III. Connective Tissue Growth Factor (CTGF)

A. Discovery

The first description of a CTGF ortholog occurred in 1988 when cDNA encoding "fibroblast-inducible secreted protein-12" (*fisp*-12) was isolated by differential screening of a cDNA library from serum-stimulated NIH 3T3 cells (30). The gene structure and predicted protein sequence of *fisp*-12 were subsequently reported in 1991 (5). Using a similar screening strategy, Brunner *et al.* (31) independently isolated the same cDNA, termed βIG-M2, from TGF-β2-stimulated mouse AKR-2B cells. *Fisp*-12/βIG-M2 comprises 348 amino acids and contains 39 Cys residues, one of which occurs in the 25-residue N-terminal signal peptide (Fig. 1) (5, 31). The human ortholog of CTGF was discovered in 1991 due to the cross-reactivity of a PDGF antiserum with 38-kDa hCTGF secreted by cultured human vein endothelial cells (HUVECs) (13). The corresponding cDNA was isolated by screening a HUVEC cDNA expression library with anti-PDGF and shown to encode a 349-amino acid protein that is 91% homologous to *fisp-12* (13) (Fig. 1). The protein was termed "CTGF" because it was both mitogenic and chemotactic for fibroblast-like cells *in vitro*. CTGF cDNAs have since been reported for pig, rat, cow, and frog (10, 32–35).

B. Structure of the CTGF gene and protein

The *fisp*-12 gene comprises five exons and four introns and spans 3.1 kb (Ref. 5; Table 2). The organization and structure of the hCTGF gene are very similar to that of *fisp*-12 except that exon 1 encodes one additional amino acid in the signal peptide (13, 36, 37). There is 80% sequence identity between CTGF and *fisp*-12 in the 300 nucleotides that lie immediately upstream of the mRNA cap site (36), and the 5′-regions of both genes contain a variety of conventional regulatory elements as well as a unique TGF-β response element (Fig. 4). *Fisp*-12 maps to the A3-B1 region of murine chromosome 10 (5) and hCTGF maps to human chromosome 6q23.1 (38).

The CTGF primary translational product comprises 349 (human, pig, cow), 348 (mouse), or 343 (*Xenopus*) residues (5, 10, 31) and is more than 90% conserved in mammals (Fig. 1 and Table 1). Most of the nonhomology occurs over the first 43 residues where the identity is only 60–65%. The secreted proteins from all five species are predicted to comprise 323 residues and to contain 38 fully conserved cysteine residues, which are evenly spread throughout the molecule except for a cysteine free-region between Asp^{167} and Asn^{198} in hCTGF. As assessed by SDS-PAGE, the molecular mass of CTGF is 36–38 kDa (5, 13, 14, 32, 39, 40). Heterogeneity in the mass of native and recombinant forms of hCTGF is due to variations in its degree of glycosylation (14, 40). hCTGF contains predicted sites for N-linked glycosylation at Asp^{28} and Asp^{225} (13) and is susceptible to Endoglycosidase F, which decreases its mass by 2–8 kDa (12, 40). Neither *fisp*-12 nor pCTGF appears to be glycosylated (5, 31, 32), suggesting that the glycan groups in hCTGF are either not functionally relevant or confer additional properties on the human ortholog.

C. CTGF mRNA production

Among the cell types that produce CTGF mRNA are fibroblasts (5, 14, 31, 35, 39, 41, 42), endothelial cells (13, 35, 43), vascular smooth muscle cells (VSMC) (35, 44), epithelial cells (45–47), chondroctyes (48), and glioblastoma cells (37). *Fisp-12*/CTGF transcripts have been detected in multiple tissues of at least four mammalian species (5, 12, 32, 35, 47, 49). Most studies have reported a single CTGF transcript of 2.4 kb, although glioblastoma cells also contain 3.5-kb and 7.0-kb transcripts (37). The 2.4-kb transcript is present in unstimulated fibroblasts (35, 39) but is rapidly induced after treatment with TGF-β or serum and is superinduced in stimulated fibroblasts in the absence of *de novo* protein synthesis (5, 31, 41). These studies collectively established that CTGF/fisp-12 are encoded by immediate early genes, although their kinetics of induction are more rapid and sustained than those of other immediate early genes (41). TGF-β also increases CTGF mRNA levels in cultured human or mouse lung mesenchymal cells (34) and in human chondrocytic cells (48), the latter of which also produce CTGF mRNA in response to bone morphogenic protein-2, a member of the TGF-β family (50). Using an enzyme-linked immunosorbent assay (ELISA), TGF-β treatment of human fibroblasts was shown to result in increased production of the CTGF protein (51). The molecular basis for the action of TGF-β on CTGF gene transcription has been atributed to nucleotides −157 to −145 of the hCTGF promoter (36). This region is a unique TGF-β-inducible element (Fig. 4) that is fully conserved in the promoter of the *fisp*-12 gene (5) but is absent from the other parologs of the gene family (Fig. 4) as well as from other immediate early genes. In addition, inhibitors of protein kinase A, but not of tyrosine kinases or protein kinase C, block TGF-β-stimulated CTGF transcription (52). Collectively, the TGF-β response element and the involvement of cAMP in CTGF mRNA production represent major mechanistic differences in CTGF gene transcription as compared with other CCN family members.

Although considerable attention has been directed toward the TGF-β inducibility of CTGF expression and the potential pathophysiological consequences thereof (53), PDGF, epidermal growth factor (EGF), and fibroblast growth factor (FGF) also stimulated hCTGF gene expression in fibroblasts (41). As compared with the effects of TGF-β on this cell type,

TABLE 2. Organization of the genes for CTGF, cyr61, and nov

	Exon 1		Intron 1 (bp)	Exon 2		Intron 2 (bp)	Exon 3		Intron 3 (bp)	Exon 4		Intron 4 (bp)	Exon 5	
	bp	aa		bp	aa		bp	aa		bp	aa		bp	aa
mCTGF/fisp12	219	21	87	223	75	250	252	84	132	212	70	371	1364	99
mCYR61	252	21	294	214	72	344	345	115	121	215	71	230	826	100
cNOV	92	23	60	229	77	150	249	83	1500	208	69	1300	1152	98
xNOV	99	15	1080	220	74	1270	249	83	700	218	72	>8000	1292	100

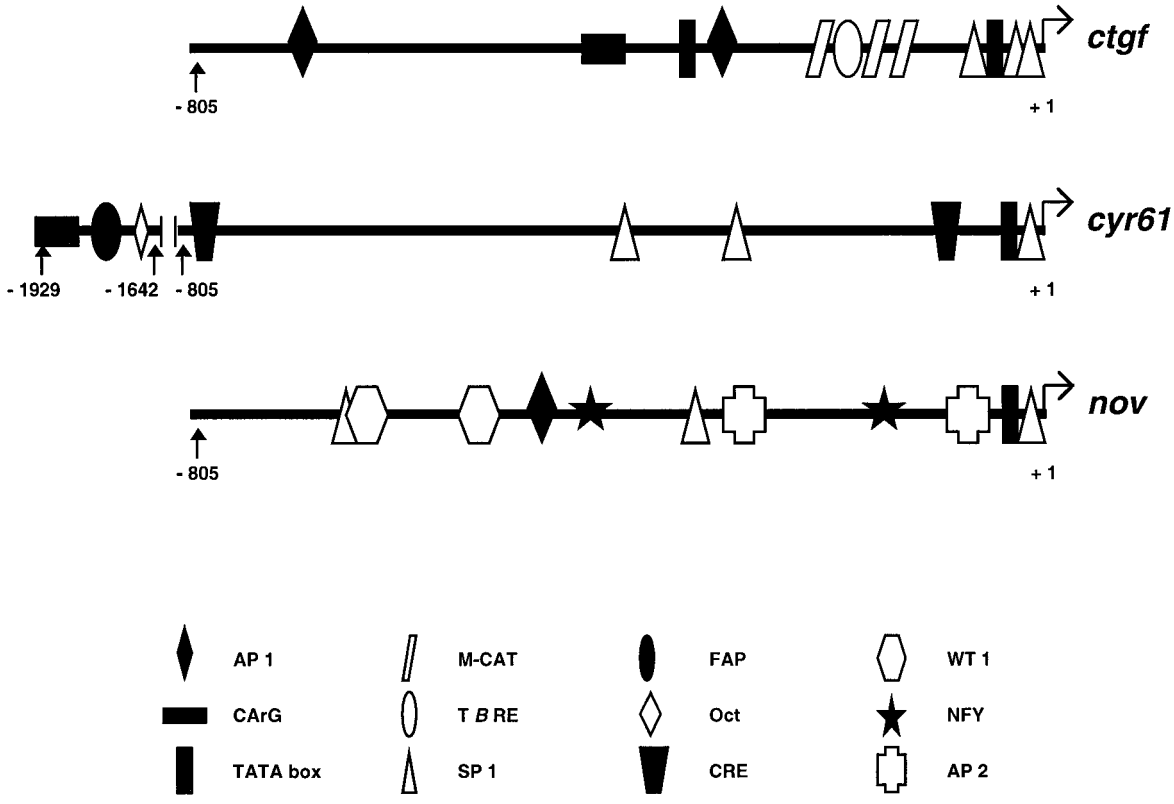

FIG. 4. Key regulatory elements in the gene promoters of hCTGF, mouse cyr61, and human nov. Note that the TGF-β response element (TβRE) is unique to CTGF.

CTGF transcription is less robust in response to these growth factors (41) and does not result in increased CTGF protein levels (51). In contrast, pancreatic cancer cells of epithelial origin demonstrate much stronger production of CTGF mRNA by EGF or TGF-α than TGF-β, although the lack of response to TGF-β was due, at least partly, to defects in TGF-β signaling (54). Phenomenologically, the lack of response to TGF-β in certain pancreatic cells is similar to that of skin epithelial cells, which (unlike fibroblasts) do not synthesize CTGF in response to local TGF-β administration *in vivo* (14). Dexamethasone treatment of cultured 3T3 cells was recently shown to stimulate CTGF expression while actually down-regulating TGF-β mRNA (55). Systemic administration of dexamethasone stimulated CTGF expression in the heart, kidney, and skin. Basal and dexamethasone-stimulated CTGF expression was strongly attenuated by tumor necrosis factor-α (TNF-α) (55), which was independently shown to suppress basal CTGF mRNA production in bovine aortic endothelial cells (BAECs), fibroblasts, and VSMCs (35). Finally, increased CTGF expression in renal epithelial cells in response to injury occurred without an increase in TGF-β expression (46). These results show, not surprisingly, that mechanisms of CTGF gene regulation may be cell specific and, in addition to the action of TGF-β, involve a variety of hormones, growth factors, and cytokines.

D. CTGF production, secretion, and processing

CTGF is present in HUVEC-conditioned medium (13) and undergoes microsomal processing (5), consistent with the presence of a signal peptide and its presumed secretion. CTGF mRNA is expressed in BAECs in which it is present at higher levels during growth than confluence (43). Similarly, expression of *fisp*-12 in cycling fibroblasts is relatively high (15). CTGF produced by human foreskin fibroblasts or mouse connective tissue cells under normal growth conditions appears to be cell associated, long lived, and secreted relatively inefficiently (39). On the other hand, *fisp*-12 was secreted efficiently from NIH 3T3 cells but was relatively unstable in the medium (5). Kireeva *et al.* (15) showed that *fisp*-12 was present in the cellular fraction, ECM, and medium and that inhibition of lysosomal acid hydrolysis increased the half-life of *fisp*-12 in the cellular fraction and stabilized steady-state *fisp*-12 levels. These data suggest that membrane-associated *fisp*-12 may form complexes with receptors on the surface of *fisp*-12-producing cells that are then internalized and degraded (15).

Various low mass (10–20 kDa) forms of pCTGF were purified from uterine secretory fluids and shown to commence between modules 2 and 3, at the beginning of module 3, or between modules 3 and 4 (10, 11) (Fig. 3). Native 38-kDa pCTGF undergoes rapid proteolytic processing by uterine fluids (11) and likely explains the presence of 38-kDa CTGF in uterine tissues (32) but not in uterine fluids (10, 11). Native low-mass CTGFs in uterine fluids are extremely stable, and their levels are strongly correlated with those of CTGF-degrading proteases (11). Although conditioned medium from serum-stimulated mouse fibroblasts did not degrade *fisp*-12 (15), 10- to 12-kDa CTGF, but not 38-kDa CTGF, was present

in conditioned medium from cycling human or mouse fibroblasts (39). Recently, Western blot analysis demonstrated 12- to 14-kDa C-terminal forms of CTGF in mouse uterine luminal flushings (47) and 18- and 24-kDa CTGF isoforms in human serum and amniotic, follicular, peritoneal, and cerebrospinal fluids (40). The presence of CTGF in sera has been confirmed by ELISA (51). Whereas serum CTGF levels in normal human subjects were less than 28 ng/ml, those of patients with biliary atresia were up to 8-fold higher (51). These results highlight potential diagnostic or prognostic applications of measuring circulating or secreted CTGF levels.

Collectively, these data suggest that qualitative and quantitative aspects of CTGF secretion may be regulated by numerous factors including stage of cell cycle, growth factor pretreatment, cell type, species and tissue of origin, and protease activity of the pericellular environment. Moreover, the presence of CTGF in a variety of body fluids suggests that they may be important reservoirs of CTGF *in vivo*.

E. CTGF-heparin interactions

Heparin-affinity chromatography has been used to purify 38-kDa rhCTGF (14) and 10- to 20-kDa pCTGF (10, 11), the latter of which are eluted from heparin by 0.8 M NaCl. ^{35}S-labeled *fisp*-12 binds to heparin-agarose beads and is eluted by 0.4 M NaCl (15). Although module 3 is a binding motif for sulfated glycoconjugates (1), residues 247–260, 274–286, and 305–328 of hCTGF bind strongly to heparin (10, 56). Residues 247–260 contain a proposed heparin-binding consensus sequence (XBBXBX) that occurs in a variety of heparin-binding proteins (57).

As with other heparin-binding growth factors, such as FGFs, heparin-binding EGF-like growth factor, amphiregulin, keratinocyte growth factor, and vascular endothelial growth factor (58–70), the biological activity of CTGF is modulated by heparin (10, 14). The binding of *fisp*-12 to heparin has been proposed as the basis for the association of *fisp*-12 with the ECM in cultured cells (15). Heparin-like molecules on the cell surface or in the ECM may constitute a high-capacity, low-affinity binding reservoir for CTGF and thereby regulate its activity, bioavailability, or stability. Indeed, the half-life of *fisp*-12 in ECM (1 h) is much less than that of cyr61 (4 h) and appears to correlate with the relative affinity of each paralog for heparin (15, 71).

F. Biological properties of CTGF

Native 38-kDa hCTGF is a mitogenic and chemotactic factor for NIH 3T3 cells *in vitro* (13). Ten-kilodalton forms of pCTGF are mitogenic for Balb/c 3T3 cells, VSMCs, and endometrial stromal cells, but not endothelial cells (10, 56). The mitogenic activity of 38-kDa rhCTGF or 10-kDa pCTGF on fibroblasts is enhanced by EGF, PDGF, basic FGF (bFGF), or IGF-I and is either enhanced or reduced by heparin according to the CTGF-heparin ratio (10, 14). In monolayer cultures of normal rat kidney (NRK) cells, 38-kDa rhCTGF stimulated DNA synthesis and induced expression of type 1 collagen, fibronectin, and α5 integrin (14), as is characteristic of the effects of TGF-β. These various biological effects are elicted by CTGF concentrations of about 1–20 ng/ml. *In vivo*, injection of TGF-β or CTGF into the dermal/subcuticular area of the skin in neonatal mice produced nodules comprising mainly connective tissue cells and ECM (14, 72), suggesting that CTGF plays a role in TGF-β-mediated formation of granulation tissue. Recombinant hCTGF is also mitogenic for human lung fibroblasts (73). However, recombinant 38 kDa *fisp*-12 did not directly stimulate DNA synthesis in HUVECs or 3T3 cells at 0.3–3 μg/ml (15), although other evidence suggests that endothelial cells do not respond mitogenically to 10-kDa CTGF, which is nonetheless mitogenic for 3T3 cells and VSMCs (10). At 0.3–3 μg/ml, the same *fisp*-12 protein enhanced the mitogenic activity of 10 ng/ml bFGF on HUVECs and NIH 3T3 cells and promoted attachment of HUVECs, NIH 3T3 cells, AKR2B cells, and mink lung epithelial cells at 5–20 μg/ml (15). These latter effects occurred at 100- to 1000-fold higher concentrations than were needed for the stimulation of mitosis, chemotaxis, or ECM production by CTGF in other studies (10, 13, 14). Nonetheless, a role for CTGF in cell adhesion is further supported by the localization of CTGF in ECM (15) and by the ability of antisense CTGF to suppress growth and migration of BAECs *in vitro* (43). While 38-kDa CTGF appears to be proteolytically cleaved into low mass derivatives (11), the relationship (if any) between processing and activity remains undefined.

Although TGF-β induces CTGF expression and CTGF mimics some of the effects of TGF-β in fibroblasts, CTGF-independent pathways of TGF-β action have been identified in these and other cell types. For example, TGF-β is a potent inhibitor of the growth of mink lung epithelial cells, whereas CTGF is not (14). In addition, while CTGF and TGF-β both stimulate DNA synthesis in monolayer cultures of NRK cells, only TGF-β is able to stimulate anchorage-independent growth (AIG) of the same cells (14, 74). AIG induced by TGF-β is antagonized by CTGF antibodies or antisense CTGF and is restored by addition of CTGF to TGF-β-stimulated cells showing that CTGF is involved in the stimulation by TGF-β of this process (74). Since CTGF was induced by TGF-β in these cells, it was proposed that both CTGF-dependent and -independent pathways are involved in the stimulation of AIG by TGF-β (74). The induction of CTGF mRNA and AIG by TGF-β in NRK cells is inhibited by elevated cAMP levels (52). This effect was reversed by addition of CTGF, but not other growth factors, and was attributed to a CTGF-specific restriction point in late G_1 of TGF-β-activated cells (52).

G. Mechanism of action of CTGF

In addition to contributing to TGF-β-mediated AIG (see above), CTGF interacts synergistically with EGF, PDGF, IGF-I, or bFGF (10, 14, 56), suggesting that it activates distinct receptors and/or signaling pathways to those used by other growth factors. Although 38-kDa hCTGF was suggested to bind PDGF receptors (13, 75), this possibility was not supported using a mitogenic form of 10-kDa murine CTGF (39). Binding studies with ^{125}I-labeled 38-kDa rhCTGF have demonstrated the presence of specific high- and low-affinity binding sites and a 280-kDa cross-linked CTGF complex (76). Although there was no clear evidence for a signal-transduc-

ing receptor, these data are nonetheless potentially very exciting. Alternatively, CTGF may function primarily as a cell adhesion molecule that regulates cell function either directly through its association with the ECM or indirectly by synergizing with other growth factors (15).

IV. Cyr61

A. Discovery of cyr61

Cyr61 (originally termed 3CH61) was described in 1985 as an immediate early gene in mouse Balb/c 3T3 cells that was induced by serum or PDGF (77). It was named cyr61 when shown to encode a cysteine-rich protein that contained 10% cysteine residues (78). The same gene, named βIG-M1, was also discovered by Brunner *et al.* (31) in their studies of TGF-β-inducible immediate early genes in mouse AKR-2B cells. The gene for cef-10, the chicken ortholog of cyr61, was described in 1989 as $pp60^{v\text{-}src}$-inducible immediate early gene in chicken embryo fibroblasts (CEFs) (79). cDNA for the human ortholog of cyr61 (hcyr61) was isolated from a 6-week-old human embryonic tissue cDNA library (80).

B. Structure of the cyr61 gene and protein

The murine cyr61 gene comprises five exons and four introns that span 3.1 kb (Table 2) and contains a serum-response element (SRE) (Fig. 4) in the 5′-region that mediates serum- or PDGF-induced gene transcription (6). Induction of cyr61 is similar to that of other immediate early genes that contain SREs, consistent with its coordinated expression with c-fos during G_0 to G_1 (81), although its transcriptional suppression is relatively inefficient (6). Although cyr61 expression is induced by TGF-β (31), its promoter lacks the novel TGF-β response element found in the promoter of CTGF (36) (Fig. 3). Human cyr61 maps to p22.3 on chromosome 1 (80, 82). The mouse cyr61 gene encodes a 379-residue protein, which, after signal peptide cleavage, yields a 355-residue protein containing 38 conserved cysteine residues (31, 78) (Fig. 1). The cysteine-free portion of cyr61 is about twice the length of that in other CCN paralogs (Fig. 1).

C. Cyr61 mRNA production

A single cyr61 transcript of 2.4 kb has been reported in mouse AKR2B cells, 3T3 fibroblasts, and PSA-1 teratocarcinoma cells (31, 77, 81, 83) and rat H19–7 embryonic neuronal hippocampal cells (84). A 1.8-kb cef-10 transcript is present in CEFs (79), and two cyr61 transcripts of 2.5 kb and 3.5–4 kb have been described in human tissues (80, 82). Although cef-10 mRNA is expressed principally in the adult lung (79), cyr61 is expressed in multiple adult tissues of the mouse and human such as heart, uterus, skeletal muscle, and lung (49, 78, 80). Transcript levels in other tissues are variable and likely due to differences in mRNA detection techniques as well as in the stage of development or differentiation of the source material. For example, human cyr61 mRNA expression is higher in the kidney of the fetus than that of the adult (80). Cyr61 mRNA is present at high levels in mouse embryos on days 9.5–14.5, whereas placental expression of cyr61 is highest on days 17.5–18.5 (83).

Cyr61 gene expression is stimulated by diverse molecular signals, which supports its role in a variety of biological processes. In mouse cells, cyr61 mRNA is rapidly induced by serum, PDGF, FGF, TGF-β, phorbol ester, or cholera toxin (31, 77, 78, 81). Serum stimulation of Balb/c 3T3 cells resulted in a rapid increase and sustained high levels of cyr61 mRNA, which were stabilized by cyclohexamide (78). Similarly, human cyr61 mRNA is present in proliferating, but not quiescent, skin fibroblasts (49). In CEFs, transcription of cef-10 is rapidly enhanced by $pp60^{v\text{-}src}$ or serum (79). In human osteoblasts, cyr61 expression is enhanced by EGF, PDGF, and interleukins-1β, -2, and -6, as well as by 1α,25-dihydroxyvitamin D_3, a mediator of osteoblast differentiation and function (85). In the rat uterus, cyr61 is estrogen-responsive and strongly induced by tamoxifen (86). In rat H19–7 cells, cyr61 is induced within 1 h by bFGF, a stimulator of neuronal differentiation in this cell type (84). This process appears to involve both mitogen-activated protein kinase-dependent and -independent pathways (84).

D. Cyr61 production and secretion

The 41-kDa mouse cyr61 protein is present at very low amounts in quiescent Balb/c 3T3 cells but reaches peak levels within 2 h of serum stimulation (78). Cyr61 is differentially distributed in the ECM, cellular fraction, or cell surface rather than the medium (71, 78). The extracellular fate and half-life of cyr61 synthesized in response to mitogenic stimulation were shown to be dependent upon the stage of the cell cycle and presence of binding moieties in the ECM and on cell surfaces such as heparin-like molecules from which cyr61 is eluted by 0.6–0.8 M NaCl (16, 49, 71).

E. Biological properties of cyr61

Recombinant murine cyr61 promotes dose-dependent attachment of HUVECs or NIH 3T3 cells to plastic surfaces coated with 3–30 μg/ml of the protein (16). Similar concentrations of cyr61 also stimulate dose-dependent chemotaxis of NIH 3T3 cells (16) and human microvascular endothelial cells (HMVEC) (87). These concentrations of cyr61 were not mitogenic for NIH 3T3 cells or HUVECs, although they did potentiate the mitogenic activity of bFGF for these cell types (16, 49). This latter effect was abolished by cyr61 antiserum and attributed to the displacement by cyr61 of bFGF from the ECM, thus increasing the effective concentration of soluble bFGF (16, 49). In mouse limb bud micromass cultures, cyr61 promoted expression of type II collagen, incorporation of sulfate, and the production of cartilage nodules (88). Moreover, cyr61 promoted the adhesion and aggregation of limb bud mesenchymal cells. Cyr61 was also a weak stimulator of DNA synthesis and proliferation of limb mesenchymal cells (88). Cyr61 was further shown to promote chondrogenesis in cell cultures seeded at subthreshold densities that do not normally undergo chondrogenic differentiation. These various responses were elicted with 0.3–5 μg/ml cyr61. Limb bud micromass cultures showed reduced levels of chondrogenesis when incubated with neutralizing cyr61 antisera, suggesting that endogenous cyr61 plays a normal physiological role in chrondrogenic differentiation (88). Finally,

cyr61 has been shown to induce neovascularization *in vivo* (87), consistent with its stimulation of directed migration of HMVECs *in vitro* (87). Stable transfection of cyr61 cDNA into RF-1 gastric adenocarcinoma cells had no effect on *in vitro* growth rate, yet produced larger tumors in immunocompromized mouse hosts as compared with a vector control, a phenomenon that was attributed to cyr61-stimulated tumor vascularization (87).

F. Mechanism of action of cyr61

A mechanistic framework for the biological properties of cyr61 has been provided by the finding that cyr61 binds to integrin $\alpha_v\beta_3$, which represents the first (and only) molecularly defined receptor for any member of the CCN family (89). The interaction of $\alpha_v\beta_3$ with cyr61 may account for its promotion of chemotaxis and growth factor-mediated DNA synthesis as well as cell adhesion since integrins are known to modulate cell migration and growth factor signaling in other systems (90). As well as demonstrating a direct interaction between the two molecules in binding assays *in vitro* (89), cyr61-mediated adhesion and migration of cultured endothelial cells were specifically inhibited by the peptide RGDS and/or antiintegrin $\alpha_v\beta_3$ (87, 89). In addition to its integrin-binding property, cyr61 appears to be localized to its site of synthesis by associating with the ECM, possibly by binding to heparin-like molecules. This interaction may limit the extent of cyr61 diffusion so that its site of action is in close proximity to its site of synthesis (15).

V. Nov

A. Discovery and structure of nov

Nov (nephroblastoma overexpressed) was first recognized as an overexpressed gene in nephroblastomas induced by myeloblastosis-associated virus type 1 (MAV-1) in day-old chicks (7, 91). Subsequently, the human (novH), mouse nov (novM), quail (novQ), and *Xenopus* (xnov) orthologs of chicken nov (novC) were isolated by hybridization screening (8, 33, 38, 92). The nov gene demonstrates a well conserved intron/exon structure that is similar to that of other paralogs in the CCN family (Table 2). In one chicken nephroblastoma, MAV-1 envelope and long-terminal repeat sequences integrated into the second exon of the novC gene at Cys^{63} (7); the first putative initiation codon downstream of this integration site is Met^{99}. The novH gene maps to chromosome 8q24.1 and appears to contain at least three transcription initiation sites in the promoter region (38). NovM maps to chromosome 15 between *D15 Mit153* and *D15 Mit 183* (92, 93).

Putative transciption factor-binding sites are present in the 5′-untranslated region of several nov genes (8, 33, 38, 92) (Fig. 4). However, SREs and the TGF-β response element are absent, suggesting that transcriptional regulation of nov is distinct from that of CTGF or cyr61. The activity of the novH promoter is repressed in the presence of the Wilms tumor suppressor gene WT-1 (94). While WT-1 binding sites are present in the novH promoter (Fig. 4), WT-1-mediated repression of novH transcription was experimentally attributed to other domains in the promoter (94).

The primary translational products of nov orthologs are predicted to comprise between 343 and 357 amino acids and to contain 38 conserved cysteine residues (Fig. 1). The signal peptide in novH appears to comprise 27 residues (8). In CEFs, an immunoreactive 46-kDa novC protein was localized to the ECM (93), although the underlying mechanisms and functional significance remain unclear. NovH produced in transfected Madin Darby canine kidney cells is a glycosylated protein that is secreted about 1.5 h after synthesis and requires 0.5 M NaCl for elution from heparin (20). Secreted novH has a half-life of more than 18 h, which is more than 10 times that of intracellular novH (20). Levels of the endogenous 46-kDa novH protein are repressed in 293 kidney cells that constitutively express WT1 (94).

B. Nov mRNA production

A single 2.2-kb novC transcript is usually detected in MAV1-induced nephroblastomas (7). An exception was a tumor in which MAV1 integrated within the novC gene itself, resulting in the production of a 2.0-kb transcript (7). The 2.2-kb novC mRNA transcript was detected at high levels in the brain and heart of day 18 chick embryos, with weak signals in muscle and intestine and nondetectable levels in liver, lung, and yolk sac. In the adult, 2.2-kb novC mRNA was expressed at high levels in brain and lung, at low levels in the spleen, and was nondetectable in heart, muscle, and liver (7). Quiescent cultures of CEFs contain a 2.2-kb novC transcript that is down-regulated during proliferation, mitogenic stimulation, or activation of viral oncogenes (95). NovH is normally expressed as a 2.5-kb transcript in human glioma cell lines and Wilms tumors, although an additional novH mRNA of 1.2 kb was observed in a single Wilms tumor (8, 37). Unlike novC, this smaller transcript was not the result of rearrangement of the novH gene (8).

There is little, if any, experimental evidence supporting a role for nov as an immediate early gene. As explained above, there are no motifs in the nov gene promoter that resemble either a SRE or a TGF-β response element. Serum stimulation of kidney-derived 293 cells or CEFs failed to increase nov mRNA levels within 90 min, even though the genes for CTGF and *jun* were rapidly activated (93). Moreover, in normal CEFs, nov was expressed in quiescent cells but down-regulated in proliferating cells (95). In contrast to cef-10, expression of $p60^{v\text{-}src}$ in CEFs resulted in transcriptional down-regulation of the novC gene (95). NovC expression was also repressed by the viral oncogenes v-erbB, v-mil, and v-crk (95). In addition, mRNA destabilization sequences in the 3′-region are absent from most nov orthologs (except xnov), suggesting that nov RNA degradation is regulated differently from that of CTGF, cyr61, and other immediate early genes.

C. Biological properties of nov

Overexpression of full-length novC cDNA was shown to inhibit the growth of CEFs grown in soft agar (7). In contrast, transformation of CEFs occurred after overexpression of an N-terminally truncated form of novC that initiated at Met^{99} and was thus comparable to that expressed after integration

of MAV-1 into the novC gene (Fig. 3) (7). This truncated form of nov also induced morphological transformation of rat embryo fibroblasts (8). Within the CCN family, the ability to function as a protooncogene is thus far unique to nov. The dramatic biological differences between the normal and truncated nov genes have led to suggestions that nov is a growth suppressor gene, truncation of which results in oncogenic activation. These interpretations are consistent with the low levels of nov mRNA in mitogen-stimulated cells and the high levels of nov mRNA in resting cells (93, 95). Although the truncated nov protein lacked the signal peptide and the IGF-binding motif in module 1, it is not known how the loss of one or both of these domains contributes (if at all) to its transforming properties.

VI. Elm1/WISP-1

Elm1 (expressed in low-metastatic type 1 cells) was initially identified by mRNA differential display as being expressed in low, but not in high, metastatic murine melanoma cells (96). The elm1 protein was subsequently predicted to comprise 367 amino acids and to conform to the CCN modular structure (97) (Figs. 1 and 3; Table 1). Elm1 transcripts of 1.8 and 5.0 kb were present in all mouse tissues analyzed (97). The 5.0-kb elm1 transcript was induced within 3 h of serum stimulation of quiescent Balb/c 3T3 cells, unlike cyr61 which was induced within 30 min in the same cells (97). Elm1 maps to mouse chromosome 15 between *D15 Mit17* and *D15 Mit3*. Southern blotting demonstrated that the elm1 gene is also present in human, monkey, rat, mouse, dog, and cow (97). Transfection of elm1 into highly tumorigenic and metastatic K-1735 M-2 cells resulted in a decreased tumor growth *in vivo* and reduction in the frequency of lung metastatic colonies (97). *In vitro*, transfected cells that expressed relatively high amounts of elm1 grew somewhat slower and to lower saturation density as compared with control cells (97). Elm1 did not exhibit mitogenic activity for NIH3T3 cells, either alone or in combination with bFGF. The growth-suppressive properties of elm1 have been tentatively linked to module 3 (97). Recently, WISP-1 was identified as the human ortholog of elm1 and shown to be a component of the Wnt-signaling pathway in transformed cells (24). WISP-1 maps to human chromosome 8q24.1–8q24.3 and exhibits tissue-specific patterns of expression (24).

VII. Heparin-Induced CCN-Like Protein (HICP)/rCop-1/CTGF-3/WISP-2

A novel 1.8-kb mRNA was identified by subtractive hybridization screening of heparin-treated and nontreated rat VSMCs and shown to encode a novel member of the CCN family that was termed HICP (98). Independent studies resulted in the isolation of cDNA for the same gene, termed rCop-1, when shown to be expressed in normal rat embryo fibroblasts or mouse 3T3 cells, but not in their transformed derivatives (99). The mouse ortholog, WISP-2, was recently identified as a unique gene that was up-regulated in Wnt-1-transformed mouse mammary epithelial cells *in vitro* (24). These molecules, together with the human ortholog, CTGF-3 (100), demonstrate an overall amino acid homology of ~30% with hCTGF and conservation of all of the cysteine residues in modules 1–3 (Fig. 1). However, all of these proteins are C-terminally truncated as compared with other CCN paralogs, resulting in a total absence of module 4 (Figs. 1 and 3). While HICP/CTGF-3/WISP-2 transcripts were present in multiple tissues (24, 98, 100), rCop-1 mRNA was not detected in any of the major organs of the mouse embryo and adult rat (99). Although this discrepancy requires further investigation, expression of rCop-1 has been linked to aging and senescence (99). HICP expression patterns, similar to those of growth arrest genes, occur at high levels in quiescent or heparin-arrested VSMCs and at very low levels during proliferation (98). Somewhat different expression patterns occur for rCop-1 in cycling mouse 3T3 cells in which rCop-1 mRNA is absent during quiescence or immediately after serum stimulation and appears only during late S phase (99). Unlike normal cells, transfection of rCop-1 into transformed cells suppressed their cell growth; this was attributed to cell death rather than growth arrest (99). In transfected cells, rCop-1 is localized primarily intracellularly and to the cell surface, but not in conditioned medium or ECM. It has been speculated (99) that the unique subcellular localization and expression pattern of rCop-1/HICP is a reflection of its role in negative growth regulation, the structural basis of which may lie in the absence of module 4. Potential tumor-suppressive properties are further suggested by the finding that WISP-2 is underexpressed in human colon tumors, unlike WISP-1 and -3, which are overexpressed (24).

VIII. WISP-3

Human WISP-3, a 354-residue protein containing 36 cysteine residues, was identified by screening expressed sequence tag databases with WISP-1 (24). The secreted WISP-3 protein is predicted to contain 34 of the 38 conserved cysteine residues found in most other CCN proteins since it lacks 4 of the cysteines normally present in module 2. It is possible that this structural difference results in unique properties and functions, although this aspect of WISP-3 biology has not yet been explored (24).

IX. Other CCN-Like Molecules

Twisted gastrulation (tsg) and short gastrulation (sog), which are involved in dorsoventral patterning in *Drosophila* embryos (101, 102), contain sequences that resemble, respectively, modules 1 and 2. As with CTGF and cyr61, tsg is eluted from heparin by 0.6–0.8 M NaCl, and the binding of tsg by somatic cells may involve a heparin-like coreceptor (103). The cytoskeletal rearrangements that take place in dorsal amnioserosa cells in the presence of tsg have been likened to those in endothelial cells during CTGF-induced chemotaxis (101). Sog, like CTGF, may be susceptible to proteolytic cleavage, yielding diffusible bioactive fragments (102). The cDNA of "small CCN-like growth factor" (SCGF) was recently isolated from an 9-week human embryo library and shown to encode a 206-residue protein that had limited structural similarity to the C-terminal region of CCN family

members, although cysteine residues occur at half the expected frequency and are poorly conserved (104). Overall, tsg, sog, and SCGF exhibit very weak alignment with the CCN family and are not genuine paralogs.

X. Regulation of Cellular Functions by the CCN Family

A. Cell cycle control

Members of the CCN family are products either of immediate early genes (CTGF, cyr61) or of putative growth arrest/suppression genes (nov, elm1, HICP). The expression of immediate early genes represents the earliest genomic response to growth factors and is likely to initiate the program leading to cell replication. Since they encode a diverse array of regulatory molecules, the induction of immediate early genes represents a central component in the proliferative response to mitogenic stimuli (105). Since CTGF and cyr61 are transcriptionally activated by TGF-β, PDGF, EGF, FGF, TPA, and cholera toxin, it is likely that the cellular responses to these diverse mitogenic stimuli are controlled, at least partly, by CTGF and cyr61. On the other hand, the expression of growth arrest-specific genes, DNA damage-inducible genes, and the MyD growth-arrest genes are associated with the negative regulation of cell growth (106). Growth arrest genes have been implicated in cellular differentiation, embryonic development, and apoptosis (107–109), and it is possible that these types of processes are similarly regulated by nov, elm1, and rCop-1.

In addition, each CCN family member appears to further regulate the cell cycle through their 1) direct mitogenic action (*e.g.*, CTGF); 2) potentiation of the mitogenic activity of other growth factors (*e.g.*, CTGF, cyr61); 3) binding of IGFs (*e.g.*, CTGF); 4) regulation of ECM synthesis (*e.g.*, CTGF, cyr61); 5) regulation of cell attachment and migration (*e.g.*, CTGF, cyr61, elm1); 6) interactions with the ECM (*e.g.*, CTGF, cyr61, nov); 7) oncogenic properties (*e.g.*, nov); and 8) regulation of a cell cycle restriction point (*e.g.*, CTGF). This broad spectrum of biological properties, many of which are interacting and interdependent, demonstrate the complexity by which the cell cycle is likely regulated by the CCN family.

B. Cell adhesion and migration

In vitro studies have shown that CTGF or cyr61 are chemotactic and promote cell adhesion (13, 15, 16, 87). Elm1 is expressed in low, but not high, metastatic cell lines and, when transfected into high-metastatic cell lines, causes them to exhibit slower rates of tumor growth and decreased incidence of metastasis (97). Truncated nov stimulates AIG and colony formation in CEFs (7). Collectively, these data demonstrate broad effects on cell adhesion and locomotion, which likely reflects the ability of CCN family members to regulate the composition of the ECM, the net balance between its synthesis and degradation, as well as to bind directly to ECM components such as integrins and heparan sulfate proteoglycans (see below).

C. ECM production

Tissue formation and cell proliferation and differentiation are dependent upon interactions between cells and the ECM. In processes such as tissue remodeling and malignancy, ECM components are degraded by proteases such as collagenase, plasmin, and matrix metalloproteases, and the ability of cells to interact with the ECM is altered or abolished. TGF-β plays a central role in ECM production since it stimulates synthesis of ECM proteins such as collagen, fibronectin, laminin, elastin, glycosaminoglycans, and other glycoproteins as well as synthesis of inhibitors of ECM degradation such as plasminogen activator inhibitor and tissue inhibitors of metalloproteases (110). In view of the TGF-β-inducibility of CTGF and cyr61 gene expression, these molecules may promote ECM deposition by stimulating synthesis of ECM components and inhibiting their degradation. While detailed studies of the regulation of ECM-degrading enzymes and their inhibitors by the CCN family have yet to be reported, initial studies have shown that 38-kDa CTGF enhances fibronectin, type IV collagen, and integrin production (14) and that cyr61 stimulates type II collagen production, albeit in a specialized cell type (88). As discussed below, numerous fibrotic disorders exhibit substantial ECM involvement and expression of CTGF. In addition, there is a striking appearance of CTGF in uterine stromal cells that are undergoing decidualization (47), a highly regulated process involving increased mitosis and synthesis and deposition of ECM molecules such as desmin, laminin, and fibronectin (111). ECM production and remodeling is also a critical component of development and differentiation during embryogenesis (112). Collectively, these data suggest that CTGF may exert diverse and important functions in normal physiology and pathological states via its net induction of ECM protein deposition and content.

XI. Biological Processes Involving the CCN Family

A. Development and differentiation

Immunohistochemical studies showed that CTGF is present in the mouse as early as embryonic days 4.5–6.5, at which time it is most abundant in the embryonic endoderm and mesoderm (47). At later stages of gestation (days 14–18), CTGF and cyr61 are both present in various tissues and organs including the cardiovascular and pulmonary systems as well as the skin and placenta (15). Secretory structures such as kidney tubules and salivary, mucous, and sebaceous glands are positive for CTGF, but not cyr61, whereas the nervous and skeletal systems are positive for cyr61, but not CTGF (15). This differential distribution may be indicative of unique roles for each factor. A role for nov during *Xenopus* development is suggested by the production of its mRNA in the oocyte at stages I, III, and IV and in the embryo at all developmental stages between egg and tadpole (33). In chickens, novC is expressed in kidney, heart, and muscle of the embryo but not of the adult (7). Other organs (*e.g.*, brain) demonstrate novC expression in both embryonic and adult life, whereas expression of novC in the lung occurs in the adult but not the embryo (7).

Evidence to date supports a role for cyr61 and CTGF in

chondrogenesis. The cyr61 protein is present in cartilage and bone in day 13–18 mouse embryos (15, 88), and cyr61 mRNA is present in a variety of developing cartilaginous structures in day 8.5–14.5 embryos (83). A role for cyr61 in normal growth and development of the cartilaginous skeleton is further supported by the ability of cyr61 to stimulate collagen synthesis, sulfate incorporation, and formation of cartilage nodules in mouse limb bud micromass cultures, and adhesion, aggregation, and growth of limb bud mesenchymal cells (88). In the case of CTGF, its mRNA is present in hypertrophic chondroctyes from day 17 mouse embryos, neonatal rabbit growth cartilage tissue and cultured cells, and human chondrocytic cell lines, but not in human osteosarcoma or mouse osteocytic cell lines (48). In cultured rabbit growth cartilage cells, peak levels of CTGF occurred during the early hypertrophic stage. CTGF mRNA levels in these cells were enhanced by treatment with TGF-β or bone morphogenic protein-2 (48). In addition to its potential role in chondrogenesis, cyr61 may also be involved in hippocampal differentiation, since it is induced in H19–7 cells by bFGF, which stimulates their differentiation into nonproliferative neuronal cells (84).

Several lines of evidence support a role for nov nephrogenesis. First, novC expression occurs in normal avian kidney cells of the embryo and neonate, yet is barely detectable in adult kidney cells (7). Second, ureteric buds, which give rise to the kidney collecting ducts, fail to demonstrate outgrowth in WT-1-null mice; as discussed above, novH promoter activity is repressed by WT-1, and a reciprocal quantitative gradient of novH and WT1 expression occurs during normal human nephrogenesis (see Ref. 94). Third, novH is localized to differentiating glomerular podocytes where it accumulates and may regulate podocyte structure or function during both pre- and postnatal life (20). Since the composition of the ECM changes during the earliest stages of metanephric induction (113) and ECM components regulate uteric bud morphogenesis, nov may also influence nephrogenesis by binding to the ECM (93).

B. Female reproductive tract function

The actions of ovarian steroids are mediated, in part, by polypeptide growth factors, which may also contribute to the uterine-embryo signaling dialogue that initiates implantation and stimulates embryonic development (114–120). Also, some growth factors are secreted into the uterine lumen or localized at the implantation site, where they may stimulate development of the embryo and placental membranes (114, 121). CTGF is present in uterine fluids of the pig and mouse, as well the uterus of the pig, mouse and human (10, 11, 13, 47, 56, 122) suggesting that it is involved in the regulation of uterine function. In uterine fluids, the levels of low mass CTGFs and CTGF proteases are highly correlated with each other and show cyclic variations during the estrous cycle (11). Moreover, there are clear differences in the levels of CTGF and CTGF protease(s) in uterine fluids at equivalent stages of the estrous cycle and early pregnancy suggesting that CTGF production or action is modified in the presence of the embryo (11, 56). A relationship between CTGF expression and TGF-β action during the periimplantation period of the pig is supported by the presence of both molecules at this time in uterine and/or embryonic tissues (11, 123–125). In addition, a direct role of CTGF on uterine cells is indicated by its stimulation of DNA synthesis in pig stromal cells *in vitro* (56).

In mice and women, CTGF is localized primarily to uterine luminal and glandular epithelial cells during the estrous cycle and during the first few days of pregnancy (47, 122). On the day of implantation in mice, epithelial staining for CTGF is strongly reduced and is followed over the next 2 days by profound staining of decidualizing endometrial stromal cells, suggesting that CTGF contributes to the decidualization process or is produced as a result of it (47). Decidualization is a highly regulated differentiation process involving increased vascular permeability, DNA synthesis, and synthesis and deposition of ECM molecules (111, 126–128). The distribution of uterine CTGF during early pregnancy in the mouse does not entirely correlate with TGF-β and its receptors, suggesting that TGF-β-independent synthesis of CTGF may be operative (47). Alternative mechanisms may involve glucocorticoids or estrogen, which stimulate CTGF and cyr61 gene transcription, respectively (55, 86). The nonconcordance of CTGF and TGF-β may also be related to the initial production of TGF-β in a latent form (129). Whatever the explanation, these data support a role for uterine CTGF at the time of implantation.

C. Angiogenesis

Angiogenesis, the formation of new capillaries from preexisting blood vessels, occurs in processes such as tumor growth, diabetic retinopathy, wound healing, and placental vascularization. It is a complex process that involves degradation of the capillary basement membrane, migration and proliferation of endothelial cells, and tube formation; it is regulated by many factors (130), including cyr61, which promotes directed migration of HMVECs via $\alpha_v\beta_3$-integrin and induces neovascularization in the rat cornea (87). Expression patterns of cyr61 support its contribution to angiogenesis in the embryo, placenta, hypertrophic cartilage, wounds, and tumors (83, 87). Since cell migration is the only component of angiogenesis to be directly stimulated by cyr61, indirect effects of cyr61, such as release and action of the angiogenic factor bFGF from ECM (16, 49), may contribute to the overall process of neovascularization. These questions and the role of other CCN family members in angiogenesis will require further study.

D. Wound repair

Many studies have established that growth factors, including TGF-β, are involved in wound healing (see Ref. 131). Collectively, CTGF and cyr61 exhibit numerous biological properties that are of potential importance in the wound healing response, including stimulation of cell proliferation, cell adhesion, chemotaxis, angiogenesis, production of ECM components, and augmentation of bFGF activity. Early studies showed that cyr61 gene expression was induced during the first hour of liver regeneration in the mouse after partial hepatectomy (132). These results are consistent with the role

of cyr61 as an immediate early gene and demonstrate that cyr61 is transcriptionally activated as a response to injury. In tissue from within subcutaneously implanted Schilling chambers in rats (133), CTGF expression peaked on day 9 of injury after peak expression of TGF-β on day 3 (41). The coordinate expression of the two factors was interpreted as a component of a growth factor cascade in which TGF-β initiated regeneration and repair and stimulated the production of CTGF, which was required later in the wound healing process (41). Somewhat different kinetics of gene transcription have been reported in full thickness wounds in which CTGF and TGF-β both exhibited peak expression 12–24 h after injury (55, 134). Although dexamethasone was shown to stimulate CTGF gene expression in uninjured tissue, it did not affect CTGF mRNA levels in wounded tissues and was attributed to the presence of TNF-α, which inhibits CTGF gene expression (35, 55). Finally, scrape wounding BSC-1 renal epithelial cells resulted in stimulation of CTGF gene expression, which reached peak levels 4 h after injury (46). CTGF was induced both in cells at the wound and some distance from it, suggesting that an inductive signal was communicated to distant cells from the initial wound site, although there were no changes in expression levels of TGF-β, PDGF, or bFGF (46).

E. Fibrotic disorders

Many fibrotic disorders are typified by excessive connective tissue and ECM formation and exhibit marked overexpression of TGF-β, which is fibrogenic and strongly linked to the pathogenesis of these diseases (135–137). In view of the biological activities of CTGF and the link between CTGF production and TGF-β action, studies have recently been initiated to examine the role of CTGF in fibrotic disorders of the skin, kidney, lung, and blood vessels. Subcutaneous injection of TGF-β into neonatal mice, which causes a rapid increase in the amount of granulation tissue comprising connective tissue cells and abundant ECM, was shown to result in enhanced levels of CTGF mRNA in connective tissue fibroblasts but not in epithelial cells or endothelial cells (14, 72). Injection of 38-kDa rhCTGF caused a very similar fibrotic reaction as TGF-β in terms of histological findings, time course, and area affected (14). This fibrotic response was specific to TGF-β and CTGF and not mimicked by EGF, PDGF, or bFGF (14, 72).

Overexpression of CTGF mRNA occurs in a variety of fibrotic skin disorders including 1) systemic sclerosis in which CTGF expression is temporally associated with the sclerotic phase and is highest in the fibroblasts of the deep dermis (42); 2) localized sclerosis in which CTGF-positive fibroblasts are scattered throughout the lesion (138); 3) keloids in which CTGF-positive fibroblasts are present throughout the lesion but concentrated in the expanding peripheral regions (138); and 4) scar tissue, eosinic fasciitis, nodular fasciitis, and Dupuytren's contracture in which CTGF was partially expressed in some of the fibroblasts (138).

In kidney fibrosis, CTGF expression is increased in inflammatory glomerular and tubulointerstitial lesions, as compared with normal kidney or noninflammatory glomerular lesions (139). Extracapillary proliferative lesions, capsular adhesions, and periglomerular fibrosis were characterized by a pronounced increase in epithelial expression of CTGF. CTGF expression was also somewhat increased in mesangial proliferative lesions of diabetic and IgA nephropathies (139). In a rat model of glomerulonephritis, CTGF mRNA was up-regulated in parietal epithelial cells and podocytes, where it was expressed in areas of crescentic extracapillary proliferation, periglomerular fibrosis, and in interstitial foci (140). Cultured mesangial cells and podocytes demonstrated enhanced CTGF mRNA in response to TGF-β (140) or under hyperglycemic conditions (141), the latter of which causes ECM deposition and induction of TGF-β1 (142).

In bleomycin-induced fibroproliferative lung disease in mice, which is characterized by up-regulation of TGF-β mRNA (143, 144), bleomycin treatment of sensitive mice resulted in a 2- to 3-fold increase in lung CTGF mRNA levels and collagen synthesis as compared with resistant mice (34). CTGF is present at higher levels in bronchoalveolar lavage fluid from patients with fibrosing alveolitis as compared with normal individuals, and lung fibroblasts from patients with scleroderma-associated fibrotic lung produce more CTGF in response to TGF-β than control cells (145). An autocrine role for CTGF in lung growth is supported by the expression of CTGF mRNA in murine lung (5) and in cultures of human or mouse lung mesenchymal cells in which it is rapidly induced by TGF-β (34) and its stimulation of mitosis and collagen synthesis in fibroblasts (73, 145).

CTGF mRNA is expressed in atherosclerotic vessels at 50- to 100-fold the level of that in normal arteries (44). In advanced atherosclerotic lesions, CTGF was localized to VSMCs and endothelial cells primarily at sites of ECM accumulation and fibrosis, including the shoulders of fibrous caps (44, 139, 146). VSMCs respond mitogenically to CTGF (10) and produce CTGF mRNA in response to TGF-β (44, 146), consistent with the presence of a CTGF autocrine loop that is initiated by TGF-β. Although the potential involvement of CTGF in stimulating intimal thickening is highly deleterious, it has been argued that CTGF-stimulated ECM production may actually stabilize the fibrous cap and reduce the chance of plaque rupture (146). Finally, the association between HICP expression and growth arrest in VSMCs (98) suggests that attenuation of HICP may contribute to hyperplastic VSMC diseases such as athero- and arteriosclerosis.

The profibrotic properties of CTGF suggest that it is an attractive therapeutic target in a variety of fibrotic disorders, especially as it operates downstream of TGF-β (53, 136). Intervention at the level of CTGF would still allow certain beneficial non-CTGF-dependent effects of TGF-β (*e.g.*, anti-inflammatory) to persist while negating its fibrogenic action. Over the next few years we can expect to see a careful evaluation of all components of the CTGF pathway—its gene, mRNA, transcription factors, protein, receptor, and second messengers—with the goal of finding optimal molecular targets for the control of CTGF-mediated fibrosis.

F. Inflammation

In addition to inflammatory kidney disease (see above), CTGF is overexpressed in inflammatory bowel diseases

(IBD) such as Crohn's disease and ulcerative colitis (147). IBD demonstrated high levels of CTGF, TGF-β, collagen type I, fibronectin, and integrin α5. CTGF was overexpressed in regions of inflammation and in noninflamed regions that were stenosed and was present in the vicinity of TGF-β-producing cells (147). CTGF was thus proposed to promote mesenchymal tissue repair and remodeling after the acute inflammatory phase and to stimulate chronic matrix deposition leading to fibrosis and stenosis (147).

G. Tumor growth

Several lines of evidence support a role for CCN molecules in tumorigenesis, the most compelling of which is the oncogenic property of N-terminally truncated nov (7). The tumorigenicity of a gastric adenocarcinoma cell line was increased when transfected with cyr61, the angiogenic properties of which support its role in tumor growth and vascularization (87). Consistent with its profibrotic properties, CTGF is overexpressed in breast cancer, pancreatic cancer, and melanomas exhibiting significant involvement of connective tissue cells (desmoplasia) (54, 148, 149). Similarly, WISP-1 and WISP-2 are strongly expressed in the fibrovascular stroma of breast tumors from Wnt-1 transgenic mice (24). CTGF is expressed in sarcoma and chondrosarcoma cells (39, 48), while cyr61 is expressed in rhabdomyosarcoma and cell lines derived from malignant melanoma, breast and colon adenocarcinoma, and bladder papilloma (87, 150). Tumors of the nervous system express CTGF, nov, and cyr61 in a complex and mainly noncorrelative pattern (37, 82).

Elevated expression of novC mRNA was a consistent finding in all MAV1- and MAV2-induced avian nephroblastomas (7, 93). While novC was expressed most highly in differentiated tumors (7), its levels are more correlated with tumor age than histological status (93). In Wilms tumors, novH is mainly overexpressed in tumors of predominantly stromal origin (8) and is positively correlated with heterotypic muscle differentiation (20). Nov expression is inversely correlated with the levels of WT-1 mRNA in some (8, 93), but not all (20), studies. Since WT-1 and several viral oncogenes repress nov expression *in vitro* (94, 95), these variable results demonstrate that the relationship between nov expression and tumorigenesis is complex and that nov transcription mechanisms in both normal and tumor cells requires further study. Despite its apparent role as a growth arrest gene in transfected cells (7), the potential role of nov in growth arrest may be cell type specific or secondary to other biological functions in tumors, thereby accounting for its overexpression in some nephroblastomas (7, 93). Nonetheless, the absence of the IGF binding motif in oncogenic nov and the coexpression of nov and IGF-II in some avian renal tumors and Wilms tumors suggest a possible link between nov and IGF in nephroblastoma (93).

The inverse relationship between nov expression and phenotype of some nephroblastomas is shared with other CCN family members in other tumors. For example, elm1 expression is inversely correlated with the incidence of metastasis and growth of melanoma cells (96, 97). In addition, inverse correlations have been reported between malignant phenotype and the level of CTGF expression in fibroblast and endothelial cell tumors (151), and the level of cyr61 expression in neuroblastoma (83) and prostate cancer (152). On the other hand, rCop-1 is underexpressed in transformed cells and is a negative regulator of the growth of transformed, but not normal, cells (99). Colon tumors demonstrate underexpression of WISP-2 and overexpression of WISP-1 and -3 while in breast tumor models both WISP-1 and -2 are involved in the Wnt signaling pathway that leads to cell transformation (24). Collectively, the individual CCN family members appear to be over-, under-, or randomly expressed in a variety of tumors, and it is currently impossible to establish a unifying hypothesis for the CCN family in tumor growth.

XII. Perspectives and Future Directions

It is clear that the initial classification of CCN proteins as immediate early gene products or growth factors is not universally true for all members of this family. As the family has grown, so has its spectrum of biological properties. In fact, the range of activities within the CCN family is now so broad that their classification on a functional basis is difficult, if not impossible. While they have been categorized by several investigators as ECM or extracellular signaling molecules, this is certainly not a distinguishing feature of the CCN family nor particularly unexpected for modular proteins. Moreover, the signaling events themselves are poorly defined at best. In view of their modular structure and presumed ability to interact with a diverse array of proteins in the pericellular environment, CCN proteins can be expected to demonstrate complex regulation in time and space. As with other bioactive proteins such as growth factors (153) and modular proteins such as TSP-1 (154), interactions with binding proteins are predicted to substantially impact the net biological properties and bioavailability of a given CCN member. While this aspect of CCN biology has not yet been systematically studied, it might help to explain some of the discrepancies and apparent contradictions that have been reported between various laboratories regarding the localization and activities of individual proteins. The likely involvement of the CCN family in complex protein-protein interactions further indicates that a careful analysis of the modules, individually and collectively, is key to understanding the relative properties of a given ortholog and its processed forms. Informative data will likely come from powerful molecular strategies such as the yeast two-hybrid system which was recently utilized to identify the binding of fibulin 1C to novH (155). In addition, the very complex molecular configuration of the CCN family highlights the importance of verifying that recombinant forms of the family members are appropriately folded and faithfully reproduce the biological properties of their native counterparts; this is no small challenge and has been largely overlooked to date. Based on other modular proteins (154), a much broader perspective is needed when considering the activities of CCN proteins, the biological processes in which they act, and the mechanisms involved.

Many additional questions remain unanswered. Are there other members of the CCN family? Do CCN proteins phys-

iologically bind IGFs and, if so, what are the consequences? Are there unique signal-transducing receptors for CCN proteins? What signaling pathways are activated when CCN proteins bind to cell surfaces? What are the identities of CTGF proteases and how are they regulated? What are the biological consequences of the absence of module 4 in rCop-1 and the absence of the cysteine residues in module 2 of WISP-3? Why is nov overexpressed in some nephroblastomas if it is a growth arrest gene? How does cyr61 promote angiogenesis? What other disease states involve the CCN family? What is the relationship between the CCN family and endocrine function? With recent interest in the field, rapid progress in answering some of these questions can be expected in the near future.

In conclusion, the CCN family comprises highly related modular proteins that have wide ranging properties that impact cellular functions such as growth, differentiation, adhesion, and locomotion. The biological properties and actions of CCN proteins are probably complex and likely reflect a dynamic equilibrium of their constituent modules with soluble, cell surface, or ECM-binding proteins. Since these conditions are hard to define molecularly and difficult, if not impossible, to reproduce *in vitro*, the challenge ahead is to understand the biological roles of the CCN family in normal and pathological processes as a function of the composition of the pericellular environment *in vivo*.

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