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The human Cyr61 gene is a transcriptional target of transforming growth factor beta in cancer cells

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Abstract

Cyr61 is a multifunctional protein that can stimulate angiogenesis and tumor growth. Its expression by many cancers and breast cancers increases with tumor grade. Cyr61 is closely related to connective tissue growth factor, CTGF. Both proteins regulate skeletal development, suggesting that they could contribute to breast cancer metastases to bone, a process regulated by TGFβ. We show that Cyr61 transcription is activated by TGFβ and that the human Cyr61 promoter contains consensus sequences that bind Smad proteins. TGFβ in the tumor microenvironment may stimulate cancer metastases to sites such as bone by increasing Cyr61 expression and secretion.

Keywords: TGFbeta; CCN; Cyr61; CTGF; Smad; promoter

1. Introduction

Cyr61 is a growth factor-inducible immediate-early gene [1] and, like the related protein connective tissue growth factor, CTGF, is a target of TGFβ [2,3]. Mouse Cyr61 was first identified as a TGFβ-inducible gene in AKR-2B fibroblasts [4], while TGFβ increases the mRNA for Cyr61 in human prostate epithelial and stromal cells [5]. Cyr61 and CTGF are members of the CCN gene family, which encodes six secreted proteins involved in cell cycle control, cell differentiation and cell survival [6] and is named from the initials of the first three family members: **C**yr61 (CCN1), **C**TGF (CCN2), and **N**OV (neuroblastoma-overexpressed, CCN3). CCNs 4–6 are the Wnt-induced secreted proteins, WISPs 1–3. The proteins have conserved domains homologous to insulin-like growth factor-binding proteins, von Willebrand factor type C repeat, thrombospondin type I repeat and cysteine-rich motifs [7]. The CCN proteins are involved in the control of skeletogenesis [8] and expressed by bone cells [9]. Secreted proteins active in the bone microenvironment are candidates for causal actions in the development of skeletal metastases, a major complication of advanced cancers of breast, prostate and other organs [10], a process that is centrally regulated by bone-derived TGFβ [11–13]. CTGF is one of a toolbox of genes overexpressed in bone-metastatic breast cancer cells [14]. Its regulation by TGFβ has been studied in detail [3,15]. Transcriptional regulation of Cyr61 by TGFβ has been less extensively investigated [16].

CCN overexpression has been reported in a wide range of tumors [7,17], and the secreted proteins can stimulate tumor growth and angiogenesis and inhibit

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apoptosis. CCN1 and the other CCNs act by complex mechanisms, including binding to multiple integrins, which may account for their cell type-specific actions [18]. Expression of CCNs 1 and 2 occurs in parallel in many cancer cells. In breast cancers, CCN1 expression, assessed as RNA or protein, consistently increased with increasing clinical grade of the primary tumor [19–21]. Some of these studies concluded that expression of CCNs 1 and 2 increased in parallel.

TGFβ binds to and activates a heteromeric cell surface receptor that phosphorylates intracellular mediators, the Smad proteins. Receptor-activated Smads bind to Smad4, and the activated complex binds to DNA at Smad-binding elements, SBEs [22–24]. TGFβ induction of CTGF mRNA requires a SBE in the promoter [3].

The published data suggest that Cyr61 is an important factor in tumor progression and is increased by factors in the microenvironment, such as TGFβ [10]. We, therefore, have investigated the molecular mechanism of TGFβ activation of the human CCN1 promoter.

2. Materials and methods

2.1. Cell culture

The human cancer cell lines MDA-MB-231 (human breast cancer) and HepG2 (human hepatocarcinoma) were originally derived from the American Tissue Culture Collection (ATCC, Manassas, VA) and maintained as recommended by the ATCC. The MDA-MB-231 cells are a bone-metastatic variant previously described [11,25]. TGFβ1 was from R&D Systems.

2.2. Analysis of mRNAs by real-time PCR

Total RNA was extracted (Rneasy mini kit, Qiagen) from HepG2 or MDA-MB-231 cells cultured in the presence of 10 ng/ml of TGFβ1 for different times. RNA was converted to cDNA (OmniScript reverse transcriptase, Qiagen), and analyzed by quantitative real-time PCR (SybrGreen, Qiagen) using a BioRad MyiQ thermocycler (annealing temperature 58 °C). Cycler output values were averaged and compared using the C_T method, where the amount of target RNA ($2^{-\Delta\Delta C_I}$) was normalized to the endogenous ATP6 (HepG2 cells) or beta-2-microglobulin (MDA-MB-231 cells) reference (ΔC_I) and related to the amount of targets in untreated cells ($\Delta\Delta C_T$) set as 1.0. The sequences of oligos used in the real-time PCR experiments were: hCCN1: forward: 5′cctcggctggtcaaagttac3′; reverse: 5′aggctccattccaaaaacag3′. hCCN2: forward: 5′gctaccacat ttcctacctagaaatca3′; reverse: 5′gacagtccgtcaaaacagattgtt3′. ATP6: forward: 5′cgccaccctagcaatatcaa3′; reverse: 5′ttaaggcgacagcgatttct3′. Beta-2-microglobulin: forward: 5′ggcgggcattcctgaag3′; reverse: 5′caatgtcggatggatgaaacc3′.

2.3. Isolation of human Cyr61 promoter and mutagenesis

DNA from a BAC plasmid containing the hCCN1 locus (RP11-290M5, Children's Hospital Oakland Research Institute) was used as a template for PCR reaction (Pfu Ultra Hot start, Stratagene) with primers from genomic sequence (accession no. AC092807) and cloned into the promoterless pGL3-basic (Promega) luciferase reporter or into pGL3-MLP vector containing a minimal promoter. Constructs containing site-directed mutations of the three SBE were generated by using the QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene) following manufacturer instructions. The three 5′CAGACA3′ SBEs 1, 2 and 3 were, respectively, mutated to 5′gtGACc3′, 5′cgGgCA3′ and 5′agGcct3′, each creating a novel restriction site, which was used to confirm the introduction of each mutation.

2.4. Analysis of the Cyr61 promoter

HepG2 cells were seeded (7×10^4 cells per well) in 12-well plates and transiently transfected with luciferase constructs 24 h later, using Exgen 500 reagent (Fermentas), following manufacturer instructions. TGFβ1 was added 24 h after transfection, and luciferase activity was assayed at 48 h using the Dual Luciferase assay kit (Promega). Triplicate luciferase activities were normalized to Renilla luciferase (phRL-CMV vector Promega). HepG2 cells were similarly transiently transfected with fragments of the hCCN1 promoter with or without mammalian expression DNAs encoding Smads 2, 3 or 4, and TGFβ treatment by the same schedule. Luciferase values were determined as the means ± SEM. of triplicates from a representative experiment and were analyzed by *t*-test (*, $P<0.1$; **, $P<0.01$; ***, $P<0.001$).

2.5. Interaction of the Cyr61 promoter with Smad3 protein

Interaction of the Cyr61 promoter with Smad3 was demonstrated by non-radioactive gel-shift experiment with a constitutively active Smad3 protein fragment. Double-stranded probe for the Cyr61 promoter was a 208 bp fragment (−260 to −53) made by PCR (Pfu Ultra Hot start, Stratagene) and cloned into pTOPO vector (Invitrogen). A 298 bp fragment was next excised from the pTOPO clone by XhoI/KpnI restriction enzyme and 3′-biotinylation according to the manufacturer's instructions (Pierce). Binding was for 20 min at room temperature in the presence of 50 ng/μl poly(d*I*–d*C*) in 1× binding buffer (LightShift™ chemiluminescent EMSA kit, Pierce) using 20 fmol of biotin-end-labeled target DNA and 500 ng of *Escherichia coli*-expressed GST-fusion proteins purified over GST-Sepharose (Amersham Pharmacia Biotech). Samples were resolved on native 4% polyacrylamide gels, pre-run for 60 min in 0.5× Tris borate/EDTA, at 100 V and transferred to Hybond XL