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Nuclear Addressing Provides a Clue for the Transforming Activity of Amino-Truncated CCN3 Proteins

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Abstract CCN3 is a founding member of the CCN (Cyr61, Ctgf, Nov) family of cell growth and differentiation regulators. These secreted proteins are key regulators in embryonic development, and are associated with severe pathologies including fibrotic diseases and cancers. CCN3 was discovered as a MAV integration site in an avian nephroblastoma. Previous work established that the amino-truncated protein expressed in this tumor was inducing morphological transformation of chicken embryo fibroblasts, whereas the full-length secreted CCN3 protein was inhibiting cell growth. Amino-truncated variants were identified in cancer cell lines. Since the lack of signal peptide was expected to alter the fate of the truncated proteins, we hypothesized that modifications of CCN3 subcellular addressing could be responsible for the oncogenic activities of CCN3. The CCN proteins are composed of four structural modules (IGFBP, TSP1, VWC, and CT). We report that amino-truncated variants of CCN3 are addressed to the nucleus and that the carboxyterminal (CT) module of CCN3 is responsible for the nuclear addressing. Furthermore, our data identify nuclear CCN3 variants as potential transcriptional regulators. In this context, the CT module confers on nuclear CCN3 proteins a negative regulatory effect on transcription. We propose that the nuclear localization of amino-truncated CCN3 proteins be correlated to oncogenicity. J. Cell. Biochem. 99: 105–116, 2006.

Key words: CCN3-NOV-CCN; nuclear localization; transcription; cell growth; tumorigenesis

The CCN family of proteins consists of six members, which participate in fundamental biological processes including cell adhesion, migration, proliferation, differentiation, and survival [Lau and Lam, 1999; Perbal, 2001; Brigstock, 2003; Planque and Perbal, 2003a; Perbal, 2004; Perbal and Takigawa, 2005]. They are involved in essential events of fetal and adult life, such as angiogenesis, skeletal development, wound repair, and inflammation. Disruption of the *CCN*1 gene is lethal at early stages of development because of failures in placental vascularization [Mo et al., 2002], while knock-out mice for CCN2 show skeletal abnormalities [Ivkovic et al., 2003]. However, the role of the CCN proteins in development is complex, as shown by the fact that CCN6-null mice do not show skeletal defects, while in human CCN6 mutations are associated with pseudorheumatoid dysplasia [Hurvitz et al., 1999; Kutz et al., 2005]. Structural alterations and abnormal expression of CCN proteins are associated with severe pathologies, such as fibrotic disorders and a large variety of cancers [Planque and Perbal, 2003a,b; Perbal, 2004; Bleau et al., 2005]. The CCN proteins share a mosaic structure with four structural modules (Fig. 1), resembling insulin-like growth factor-binding proteins (IGFBP), the Von Willebrand factor type C repeat (VWC), the thrombospondin type 1 repeat (TSP1), and the C-terminal

Abbreviations used: GFP, green fluorescent protein; NLS, nuclear localization signal.

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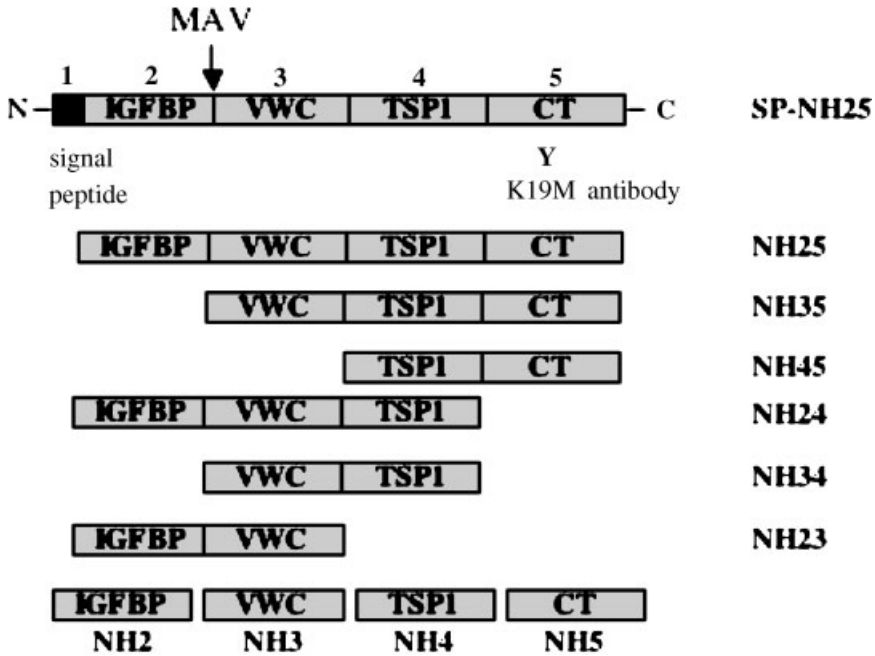

Fig. 1. Schematic diagram depicting the modular organization of the CCN3 protein and the nomenclature used in this study. The amino-truncated variant resulting from the MAV-insertion in a nephroblastoma corresponds to NH35.

domain (CT) of several extracellular proteins [Planque and Perbal, 2003a,b; Perbal, 2004] (Fig. 1).

CCN3 was initially described as *nov*, a gene overexpressed in avian myeloblastosis-associated virus (MAV)-induced nephroblastomas [Joliot et al., 1992]. It is now well established that in addition to its antiproliferative activity originally observed on chicken embryo fibroblasts (CEF) in culture [Joliot et al., 1992], full-length secreted CCN3 protein also inhibits the growth and reduces the tumorigenicity of a large variety of human cancer cell lines, including glioblastoma (G59), choriocarcinoma (Jeg3), and musculoskeletal Ewing's sarcoma (TC71) [Gupta et al., 2001; Gellhaus et al., 2004; Benini et al., 2005; Planque et al., 2005]. Downregulation of CCN3 expression has also been associated with poor tumor prognosis in a variety of cancers [Chevalier et al., 1998; Manara et al., 2002; Planque and Perbal, 2003b; Jiang et al., 2004; Perbal, 2004; Xie et al., 2004; Planque et al., 2005; McCallum et al., in press].

In an avian nephroblastoma, integration of the MAV proviral genome within the *ccn3* gene resulted in the synthesis of an amino-truncated CCN3 protein, deprived of signal peptide and of the IGFBP module (Fig. 1). Forced expression of this truncated protein induced morphological transformation of CEF in culture, whereas the full-length secreted CCN3 protein inhibited CEF growth [Joliot et al., 1992]. An amino-truncated CCN3 protein, with a size of 32 kDa, was also detected in the nucleus of HeLa and 143 osteosarcoma human cancer cells [Perbal, 1999]. Truncated CCN variants have been identified in several other tumors [Planque and Perbal, 2003b; Cervello et al., 2004; Perbal, 2004]. These observations suggested that the expression of truncated CCN proteins was correlated with tumorigenesis [Perbal, 2001, 2004; Planque and Perbal, 2003b]. Since the amino-truncated CCN3 proteins do not contain the secretory signal peptide, their intracellular addressing was expected to be modified.

In this study, we determined the subcellular localization of truncated CCN3 constructs tagged with the green fluorescent protein (GFP) and analyzed the transcriptional activity of various CCN3 variants. Our results establish that, in the absence of signal peptide, the CT module of CCN3 directs the truncated CCN3 proteins to the cell nucleus where they demonstrate a negative effect on transcription via the CT module. These findings suggest that the CT

module is critical to the functions of nuclear CCN3 proteins.

MATERIALS AND METHODS

Constructs

Figure 1 outlines the series of constructs and nomenclature used in this study. The pcDNA-CCN3 vectors were obtained by cloning PCR-amplified CCN3 sequences between the EcoRI and XbaI sites of pcDNA6/V5-His A vector (Invitrogen). The EGFP cDNA (Clontech) was PCR-amplified (5′ TATTGGATCCGCCAC-CATGGTGAGCAAGGGC and CGCCGAATTC-GAGTCCGGACTTGTACAGCTC), digested by BamHI and EcoRI, and inserted upstream to the CCN3 sequences to provide a green fluorescent tag. Myc-tagged CCN3 variants were constructed by inserting, upstream to the CCN3 sequences, the 6 Myc tag repeats contained in the pCMS2 + MT vector (kind gift of Professor K. Lyons). For this purpose, the EcoRI site of the vector was modified into 5′ ATTTAGAAT-TCGGCGGAT 3′ to allow in frame cloning. To construct the red fluorescent tagged CCN3 variant, NH35 DNA was PCR-amplified with 5′ TATTGAATTCGCCACCATGGCGGTAGAG-GGAGATAAC 3′ and 5′ GGACGGATCCGCC-ATTTTCCCTCTGGTAGT 3′ amplimers, digested with EcoRI and BamHI, and inserted in the pDsRedN1 vector (Clontech), upstream to the Dictosoma striata Red protein coding sequence.

The putative CCN3 NLS (5′ TCGAGAGC-AGCCAACAGATAAGAAAGGAAAAAAGTGT-CTCCGCACCAAGAAGTCACTCAAAGCCGA-TATCTAGG 3′) contained in the CT module, was generated by oligonucleotide synthesis (Eurogentec), digested by EcoRI and XhoI, and inserted in the EcoRI-XhoI digested pEGFPc1.

For the transactivation assays in mammalian cells, the CCN3 coding sequences were cloned in frame with the DBD of GAL4 contained in the EcoRI-XbaI digested pFA-CMV vector (Stratagen), previously modified between the EcoRI and HindIII sites (first strand: 5′ AATTGAATTCGATATCTCTAGAA 3′).

All the PCR-amplified clones were sequenced to ensure that no mutation occurred during the amplification.

Cell Culture and Transfections

The BHK21 (baby hamster kidney 21, ATCC CCL10) and G59 (Glioblastoma 59, isolated and provided by Dr. M. Westphal, Hamburg) cell lines were grown in Dulbecco's modified Eagle medium (Sigma) supplemented with 10% fetal bovine serum (FBS) (Sigma) and L-Glutamin-Penicillin-streptomycin solution (Sigma). Jeg3 cells (ATCC HTB-36) were grown in minimal essential Eagle medium (Sigma) supplemented as above. Cell transfections were performed for 48 h in six-well plates in 3 ml of completed medium. For each well, 2 μg of DNA were incubated with 6 μl of EXGEN500 (Euromedex) in a final volume of 100 μl for 15 min at room temperature, according to the manufacturer's instructions.

Immunostaining

Cells were seeded on glass coverslips. Forty eight hour after transfection, cells were fixed in 4% paraformaldehyde in Phosphate Buffer Saline (PBS) for 30 min at 4°C, rinsed with PBS, permeabilized for 5 min with PBS/0.1% Triton X-100. Coverslips were sequentially, incubated in PBS/5% FBS for 1 h at room temperature, incubated in primary antibodies for 1 h in PBS/5% FBS, rinsed four times in PBS, incubated with secondary antibodies for 30 min, rinsed four times in PBS, incubated 5 min with PBS/Hoechst 33258 (Sigma) (1 mg in 250 ml), rinsed in PBS, and finally mounted in Fluoro Gard antifade reagent (Biorad). Primary antibodies: K19M rabbit anti-CCN3 polyclonal serum (1:1,500); mouse monoclonal anti-HIS (Roche) (1:400); mouse monoclonal anti-c-Myc (Roche) (1:500). Secondary antibodies: Alexa-Fluor 594 chicken anti-rabbit; AlexaFluor 488 goat anti-mouse (Molecular Probes) (1:2,000).

Western Blotting

Cells were lysed in 50 mM Tris hydrochloride, pH 8.0, 150 mM NaCl, 5 mM EDTA, and 2% NP40, containing protease inhibitors (Cocktail Tablets, complete, Roche, 1 tablet for 50 ml) and phosphatase inhibitors (50 mM NaF, 2 mM sodium orthovanadate) for 30 min at 4°C. Cell debris were removed by centrifugation at 15,000*g* for 30 min. The protein content of cellular lysates was measured using the BCA Protein Assay kit (Pierce). Laemmli buffer—5% 2-mercaptoethanol was added to 60–100 μg of proteins, boiled for 10 min, and subjected to electrophoresis under reducing conditions in a 12.5% polyacrylamide gel, prior transfer to nitrocellulose (Hybond-C extra, Amersham). The membrane was saturated for 1 h at room temperature with 5% nonfat milk in PBST

(PBS, 0.2% Tween 20), incubated with anti-c-myc antibodies (1:2,000 in PBST, Roche) for 1 h at room temperature, washed extensively with PBST, and incubated with anti-mouse IgG antibodies conjugated with peroxidase (1:10,000, Southern Biotechnology Associates, Inc.) for 1 h at room temperature. After washing with PBST, revelation was performed using a chemiluminescent substrate (Pierce) as recommended by the manufacturer.

Imaging and Quantification

Fluorescent signals were captured with a 5-MHz Micromax 1300Y interline charge-coupled device (CCD) camera (Roper Instruments) as 16-bit images using identical parameters with the Metamorph software (Universal Imaging). For quantification, the images were captured with settings where the intensity of GFP fluorescence was linear. ImageJ version 1.32 software was used to quantify the intensity of GFP fluorescence in the nucleus and the cytoplasm. Relative nuclear localization was reported as the ratio of the intensity of GFP fluorescence in the nucleus and in the cytoplasm (nuclear/cytoplasmic ratio). Statistical significance was evaluated by a *t*-test.

Transactivation Assay in Mammalian Cells

Forty-eight hour after transfection, cells were lysed in the Promega reporter lysis buffer. Fire Fly Luciferase (pFR-Luc, Stratagen) and β-galactosidase activities were measured in a LB Lumat 9507 (Berthold technologies), using respectively the luciferase assay kit by Promega and the Galacto-Star system (Applied Biosystems).

RESULTS

CCN3 Proteins With Deletion of the Signal Peptide Are Addressed to the Cell Nucleus

Amino-truncated CCN3 proteins were previously detected in MAV-induced nephroblastoma cells, in the nucleus of HeLa and 143 osteosarcoma cells, and in normal brain tissues [Joliot et al., 1992; Perbal, 1999; Su et al., 2001]. Furthermore, CCN3 staining was observed in the nucleus of Jeg3 choriocarcinoma cells and C6 glioma cells [Gellhaus et al., 2004, Fu et al., unpublished data]. A few cases of osteosarcomas and Ewing's sarcomas also showed a CCN3 nuclear labeling [Manara et al., unpublished data]. Western blotting of glioma G59-540 and adrenocortical tumor NCI 295R cellular extracts indicated that in addition to the large amounts of full-length CCN3 protein, an amino-truncated CCN3 variant was also produced in these cells [Kyurkchiev et al., 2004]. Since we could not identify alternative splicing of ccn3 RNA in these cells, our observations suggested that post-translational processing of the CCN3 protein was responsible for the accumulation of truncated variants. In order to unequivocally establish whether a relationship might exist between the expression of truncated CCN3 variants and the detection of CCN3 proteins in the nucleus of tumor cells, we analyzed the fate of various CCN3 recombinant proteins by immunocytochemistry.

Baby Hamster Kidney 21 (BHK21) cells were transiently transfected with the NH35 plasmid DNA which encodes an amino-truncated CCN3 protein similar to the one expressed in MAV-induced nephroblastoma and in tumor cells. Immunocytochemistry performed with the K19M antibody indicated that the amino-truncated CCN3 protein was localized in the nucleus of transfected cells (Fig. 2). In control cells transfected with full-length CCN3 expression vector (SP-NH25), the CCN3 protein was detected at the cell membrane and in the cytoplasm (Fig. 2). Since the expression of a full-length CCN3 protein deprived of signal peptide (NH25) also resulted in nuclear staining (Fig. 2), these observations indicated that the absence of secretory signal peptide permitted nuclear addressing of CCN3 variants.

The CT Module of CCN3 Directs Amino-Truncated CCN3 Proteins to the Nucleus

To further investigate which part of the CCN3 protein is involved in the nuclear localization, we constructed a series of plasmids in which the GFP ORF was cloned in frame with different combinations of the CCN3 modules (Fig. 1). The autofluorescence of GFP permitted the detection of the fusion proteins and established that all the recombinants that contained the CT module (GFP-NH25, -NH35, -NH45, -NH5) were addressed to the nucleus (arrows in Fig. 3, left column). These nuclear proteins were also detected by the K19M antibody (Fig. 3, right column). Staining of cell nuclei with DAPI (Fig. 3, middle column) allowed to confirm the nuclear subcellular localization of the proteins. On the contrary, proteins lacking the CT module (GFP-NH24, -NH34) were detected only

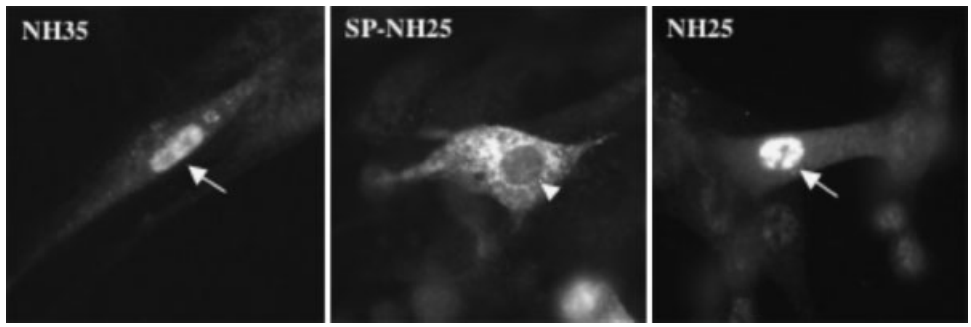

Fig. 2. Immunocytolabeling of CCN3 recombinant proteins by K19M antibodies in transiently transfected BHK21 cells. A nuclear staining is observed in cells transfected with NH35 and NH25 (arrows), in contrast to the transfected cells expressing the full-length secreted form of CCN3 (SP-NH25) (arrowhead).

in the cytoplasm, as the control GFP alone (compare panels M, P, and S). The nuclear localization of amino-truncated CCN3 variants was also observed in cells transfected with Myc-tagged CCN3 variants (Fig. 4A). Recombinant CCN3 variants with the expected size were indeed produced and could be detected in transiently transfected cells (Fig. 4B). Cells transfected with a plasmid expressing a NH35-CCN3 recombinant protein tagged at its carboxyterminus with the Discosoma striata Red protein (DsRed), also showed nuclear addressing of the truncated CCN3 protein (Fig. 5). Altogether, these results indicated that the subcellular localization of CCN3 variants in transfected cells was affected neither by the nature nor the position of the various tags used in this study.

Considering that the NH25 protein—which is deprived of signal peptide—was addressed to the nucleus, and that the NH24 protein—which is deprived of both signal peptide and CT module—was localized in the cytoplasm, we concluded that, in the absence of signal peptide, the CT module was responsible for nuclear addressing. Moreover, the CT module alone (NH5) was also addressed to the nucleus (Fig. 3, panels J, K, L). A putative nuclear localization signal (NLS) (PTDKKGKKCLRTKKSLKAIHLQFK) is localized at position 280–303 of the CT module. When this basic amino acids-rich region was cloned downstream to the GFP coding sequence, the recombinant GFP protein was partially addressed to the cell nucleus (Fig. 6A). Quantitative analysis of the nuclear and cytoplasmic fluorescence signals showed that the putative NLS localized in the CT module was indeed able to drive nuclear addressing (Fig. 6B). Inasmuch as a slight cytoplasmic staining was also observed with the GFP-NH5, NH35, and NH25 (Fig. 3), nuclear addressing of these proteins is not fully efficient. It is possible that fine-tuning of biological effects associated to the nuclear localization of CCN3 variants requires a relatively weak NLS activity.

Nuclear Variants of CCN3 Act as Transcription Repressors

CCN3 physically interacts with the rpb7 subunit of RNA polymerase II in a yeast two-hybrid assay [Perbal, 1999] and the CT module of CCN3 binds to a specific sequence in the human promoter of the Plasminogen Activator Inhibitor-2 (PAI-2) [Mahony et al., 1999]. To investigate the potential role of CCN3 in the regulation of transcription, quantitative assays were performed in mammalian cells. We used pFA-CMV-derived plasmids that express amino-truncated CCN3 variants fused in frame with the DNA binding domain of Gal4 (pFA-NH). A six histidine tag (6HIS) was added at the C-terminal part of the fusion proteins to allow immunological detection of the recombinant proteins. The use of anti-6HIS antibodies confirmed that all the chimaeric proteins were synthesized at satisfactory levels and were localized in the nucleus of transfected cells (not shown). For the transactivation assays, cells were co-transfected with one of the pFA-NH expression plasmids, the pFR-Luc reporter plasmid in which the luciferase gene transcription is under the control of 5 Gal4 binding sites, and a lacZ vector for β-galactosidase normalization. The results obtained in BHK21 cells established that amino-truncated variants

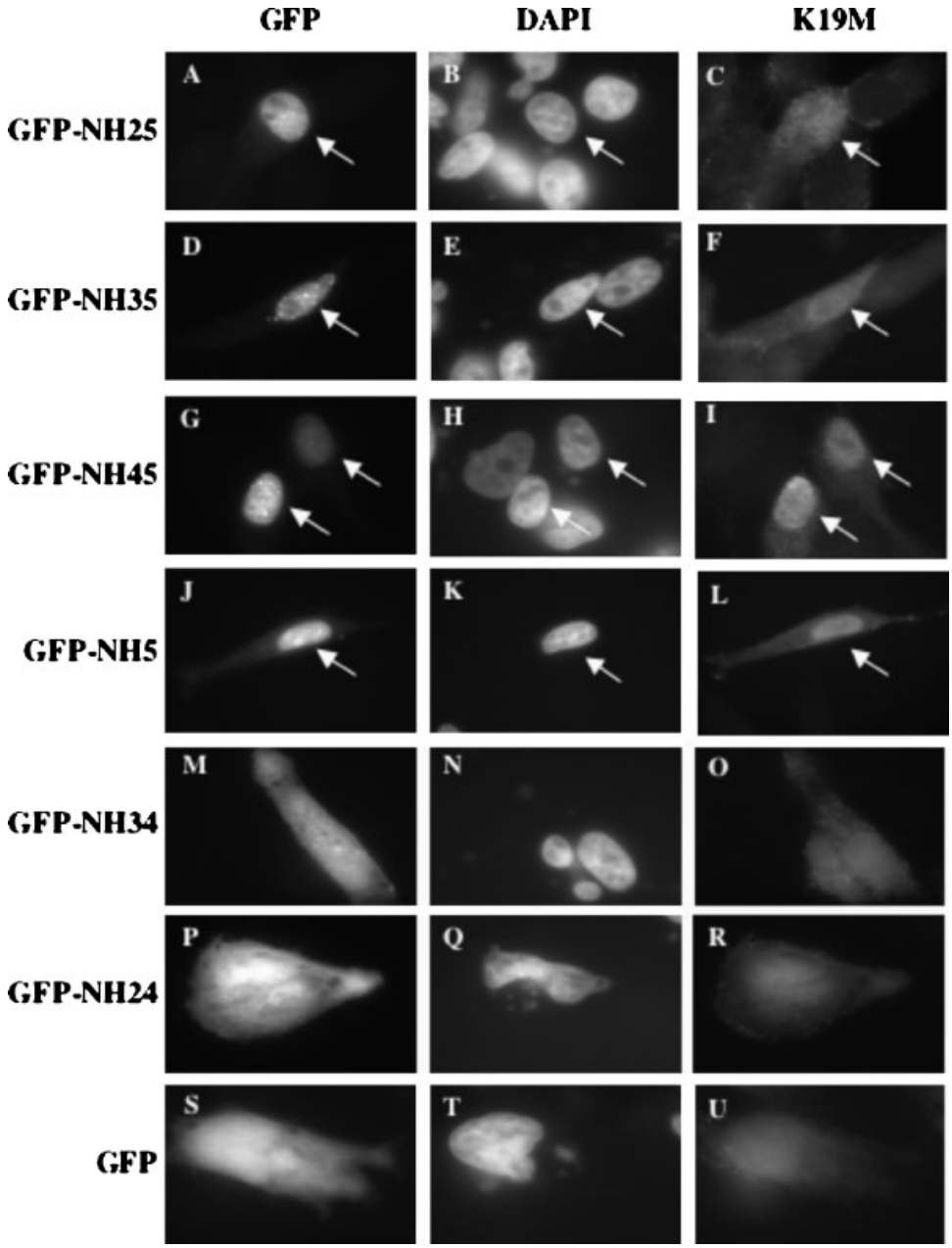

Fig. 3. Subcellular localization of GFP-tagged CCN3 proteins in transiently transfected G59 cells. GFP, direct detection of fluorescence. DAPI, staining for nucleus. K19M, alexafluor 594 staining anti K19M antibodies. The arrows show the nucleus of transfected cells.

similar to the ones expressed in MAV-induced nephroblastoma and in tumor cells can inhibit transcription in mammalian cells (Fig. 7A). Furthermore, the successive deletions of the modules IGFBP, VWC, and TSP1, and the 76% reduction of luciferase activity observed with the NH5 construct alone indicated that the inhibitory domain lies in the CT module (Fig. 7A). Furthermore, the NH24 construct which is lacking the CT module did not inhibit transcription. Dose-response experiments confirmed that the inhibitory effects of CT were specific. Indeed, the level of transcription inhibition induced by the CT module increased with the quantity of NH35 DNA used in the experiment (Fig. 7B).

Similar results were obtained with Jeg3 choriocarcinoma (Fig. 7C) and G59 glioblastoma

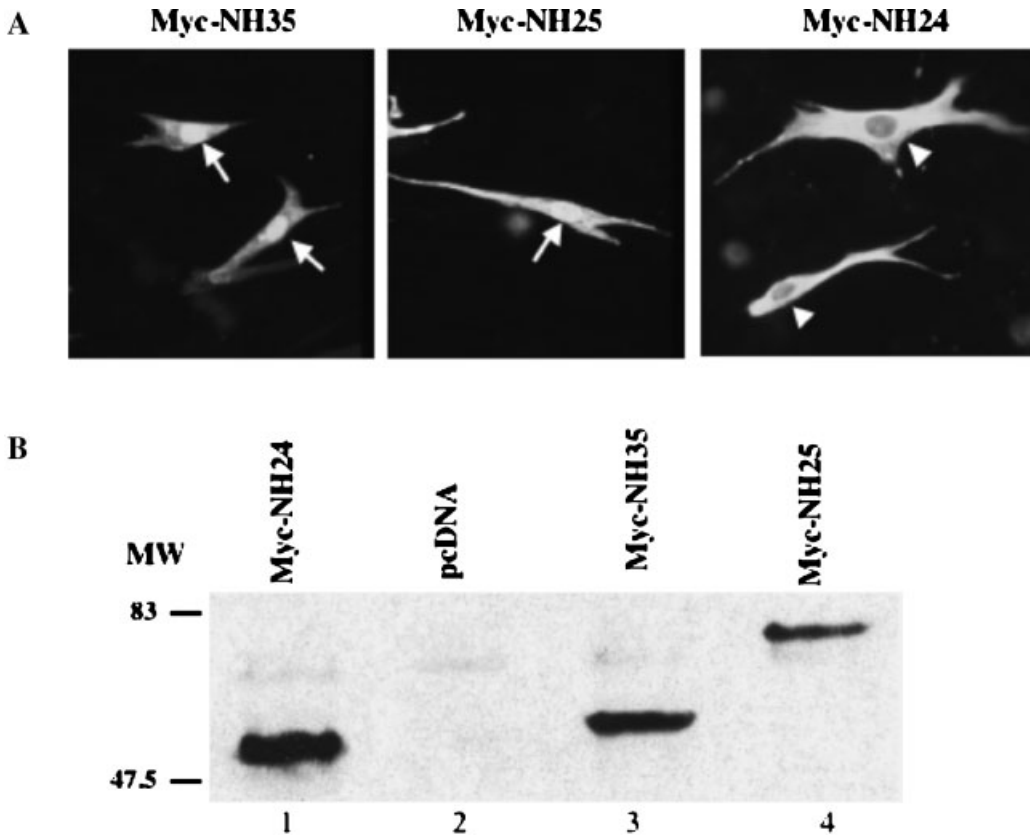

Fig. 4. **A**: Immunocytolabeling of CCN3 recombinant proteins tagged at their N-terminal part with a Myc epitope. Detection by Myc antibodies in transiently transfected BHK21 cells. A nuclear staining is observed in cells transfected with NH35 and NH25 (arrows), in contrast to the transfected cells expressing NH24 (arrowhead). **B**: Western blot analysis of CCN3 recombinant proteins expressed in transiently transfected G59 cells. G59 cells were collected 48 h after transfection with plasmid DNA expressing tagged CCN3 constructs. Cellular proteins were analyzed by Western blotting with cMyc antibodies. Broadrange molecular weight markers were from Biolabs. Cells were transfected with Myc-NH24 (lane 1), pcDNA control vector (lane 2), Myc-NH35 (lane 3), and Myc-NH25 (lane 4).

cells (data not shown), indicating that the effects of the CCN3 variants on transcription are observed in different cellular contexts.

In conclusion, the presence of the CT module (contained in NH5, NH25, NH35, NH45) resulted in a downregulation of the reporter promoter activity. These results were in agreement with the data that we obtained in yeast (not shown), and suggested that the nuclear CCN3 variants detected in tumors act as negative transcriptional regulators.

DISCUSSION

A growing body of evidence supports the idea that the full-length secreted CCN3 protein acts

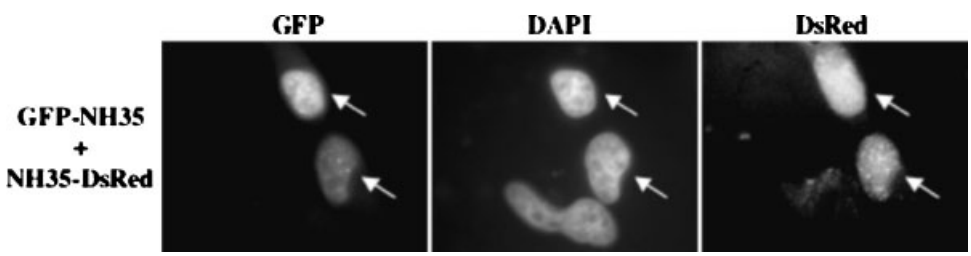

Fig. 5. Nuclear localization of GFP- and DsRed-tagged NH35 CCN3 proteins in transiently transfected G59 cells. GFP, direct detection of green fluorescence; DAPI, staining for nucleus. DsRed, direct detection of red fluorescence. The arrows show the nucleus of transfected cells.

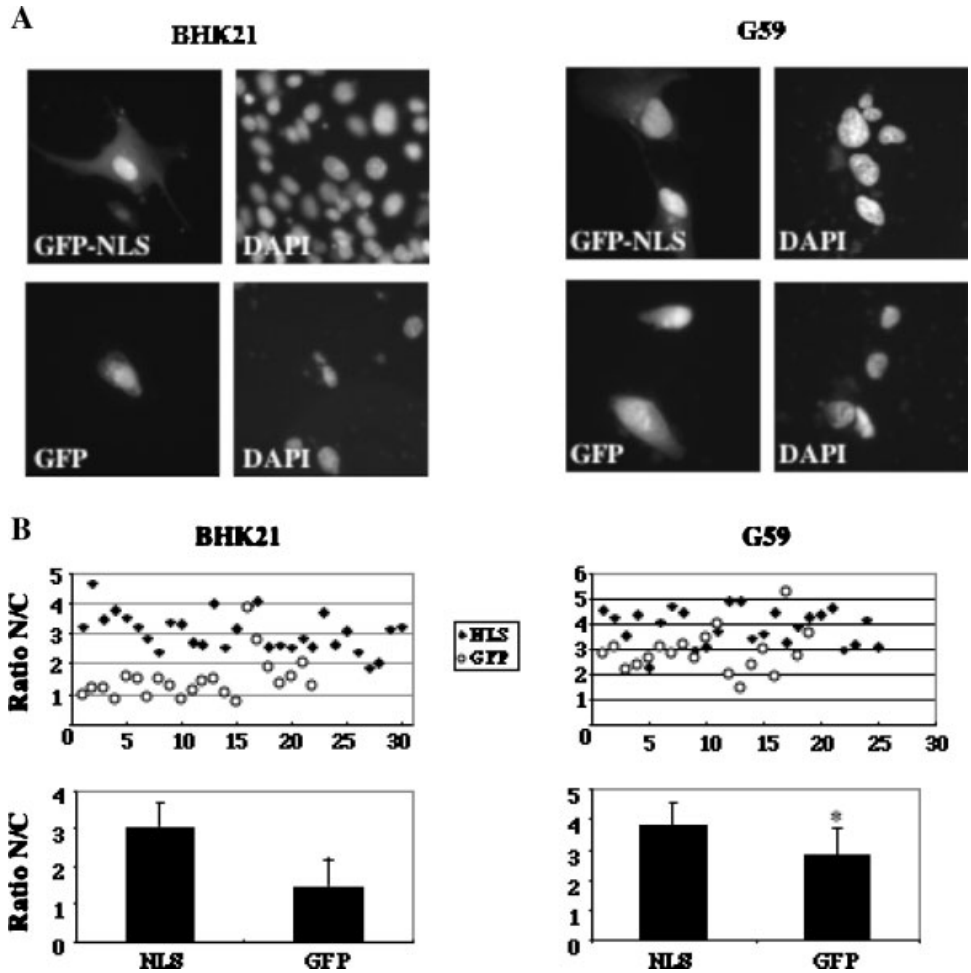

Fig. 6. Distribution of the GFP fluorescence in BHK21 and G59 cells transiently transfected with GFP-NLS and GFP alone. **A**: Fluorescence microscopic images of transfected cells. **B**: The GFP signal was measured using the ImageJ software on 16-bit images recorded with the Metamorph software. For each one of the individual cells that were considered, the relative nuclear localization value was expressed as the ratio of the GFP fluorescence signals measured in the nucleus and in the cytoplasm (nuclear/cytoplasmic N/C ratio). The upper histograms represent the N/C ratio for each one of the individual cells. The lower histograms represent the mean values of N/C ratios for the total number of cells. Statistical significance was evaluated by a *t* test, $P = 0.021\%$ in G59 cells (*).

as a negative regulator of cell proliferation [Joliot et al., 1992; Gupta et al., 2001; Gellhaus et al., 2004; Benini et al., 2005; Bleau et al., 2005; Planque et al., 2005; Bleau et al., submitted], whereas amino-truncation of CCN3 has been associated to oncogenic transformation [Joliot et al., 1992] and cancer development [Planque and Perbal, 2003a; Perbal, 2004].

In this study, we showed that CCN3 proteins deprived of signal peptide are directed to the nucleus where they might be involved in the negative regulation of transcription. Nuclear CCN3 proteins were detected by immunofluorescence in several human cancer cell lines, including HeLa, osteosarcoma 143 and choriocarcinoma Jeg3, in rat C6 glioma cells, as well as in some cases of osteosarcomas and Ewing's sarcomas [Perbal, 1999, 2001; Gellhaus et al., 2004; Fu et al., unpublished data; Manara et al., unpublished data]. Furthermore, electron microscopy studies detected CCN3 both in the cytoplasm and at the nuclear pores of adrenocortical tumor cells [Thomopoulos et al., 2001]. In HeLa and 143 cells, Western blot analysis revealed an amino-truncated nuclear CCN3 protein containing the CT module [Perbal, 1999]. An amino-truncated variant of CCN3 was also expressed in a MAV-induced nephroblastoma [Joliot et al., 1992]. In the latter case, it was shown that this variant protein induced

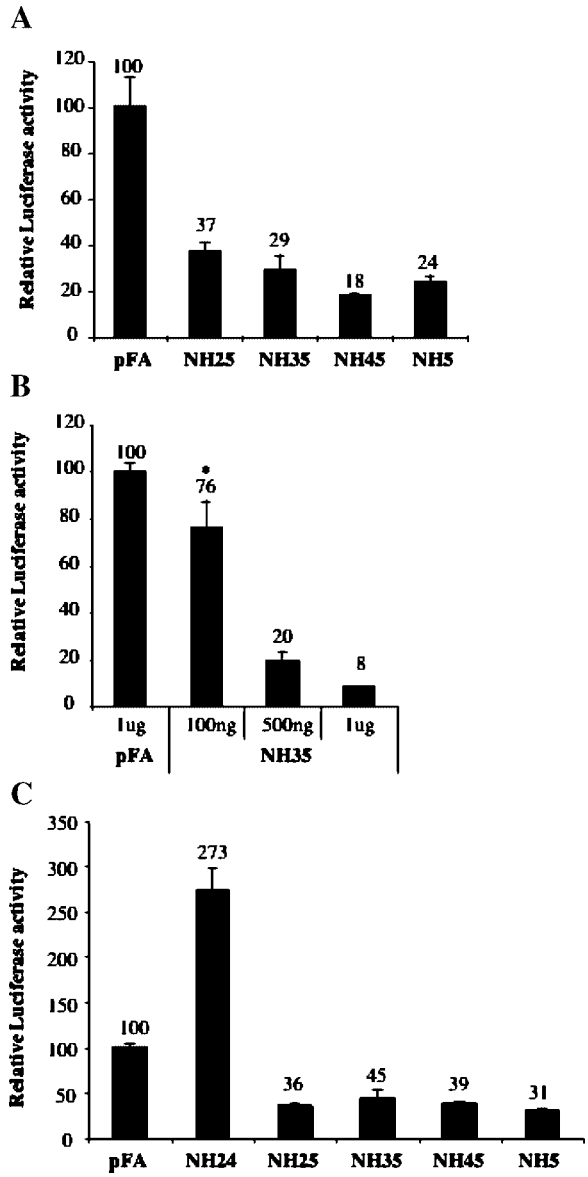

Fig. 7. Transcription assays in mammalian cells. **A**: BHK21 cells were transiently cotransfected with 1 μg of each pFA-NH expression plasmid, 1 μg of the pFR-Luc reporter plasmid, and 50 ng of the pcDNA3-LacZ plasmid. The results were normalized with respect to β-galactosidase activity. Luciferase activities were expressed relative to the values for the pFR-Luc with the pFA vector (set to a value of 100%). Standard deviations (bars) were calculated from transfections performed in duplicate. The mean value is indicated above each histogram. Statistical analyses: $P < 0.02$. Two independent experiments were performed. **B**: BHK21 cells were cotransfected with 1 μg of pFR-Luc and increasing amounts of NH35. The total amount of DNA used for each transfection was kept constant by adjustment with pFA DNA. $P < 0.02$ except for *, $P = 0.07$. **C**: Human choriocarcinoma Jeg3 cells were transfected as described in the panel A ($P < 0.02$).

morphological transformation of normal CEF in culture [Joliot et al., 1992]. These observations suggested that truncated nuclear CCN3 proteins were responsible, at least in part, for abnormal growth of tumor cells.

The results reported in this study suggest that nuclear CCN3 variants participate to the regulation of transcription. Even though the CT domain of CCN3 interacts with the promoter of the plasminogen activator inhibitor-2 (PAI-2) [Mahony et al., 1999], we found that CCN3 did not regulate directly the expression of a reporter gene driven by the PAI2 promoter (data not shown). These results indicate that CCN3 might act in concert with other regulatory proteins that are involved in the control of transcription, in a similar manner as general co-repressors of transcription. CCN3 could act as a docking protein between the basal transcription machinery and chromatin remodeling complexes. The physical interaction of CCN3 with subunit 7 of RNA polymerase II [Perbal, 1999], and the colocalization of CCN3 with ICP4 in Herpes Simplex-infected cells [Perbal, 2001], are in favor of CCN3 acting on the transcription complex.

Interestingly, CCN2/CTGF, another member of the CCN family, was routed to the perinuclear compartment of mesangial cells when added ectopically, and was able to stimulate general transcription in cell-free systems [Wahab et al., 2001]. These results therefore indicated that nuclear import also occurs with other CCN proteins.

Nuclear localization of secreted regulatory proteins is not an epiphenomenon. Transport of secreted or transmembrane proteins to the cytoplasm and nucleus has been documented for a variety of protein families including growth factors, hormones, cytokines, transmembrane receptors [Olsnes et al., 2003]. The nuclear import of Insulin-like growth factor binding proteins (IGFBP)-3 and -5 is dependent on a NLS pathway mediated by importin-β [Lee and Cohen, 2002]. In the case of fibroblast growth factor family members (FGF-1, -2, and -3), nuclear addressing also involves NLS-dependent pathways [Olsnes et al., 2003]. In this work, we have identified a basic amino acid-rich sequence in the CCN3 protein that can drive recombinant GFP protein to the nucleus, therefore suggesting that an NLS-dependent pathway mediates the nuclear import of CCN3 variants.

The relatively low efficiency of the CT NLS might provide a mean for fine-tuning the amount of nuclear variants that are showing a marked inhibitory action on transcription. It is quite possible that the nuclear CCN3 variants act as a dominant system since even in cancer cells that contain the amino-truncated nuclear CCN3 variant, the full length extracellular CCN3 protein is the predominating component. In addition, the effects of both types of proteins likely requires distinct signaling pathways involving receptors and transporters whose bioavailabilty may be dependent upon physiological environment.

A growing body of evidence shows that a large number of secreted regulatory proteins directly regulate transcription. FGF2 was shown to stimulate transcription in cell-free systems [Nakanishi et al., 1992]. Externally added lactoferrin was described to enter cells, bind to specific sequences in DNA, and to activate transcription [He and Furmanski, 1995]. The transmembrane receptor for the epidermal growth factor (EGFR) was reported to be localized in the nucleus of high proliferative cells, to contain a transcription transactivation domain in its C-terminal part, and to bind to AT-rich sequences found in the promoter of the Cyclin *D1* gene [Lin et al., 2001]. IGFBP-3 was found to interact with the nuclear retinoid X receptor (RXR) [Lee and Cohen, 2002], which is a member of the family of transcription factors that bind to steroid hormones. IGFBP-3 and RXR ligands may cooperate in inducing gene transcription leading to apoptosis [Liu et al., 2000]. The nuclear accumulation of FGF2 correlates with transcription of ribosomal genes [Olsnes et al., 2003].

Amino-truncated CCN3 variants deprived of signal peptide might be generated by chromosomal rearrangements and translocations that occur frequently in cancer cells. Alternatively, one cannot exclude the possibility that intra-cellular amino-truncated CCN3 might be generated by proteolysis of the secreted full-length CCN3 and internalization of the resulting fragment [Perbal et al., 1999; Perbal, 2001]. It is well known that cancer cells show increased proteolytic activities [Perbal, 1984]. Along this line, it will be interesting to identify the proteolytic system, which is responsible for the well documented cleavage of CCN proteins between modules 2 and 3, and to establish whether the activity of this proteolytic system is increased upon cell transformation.

Amino-truncated CCN3 proteins have also been detected in normal tissues including myoblasts, cerebrospinal fluid, normal brain tissues, and astrocytes [Su et al., 2001; Fu et al., 2004; and our unpublished results].

Considering that the secreted full-length CCN3 protein possesses antiproliferative activity, and that truncated CCN3 variants promote increased proliferation, we propose a model in which an unbalanced production of CCN3 isoforms might ultimately contribute to oncogenic transformation. Recent data obtained with Jeg3 choriocarninoma cells support this model. A nuclear CCN3 staining was detected in the tumorigenic Connexin43-negative Jeg3 cells. Upon stable expression of Cx43 and decreased tumorigenicity of these cells, the CCN3 protein was then detected in the cytoplasm and at the plasma membrane where it is colocalized with Cx43 [Fu et al., 2004; Gellhaus et al., 2004].

Nuclear addressing provides a strong support to the transforming activity of the amino-truncated CCN3 variants expressed in tumor cells. Whether other CCN variants whose expression has been associated with tumorigenesis [Planque and Perbal, 2003b; Cervello et al., 2004; Perbal, 2004] also show a similar subcellular localization is a challenging question.

The dual situation that we described for CCN3 proteins resembles the case of FGF3, in which secreted and nuclear forms show opposite effects on the cell cycle. Whereas the secreted FGF3 stimulates cell growth and transformation, the nuclear form inhibits DNA synthesis and proliferation [Kiefer and Dickson, 1995], through a physical interaction with the NoBP protein [Reimers et al., 2001].

To date, studies carried out to understand the roles of the CCN proteins have mostly focused on deciphering outside in signaling pathways. The studies presented here open new fields of investigation into the regulatory functions played by the CCN proteins. Deciphering the mechanisms underlying the nuclear localization of the CCN3 proteins and identifying their functions will be of prime importance for the understanding of the various functions attributed to these new regulators of cell growth and differentiation.

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