BITS AND BYTES

Alternative splicing of CCN mRNAs …. it has been upon us

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Abstract Variant CCN proteins have been identified over the past decade in several normal and pathological situations. The production of CCN truncated proteins have been reported in the case of CCN2(ctgf), CCN3(nov), CCN4(wisp-1) and CCN6(wisp-3). Furthermore, the natural CCN5 is known to miss the C-terminal domain that is present in all other members of the CCN family of proteins. In spite of compelling evidence that assign important biological activities to these truncated CCN variants, their potential regulatory functions have only recently begun to be widely accepted. The report of CCN1(cyr61) intron 3 retention in breast cancer cells now confirms that, in addition to well documented post-translational processing of full length CCN proteins, alternative splicing is to be regarded as another effective way to generate CCN variants. These observations add to a previous bulk of evidence that support the existence of alternative splicing for other CCN genes. It has become clearly evident that we need to recognize these mechanisms as a means to increase the biological diversity of CCN proteins.

Keywords CCN1 · CCN2 · CCN3 · CCN4 · CCN5 · CCN6 · Alternative splicing · Variant proteins

Introduction

The production of CCN proteins lacking one or more of the four structural modules (IGFBP, VWC, TSP and CT) that constitute the prototypic CCN protein (Bork 1993;

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Holbourn et al. 2008) has already been discussed (Perbal 2001, 2004; Planque and Perbal 2003).

Aside from the natural CCN5 protein that does not contain a CT domain (Perbal 2001), several other examples of truncated CCN proteins were identified in biological fluids, cell culture lysates, cell culture medium, and normal or tumor tissues (Perbal 2001, 2006; Leask and Abraham 2006; Holbourn et al. 2008).

In the case of CCN2, the production of short variants in pig uterine flushings was hypothesized to result from proteolytic digestion of the full length protein (Brigstock et al. 1997). Indeed, inter-domain regions that show much less organization than the modules themselves (Holbourn et al. 2008) may be the target for proteolytic activity as shown by the cleavage of CCN2 by the MMP2 metalloproteinase at the junction between VWC and TSP1 modules (Dean et al. 2007).

A truncated CCN3 variant deprived of both the IGFBP domain and the signal peptide that drives secretion of the CCN proteins (Joliot et al. 1992; Perbal 2001, 2004) was expressed in nephroblastoma tumor cells, because of myeloblastosis associated virus (MAV) insertional mutagenesis in the genome of the blastemal target chicken cells (Li et al. 2006).

CCN3 truncated isoforms that are deprived of the IGFBP and VWC modules and contain only the last two C-terminal modules (TSP1 and CT) were identified in most cultured cells and tissues which also express the full length protein (Perbal 1999, 2006; Su et al. 2001; Kyurkchiev et al. 2004; Bleau et al. 2007; Vallacchi et al. 2008).

N-terminal sequencing of the truncated CCN3 protein that is contained in the cell culture medium of insect cells infected with a recombinant CCN3 baculoviral vector (Perbal et al. 1999) established that it was generated by a proteolytic cleavage occurring between domain II (VWC)

and III (TSP1) of the full length CCN3 protein. Since the cleavage site was found to be identical to that used in the case of CCN2 , we suggested that a common specific protease might be involved in the processing of CCN proteins which generated these variants.

Production of CCN variants via alternative splicing

The production of rearranged CCN variants as a result of alternative splicing has also been documented for many times over several years.

Indeed, a CCN4 protein lacking the VWC module II (WISP1v) was detected in scirrhous gastric carcinoma cells. This variant is encoded by a 840 nucleotide alternatively spliced mRNA species, missing the 260 nucleotides of exon 3 that encode VWC module sequences in the wild type mRNA species (Tanaka et al. 2001).

Both a similarly spliced message and rearranged variant CCN4 protein were detected in invasive cholangio-carcinoma (Tanaka et al. 2003) and in the human chondrosarcoma-derived chondrocytic cells HCS-2/8 (Yanagita et al. 2007). Interestingly, another spliced variant expressed in these cells was found to encode a single IGFBP module in which eight authentic aminoacids at the C-terminus were replaced by 14 other residues (Yanagita et al. 2007).

In addition to the expected full length transcript of WISP1/CCN4 (1,204 bp), two shorter transcripts of 943 bp and 750 bp were identified in the human hepatoma HuH-6 and HA22T/VGH cell lines (Cervello et al. 2004). Sequence analysis of the purified 943-bp fragment revealed that this variant lacks exon 3. Since the joining of exons 2 and 4 did not result in any reading frame shift, the variant mRNA species also encoded a CCN4 protein lacking the VWC module as previously described by Tanaka et al. (2001, 2003)

Exons 3 and 4 were not contained in the CCN4 750-bp spliced variant, and the frameshift that was created by the joining of exons 2 and 5 in this RNA resulted into a premature translation arrest 38 residues downstream. As a consequence, the CCN4 variant protein expressed by this spliced message encoded only the IGFBP module.

A longer CCN4 transcript containing an insertion of 64 bp between exons 3 and 4 was detected In HuH-6 and HuH-7 human hepatoma cells. The insertion of this short stretch caused a frameshift at residue 197 that resulted in a premature translation stop 22 residues downstream. As a consequence, a half CCN4 protein containing only the two N-proximal IGFBP and VWC domains, was encoded by this spliced mRNA species.

The first evidence suggesting the existence of CCN1 (cyr61) alternatively spliced messages was obtained in my laboratory in the course of a study aimed at identifying the chromosomal localization and expression of CCN1 in human neuroblastoma and glioblastoma cell lines (Martinerie et al. 1997). Because the full length cDNA clone mapped only one chromosomal location at 1p22-p31, we proposed that the additional 3.5 kb CCN1 mRNA species that was detected in a few cell lines in addition to the "canonical" 2.5 kb mRNA likely corresponded to an alternatively spliced message.

Since the focus of our studies was on the CCN3 gene and protein, we did not pursue the identification of this additional mRNA species.

A few years later, another example of an alternatively spliced CCN1 mRNAs species was documented in the case of serum-induced normal human fibroblasts, which were shown to express a CCN1 message in which an in frame deletion within exon 4 resulted in the production of a CCN1 protein deprived of the TSP1 module (Leng et al. 2002).

The results that have now been reported by Hirschfeld et al. (2009) confirm the existence of alternatively spliced CCN1 mRNA species. Interestingly, the new message that is identified in human breast cancer cell lines resulted from retention of the 131 nucleotide intron 3 that separates the exons encoding the VWC and TSP1 domains of CCN proteins. Since this intron contains two stop codons, the authors propose that the alternatively spliced message does not encode a full length protein.

The organization of the various alternatively spliced CCN mRNAs identified thus far is depicted in Fig. 1.

In the case of CCN3, several observations suggested that variant CCN3 proteins might be produced in a regulated way in both normal and pathological conditions.

Large amounts of a CCN3 32–38 kDa doublet were detected in the brain lysates of adult rats in which small amounts of the full length CCN3 protein were detected (Su et al. 2001). Since these short variants were detected by the K19M antibody— that was raised against the C-terminal end of CCN3—we assumed that they were composed of the two last domains (TSP1 and CT) and that they might be generated through alternative splicing.

In transfected glioma cells producing exogenous CCN3, a large amount of a half CCN3 protein was also detected in the cytoplasmic fraction in addition to the truncated form that is usually secreted in the culture medium of cells producing CCN3 (Kyurkchiev et al. 2004).

Although we did not investigate the mechanisms leading to the production of the intracellular variant CCN3 species, all these findings suggested the existence of two CCN3 variant species that might result from two different mechanisms: post-translational processing of the secreted full length protein and alternative splicing leading to the intracellular short species.
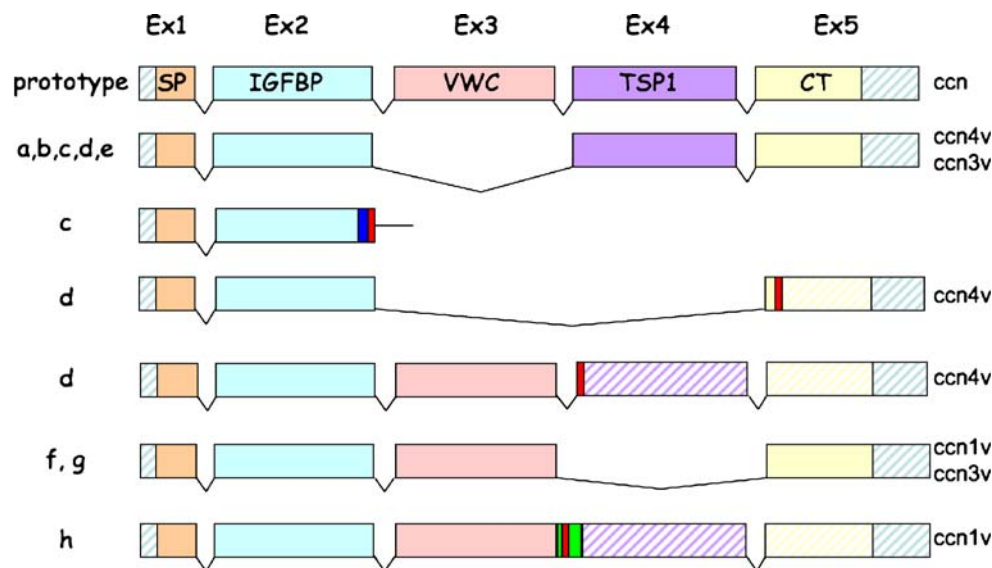
Fig. 1 Schematic organization of the various CCN spliced variants which have been described thus far. The prototypic structure of the CCN proteins (except fro CCN5 lacking CT) is represented on the top, with the five exons that encodes, the signal peptide (SP) and the Von Willebrand (VWC), thrombospondin (TSP1) and Cterminal (CT) domains (see Holbourn et al. 2008). Alternative splicing leading to variant proteins has been identified for CCN1 CCN4, CCN3. In the case of ccn1 and ccn4, the structure of the alternatively spliced messages has been established. Variant proteins resulting from alternative splicing have been identified in scirrhous gastric carcinoma (**a**), cholangiocarcinoma (**b**), HCS-2⁄8 human chondrosarcoma-derived chondrocytic cells (**c**), human hepatoma cells (**d**) Ewing's tumors (**e**), human fibroblasts (**f**), Wilm's tumors and normal human embryonic kidneys (**g**), human breast tumors (**h**). See text for details

Aside from these two pieces of indirect evidence, we recently identified CCN3 variant proteins that most likely result from alternative splicing. In addition to the full length CCN3 protein, Wilm's tumors and normal human embryonic kidneys also expressed a CCN3 variant that was deprived of the TSP1 domain (Subramaniam et al. 2008). Also, 50% of Ewing's tumor cells were found to express a truncated CCN3 protein species lacking the VWC module (Perbal et al. Submitted).

Biological significance of CCN alternative splicing

As previously discussed (Perbal 2001, 2004) the multi-modular structure of the CCN proteins raises an interesting question as to the contribution of each module to the biological function(s) of the fully assembled protein. Either the activities of each module sum up or they confer on the whole protein specific functions that might substitute or add to the function of the individual modules.

The present consensus is to view the biological properties of the various CCN proteins as the result of both individual module activities and functional interactions between different modules (Leask and Abraham 2006; Yeger and Perbal 2007; Irvine et al. 2008).

In view of the complex array of regulatory factors and receptors that physically interact with CCN proteins, the production of variants deprived of one or more elementary module is expected to have profound biological effects. Not only can variant CCNs titrate receptors and other partners interacting with each individual module, and thereby interfere with biological activity of the full length proteins, but also, the absence of a single module might induce conformational changes that could potentially modulate, either positively or negatively, the intrisic biochemical functions of the resulting CCN protein.

Various biological activities were assigned to the CCN variants. The amino-proximal half of CCN2 (which contains only the IGFBP and VWC domains) was reported to be an effective surrogate biomarker for fibrosis (Leask et al. 2009) and to mediate both myofibroblast differentiation and collagen synthesis whereas the C-terminal half (composed of the TSP1 and CT domains) mediated fibroblast proliferation (Grotendorst and Duncan 2005). More recently, the recombinant IGFBP and VWC modules were reported to display stronger binding to aggrecan compared to recombinant TSP1 and CT modules (Aoyama et al. 2009).

In the case of CCN1, a mutant lacking the CT domain was unable to promote cell adhesion but had conserved the chemotactic and growth-factor promoting activities of the full CCN1 protein (Grzeszkiewicz et al. 2001)

The amino-truncated version of CCN3 (containing the three first modules) that was cloned from MAV-induced nephroblastoma exhibited transforming properties in chicken embryo fibroblasts whereas, the full length CCN3 showed growth inhibitory effect (Joliot et al. 1992; Planque et al. 2006).

Since half proteins show such specific biological properties, it is surprising that neither Hirschfeld and colleagues, nor the reviewers who evaluated their manuscript, considered the possibility that the truncated protein likely expressed from the CCN1 mRNA species which retain intron3—as stated by the authors themselves—might play a critical role in the breast cancer cells that contains this spliced variant. The use of domain specific CCN1 antibodies that are available would have permitted to address this important question. This is especially critical in the context of this work, since the authors reported exon 3 skipping as a way to produce the full length active CCN1 protein (Hirschfeld et al. 2009).

In other words, the strong correlation that was observed between the intron 3 skipping and the invasive breast cancer phenotype might have resulted from the production of a full-length CCN1 in these cells, whereas in non-cancerous tissues, only the amino-proximal half CCN1 was expressed.

These authors also report that the switch from intron retention to intron skipping was induced by hypoxia.

This observation is in line with other results showing that alternative splicing of CCN mRNAs is tightly regulated and might affect the tumorigenic potential of cancer cells.

Hence, the use of domain-specific antibodies (Lazar et al. 2007) allowed us to establish that CCN3 variants lacking the TSP1 domain were expressed in Wilm's tumors and that in normal kidneys, the production of CCN3 lacking the TSP1 domain is developmentally regulated (Subramaniam et al. 2008).

Furthermore, we established that in the case of Ewing's tumors, the increased level of variant CCN3 in tumor cells reduces their tumorigenic potential, and results in better outcome.

Conclusion

The existence of alternative splicing leading to the production of variant CCN proteins is well documented and should be regarded as a means to increasing the biological functions of this fascinating family of proteins.

Whether the variant forms that were detected in normal and pathological conditions antagonize or synergize the functions of full length CCN proteins remains to be clarified. In some cases, the biological activities of variant proteins were associated with particular phenotypes, especially in the tumor cells in which these variants were identified. However, in other cases the origin of the variants is not clearly established even though it is highly probable that alternative splicing is responsible for their production.

When we first established that CCN3 was involved in the development of the human brain (Su et al. 2001), we used the K19M polyclonal antipeptide that is directed against the C-terminal end of CCN3 (Chevalier et al. 1998). As a consequence, immunocytochemistry experiments performed with this antibody did not permit us to distinguish between a positive staining due to the presence of a full-length or an amino-truncated CCN3 protein. Neither was the K19M antibody able to identify the variant forms lacking one particular domain. Only the detection of large quantities of a low molecular weight CCN3 variant in rat brain lysates (Su et al. 2001) raised the possibility that alternative splicing was involved and that the short amino-truncated CCN3 might play a critical role in this tissue at this particular developmental stage.

The recent results that we have obtained in the course of study performed with Ewing's tumors and Wilm's tumors, established that domain-specific antibodies such as those we derived for CCN3 are invaluable tools to identify all the CCN isoforms contained in cells and tissues.

The existence of variants generated by both posttranslational processing and alternative splicing can no longer be ignored by the scientific community.

Future progress and understanding of the role of CCN proteins in normal and pathological conditions will rely on the thorough characterization of all isoforms that requires the use of appropriate biological tools.

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