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Review

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CCN3 and calcium signaling

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Abstract

The CCN family of genes consists presently of six members in human (CCN1-6) also known as Cyr61 (Cystein rich 61), CTGF (Connective Tissue Growth Factor), NOV (Nephroblastoma Overexpressed gene), WISP-1, 2 and 3 (Wnt-1 Induced Secreted Proteins). Results obtained over the past decade have indicated that CCN proteins are matricellular proteins, which are involved in the regulation of various cellular functions, such as proliferation, differentiation, survival, adhesion and migration. The CCN proteins have recently emerged as regulatory factors involved in both internal and external cell signaling. CCN3 was reported to physically interact with fibulin-1C, integrins, Notch and S100A4. Considering that, the conformation and biological activity of these proteins are dependent upon calcium binding, we hypothesized that CCN3 might be involved in signaling pathways mediated by calcium ions.

In this article, we review the data showing that CCN3 regulates the levels of intracellular calcium and discuss potential models that may account for the biological effects of CCN3.

Review

Introduction

The control of normal cell biology, from life to death involves an extremely complex array of interconnected signaling pathways, which govern inward and outward communication. Over the two past decades, a plethora of proteins have been found to participate in these fundamental regulatory circuits. In most cases, alterations of signaling result in pathological conditions. For example, many of the proteins discovered as oncogenes and tumor suppressors in cancer cells were shown to be key signaling molecules.

Signaling is pivotal to the coordinated response of cells in tissues and organs within the whole body. It is often considered that cell populations function as « societies » and that intercellular communication is pivotal to harmonious development during life and to intracellular biological modifications leading to cell death. Efficient coordination is an absolute requirement to safe functioning

It is critical to study and understand cross talking in cell population and to identify messengers that allow integrated responses. Unfortunately, very little is known presently about the processes that coordinate the various cellular signaling pathways.

An increasing amount of data points to matricellular proteins as major players in global control. Members of the

CCN family of proteins have recently emerged as important matricellular regulatory factors involved in both internal and external cell signaling.

The CCN family of proteins

Bork coined the CCN acronym in 1993 [1], soon after our discovery of a new gene –*nov*– presently designated CCN3 [2] whose expression was enhanced in all myeloblastosis associated virus (MAV)-induced nephroblastomas [3], which represent a unique animal model of the Wilms' tumor in human [4].

Analysis of the predicted primary structure of CCN3 indicated that it was structurally related to two other proteins (CYR61/CCN1 and CTGF/CCN2) that had been previously described as « immediate early » proteins showing mitogenic activities. The concept of a protein family was sustained by the fact that these three proteins were sharing a common mosaic organization with four structural modules sharing identity with Insulin like growth factor (IGF)-binding proteins (IGFBPs), Von Willebrand factor type C repeat, thrombospondin type 1 repeat, and secreted regulatory factors containing a cystin knot motif involved in dimerization.

The discovery of CCN3 provided the first evidence for a member of the CCN family of proteins showing antiproliferative activity on chicken embryo fibroblasts (CEF). Interestingly, the aminotruncated version of CCN3 expressed in one MAV-induced nephroblastoma was stimulating growth and inducing morphological transformation of the CEF [3].

Since then, three new members of the CCN family have been identified. CCN4 and CCN5, which were originally designated elm1 and rCOP1 respectively [5,6], were also described as rodent proteins devoted of an antiproliferative activity. Their human counterpart were identified a few years later as WINT-induced secreted proteins (WISP), with a new member (CCN6) originally identified from EST libraries and designated WISP3.

All CCN proteins except CCN5 – which lacks the CT module – show a similar multimodular organization.

The *ccn* genes are expressed in a wide variety of tissues. Expression of CCN proteins has been associated to several fundamental biological processes such as angiogenesis, chondrogenesis, wound healing [7–9]; their expression is detected during muscular differentiation, nervous system development and bone remodeling with an enhancement of CTGF correlated to several fibrotic situations [7–9]. Aberrant *ccn* gene expression is also associated to tumorigenesis [10], and has been shown to provide useful markers for tumor typing and prognosis.

Considering the variety of sometimes opposite biological functions attributed to the CCN proteins and the tight spatio-temporal regulation of their expression in many tissues of different origins, we proposed that the CCN proteins interact with several different partners and that the resulting combinatorial events are responsible for the variety of their functions [7].

CCN3 interacts with several different proteins

To identify the potential partners of CCN3, we first used the yeast two-hybrid strategy [11,12]. Two types of considerations dictated our choice of the full length CCN3 protein as bait. Firstly, each individual module was potentially interacting with several factors whose participation is essential to the biological functions of the families of proteins that contain these modules. Therefore, interaction with many of the potential interactions taking place with individual modules might not be directly relevant to the functions of CCN3. Secondly, and more importantly, some interactions critical for the functions of CCN3 might require the tertiary structure of the full-length protein.

Several different proteins were found to physically interact with CCN3 in the yeast assay especially fibulin-1C, integrins, Notch and S100A4 [7]. The physical interaction of CCN3 with these proteins has been checked either by GST-pull down assay, co-immunprecipitation, co-immunolocalization, or functional interdependence. The proteins interacting with CCN3 were either secreted, or localized in the cytosol, or localized in the nucleus of various cell types.

In as much as the conformation and biological activity of fibulin-1C, integrins, Notch and S100A4 were dependent upon calcium, we hypothesized that CCN3 might be involved in signaling pathways involving calcium ions.

Considering the considerable importance of calcium as a second messenger, these observations open new avenues for understanding the role of CCN proteins in cell growth regulation.

CCN3-Fibulin-1C and calcium

Yeast two-hybrid screens indicated that CCN3 interacted with the calcium-binding extracellular matrix glycoprotein fibulin-1C [11]. Fibulin-1C is associated with various connective tissues, basement membranes and blood; it was also reported to interact with extracellular matrix proteins such as fibronectin, laminin, and fibrinogen [13]. Although a distinct function for fibulin-1C has not yet been described, evidence has emerged to indicate that it may regulate cell adhesion and migration along protein fibers within the extracellular matrix and play a role in homeostasis and thrombosis [14]. Moreover, fibulin-1C

exhibits calcium-dependent binding and its functions were reported to dependent upon the calcium binding [11].

The identification of an interaction between fibulin-1C and CCN3 provides clue for possible interaction of CCN in signaling pathways involving extracellular matrix, cytoskeleton proteins and calcium. The C-terminal part of CCN3 is involved in the interaction with fibulin-1C. Truncated recombinant protein that represented only the C-terminal portion of CCN3 was able to interact with fibulin-1C. Similar to CCN3, fibulin-1C has a multimodular structure and contains a portion of nine calcium-binding type II EGF-like modules [13]. Using these modules, fibulin-1C is able to interact with the extracellular domain of the heparin-binding EGF-like growth factor precursor suggesting an important role during extracellular matrix formation in wound healing [15]. Binding of CCN3 to fibulin-1C involves this EGF-like module as previously reported for fibronectin [16]. This interaction might induce modification in calcium levels in the neighborhood of the cells.

CCN3 – Integrins and calcium

Integrins are cell surface receptors that are involved in adhesive interactions. They interact with several extracellular matrix proteins and other cell surface receptors. Integrins are heterodimeric transmembrane proteins that are composed of non-covalently associated α and β subunits [17]. The activation of integrins has been associated with conformational changes in the extracellular domains, which leads to an increased affinity for the ligand binding. Integrin-ligand interactions are dependent upon divalent cations. Some cations can enhance the affinity for the ligand, while others can suppress or lower it. For many integrins, Mg^{2+} and Mn^{2+} can induce conformational changes associated with a higher affinity for the ligand. On the contrary, Ca^{2+} often has an inhibitory effect on ligand binding. For instance, for integrin $\alpha_v\beta_3$, the increase in affinity for the ligand mediated by Mg^{2+} needs the chelation of Ca^{2+} [18].

It has been shown that extracellular Ca^{2+} can modulate the function of integrins but the Ca^{2+} binding sites on integrins are poorly characterized. To date, two distinct classes of Ca^{2+} binding sites were identified on integrins: a low and a high affinity-binding site for Ca^{2+}. For integrins $\alpha_5\beta_1$ and β3, a high affinity site for Ca^{2+} promotes the binding to the ligand, and a low affinity site seems to compete with a Mg^{2+} site [19,20]. Studies on integrin $\alpha_{IIb}\beta_3$ suggested that a high affinity Ca^{2+} site is involved in the heterodimerization of the two subunits. Since several integrins exhibit a high affinity Ca^{2+} binding site, this suggests a possible common role in heterodimerization of the two subunits. Furthermore, the binding of Ca^{2+} to a high affinity site seems to be necessary for ligand binding.

It was recently shown that CCN3 physically interacts with integrins $\alpha_V\beta_3$ and $\alpha_5\beta_1$ [21]. CCN3 acts on endothelial cells to stimulate pro-angiogenic activities, and supports cell migration and adhesion through different cell surface receptors including integrins $\alpha_V\beta_3$, $\alpha_6\beta_1$ and $\alpha_5\beta_1$. Extracellular Ca^{2+} could modulate CCN3-integrins interactions and thus, play a role in the functions of CCN3 in angiogenesis.

Intracellular Ca^{2+} is also involved in the intracellular integrin-pathways leading to integrin-mediated adhesion (for review [22]). The Ca^{2+} increase induced by the activation of integrins by their ligands [23] can activate the protease calpain. Studies on migrating CHO cells transfected with β1 and β3 integrins suggest that the Ca^{2+}-activated calpain could be involved in the dissociation between integrins and cytoskeleton, and thereby could influence the binding of integrins to their ligands. Since CCN3 modulates the Ca^{2+} influx in some cells, CCN3 could also acts on integrins function in an indirect manner by activating the calpain protease via Ca^{2+} entry. It would be of prime interest to determine if CCN3 modifies the Ca^{2+} influx in endothelial cells that could be linked to the migration of these cells.

CCN3 – Notch and calcium

CCN3 has been found to interact with Notch1 and to activate downstream effectors of the Notch pathway [24]. Notch1 is a member of a family of highly conserved transmembrane receptors, which are involved in fundamental biological processes during embryonic development such as differentiation, proliferation and apoptosis [25].

Notch receptors are synthesized in the form of an approximatively 300 to 350 kDa transmembrane precursor. This single protein is cleaved in the trans-Golgi network to form a heterodimeric receptor, consisting of a N-terminal extracellular subunit and a C-terminal transmembrane domain [25]. The extracellular subunit of various Notch receptors contains several tandemly repeated EGF (Epidermal growth Factor) modules. The N-terminal part of the receptor acts to restrain receptor activation in absence of ligand, and the structural integrity of the EGF repeats is dependent upon the presence of Ca^{2+}. The N- and C-terminal parts of the Notch heterodimer are non-covalently associated [26]. The association is stabilized in the presence of Ca^{2+} and destabilized by EDTA, resulting in the activation of Notch target genes [26]. These results suggest a regulation of the Notch activity implying a Ca^{2+}-dependent interaction between the extracellular and the transmembrane parts of the Notch receptor, prior to activation.

Although CCN3 physically interacts with the EGF-like repeats of Notch1, this binding is Ca^{2+}-independent, since co-immnunoprecipitation of CCN3 and Notch1 was maintained in buffer without Ca^{2+} and in buffer containing EGTA [24].

CCN3-S100A4 and calcium

Our recent studies demonstrated that CCN3 physically interacts with the calcium binding protein S100A4 [27]. This provides a clue for the detection of CCN3 at sites where S100A4 was reported to be expressed in normal conditions and where calcium is known to play critical roles.

S100A4 belongs to the group of S100 proteins, one of the largest subfamilies of the EF-hand proteins, which bind calcium selectively and with high affinity [28,29]. The S100 proteins are thus thought to modulate the propagation of calcium signals [30]. The human S100A4 gene was reported to be frequently rearranged through deletions, duplications and translocations, and is altered in several cancers [31]. Recently, interest has focused on S100A4 due to its implication in tumor progression and metastasis.

Binding of calcium to S100A4 proteins induces conformational changes, which result in exposure of new binding sites at their surface, and consequently allows for interaction with target proteins [32]. Thus, the interaction of CCN3 with S100A4 might be dependent upon local intracellular calcium concentration. The interaction of CCN3 with S100A4, as revealed with the yeast two hybrid system and GST pull down assay *in vitro*, strongly suggests that these two proteins can interact *in vivo* [27]. In support to this hypothesis, studies performed with CCN3 and S100A4 indicated that their site of expression showed significant overlap [7,33–36].

Involvement of CCN3 and S100A4 in tumorigenesis

CCN3 is expressed in many different types of tumors and shows positive or negative effects on tumorigenesis and metastasis [10]. On the other hand, the elevated expression of S100A4 in tumor metastasis suggests a role in tumor progression [37]. Since S100A4 itself is not able to initiate tumors, it was proposed that it might act in cooperation with other oncogenes [38]. The interaction of CCN3 with S100A4 makes it interesting to check whether these two proteins act in synergy or antagonize during tumorigenesis.

In one hand, CCN3 proteins were detected in the extracellular matrix, conditioned cell culture medium, cytoplasm and nucleus [7,10,12], in the other hand, S100A4 has been essentially described as a cytoplasmic protein, although it was also found to be secreted [39]. These results therefore suggest that CCN3-S100A4 could interact intra and extra cellularly, and that this interaction might be critical during development, both in physiological and pathological conditions.

Biological effects of CCN3-S100A4 interaction

S100A4 protein is now known to be involved in the regulation of cell motility and cell cycle progression, intercellular adhesion, angiogenesis, and metastatic properties of cancer cells. The interaction of CCN3 with S100A4 should therefore be helpful in deciphering the biological function of CCN3 and extends the roles of CCN proteins to S100 family.

Regarding cell motility, CCN3 was reported to decrease the adhesive capacity and increase the motility of Ewing's transfected cells [40]. It was suggested that S100A4 protein affects the assembly of the cytoskeleton [41,42]. These findings suggest that CCN3, through its interaction with S100A4, might alter cytoskeletal organization and facilitate cell motility.

The expressions of S100A4 and E-cadherin, which suppress tumor invasion, were reported to be inversely regulated in a series tumor cell lines [43]. It was suggested that the invasiveness of tumors expressing S100A4 might be at least partially induced by the abrogation of E-cadherin expression [44]. The interaction of CCN3 with S100A4 sheds new light on the potential role of CCN proteins as matricellular regulators of cell proliferation. Down-regulation of S100A4 resulted in a decrease in metalloproteinases expression and consequently in a reduction in migration process [45,46]. The interaction of CCN3 with S100A4 suggests that CCN3 might also be involved in the dysregulation of metalloproteinases expression, and may participate in a large array of connections involving several different proteins of the extracellular matrix.

Recently, an association between S100A4 and cell proliferation has been postulated. S100A4 expression was associated with elevated levels of wild-type p53. It was suggested that the physical interaction between wild-type p53 and S100A4 might result in a stimulation of the cells to enter the S phase [47,48]. The transfection of S100A4-negative cells with S100A4 constructs led to clonal death that was prevented by co-transfection with the anti-apoptotic gene *bcl-2*, which control calcium entry in different subcellular compartments [49]. CCN3-S100A4 interaction raises the possibility that CCN3 might promote apoptosis through its interaction with the S100A4-p53 complex. This would provide a clue (an explanation) for the dual role of CCN3 in proliferation and differentiation [6].

Recent findings established that CCN3 acts directly upon endothelial cells to stimulate pro-angiogenic activities, and induces angiogenesis *in vivo* [21]. Interestingly, S100A4 also promotes angiogenesis *in vivo* by preventing the anti-angiogenic effect of THBS1 [50]. In addition, preliminary results suggest that S100A4 protein may act directly as an angiogenic factor [51]. Whether CCN3 promotes angiogenesis directly, through its interaction with S100A4 or otherwise remains unclear.

CCN3-S100A4 interaction provides another pathway for stimulating calcium entry induced by CCN3: S100A4 could modulate cellular calcium levels via its roles in calcium uptake, transport and buffering [52]. The CCN3-S100A4 interaction might change the affinity of S100A4 for calcium and cause a release from S100A4. Because of its limited binding capacity for calcium, S100A4 might not be the main effector of calcium increase in cell upon induction by CCN3. In conclusion, the interaction of CCN3 with S100A4 might account at least in part for the association of CCN3 with tumorigenesis and metastasis.

CCN3 and calcium signaling

Because CCN3 is expressed in various tissues where the calcium metabolism and ion transport are essential [7] and that it physically interacts with proteins whose activity depended on calcium binding, we hypothesized that CCN3 might have a direct action on intracellular calcium mobilization. We used dynamic intracellular calcium fluorimetry [53] to examine the potential role of CCN3 in affecting intracellular calcium level in human tumoral cells (fig. 1).

The application of CCN3 resulted in a transient increase of the intracellular calcium concentration in G59 glioblastoma cells (fig. 1A). The control GST protein alone was without effect on the intracellular calcium level (data not shown). A similar transient increase in calcium was also seen with human neuroblastoma SK-N-SH cells upon CCN3 delivery [27]. In these experimental conditions, the calcium response reached a maximum and sustained effect (fig. 1B). Toxic effects (including the cells blowing up) were seen when higher concentrations of the GST fusion protein were used. To determine the origin of this transient calcium increase, experiments were carried out in the absence of extracellular calcium. Under these conditions, lower transient calcium increase in G59 cells persisted (fig. 1C). Furthermore, the addition of calcium to EGTA containing medium induced a sustained increase of intracellular calcium, suggesting a mixed interaction of CCN3 with the stimulation and release of intracellular calcium from the internal store and with the entry of extracellular calcium in G59 cells (fig. 1D). The addition of blockers of voltage-dependant calcium and/or sodium channels (verapamil and flunarizine) did not modify the transient calcium increase induced by GST-CCN3 in this cell line [27]. Finally, no increase was induced by the GST-fibulin-1C fusion protein, which contained the portion of fibulin-1C interacting with CCN3 [11] (fig. 1E). Conversely, a transient increase in intracellular calcium was also induced when another member of CCN family, GST-CCN2, was applied to G59 cells, but the effect of CCN2 was slightly lower than that of CCN3 at the same dose (fig. 1F). The presence of fibulin-1C or CCN2 did not modify the calcium response induced by CCN3, suggesting that these proteins did not compete with CCN3.

An interesting aspect of the relation between CCN3 function and calcium signaling was raised by the pronounced increase in intracellular calcium concentration, which was transiently induced in CCN3 treated G59. In these cells, both entry and the mobilization of the internal calcium store were induced by the addition of CCN3. Because the inhibitory effect of EGTA upon the calcium increase was reversed after the addition of $CaCl_2$, CCN3 is unlikely to be an ionophore by itself.

The uptake of extracellular calcium induced by CCN3 proceeded even when voltage dependent channels were blocked. Therefore, it is tempting to speculate that it involves store operated calcium channels, a heterogeneous subset of plasma membrane calcium ion channels. The best characterized channel of this type was originally described in mast cells in which the depletion of calcium induced a sustained calcium inward current that was not voltage activated and therefore termed I_{CRAC} (for calcium release activated calcium) [54–56]. A direct stimulation of these channels or of transient receptor potential (TRP) family of non-selective cation channels [57] could be postulated as a possible mode of action of CCN3.

The mobilization of intracellular calcium stores by CCN3 in G59 cells might involve specific receptors and IP3 activated channels. The transient calcium increase induced by CCN3 is sufficient to activate potassium current ($B_{K/Ca}$) as recorded by whole-cell patch clamp on G59 cells (unpublished data). Along this line, the interaction of CCN1, CCN2, and CCN3 proteins [58–60] with integrins might be of relevance to induce a transient increase of intracellular calcium.

As discussed above, CCN3 can interact with fibulin-1C and Notch1. It will be interesting to determine whether the regulation of intracellular calcium concentrations by CCN3 affects in any way its association with these proteins and their biological activities.

The interaction of CCN3 with S100A4 and its ability to induce a transient increase of intracellular calcium by two

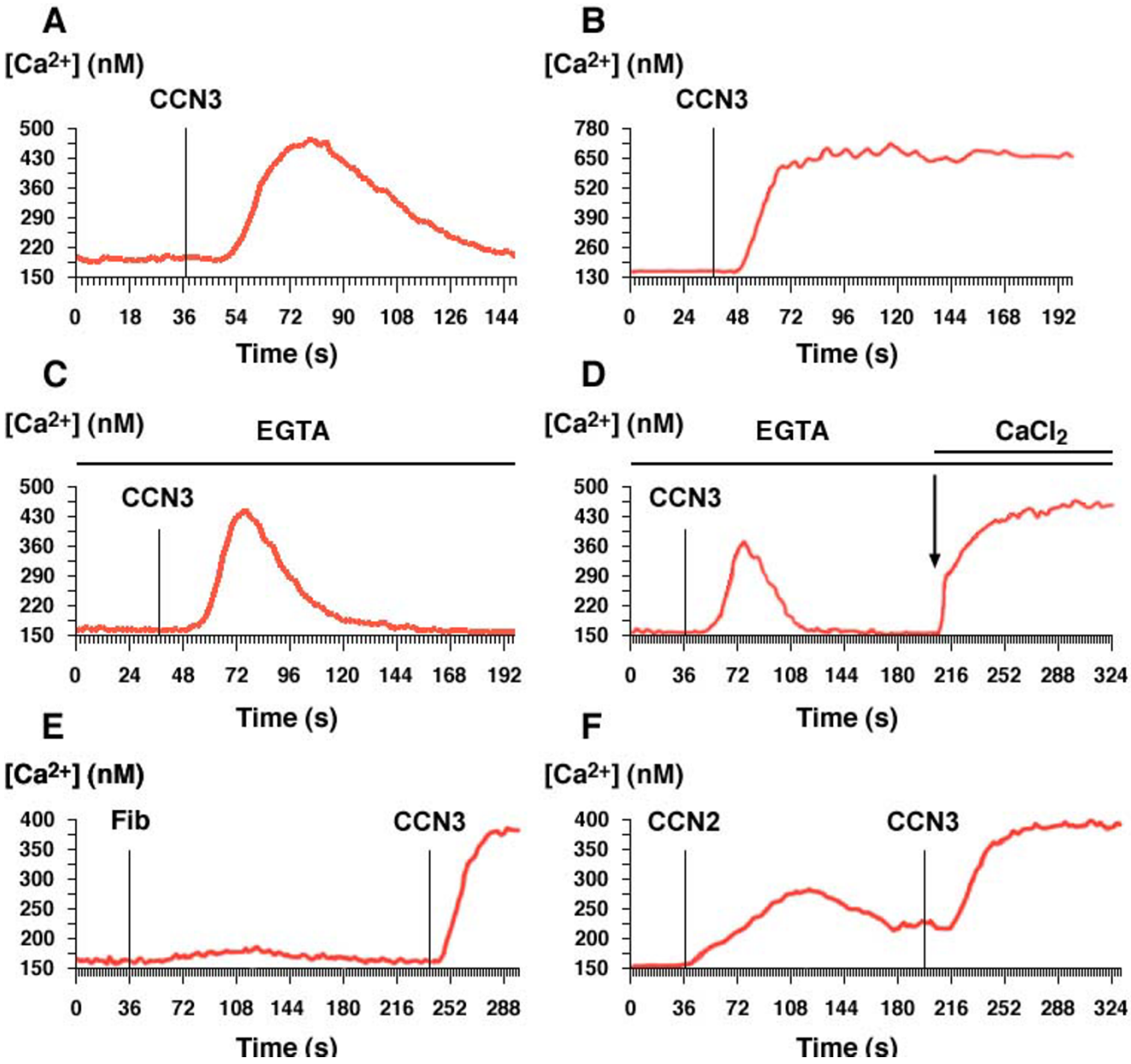

Figure 1

Effect of CCN proteins on intracellular calcium levels in G59 glioblastoma cells. GST-CCN3 (2 μg/ml, Fig. 1A or 10 μg/ml, Fig. 1B) was applied on G59 cells in the absence of EGTA. In the presence of 10 mM EGTA, GST-CCN3 (2 μg/ml) was added (Fig. 1C) and 12 mM $CaCl_2$ was overloaded (Fig. 1D). The effects of fibulin-1C and GST-CCN2 (5 μg/ml) were compared to that of 5 μg/ml GST-CCN3 (Fig. 1E,1F)

different mechanisms add another degree of variety to the pleiotropic biological properties of the CCN proteins.

A growing body of evidence indicates that CCN3 is implicated in many fundamental aspects of cellular activity. Our observations suggest for the first time that CCN proteins functionally crosstalk with calcium related physiological and pathological processes and open new perspectives in understanding the functions of CCN proteins in normal and pathological conditions. The interaction of CCN3 protein with numerous potential partners

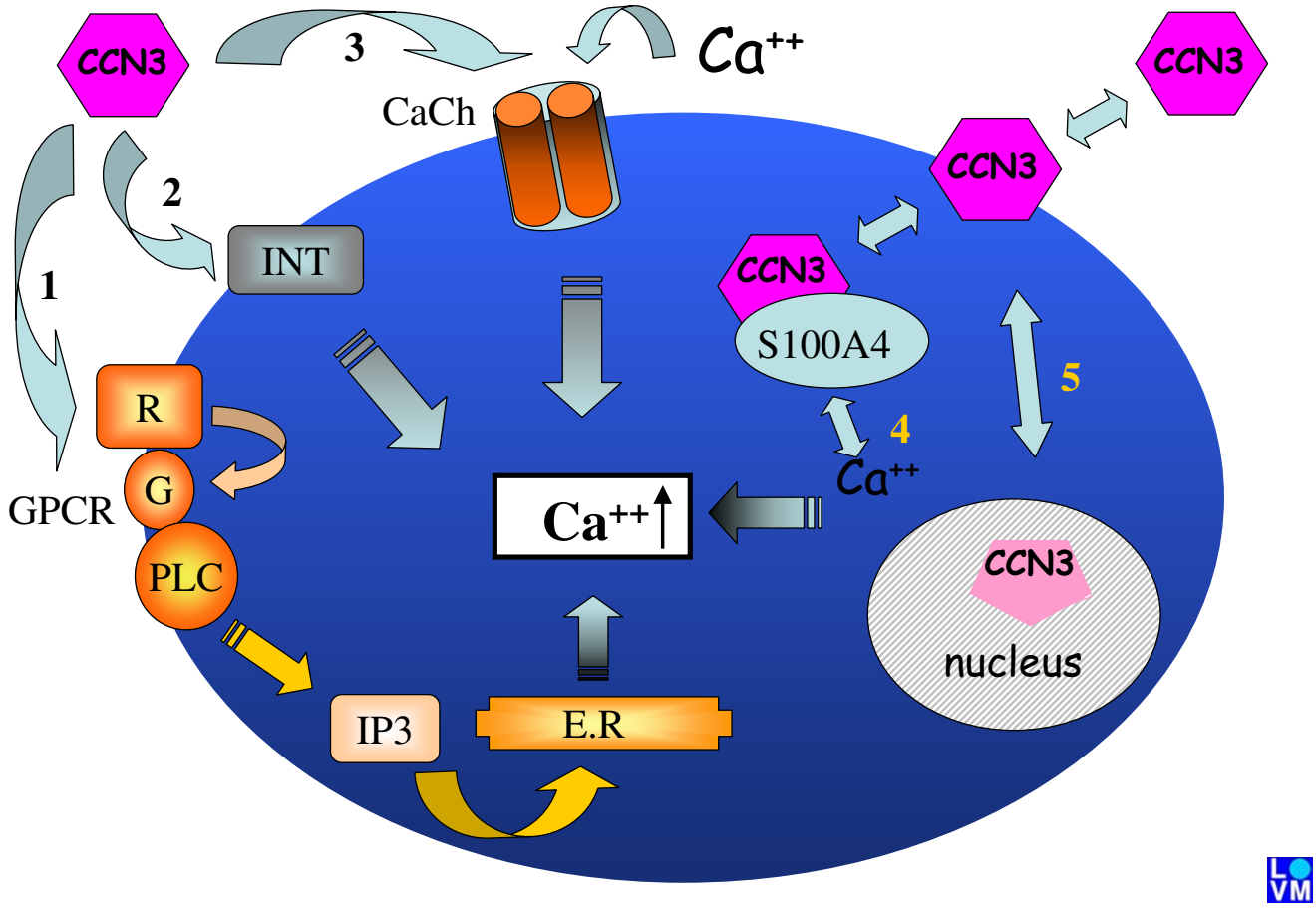

Figure 2
Schematic representation of different signaling pathways that may account for the calcium-related functions of CCN3. 1. Activation of GPCRs 2. Binding to integrins 3. Opening of voltage-independent calcium channels 4. Interaction with calcium binding proteins inside the cell 5. Modification of the calcium status inside the nucleus and modulation of gene expression

could explain the multiplicity of functional implications of CCN3 in cellular calcium signaling.

The multimodular organization of the CCN proteins provides a structural base that may account for its interaction with different target proteins. This type of multimodular organization is not unique to the CCN proteins. For example, the Caspase proteins family also show a modular organization [61]. They contain different domains (DD, CARD, etc.) that can be bound by adaptative proteins such as FADD and Apaf-1 [62] or BAR [63], which are involved in controlling apoptosis.

The regulation of intracellular calcium levels can be achieved by several ways. Recently, a PKC binding protein – enigma homolog (ENH)- has been reported to specifically interact with both PKCε and N-type Ca^{2+} channels, thereby forming a PKCε-ENH-Ca^{2+} channel macromolecular complex [64] that facilitated modulation of N-type Ca^{2+} channel activity by PKC.

The increased intracellular calcium level induced by CCN3 might also involve different voltage-independent calcium channels, among which receptor operated calcium channel and capacitative calcium entry channel may be of considerable importance in regulating this calcium influx.

Conclusions

We have hypothesized [7] that CCN3 might function as an adaptative protein by interacting with different regulatory proteins. Thus, CCN3 was proposed to coordinate signaling pathways by bringing together regulators, which require calcium for their biological effects.

In figure 2 we have summarized non-exclusive possible pathways that may account for the calcium-related functions of CCN3.

In the first case, CCN3 is shown to interact with members of the G Protein-Coupled Receptors (GPCR) family whose activation is known to result in Inositol 1,4,5-triPhosphate (IP3) production and calcium release from endoplasmic reticulum. IGFBPs were recently reported to specifically recognize these receptors and induce a transient increase of intracellular calcium [65]. The possibility that the IGFBP module is responsible for its effects on cellular calcium is under current investigation.

The second pathway by which CCN3 could induce the calcium increase involves binding to integrins. Effects of integrins on calcium have been extensively documented. The physical interaction of CCN3 with integrins makes it a major possibility.

In the third case, interaction of CCN3 with voltage-independent channels would lead to an uptake of calcium from external sources. As discussed above, store operated channels are among the likely candidates.

The fourth level of action would imply S100A4 whose physical interaction with CCN3 might interfere with its ability to bind calcium ions and play its buffer functions. We are presently in process of determining whether other proteins of the S100 family are also interacting with CCN3.

In the fifth case, translocation of CCN3 to the nucleus [66], is thought to account for the detection of the truncated CCN3 nuclear isoforms [12]. The importance of calcium in the regulation of gene expression is well established. It has been proposed that CCN3 play a direct role in the regulation of transcription by interacting with the rpb7 subunit of RNA polymerase II [12]. The levels of intracellular calcium ions might therefore be a key factor in controlling the interactions of CCN3 with its nuclear partners.

In any case, the links that we have established between CCN proteins and calcium signaling opens new avenues that should help to decipher the role of these regulatory proteins in processes governing cell growth control, development, and normal and pathological physiology.

Competing interests

None declared.

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