CCN3 (NOV) Interacts with Connexin43 in C6 Glioma Cells

POSSIBLE MECHANISM OF CONNEXIN-MEDIATED GROWTH SUPPRESSION*

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Many tumor cells exhibit aberrant gap junctional intercellular communication, which can be restored by transfection with connexin genes. We have previously discovered that overexpression of connexin43 (Cx43) in C6 glioma cells not only reduces proliferation but also leads to production of soluble growth-inhibitory factors. We identified that several members of the CCN (Cyr61/nectin) family are up-regulated following Cx43 expression, including CCN3 (NOV). We now report evidence for an association between CCN3 and Cx43. Western blot analysis demonstrated that the 48-kDa full-length CCN3 protein was present in the lysate and conditioned medium of glioma cells transfected with Cx43 cells, as well as primary astrocytes, but not in C6 parental and human glioma cells. Immunocytochemical examination of CCN3 revealed diffuse localization in parental C6 cells, whereas transfection of C6 cells with Cx43 (C6-Cx43) or with a modified Cx43 tagged to green fluorescent protein on its C terminus (Cx43-GFP) resulted in punctate staining, suggesting that CCN3 co-localizes with Cx43 in plaques at the plasma membrane. In cells expressing a C-terminal truncation of Cx43 (Cx43Δ244–382), this co-localization was lost. Glutathione S-transferase pull-down assay and co-immunoprecipitation demonstrated that CCN3 was able to physically interact with Cx43. In contrast, CCN3 was not found to associate with Cx43Δ244–382. Similar experiments revealed that CCN3 did not co-localize or associate with other connexins, including Cx40 or Cx32. Taken together, these data support an interaction of CCN3 with the C terminus of Cx43, which could play an important role in mediating growth control induced by specific gap junction proteins.

Gap junctions have long been thought to play an integral part in cellular growth control, both in normal and tumor cells. Loewenstein and Ranno (1) were first to observe a decrease in gap junctional intercellular communication (GJIC) in tumor cells when compared with their nontumorigenic counterpart cells. Since then, many tissues and cell lines have been analyzed in which the hypothesis of GJIC down-regulation in tumor tissues is upheld (2–4). Furthermore, this tumor-suppressive concept has been reinforced by the observation that treatment of different cell types with tumor-promoting agents, such as 12-O-tetradecanoylphorbol 13-acetate, inhibits GJIC (5–7), and 12-O-tetradecanoylphorbol 13-acetate antagonists, such as retinoic acid, are capable of reversing these effects (8). In addition, GJIC is also modulated by oncogenes (9–11). In many cases, loss of GJIC correlates with an increase in growth rate in vitro and tumor formation in vivo (2). The tumorigenic phenotype can be decreased by transfection with specific connexins, such as Cx43 (12–17) or Cx26 (15). In contrast, other connexins fail to exhibit this growth-suppressive phenotype (2).

Functional gap junctions may not be completely necessary for growth-regulating effects, but simply the expression of connexin genes may be sufficient. Supporting evidence shows that medium conditioned by C6 cells transfected with Cx43 is capable of suppressing the growth rate of the control tumor cells (18). Goldberg et al. (17) suggested that cells transfected with connexin genes may secrete soluble growth-inhibitory factors into the surrounding environment in order to potentiate any effects on growth of the neighboring cells. Recently, several reports have described that Cx43 plays a role in suppressing tumor growth independent of gap junction formation (19–22). The C-terminal domain of Cx43 seems to be important in this tumor-inhibiting effect (20). Cx43 may also regulate the cell cycle by acting on the S-phase kinase-associated protein (21).

To date, the mechanism by which connexins may suppress growth of tumor cells is unknown. Connexins may elicit growth-altering effects by influencing the expression of other downstream genes. In the pursuit of genes possibly regulated by connexins, we identified members of the CCN (Cyr61/nectin) family (23). These genes are involved in numerous cellular processes including cell proliferation, differentiation, and development (reviewed in Ref. 24). They have also been shown to play a role in tumorigenesis and growth control. One of these, CCN3 (NOV), showed substantial increase in expression in C6 glioma cells following transfection with Cx43, and immunocytochemistry indicated co-localization at gap junction plaques (25). Here we show that Cx43 interacts with CCN3, as demonstrated by glutathione S-transferase (GST) pull-down and co-immunoprecipitation. Furthermore, this association is lost when the C terminus of Cx43 is deleted. It is also connexin-specific, since both Cx40 and Cx32 were not found to associate with CCN3. In addition, CCN3 is secreted from C6 cells transfected with Cx43. These findings have implications for understanding the tumor-suppressive role of connexins.

Received for publication, April 9, 2004, and in revised form, June 15, 2004
Published, JBC Papers in Press, June 21, 2004, DOI 10.1074/jbc.M403952200

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* This research was supported by grants from the Canadian Institutes of Health Research (to C. C. N.) and from the Ligue Nationale contre le Cancer (Comité du Cher) (to B. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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‡The abbreviations used are: GJIC, gap junctional intercellular communication; GST, glutathione S-transferase; PBS, phosphate-buffered saline; CIP, calf intestinal phosphatase.

This paper is available online at http://www.jbc.org
mouse (Chemicon), and GFP (mouse (Chemicon)). Horseradish peroxidase-labeled secondary antibodies were used at a 1:10,000 dilution (Cedarlane, Hornby, Canada), whereas fluorescence-labeled secondary antibodies were diluted 1:500 (Molecular Probes, Inc., Eugene, OR).

**Retroviral Infection of C6 Cells**—The retroviral vector AP2-Cx40GFP was generated and used as previously described for AP2-Cx43GFP (27). The AP2-Cx43Δ3244–382-GFP vector was generously provided by Dr. Dale Laird (University of Western Ontario, London, Canada). Delivery of genes was performed by exposing 60% confluent cultures of C6 cells to viral medium for 24 h.

**GST Pull-down Assay**—A fusion protein of GST and full-length human CCN3 was produced in E. coli BL21 and solubilized with 1% SDS using a protocol modified from Ref. 28. The fusion protein was purified with glutathione-Sepharose beads (Amersham Biosciences) and incubated with cell lysates for 2.5 h at 4 °C. The beads were subsequently washed in PBS, and isolated protein was subjected to SDS-PAGE followed by immunoblot analysis.

**Immunocytochemistry**—Cells grown on coverslips were fixed in 80% methanol at −20 °C for 10 min followed by rehydration in PBS (pH 7.4). Coverslips were blocked in 10% normal goat serum and 1% bovine serum albumin in PBS for 30 min and incubated with primary antibody for 1 h. After three washes in PBS, secondary antibody was applied for 1 h, followed by further washes. 4’,6-Diamidino-2-phenylindole was used to stain nuclei where indicated. Coverslips were mounted onto glass slides using Vectashield fluorescent mounting medium (Vector Laboratories, Burlingame, CA) and viewed under a fluorescent microscope (Axioskop 2; Zeiss).

**Protein Isolation and Immunoprecipitation**—Cells on a confluent 100-mm plate were lysed in 800 μl of radioimmune precipitation lysis buffer (0.1% SDS, 1% IGEPA, 0.5% Sarkosyl, 50 mM Tris-HCl (pH 8.0), 150 mM NaCl) supplemented with MiniComplete protease inhibitors (Roche Applied Science) and phosphatase inhibitors (Sigma). Protein concentration was determined using the BCA protein quantification kit (Pierce). Following preclearing with 10 μl of preimmune serum and 10 μl of goat A-Sepharose (Sigma) at 4 °C for 30 min, cell lysates were incubated with 10 μl of antiserum for 1 h and 20 μl of protein A beads for 2 h at 4 °C with gentle agitation. Beads were washed three times in radioimmune precipitation buffer and boiled in SDS sample buffer to release bound proteins. For alkaline phosphatase treatment, half of the lysate collected from a 100-mm plate was adjusted to pH 7.5 and incubated with 4 μl of calf intestinal phosphatase (CIP; Sigma) at 30 °C.

**Fig. 2.** Analysis of CCN3 protein secreted into the conditioned medium of C6 and C6-Cx43 cells. A, equal amounts of protein (13 μg) precipitated from conditioned media were analyzed by Western blotting. Four bands of 56, 51, 46, and 27 kDa were present in medium conditioned by C6-Cx43. No slowly migrating CCN3 proteins were detected in the conditioned medium of C6 cells except for the 27-kDa isoform (open arrow). To permit better resolution of the three high molecular weight bands that are similar in size (solid arrow), proteins were separated on an 8% polyacrylamide gel for a longer period of time in B. Only the upper portion of the Western blot is shown here.
for 15 min. CIP-treated lysate was compared with the untreated half by Western analysis. Precipitation of Protein from Conditioned Culture Medium—Conditioned media were collected following culturing of C6 and C6-Cx43 cells in serum-free media for 24 h. Methanol precipitation (29) was performed on 200 μl of each sample. Briefly, 3 volumes of methanol, 1 volume of chloroform, and 4 volumes of H2O were added, mixed well, and centrifuged at maximum speed for 1 min. Upper aqueous phase was removed without disturbing the protein at the interphase. Subsequently, 8 volumes of methanol were added and vortexed to mix. Following precipitation at −20 °C for 30 min, samples were centrifuged at maximum speed for 15 min to pellet the protein. Proteins were then air-dried, resuspended in radioimmune precipitation buffer, and quantitated using the BCA kit (Pierce).

Western Blotting—Protein samples were boiled for 5 min in SDS sample buffer and separated on a 10% polyacrylamide gel. Subsequently, proteins were transferred to polyvinylidene difluoride membrane (Amersham Biosciences) at 100 V for 1 h. Following incubation with primary antibody at 4 °C overnight and horseradish peroxidase-linked secondary antibody for 1 h at room temperature, detection was achieved with SuperSignal chemiluminescent substrate (Pierce) on x-ray film.

**RESULTS**

A 48-kDa Isoform of CCN3 Appears in C6 Cells Transfected with Cx43—Lysates of C6 and transfected cells were analyzed for CCN3 expression with Western blotting. The appearance of a 48-kDa isoform of CCN3 was readily apparent in C6-Cx43 cells, whereas it was absent in parental C6 cells (Fig. 1A, solid arrow). In C6 cells virally infected with Cx43 carrying a C-terminal GFP tag, this 48-kDa CCN3 was also observed, although at a lower level compared with C6-Cx43 cells. On the contrary, C6 cells infected with a retroviral construct of Cx43 deleted on the C terminus at amino acid 244 and fused C-terminally with GFP (Cx43∆244–382-GFP) was also examined and found to express no 48-kDa CCN3 isoform (Fig. 1A).

In addition, a 27-kDa band was abundant in all cell lines examined (Fig. 1A, open arrow). The level of this truncated CCN3 isoform rose in C6-Cx43 cells, whereas no noticeable up-regulation could be observed in other transfecant cells. Both the 48- and 27-kDa bands disappeared following preabsorption...
of CCN3 IgG with specific immunogen peptide used to raise the antibody (Fig. 1B, arrows). Other bands that were present in all samples represented nonspecific cross-reactivity of the polyclonal antibody, since these were still observed following pre-absorption. The size of CCN3 isoforms detected here corresponds with those identified in previous studies (30–32).

**CCN3 Is Present in Medium Conditioned by C6-Cx43 but Not C6 Cells—**Conditioned medium collected from C6 and C6-Cx43 cells was concentrated by methanol precipitation and subjected to Western blot analysis. Three bands were detected in medium conditioned by C6-Cx43 that were absent in the C6 sample (Fig. 2A, solid arrow). Better separation on SDS-PAGE resolved their molecular masses to be 56, 51, and 46 kDa, respectively (Fig. 2B). The 51-kDa protein probably corresponds to the 52-kDa isoform of CCN3 observed in serum (33). The 46-kDa isoform has been found in various samples including rat brain extract and vascular endothelial cells (30, 31). The increase in molecular mass of CCN3 in conditioned medium is not unexpected. Chevalier et al. (34) previously noticed an increase of ~4 kDa in apparent molecular mass as CCN3 was secreted from the cell, suggesting progressive N-glycosylation. In addition, a 27-kDa isoform was present in both conditioned medium samples at a similar level (Fig. 2A, open arrow). Following peptide neutralization, all four CCN3 bands were removed, confirming their specificity (Fig. 2A).

**CCN3 Co-localizes with Cx43—**Immunocytochemistry was used to determine the subcellular localization of CCN3 in C6 and C6-Cx43 cells. Previous reports have demonstrated that CCN3 is in general diffusely localized within various cell types (34–37). In the current study, we used a new rabbit polyclonal antibody that showed a diffuse staining pattern of CCN3 in the cytoplasm (Fig. 3B). On the other hand, C6-Cx43 cells exhibited punctate staining (Fig. 3, E and H). Co-labeling with Cx43 and CCN3 antibodies revealed that CCN3 co-localized with Cx43 in gap junction plaques (Fig. 3, F and I).

**CCN3 Physically Interacts with Cx43—**To examine whether physical interaction exists between co-localized CCN3 and Cx43, a fusion construct of GST and full-length human CCN3 (GST-CCN3) was employed (38). The GST-CCN3 fusion protein immobilized on glutathione beads sequestered Cx43 from the C6-Cx43 cell lysate (Fig. 4A). No Cx43 was detected in the lysate of C6 parental cells subjected to the GST pull-down assay, probably because the level of Cx43 in C6 cells was too low to be detected. Negative control experiments were conducted with GST protein only and showed no pull-down of Cx43 (data not shown).

The physical association of CCN3 with Cx43 was verified...
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Figure 6. Western analysis to assess the role of Cx43 C terminus in its interaction with CCN3. A, Cx43 Δ244–382-GFP failed to co-immunoprecipitate (IP) with CCN3. Lysates of C6-Cx43GFP and C6-Cx43Δ244–382-GFP were subjected to co-immunoprecipitation with CCN3 antibody. GFP antibody was used to visualize the bands. Whereas Cx43GFP and Cx43Δ244–382-GFP were expressed at similar levels (compare lanes 1 and 3), only Cx43GFP was co-immunoprecipitated with CCN3 (compare lanes 2 and 4). B, CCN3 interacts with both the phosphorylated and unphosphorylated Cx43. GST pull-down assay was performed with C6-Cx43 lysate treated with CIP. Cx43 was pulled down by GST-CCN3 both in the presence (lane 3) and absence (lane 4) of phosphorylation. C, all three Cx43 species (NP, P1, and P2) were co-immunoprecipitated with CCN3 (lane 3). Dephosphorylation by CIP prior to immunoprecipitation did not abolish the ability of Cx43 to interact with CCN3. In B and C, crude lysates were presented as control to illustrate the phosphorylation profile of untreated and treated Cx43 (lanes 1 and 2, respectively).

Interaction with CCN3 Occurs via the C Terminus of Cx43 but Is Phosphorylation-independent—To delineate the domain of Cx43 that interacts with CCN3, the aforementioned retroviral construct of Cx43 deprived of its C-terminal tail (Cx43Δ244–382-GFP) was utilized. C6-Cx43Δ244–382-GFP cells were examined for CCN3 localization in parallel with those expressing full-length Cx43GFP (C6-Cx43GFP). Immunocytochemical analysis revealed punctate staining of CCN3 co-localizing with Cx43GFP epifluorescence (Fig. 5, A–C). In contrast, although the level of gap junction plaque formation in C6-Cx43Δ244–382-GFP cells was comparable with that of C6-Cx43GFP, CCN3 was present only as diffuse cytoplasmic staining rather than localizing to areas where C6-Cx43Δ244–382-GFP accumulated (Fig. 5, D–F).

Whereas Cx43GFP co-immunoprecipitated with CCN3 antibody, Cx43Δ244–382-GFP failed to co-immunoprecipitate, indicating that C-terminal truncation of Cx43 abolished its interaction with CCN3 (Fig. 6A). Since the C terminus of connexins contains numerous sites of phosphorylation (39), alkaline phosphatase CIP treatment was conducted to study the effect of dephosphorylation on the CCN3-Cx43 interaction. Both the phosphorylated (Cx43-P1) and dephosphorylated Cx43 (Cx43-NP) in C6-Cx43 lysates were pulled down by GST-CCN3 (Fig. 6B). Co-immunoprecipitation of CIP-treated Cx43 (NP) with CCN3 (Fig. 6C) concurred with the GST pull-down study, suggesting that the interaction of Cx43 with CCN3 was not critically dependent on the phosphorylation state of Cx43. Although the most highly phosphorylated species of Cx43 (Cx43-P2) did not appear to be pulled down by GST-CCN3 (Fig. 6B), a co-immunoprecipitation experiment that is considered more physiologically relevant demonstrated the ability of Cx43-P2 to associate with CCN3 in vivo (Fig. 6C). The failure of Cx43-P2 to interact with GST-CCN3 might reflect the lack of post-translational modification (such as glycosylation) of CCN3 in the bacterial expression system, or steric hindrance might have resulted from the GST tag.

CCN3 Does Not Interact with Cx40 or Cx32—In order to investigate whether interaction with CCN3 is specific to Cx43 or shared among other members of the connexin family, we studied Cx40 and Cx32, members of the α and β subfamilies, respectively. Immunocytochemical revealed that CCN3 was not co-localized with Cx40GFP (Fig. 7, A–C). In accordance with this observation, Cx40GFP failed to co-immunoprecipitate with CCN3 (Fig. 8A).

Immunocytochemical examination of CCN3 in C6-Cx32 cells revealed a diffuse cytoplasmic localization rather than the distinct pattern of punctate membrane staining for Cx32 (Fig. 7, D and E). No co-localization of CCN3 with Cx32 was observed (Fig. 7F). Furthermore, no co-immunoprecipitation of Cx32 was detectable following incubation with CCN3 antibody (Fig. 8B). Since the physiological level of CCN3 in C6-Cx32 might be too low to allow for successful co-immunoprecipitation, a GST pull-down assay was carried out to assess the biochemical potential of CCN3 to interact with Cx32. In keeping with the co-immunoprecipitation data, Cx32 failed to interact with the GST-CCN3 fusion protein in vitro (Fig. 8C).

CCN3 in Primary Astrocytes and Human Glioma Cells—To demonstrate that the expression, localization, and interaction pattern of CCN3 presented here is not an artifact of Cx43 overexpression, primary astrocytes were examined as the normal counterpart of C6 glioma cells. Western blot analysis confirmed that the 48-kDa full-length isoform of CCN3 was also expressed in primary rat astrocytes (Fig. 9A, arrow). In parallel to C6-Cx43, endogenous Cx43 was co-immunoprecipitated with CCN3 antibody in primary rat astrocytes (Fig. 9B). Mouse astrocytes were used to study the immunolocalization of CCN3 because of the availability of Cx34 knockout cells. In wild type astrocytes, double labeling immunocytochemistry indicated that CCN3 co-localized with Cx43 (Fig. 10A). The overall pattern of CCN3 labeling did not change in Cx43 knockout astrocytes (Fig. 10B).

Human glioma cells were found to vary in their expression of CCN3 and Cx43. Of the six cell lines examined (CRL-1718, SF188, SF539, U251, U87, and XF498), most expressed low levels of Cx43 except for SF539 and XF498, which had relatively higher amounts of Cx43 protein as detected by Western blotting. All of these lines expressed the 27-kDa but not the 48-kDa isoform of CCN3 (data not shown). The immunolocalization of CCN3 in human glioma cells was similar to C6 cells, in which CCN3 was localized diffusely to the cytoplasm instead of displaying any membrane-associated punctate pattern. Even in XF498 cells where both CCN3 and Cx43 protein were expressed, there was no apparent co-localization (Fig. 10C). A previous study demonstrated that in human glioma cells expressing a high level of Cx43 protein, the Cx43 was predomi-
nantly confined in the cytoplasm, whereas few gap junction plaques were formed at the plasma membrane (26).

**DISCUSSION**

The association of connexin expression and its resultant GJIC has long been heralded as being important for cell growth control. However, elucidation of possible mechanisms by which such growth is controlled has remained elusive and speculative at best. Whereas correlation of decreased connexin expression and GJIC in association with increased tumorigenic phenotype can readily be found, several well documented exceptions exist. These include the following: 1) some tumors show a high level of connexin expression and GJIC; 2) some connexins act as tumor suppressors, whereas others do not; 3) forced expression of some connexins decreases growth *in vivo*, but not *in vitro* (40, 41); and 4) forced connexin expression can decrease growth...
without enhancing GJIC (20). One is therefore driven to search for other mechanisms by which connexins may alter growth. One avenue we have pursued concerns effects mediated by alteration in expression of other genes that are involved in growth control. Using this approach, we have identified an interaction of Cx43 with CCN3 and propose that such interactions of connexins with other proteins could underlie their role as growth suppressors.

CCN3 was first identified as being overexpressed in chick nephroblastomas induced by myeloblastosis-associated virus type 1 (42). It is a member of the CCN family, along with CCN1 (CYR61), CCN2 (CTGF), and CCN4, -5, and -6 (WISP 1, 2, and 3) (46). These proteins share 40–95% sequence homology, and all are rich in cysteine. CCN proteins generally contain four structural module domains (44, 45). They possess an N-terminal secretory signal, consistent with these proteins being secreted from the cell into the extracellular space (46). The other domains include an insulin-like growth factor binding domain near the N terminus (46, 47), a von Willebrand factor type C repeat (48), a highly variable region devoid of cysteine residues (45), a thrombospondin type 1 repeat domain, and a C-terminal cysteine-rich region (49).

The current study demonstrates an interaction between CCN3 and Cx43 and suggests that this interaction is 2-fold. First, Cx43 is involved in regulating the level of CCN3 expression. The full-length 48-kDa isoform of CCN3 is present in Cx43-expressing cells (C6-Cx43 and C6-Cx43GFP) but not in parental C6 cells. The absence of this isoform in C6-Cx43Δ244–382-GFP cells implies that the C-terminal tail of Cx43 participates in enhancing the accumulation of full-length CCN3, either by promoting transcription/translation or preventing degradation. Second, Cx43 recruits CCN3 to the gap junction plaques, where they physically interact. C6 and C6-Cx43 cells demonstrate unique localization of CCN3 protein within each cell. Whereas CCN3 is diffusely present in C6 cells, it becomes distinctly localized to areas of Cx43-containing gap junction plaques in C6-Cx43 cells as well as C6-Cx43GFP and primary astrocytes that express high levels of Cx43. It appears that these two levels of interaction might be causally linked, considering that redistribution of CCN3 coincides with the appearance of the 48-kDa isoform in C6-Cx43, C6-Cx43GFP, and primary astrocytes. In addition, the ability of full-length CCN3 to bind Cx43 has been demonstrated with the GST-CCN3 pull-down assay. However, the present data do not exclude the possibility that other CCN3 isoforms might also associate with Cx43.

The biological functions of CCN3 have been suggested to depend on the size of isoforms expressed, their relative amount, and their subcellular localization (50). Various forms of CCN3 have been reported, including 46-, 38-, and 18-kDa isoforms in rat total brain extract (30) as well as two CCN3 doublets of 46–48 and 28–30 kDa in endothelial cells (31). Previous reports using different antibodies have also noted nuclear localization of CCN3 in various cell types (35, 51). Examination of the CCN3 protein sequence reveals a KGKKCLRTKK8 motif, which has been implicated in the nuclear localization of FGF3 (35). In addition, CCN3 was found to bind the Rpb7 subunit of RNA polymerase II, implicating a role in transcriptional regulation (35). These aforementioned cells expressed a truncated 31/32-kDa isoform of CCN3, compared with the 48-kDa full-length protein (35). Whereas the full-length CCN is believed to be responsible for growth suppression, truncated CCN3 has been associated with growth-promoting activities (42). We have observed a 27-kDa isoform in all cell lines examined. The significance of this truncated protein in the glial system remains to be established.

On the other hand, expression of the full-length CCN3 might have profound functional implications. The present study reveals that C6-Cx43 cells, which exhibit reduced growth and tumor formation, express a full-length protein of 48 kDa, in contrast to the parental C6 cells. As well, primary astrocytes express this CCN3 isoform, which is absent in human glioma cells. The full-length CCN3 has been shown to possess antiproliferative activity in glial cells. The expression of CCN3 in human glioma G59 cells significantly suppressed their growth in vitro as well as tumor size in vivo (51). Full-length CCN3 was detected in the conditioned medium of these transfected cells (51) and has also been detected in the conditioned medium of vascular endothelial cells that express CCN3 (31). With regard to connexins, not only does the expression of Cx43 decrease growth in C6 cells (13, 52), but medium conditioned by these transfected cells causes the same effect (18). Thus, the growth-suppressive capacity of Cx43 may be mediated by the secretion of soluble growth-inhibitory factors into the surrounding medium (17, 18). The detection of full-length CCN3 in a medium conditioned by C6-Cx43, together with the negative growth regulation known of this protein, has led us to propose that CCN3 may account for the growth-suppressive effects.
observed in the conditioned medium of C6-Cx43 cells. If CCN3 was indeed a downstream effector of Cx43-induced tumor suppression via its physical association with Cx43, the requirement of the Cx43 C terminus in such interaction would be consistent with the established importance of this domain in growth suppression.

Connexins have been found to bind various other proteins, such as calmodulin (53), microtubules (54), v-Src (55), and zonula occludens-1 (56–58). Evidence is accumulating that connexins and interacting partners form multiprotein complexes termed Nexus, where enzymes or signaling molecules can be recruited to specific membrane subdomains, or concentrated to achieve efficient reaction kinetics (reviewed in Refs. 59 and 60). Here we identify CCN3 as a putative scaffolding protein or effector molecule up-regulated by Cx43 expression and redistributed to the Nexus. Since the C terminus displays much diversity among connexin members, its involvement may underlie the connexin specificity of the Cx-CCN3 interaction. However, the specific site(s) of interaction between CCN3 and the C terminus of Cx43 is yet to be identified.

In summary, CCN3 has been described as a matricellular protein, being found within the cell and in the extracellular matrix. Here we describe distinct compartmentalization of some of the cellular CCN3, namely sequestered at Cx43-containing gap junction plaques in C6-Cx43 cells and astrocytes. The interaction with CCN3 is dependent upon the C-terminal containing gap junction plaques in C6-Cx43 cells and astrocytes. Here we identify CCN3 as a putative scaffolding protein or recruited to specific membrane subdomains, or concentrated to zonula occludins-1 (56).

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J. Biol. Chem. 2004, 279:36943-36950.
doi: 10.1074/jbc.M403952200 originally published online June 21, 2004

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