J Membrane Biol (2007) 217:53–61
DOI 10.1007/s00232-007-9051-y

Control of Intracellular Localization and Function of Cx43 by SEMA3F

Yumi Kawasaki · Aya Kubomoto · Hiroshi Yamasaki

Received: 1 May 2007 / Accepted: 14 May 2007 / Published online: 31 July 2007

Abstract Connexin genes are considered to form a family of tumor-suppressor genes. However, the mechanism of connexin-mediated growth control is not well understood. We now provide several lines of evidence which suggest that SEMA3F, a member of the class 3 semaphorin family, which is also reported to be a tumor suppressor, controls the intracellular localization and function of connexin 43 (Cx43). We employed a series of rat liver epithelial cell lines, among which we previously found that the level of expression of malignant phenotypes (IAR20 < IAR27E < IAR6-1 < IAR27F) is inversely related to that of gap junctional intercellular communication (GJIC). When we immunostained SEMA3F and Cx43 in these cell lines, the extent of immunostaining in the plasma membrane of both proteins decreased in the order of IAR20 > IAR27E > IAR6-1 > IAR27F, suggesting a close relationship between Cx43 and SEMA3F. Further studies revealed a partial colocalization of SEMA3F and Cx43 in the plasma membrane of IAR20 cells. We also found that both SEMA3F and Cx43 moved from the cytoplasm to the plasma membrane in a mouse papilloma cell line when E-cadherin became functional after transferring the cells from low- to high-calcium conditions. When SEMA3F gene expression was inhibited by siRNA in IAR20 cells, Cx43 localization in the plasma membrane and GJIC ability were reduced. Moreover, we found that SEMA3F binds with the cytoplasmic loop domain of Cx43, employing the yeast two–hybrid complementation and screening assays. Taken together, these results strongly suggest that SEMA3F directly associates with Cx43 and controls its intracellular localization and function.

Keywords Connexin · Cx43 · Gap junction · Semaphorin 3F · Cadherin · Yeast two-hybrid system · siRNA

Y. Kawasaki · A. Kubomoto · H. Yamasaki (✉)
Department of Bioscience, School of Science and Technology, Kwansei Gakuin University, 1-2, Gakuen, Sanda, Hyogo 669-1337, Japan
e-mail: hyamasaki@ksc.kwansei.ac.jp

Introduction

Gap junction channels mediate direct exchange of ions and other small molecules (M_r <1,000), including second messengers such as cyclic adenosine monophosphate, inositol 1,4,5-trisphosphate and Ca^{2+} between adjacent cells (Bruzzone, White & Paul, 1996). A gap junction channel consists of two hemichannels (connexons), which are composed of six transmembrane proteins called "connexins." So far, 21 connexin genes have been reported in humans (Sohl & Willecke, 2003). The structures of connexin proteins are similar, consisting of a short intracellular N-terminal region, four membrane-spanning domains, two extracellular loops, a cytoplasmic loop and a C-terminal cytoplasmic tail. The length of the C-terminal tail and the amino acid sequences of the cytoplasmic loop domain are relatively more varied than other domains among these connexins.

Gap junctional intercellular communication (GJIC) is considered to play an important role in the maintenance of tissue homeostasis and coordinated cell growth (Loewenstein & Kanno, 1966; Yamasaki & Naus, 1996; Trosko & Ruch, 2002; Mesnil et al., 2005; Leithe, Sirnes & Rivedal, 2006). Inhibition of GJIC is associated with disrupted cell growth control, and almost all cancer cells show reduced GJIC ability and/or do not communicate with surrounding normal cells (Yamasaki et al., 1987, 1999a b, c; Yamasaki & Naus, 1996; Mesnil, Montesano & Yamasaki, 1986).

Numerous studies have shown that growth control of GJIC-deficient cells can be recovered by transfection of connexin genes (*reviewed by* Yamasaki & Naus, 1996). Thus, connexin genes form a family of tumor-suppressor genes. However, the mechanism of growth control by connexin genes is not known.

We have previously reported that connexin gene mutations are rarely found, but aberrant intracellular localization of connexins is often seen in tumors and in transformed cell lines (Krutovskikh et al., 1994; Yamasaki et al., 1999a, b, c). We then reported that intracellular localization of Cx43 as well as Cx26 is controlled by functional E-cadherin in a murine papilloma cell line (Jongen et al., 1991; Hernandez-Blazquez et al., 2001). However, since there is no evidence that connexins and E-cadherin bind to each other directly, we are interested in identifying a factor(s) which links the functions of these two intercellular junction molecules. Semaphorin (SEMA) 3F, a member of the class 3 semaphorin family, is another putative tumor-suppressor molecule localized at cell-cell contact areas (Roche et al., 1996; Kessler et al., 2004; Xiang et al., 2002; Brambilla et al., 2000), which was also reported to control function of E-cadherin (Nasarre et al., 2005).

In order to examine whether SEMA3F and connexins are functionally related, we used rat liver epithelial cell lines in which the level of GJIC and the degree of transformed phenotype expression are well studied. In this series of established rat liver epithelial cell lines, the level of GJIC and the degree of transformed phenotype expression are inversely related (Mesnil et al., 1986). The non-tumorigenic rat liver epithelial cell line IAR20 expresses a high level of Cx43 and has a high level of GJIC, but transformed and tumorigenic cell lines (IAR6-1, IAR27E and IAR27F) showed less Cx43 plaques in the plasma membrane and less GJIC ability. In these cell lines, we found that intracellular localization of SEMA3F was similar to that of Cx43 and that its expression was inversely related to GJIC levels. Further studies suggested that SEMA3F binds to the Cx43 loop domain in the yeast two-hybrid assay, suggesting that SEMA3F may be a specific partner of Cx43 and play a role in the regulation of normal function and localization of Cx43.

Materials and Methods

Cell Lines and Cell Culture

The rat liver epithelial cell lines used in this study (IAR20, IAR27 and IAR6-1) were isolated as described previously (Montesano et al., 1977, 1980). IAR27E (epithelioid) and IAR27F (fibroblastic) were cloned from the parental IAR27 cell line as described previously (Mesnil et al., 1994). The P3/22(E) cell line, which expresses endogenous E-cadherin gene, was cloned from the parental P3/22 cells (Klam et al., 1999).

The rat epithelial cell lines were cultured in Dulbecco's Eagle medium supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin in a 5% CO_2 incubator at 37°C. The basal medium used for cultures of P3/22(E) cells was Eagle minimal essential medium without $CaCl_2$ as modified by Jongen et al. (1991). This medium was then supplemented with 10% FBS treated with Chelex 100 resin (Bio-Rad Laboratories, Richmond, CA) to remove calcium. $CaCl_2$ was then added to the medium to attain a final concentration of 0.05 mM (low calcium, LC) or 1.2 mM (high calcium, HC). P3/22(E) cells were maintained in LC medium before use for experiments.

Immunohistochemical Staining

Cells were grown on a cover glass. At confluence, cultures were washed twice with phosphate-buffered saline (PBS) and then fixed in methanol for at least 30 min. All treatments were done at room temperature. The cover glass was washed twice with PBS and incubated in PBS containing 0.2% Triton X-100 for 20 min. Then, cells were blocked with Blocking Ace (Dainippon Sumitomo Pharma, Osaka, Japan) for 1 h and incubated with a mixture of mouse anti-Cx43 (1:800 diluted with PBS; Zymed/Invitrogen, Carlsbad, CA) and rabbit anti-class 3 semaphorin antibodies (1:300 diluted with PBS; Santa Cruz Biotechnology, Santa Cruz, CA) in PBS overnight at 4°C. After washing three times with PBS, the cells were incubated with a mixture of Alexa-568 anti-mouse and Alexa-488 anti-rabbit secondary antibodies (1:300 diluted with PBS; Molecular Probes/Invitrogen, Carlsbad, CA) for 1 h at room temperature. After washing with PBS, the cover glass was mounted on a slide glass with 0.1% *n*-propylgallate/PBS. Then, the cells were observed under a fluorescence microscope (Bx50; Olympus, Tokyo, Japan) and a confocal laser scanning microscope (LSM510 META; Carl Zeiss, Oberkochen, Germany). The same protocol was used for staining of E-cadherin (anti-E-cadherin antibody, 1:800 diluted with PBS; Zymed/Invitrogen).

Reverse Transcriptase-Polymerase Chain Reaction

Total RNA from cultured cells was isolated by ISOGEN according to the protocol supplied by the furnisher (Nippon Gene, Tokyo, Japan). The RNA concentration was measured by GeneQuant pro (GE Healthcare, UK), and reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using the one-step kit AccessQuick RT-PCR system (Promega, Madison, WI) by GeneAmp PCR system

9700 (Applied Biosystems, Foster City, CA). The primers used were as follows: glyceraldehyde-3-phosphate dehydrogenase (G3PDH) (F) 5′-ACCACAGTCCATGCCATCAC-3′, (R) 5′-TCCACCACCCTGTTGCTGTA-3′; Cx43 (F) 5′-AGGAGTTCAATCACTTGGCG-3′, (R) 5′-GCAGGATTCGGAAAATGAAA-3′; SEMA3A (F) 5′-AGACTCACTTGTACGCCTGTGG-3′, (R) 5′-AAGATAGCAAAGTCTCGTCCCAT-3′; SEMA3B (F) 5′-TGAACCCTGAGCACCCTGAGC-3′, (R) 5′-ACCCACAAACAGGCGTCCA-3′; SEMA3F (F) 5′-CCGAGGATCTGCAGTGTGTG-3′, (R) 5′-GACAGTGGTGAGGCGGTAGG-3′. The amplification program consisted of one cycle of 48°C for 45 min and 95°C for 2 min; 25 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 30 s; and one cycle of 72°C for 5 min. PCR products were analyzed on a 2% agarose in Tris-Acetate-EDTA (TAE) buffer.

Small Interfering RNA Treatment

To knock down SEMA3F gene expression by siRNA, we selected the target sequence with the aid of an small interfering RNA (siRNA) design support system (Takara Bio, Shiga, Japan). The sequence 5′-AACCTTTACGCCATCGATGAAGT-3′ (position 1268, XM_236623) was used as the target. For cloning into a pSilencer 2.1 siRNA expression vector, each DNA oligonucleotide insert sequence was decided using an insert design tool (Ambion, Austin, TX). The insert sequence pairs designed were as follows: 5′-GATCCGCCTTTACGCCATCGATGAATTCAAGAGATTCATCGATGGCGTAAAGGTTTTTTGGAAA-3′ (sense) and 5′-AGCTTTTCCAAAAAACCTTTACGCCATCGATGAATCTCTTGAATTCATCGATGGCGTAAAGGCG-3′ (antisense). Based on the protocol supplied for the Silencing^{TM} siRNA Construction kit (Ambion), the double-stranded DNA (dsDNA) oligonucleotides including the siRNA target sequence were inserted into the pSilencer 2.1 vector. The SEMA3F siRNA vector was transfected into cells with siPORT^{TM} XP-1 Transfection Agent (Ambion). After 24 h, hygromycin was added to the cultures and the cells were subcultured when confluent. After two or three passages in the presence of hygromycin, the cultures were used for experiments without cloning.

Western Blotting

Cells were grown to confluence and directly lysed in a lysis buffer (60 mM 4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid [HEPES, pH 7.2], 1% Triton X-100, 1% NP-40, protease inhibitor cocktail [Sigma-Aldrich, St. Louis, MO]) after washing with PBS. Total protein of the lysate was diluted with 2 × sodium dodecyl sulfate (SDS) sample buffer and boiled at 95°C for 5 min before electrophoresis in 10% SDS-polyacrylamide gel electrophoresis gels. After transfer to polyvinylidene difluoride membranes, the blots were washed four times with PBS and blocked with the blocking buffer (Odyssey kit; LI-COR, Lincoln, NE) for 1 h before incubation with the primary antibodies against SEMA3F (1:300; Chemicon International, Temecula, CA), Cx43 (1:500, Zymed/Invitrogen) and β-actin (1:2,000, Sigma-Aldrich). After incubation with the respective secondary antibodies (Odyssey kit) for 1 h at room temperature, the bands were scanned by the Odyssey Infrared Imaging System (LI-COR).

Measurement of GJIC

GJIC was measured by the scrape loading assay (El-Fouly, Trosko & Chang, 1987). A scrape line was made with a toothpick on a monolayer of cultured cells in a 0.05% solution of Lucifer yellow and 0.01% Texas red. Immediately after scraping, the culture was washed with PBS and dye transfer was observed under a fluorescence microscope (Bx10, Olympus).

Yeast Two-Hybrid Complementation and Screening Assay

The Matchmaker two-hybrid system 3 (Clontech Laboratories, Mountain View, CA) was used according to the supplied protocol to confirm interaction between Cx43 and SEMA3F. The human Cx43 cytoplasmic loop domain (position aa 96-167) was fused to the GAL4 binding domain (BD), and the human SEMA3F C-terminal tail (position aa 639–785), which is a specific domain for SEMA3F, was fused to the GAL4 activation domain (AD). The yeast strain AH109 was a kind gift from Dr. Omori (Akita University, Akita, Japan). The vector pAS2-1 containing the Cx43 cytoplasmic loop domain and the vector pGAD10 containing the SEMA3F C-terminal domain were cotransformed into yeast strain AH109 and grown on SD-Trp-Leu-Ade-His/X-α-Gal plates two or three times. pGADT7-T (AD/T antigen), pGBKT7-53 (DNA-BD/p53) and pGBKT7-Lam (DNA-BD/laminC) were used as controls.

To confirm whether SEMA3F interacts with Cx26, the same complementation protocol was used. The recombinant plasmid construct for the BD consisting of the human Cx26 cytoplasmic loop domain (position aa 95–129) cloned into the vector pAS2-1 was kindly provided by Dr. Omori.

To screen a human heart cDNA library, the supplied protocol for the Matchmaker two-hybrid system 3 was used. The Matchmaker human heart cDNA library fused to the GAL4 AD was purchased from Clontech Laboratories. Vector pAS2-1 containing the human Cx43 cytoplasmic loop domain and the amplified library in vector pGAD10

were cotransformed into yeast strain AH109 and grown on SD-Trp-Leu plates. At least five independent transformants were picked from each plate and replicated on SD-Trp-Leu-Ade-His/X-α-Gal plates. Positive colonies were restreaked on SD-Trp-Leu-Ade-His/X-α-Gal plates two or three times. Then, the plasmid DNA was isolated from each positive yeast colony according to the yeast protocol handbook (Clontech Laboratories). To determine the identity of the interacting clones, the PCR-amplified pGAD10 inserts were sequenced using standard techniques. The identities of the inserts were determined by comparing the cDNA sequences with a BLAST search.

Results

Cx43 and class 3 Semaphorins Are Similarly Expressed in the Plasma Membrane and Only at Cell-Cell Contact Areas in IAR Cell Lines

Among IAR cell lines established from rat liver epithelial cells, IAR20 is nontumorigenic but IAR6-1, IAR27F and IAR27E showed various degrees of transformed phenotypes. We previously found that the level GJIC is inversely related with the degree of transformed phenotypes among these cell lines (Mesnil et al., 1986). When Cx43 and class 3 semaphorins were immunostained in these cells, we found that their intracellular localization was very similar (Fig. 1). While both class 3 semaphorins and Cx43 were mostly localized in the plasma membrane in IAR20 cells, their localization in the plasma membrane was reduced in other cell lines in the order of IAR27E > IAR6-1 > IAR27F.

In order to see whether Cx43 is colocalized with class 3 semaphorins in the plasma membrane, we double-immunostained IAR20 cells with the antibody raised against the SEMA domain of SEMA3A. From observation under the laser scanning microscope (Fig. 2a) and from comparison of their signal intensity in the plasma membrane (*data not shown*), a partial colocalization of class 3 semaphorins and Cx43 was confirmed.

Since a major function of connexins is to form gap junctions, they are considered to be localized at the cell-cell contact area. We therefore examined whether both Cx43 and class 3 semaphorins are localized in the plasma membrane only at the cell-cell contact area. After immunostaining of IAR20 cells, we prepared serial sections of three-dimensional images of cell-cell contact areas and noncontact areas under the laser scanning microscope. Our results showed that class 3 semaphorins and Cx43 are indeed located only at cell-cell contact areas and not in the plasma membrane, which is not in direct contact with adjacent cells (Fig. 2b).

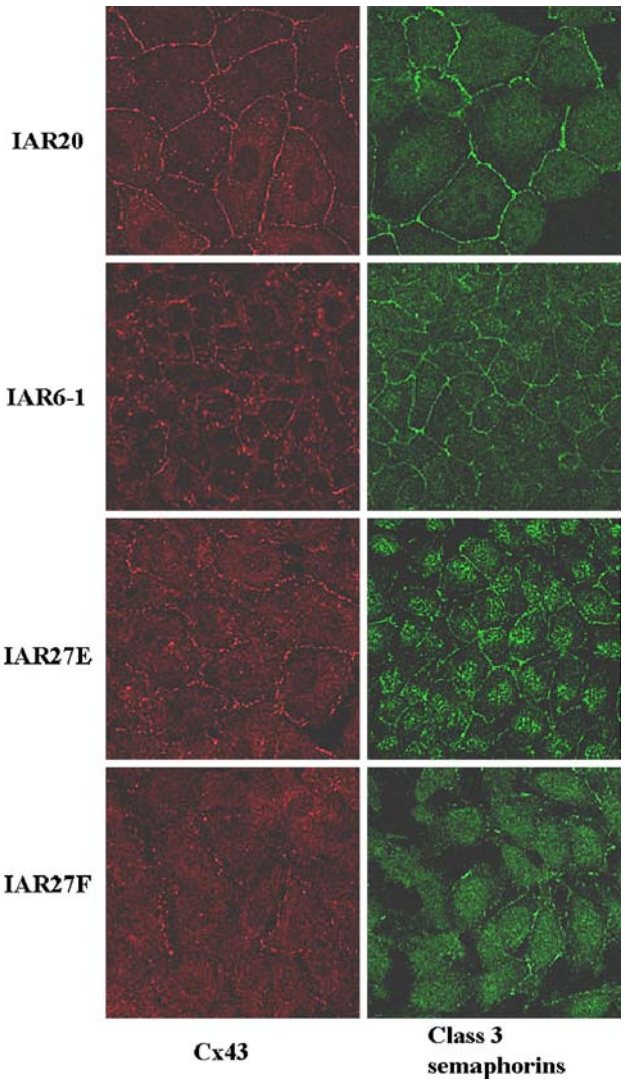

Fig. 1 Localization of Cx43 and class 3 semaphorins in IAR cell line series (IAR20, 6-1, 27E and 27F)

SEMA3F Is Expressed in All IAR Cell Lines and Controls Intracellular Localization and Function of Cx43

Expression of three members of class 3 semaphorins, SEMA3A, SEMA3B and SEMA3F, in IAR cell lines was examined by RT-PCR (Fig. 3). SEMA3A was expressed in IAR20 and IAR27E cell lines, and SEMA3B was not expressed in any IAR cell line. On the other hand, SEMA3F was expressed in all IAR cell lines. Thus, we consider that it is SEMA3F which is expressed and localized in a similar fashion to Cx43 in these cell lines.

In order to examine if SEMA3F and Cx43 are functionally related, we knocked down the expression of SEMA3F of IAR20 cells by siRNA (Fig. 4a). When SEMA3F expression was inhibited by siRNA, localization of Cx43 in the plasma membrane as well as GJIC level were reduced

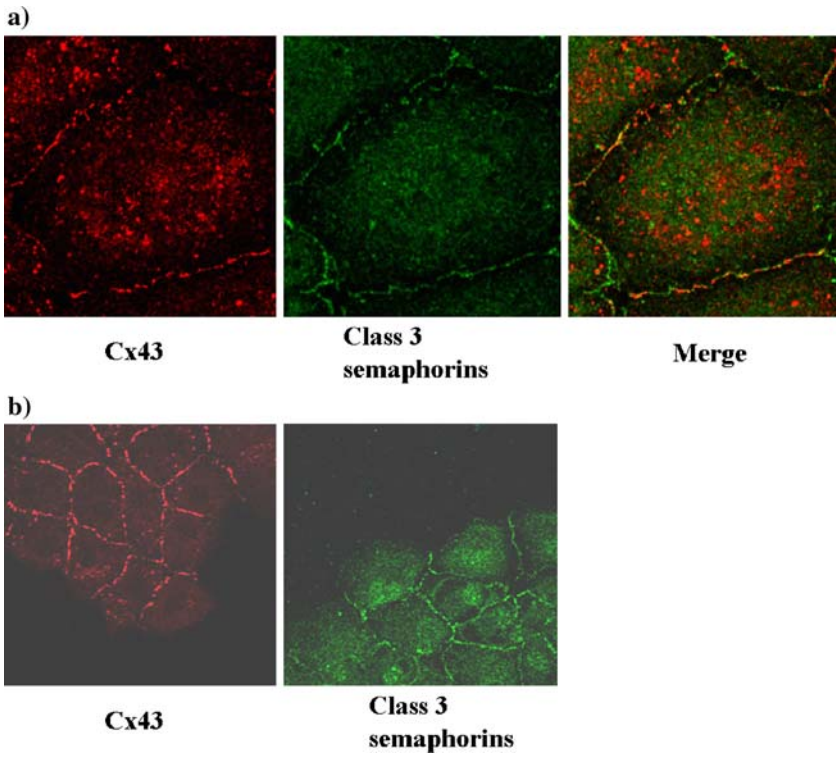

Fig. 2 Localization of Cx43 and class 3 semaphorins in the plasma membrane of IAR20 cells. **a** Double-immunostaining for class 3 semaphorins and Cx43. **b** Localization of class 3 semaphorins and Cx43 in the plasma membrane in the presence or absence of direct cell-cell contact. Cross-sectional observations of these images confirmed that class 3 semaphorins and Cx43 are absent from the plasma membrane in any sections of the image

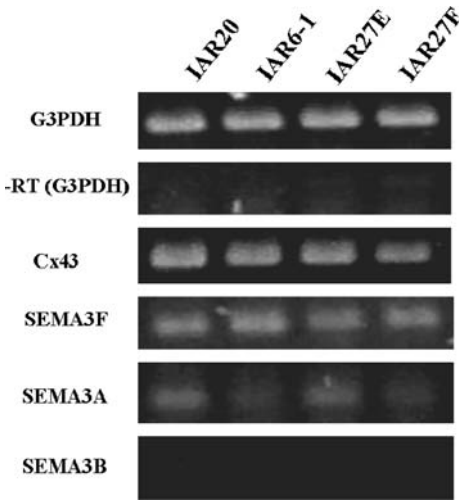

Fig. 3 Expression of class 3 semaphorin family (SEMA3A, SEMA3B and SEMA3F) and Cx43 genes in IAR cell line series

(Fig. 4b,c). These results indicate that SEMA3F is involved in control of intracellular localization and function of Cx43.

Localization of Class 3 Semaphorins and Cx43 Is E-Cadherin-Dependent

We have previously shown that E-cadherin was essential for intracellular transport of connexins in mouse epidermal cells. Thus, only after the E-cadherin gene was transfected into a mouse papilloma cell line did Cx43 move from the cytoplasm at low calcium to cell-cell contact areas at high calcium and was GJIC ability recovered (Jongen et al., 1991; Hernandez-Blazquez et al., 2001). In order to see whether intracellular localization of class 3 semaphorins is also E-cadherin-dependent, we examined its localization in P3/22(E) cells (E-cadherin-expressing mouse papilloma cells) in LC and HC media. We found that class 3 semaphorins remained in the cytoplasm at low calcium but moved to cell-cell contact areas at high calcium together with Cx43 and E-cadherin (Fig. 5), suggesting that the intracellular localization of not only Cx43 but also class 3 semaphorins is E-cadherin-dependent.

SEMA3F Binds with Cx43 in the Yeast Two-Hybrid Complementation Assay

Our results described above suggested that SEMA3F is closely associated with Cx43 and that it may mediate E-cadherin-mediated control of Cx43. Therefore, we examined whether SEMA3F directly binds to Cx43. Since Cx43 is properly localized in the plasma membrane even without its C-terminal domain (Omori & Yamasaki, 1999), we hypothesized that such a putative binding site may reside in the cytoplasmic loop domain. Thus, we prepared the cytoplasmic loops of Cx43 and Cx26 as the baits and examined their possible interaction with SEMA3F, employing a highly stringent two-hybrid assay (yeast strain AH109 and four reporters, ADE2, HIS3, LacZ and MEL1).

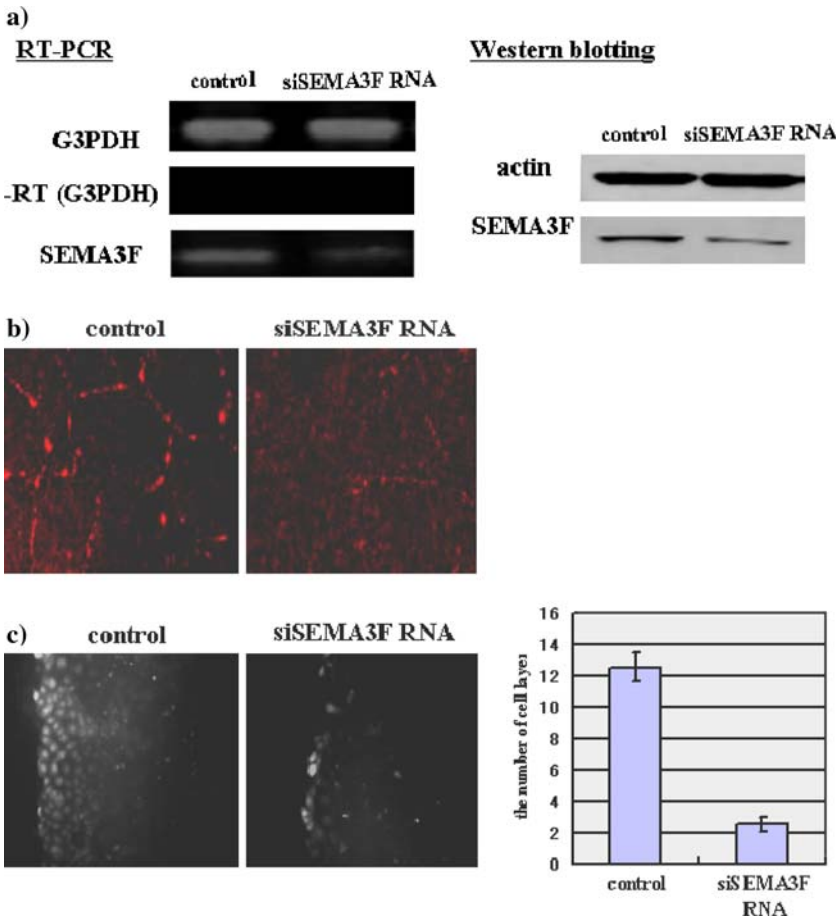

Fig. 4 Effect of SEMA3F gene expression knockdown by siRNA on intracellular localization and function of Cx43 in IAR20 cells. **a** SEMA3F mRNA and protein expression. **b** Immunostaining of Cx43. **c** GJIC level measured by the scrape-loading method as described in the text

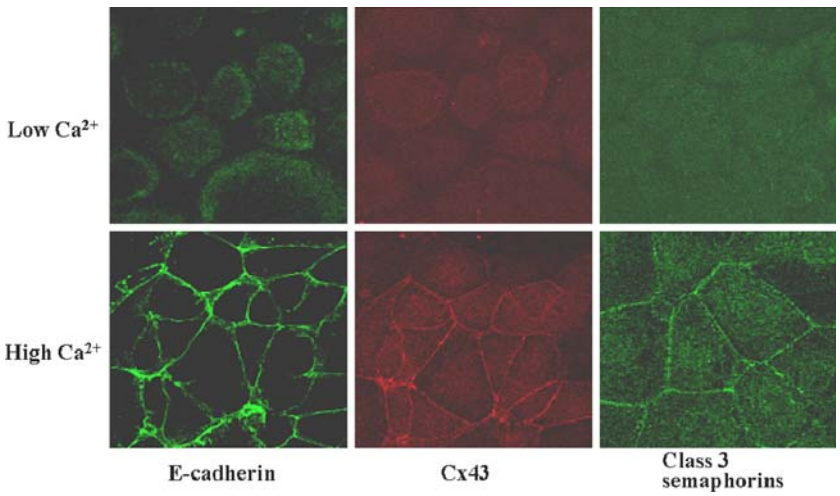

Fig. 5 Localization of class 3 semaphorins and Cx43 in mouse papilloma cell line P3/22(E) in LC and HC culture media

In such cotransformation assays, SEMA3F was indeed found to interact with the human Cx43 cytoplasmic loop but not with Cx26 or P53 (Table 1).

In order to see whether SEMA3F can be picked up as a candidate protein from the cDNA library by the same Cx43 bait, we performed the two-hybrid screening assay employing the human heart cDNA library. Among a number of candidate genes cloned in the screening, we found that SEMA3F represents one of those which show strong interaction with the bait in the yeast two-hybrid complementary assay (Table 1). These results reinforce our idea that SEMA3F is a specific binding partner of Cx43.

Table 1 Characterization of the interaction of the Cx43 cytoplasmic loop domain with SEMA3F and other candidates by the yeast two-hybrid complementation assay

Gene (accession number)	Interaction[a]
SEMA3F (U33920)	++
RBT-1 (AF192529)	++
HSJ2 (AB015798)	++
Natriuretic homologue (M25296)	+
AP43-1 (AL833672)[b]	+

Candidate genes which bind to the bait, cytoplasmic domain of Cx43, were identified by the yeast two-hybrid screening assay from the human heart cDNA library as described in the text

[a] We confirmed that the interaction of positive control, i.e., p53 and SV40 T antigen was ++. We also found that SEMA3F does not interact with the cytoplasmic loop domain of Cx26

[b] Only a part of the sequence of the gene AL833672 was found in this clone

Discussion

In this study, we examined a possible relationship between Cx43 and a member of class 3 semaphorins, SEMA3F, since both are considered to be tumor suppressors and their functional relationship with E-cadherin was reported (Roche et al., 1996; Kessler et al., 2004; Xiang et al., 2002). Our present study showed a very close localization of these two proteins at the plasma membrane in a series of rat liver epithelial cell lines. The expression levels of both proteins were inversely related with the level of transformed phenotypes of these cell lines, supporting their role in tumor suppression. When expression of SEMA3F is reduced in IAR20 cells, stable localization of Cx43 in the plasma membrane and GJIC are inhibited, suggesting a role of SEMA3F in controlling Cx43.

Our results from the yeast two-hybrid complementation assay also indicate that SEMA3F is directly associated with the Cx43 cytoplasmic loop domain. Moreover, when the human heart cDNA library was screened with the Cx43 cytoplasmic loop as the bait in yeast two-hybrid assays, the SEMA3F gene was isolated as one of the candidate genes. While these results strongly indicate that SEMA3F directly interacts with Cx43, our attempt to coimmunoprecipitate them was unsuccessful (*data not shown*). These results leave some doubt about their direct binding. However, considering strong evidence from other results supporting a close relationship between SEMA3F and Cx43 and the fact that they are directly bound in the yeast, it is possible that their binding was not strong enough to tolerate coimmunoprecipitation.

For studying protein expression of SEMA3F, we used the antibody raised against the SEMA domain of the SEMA3 family. Therefore, there was uncertainty whether SEMA3F itself or other SEMA3 members is associated with intracellular localization and function of Cx43. We concluded that SEMA3F, rather than other SEMA3 members, controls Cx43 since only SEMA3F is expressed in all IAR cell lines, SEMA3F-specific siRNA expression downregulated the function of Cx43 and SEMA3F was found to bind directly with Cx43 in the yeast.

A number of proteins which bind to the C-terminal domain of Cx43 have been reported (Toyofuku et al., 1998; Giepmans & Moolenaar, 1998; Butkevich et al., 2004; Fu et al., 2004; Gellhaus et al., 2004). On the other hand, we have previously shown that intracellular trafficking and GJIC ability of Cx43 are not reduced when most parts of its cytoplasmic C-terminal region are deleted (Omori & Yamasaki, 1999). We therefore considered that the C-terminal domain of Cx43 plays important roles in its regulation rather than its intrinsic ability. In addition, we have shown that E-cadherin controls intracellular trafficking of Cx26, which has a very short cytoplasmic C-terminal domain (Hernandez-Blazquez et al., 2001), suggesting the importance of a cytoplasmic domain other than the C-terminal region of connexins. The importance of interaction of the cytoplasmic loop domain and the C-terminal tail of Cx43 for its permeability has long been proposed (Duffy et al., 2002; Morley, Taffet & Delmar, 1996; Seki et al., 2004). Recent studies have shown that aquaporin-0 binds to the cytoplasmic loop domain of Cx45.6 in the eye lens (Yu & Jiang, 2004; Yu et al., 2005). However, this interaction had no effect on GJIC ability of Cx45.6 (Yu et al., 2005). Our data showing that SEMA3F binds to the cytoplasmic loop domain and regulates intracellular trafficking and GJIC of Cx43 indicate an additional important role of this domain in the control of connexin functions.

During the course of this study, we found that N-cadherin controls the intracellular localization and function of Cx43 in HeLa cells. Our preliminary studies also demonstrated that N-cadherin controls the localization and function of Cx43 in IAR20 and IAR6-1 cells (*unpublished results*). Similar N-cadherin-dependent control of Cx43 localization has recently been reported from studies using HeLa cells (Shaw et al., 2007). Control of connexin function by E-cadherin has already been reported from several laboratories, including ours (Mege et al., 1988; Jongen et al., 1991; Meyer et al., 1992). Since both E-cadherin and N-cadherin are able to control the intracellular localization of connexins, control of intracellular trafficking and function of connexins by functional cadherins may be a general mechanism.

In our previous study exploring the mechanisms by which E-cadherin controls connexin trafficking, we demonstrated that E-cadherin controls intracellular trafficking of Cx43 and Cx26 from the endoplasmic reticulum to the plasma membrane via the Golgi apparatus in mouse papilloma cells (Hernandez-Blazquez et al., 2001). These results indicated that E-cadherin provides signals for

intracellular trafficking and/or stable plasma membrane localization of connexins and that an intermediate protein(s) which directly binds to connexins may exist. Since intracellular localization of not only Cx43 but also SEMA3 members was E-cadherin-dependent in a mouse cell line and since Cx43 can bind to SEMA3F directly, it may be hypothesized that E-cadherin controls Cx43 with the aid of SEMA3F.

References

Brambilla E, Constantin B, Drabkin H, Roche J (2000) Semaphorin SEMA3F localization in malignant human lung and cell lines. A suggested role in cell adhesion and cell migration. Am J Pathol 156:939–950

Bruzzone R, White TW, Paul DL (1996) Connections with connexins: the molecular basis of firect intercellular signaling. Eur J Biochem 238:1–27

Butkevich E, Hulsmann S, Wenzel D, Shirao T, Duden R, Majoul I (2004) Drebrin is a novel connexin-43 binding partner that links gap junctions to the submembrane cytoskeleton. Curr Biol 14:650–658

Duffy HS, Sorgen PL, Girvin ME, O'Donnell P, Coombs W, Taffet SM, Delmar M, Spray DC (2002) pH-dependent intramolecular binding and structure involving Cx43 cytoplasmic domains. J Biol Chem 277:36706–36714

El-Fouly MH, Trosko JE, Chang CC (1987) Scrape-loading and dye transfer. A rapid and simple technique to study gap junctional intercellular communication. Exp Cell Res 168:422–430

Fu CT, Bechberger JF, Ozog MA, Perbal B, Naus CC (2004) CCN3 (NOV) interacts with connexin43 in C6 glioma cells: possible mechanism of connexin-mediated growth suppression. J Biol Chem 279:36943–36950

Gellhaus A, Dong X, Propson S, Maass K, Klein-Hitpass L, Kibschull M, Traub O, Willecke K, Perbal B, Lye SJ, Winterhager E (2004) Connexin43 interacts with NOV: a possible mechanism for negative regulation of cell growth in choriocarcinoma cells. J Biol Chem 279:36931–36942

Giepmans BN, Moolenaar WH (1998) The gap junction protein connexin43 interacts with the second PDZ domain of the zona occludens-1 protein. Curr Biol 8:931–934

Hernandez-Blazquze FJ, Joazeiro PP, Omori Y, Yamasaki H (2001) Control of intracellular movement of connexins by E-cadherin in murine skin papilloma cells. Exp Cell Res 114:545–555

Jongen WMF, Fitzferald DJ, Asamoto M, Piccoli C, Slaga TJ, Gros D, Takeichi M, Yamasaki H (1991) Regulation of connexin43-mediated gap junctional intercellular communication by Ca^{2+} in mouse epidermal cells is controlled by E-cadherin. J Cell Biol 114:545–555

Kessler O, Shraga-Heled N, Lange T, Gutmann-Raviv N, Sabo E, Baruch L, Machluf M, Neufeld G (2004) Semaphorin-3F is an inhibitor of tumor angiogenesis. Cancer Res 64:1008–1015

Klam RC, Fitzgeral DJ, Piccoli C, Slaga TJ, Yamasaki H (1999) Gap-junctional intercellular communication in epidermal cell lines from selected stages of SENCAR mouse skin carcinogenesis. Cancer Res 49:699–705

Krutovskikh V, Mazzoleni G, Mironov N, Omori Y, Aguelon AM, Mesnil M, Berger F, Partensky C, Yamasaki H (1994) Altered homologous and heterologous gap-junctional intercellular communication in primary human liver tumors associated with aberrant protein localization but not gene mutation of connexin 32. Int J Cancer 56:87–94

Leithe E, Sirnes S, Rivedal E (2006) Downregulation of gap junctions in cancer cells. Crit Rev Oncog 12:225–256

Loewenstein WR, Kanno Y (1966) Intercellular communication and the control of tissue growth: lack of communication between cancer cells. Nature 209:1248–1249

Mege RM, Matsuzaki F, Gallin WJ, Goldberg JI, Cunningham BA, Edelman GM (1988) Construction of epithelioid sheets by transfection of mouse sarcoma cells with cDNAs for chicken cell adhesion molecules. Proc Natl Acad Sci USA 85:7274–7278

Mesnil M, Asamoto M, Piccoli C, Yamasaki H (1994) Possible molecular mechanism of loss of homologous and heterologous gap junctional intercellular communication in rat liver epithelial cell lines. Cell Adhes Commun 2:377–384

Mesnil M, Crespin S, Avanzo JL, Zaidan-Dagli ML (2005) Defective gap junctional intercellular communication in the carcinogenic process. Biochim Biophys Acta 1719:125–145

Mesnil M, Montesano R, Yamasaki H (1986) Intercellular communication of transformed and non-transformed rat liver epithelial cells. Exp Cell Res 165:391–402

Meyer RA, Laird DW, Revel JP, Johnson RG (1992) Inhibition of gap junction and adherens junction assembly by connexin and A-CAM antibodies. J Cell Biol 119:179–189

Montesano R, Bannikov G, Drevon C, Kuroki T, Saint-Vincent L, Tomatis L (1980) Neoplastic transformation of rat liver epithelial cells in culture. Ann N Y Acad Sci 349:323–331

Montesano R, Drevon C, Kuroki T, Saint-Vinsent L, Handleman S, Sanford KK, DeFeo D, Weinstein BJ (1977) Test for malignant transformation of rat liver cells in culture: cytology, growth in soft agar, and production of plasminogen activator. J Natl Cancer Inst 59:1651–1658

Morley GE, Taffet SM, Delmar M (1996) Intramolecular interactions mediate pH regulation of connexin43 channels. Biophys J 70:1294–1302

Nasarre P, Kusy S, Constanin B, Castellani V, Drabkin HA, Bagnard D, Roche J (2005) Semaphorin SEMA3F has a repulsing activity on breast cancer cells and inhibits E-cadherin-mediated cell adhesion. Neoplasia 7:180–189

Omori Y, Yamasaki H (1999) Gap junction proteins connexin32 and connexin43 partially acquire growth-suppressive function in HeLa cells by deletion of their C-terminal tails. Carcinogenesis 20:1913–1918

Roche J, Boldog F, Robinson M, Robinson L, Varella-Garcia M, Swanton M, Waggoner B, Fishel R, Franklin W, Gemmill R, Drabkin H (1996) Distinct 3p21.3 deletions in lung cancer and identification of a new human semaphorin. Oncogene 12:1289–1297

Seki A, Duffy HS, Coombs W, Spray DC, Taffet SM, Delmar M (2004) Modifications in the biophysical properties of connexin43 channels by a peptide of the cytoplasmic loop region. Circ Res 95:e22–e28

Shaw RM, Fay AJ, Puthenveedu MA, von Zastrow M, Jan YN, Jan LY (2007) Microtubule plus-end-tracking proteins target gap junctions directly from the cell interior to adherens junctions. Cell 128:547–560

Sohl G, Willecke K (2003) An update on connexin genes and their nomenculture in mice and man. Cell Commun Adhes 10:173–280

Toyofuku T, Yabuki M, Otsu K, Kuzuya T, Hori M, Tada M (1998) Direct association of the gap junction protein connexin-43 with ZO-1 in cardiac myocytes. J Biol Chem 273:12725–12731

Trosko JE, Ruch RJ (2002) Gap junctions as targets for cancer chemoprevention and chemotherapy. Curr Drug Targets 3:465–482

Xiang R, Davalos AR, Hensel CH, Zhou XJ, Tse C, Naylor SL (2002) Semaphorin 3F gene from human 3p21.3 suppresses tumor formation in nude mice. Cancer Res 62:2637–2643

Yamasaki H, Naus CCG (1996) Role of connexin genes in growth control. Carcinogenesis 17:1199–1213

Yamasaki H, Hollstein M, Mesnil M, Martel N, Aguelon AM (1987) Selective lack of intercellular communication between transformed and nontransformed cells as a common property of chemical and oncogene transformation of BALAB/c 3T3 cells. Cancer Res 47:5658–5664

Yamasaki H, Krutovskikh V, Mesnil M, Tanaka T, Zaidan-Dagli ML, Omori Y (1999a) Role of connexin (gap junction) genes in cell growth control and carcinogenesis. C R Acad Sci III 322:151–159

Yamasaki H, Omori Y, Krutovskikh V, Zhu W, Mironov N, Yamakage K, Mesnil M (1999b) Connexins in tumour suppression and cancer therapy. Novartis Found Symp 219:241–260

Yamasaki H, Omori Y, Zaidan-Dagli ML, Mironov N, Mesnil M, Krutovskikh V (1999c) Genetic and epigenetic changes of intercellular communication genes during multistage carcinogenesis. Cancer Detect Prev 23:273–279

Yu XS, Jiang JX (2004) Interaction of major intrinsic protein (aquaporin-0) with fiber connexins in lens development. J Cell Sci 117:871–880

Yu XS, Yin X, Lafer EM, Jiang JX (2005) Developmental regulation of the direct interaction between the intracellular loop of connexin 45.6 and the C terminus of major intrinsic protein (aquaporin-0). J Biol Chem 80:22081–22090