The Gap Junction-independent Tumor-suppressing Effect of Connexin 43*

Received for publication, May 14, 2003, and in revised form, August 20, 2003
Published, JBC Papers in Press, September 2, 2003, DOI 10.1074/jbc.M305072200

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The gap junction gene connexin 43 (Cx43) showed tumor-suppressing effects on various tumor cell lines. We have previously demonstrated that Cx43 inhibited expression of S phase kinase-associated protein 2 (Skp2), the human F-box protein that regulates the ubiquitination of p27. Cx43 did not alter the mRNA level of SKP2, but it promoted the degradation of the Skp2 proteins (Zhang, Y. W., Nakayama, K., Nakayama K. I., and Morita, I. (2003) Cancer Res. 63, 1623–1630). In this study, we showed that the specific gap junction inhibitor 18 β-glycyrrhetinic acid did not influence the inhibitory effect of Cx43 on Skp2 expression. Further, the deletion mutation analyses demonstrated that the C-terminal domain of Cx43 did not form gap junctions was sufficient to inhibit expression of Skp2, whereas the N-terminal domain that formed the gap junctions did show such an effect. Like the full-length Cx43, the C-terminal domain also increased the protein instability of Skp2, whereas the N terminus did not. Moreover, the C-terminal domain was as effective as the full-length Cx43 in inhibiting cell proliferation; however, the N-terminal domain did not show any inhibitory effect on cell proliferation. Therefore, these data revealed a gap junction-independent pathway for Cx43 to inhibit tumor growth by suppressing the Skp2 expression.

As one of the cell-cell interaction structures, the gap junctions (GJs) provide direct transfer of small hydrophilic molecules (e.g. ions and metabolites) up to ~1 kDa in size from the cytoplasm of one cell to that of adjacent cells. This process is called gap junctional intercellular communication (GJIC). GJs are composed of connexin (Cx) proteins. Currently, at least 14 Cxs have been cloned and identified (1, 2).

A large number of studies have indicated that certain Cxs (e.g. Cx26, Cx32, and Cx43) have tumor-suppressing effects (3–5). Either the Cx-modulated GJs or the Cx protein itself contributed to the effects of tumor growth inhibition (4). It seems that the Cx species and the cell types used determined whether the GJs or the Cx proteins regulate the tumor growth (4, 6).

Recently, we reported that Cx43 suppressed expression of S phase kinase-associated protein 2 (Skp2), the human F-box protein that regulates p27 ubiquitination (7); through it, Cx43 increased the level of p27 and inhibited cell proliferation of the osteosarcoma U2OS cells (5, 7). Here we report that Cx43 inhibited Skp2 expression via a GJ-independent pathway.

EXPERIMENTAL PROCEDURES

Construction of the Cxs Mutants—The rat heart cDNA library (TaKaRa, Tokyo, Japan) was used to generate the Cx43 mutants by PCR. The primers will be provided upon request. TaKaRa Ex Taq polymerase (TaKaRa) was used to attach an additional A at the 3′ end of the PCR products. Then the PCR products were ligated into the pcDNA3.1/V5-HisTOPO (Invitrogen) vector using the TA cloning techniques. Sense insertion and the DNA sequence were confirmed by both restriction enzyme reaction and sequencing.

Cell Culture and Transfection—U2OS and COS-7 cells were cultivated in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum. Cells were transfected using the FuGENE 6 reagent (Roche Applied Science), as recommended by the manufacturer.

Cell Proliferation—Cell proliferation was estimated by counting the cell number as previously described (5).

GJIC Analysis—A cell-coupling assay was performed as previously described using the gap fluorescence recovery after photobleaching (gap-FRAP) technique (8, 9). In brief, the cells were rinsed twice with PBS containing 1.25 mM CaCl2 and 0.5 mM MgCl2 (PBS (−)) and stained with 5–10 μM 5,6-carboxyfluorescein diacetate (CFDA) in PBS (−) for 10–15 min. The cells were then rinsed five times with PBS (+) to remove the extra dye and covered with PBS (+) for FRAP analysis. The fluorescence of several randomly selected cells that had been stained with CFDA was bleached with 488-nm 600 mW visible laser beams, and then the recovery of fluorescence was monitored over the subsequent 15 min. An unbleached cell served as the 100% fluorescence control and was used to correct for the loss of fluorescence due to background leakage and photobleaching. This technique involves analyzing the increase of fluorescence over time in a photobleached living cell adjacent to unbleached dye-labeled living cells, so it is able to yield quantititative data. The fluorescence recovery of the photobleached cell reflects the level of GJIC that existed between the photobleached cell and neighbor cells.

Immunofluorescence Staining—Immunofluorescence staining was performed according to a modified procedure (5, 8). The cells were seeded into 35-mm glass-cover dishes 1 day prior to transfection with the Cx43 mutants. After 48 h of transfection, the cells were rinsed twice with PBS and fixed in cold methanol/acetone (1:1) for 15 min at room temperature. After thorough washing, the cells were blocked in PBS containing 10% fetal bovine serum and 0.2% saponin for 10–15 min. The cells were then rinsed five times with PBS (+) to remove the extra dye and covered with PBS (+) for FRAP analysis. The fluorescence of several randomly selected cells that had been stained with CFDA was bleached with 488-nm 600 mW visible laser beams, and then the recovery of fluorescence was monitored over the subsequent 15 min. An unbleached cell served as the 100% fluorescence control and was used to correct for the loss of fluorescence due to background leakage and photobleaching. This technique involves analyzing the increase of fluorescence over time in a photobleached living cell adjacent to unbleached dye-labeled living cells, so it is able to yield quantititative data. The fluorescence recovery of the photobleached cell reflects the level of GJIC that existed between the photobleached cell and neighbor cells.

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The abbreviations used are: GJ, gap junction; GJIC, gap junctional intercellular communication; BFA, brefeldin A; Cx, connexin; GA, 18 β-glycyrrhetinic acid; Skp2, S phase kinase-associated protein; PBS, phosphate-buffered saline; FL, full-length.
The Specific GJ Inhibitors Did Not Alter the Effects of Cx43 on Skp2 Expression—In our previous study (5), we reported that stable-transfection of Cx43 elevated the level of the cyclin-dependent kinase inhibitor p27 in the human osteosarcoma U2OS cells. We recently demonstrated that Cx43 suppressed the expression of Skp2 (7), the human F-box protein that regulates the ubiquitin-dependent p27 degradation. Because both the GJ-dependent and the GJ-independent pathways were involved in the Cx43-induced p27 up-regulation (5), we then asked whether Cx43 inhibited expression of Skp2 via the GJ-dependent pathway or not. We first examined effects of two GJ inhibitors on the down-regulated Skp2 expression by Cx43. Brefeldin A (BFA) blocks the transfer of the Cx43 proteins from the Golgi apparatus to the cellular membranes, thus preventing formation of both the GJs and the hemi-channels by Cx43 (5). However, 18β-glycyrrhetinic acid (GA) specifically inhibits the GJ function whereas it does not affect the GJ formation (5). Thus, both BFA and GA significantly inhibited the GJIC level of the Cx43-transfected cells (Fig. 1A) (5). Then we examined the expression of Skp2 in the presence of these two GJ inhibitors. As reported (7), the level of Skp2 was clearly reduced in the Cx43-transfected cells (Fig. 1A, lanes 1–3). Nevertheless, treatment with neither BFA nor GA changed the reduction in the level of Skp2 in the Cx43-transfected cells (Fig. 1A, lanes 3–5). Because Cx43 reduced the level of Skp2 via increasing the protein degradation (7), we also examined effects of BFA and GA on the Skp2 protein stability. Fig. 1B showed that the Skp2 degradation was clearly enhanced when the Cx43-transfected cells were treated with the protein synthesis inhibitor cycloheximide. However, BFA or GA did not alter the enhanced effect of Cx43 on the degradation of Skp2. Thus, these data suggest that Cx43 inhibited the Skp2 expression likely through a GJ (also including the hemi-channel-)independent pathway, which may be ascribed from the biological role of the Cx43 protein itself.
The Cytoplasmic Carboxyl Terminal Domain Was the Active Part of Cx43 in Suppressing Skp2 Expression—Cx43, like other connexin proteins, goes through the cellular membranes four times, leading to a short N-terminal and a relatively long C-terminal domain within the cytoplasm (3) (Fig. 2A). The above mentioned data indicate that the inhibitory effect of Cx43 on Skp2 expression is likely GJ-independent (Fig. 1). To confirm this hypothesis, we generated four deletion mutants that contained part of either N-terminal or C-terminal domain of Cx43. All these mutants were tagged at the C terminus with an additional His6 tail. To eliminate the possibility that the His6 tail might influence the function of these mutants, we generated the full-length (FL) construct of Cx43, which was also tagged with the His6 tail at the C terminus (Fig. 2A).

We first analyzed the cellular distribution of these mutants. In comparison with the parental cells that did not show any GJ staining in the membranes (Fig. 2B, a), the un-tagged Cx43 proteins, the FL and the N3 constructs (all contain the four trans-membrane domains of Cx43) went into the membranes and formed GJs (Fig. 2B, b–d). However, the N1, N2, and C mutants (all contain only a part of the four trans-membrane domains of Cx43) did not form GJs (Fig. 2B, e–g).

Next we examined the GJIC levels in cells transiently transfected with these mutants. Like Cx43, the FL and the N3 constructs showed very high level of GJIC, whereas the N1, N2, and C mutants did not (Fig. 3). Together with Fig. 2, these data indicate that the four trans-membrane domains are necessary for the formation of functional GJs.

Immunoblotting with the monoclonal anti-His6 antibodies demonstrated that cells transfected with these constructs expressed the similar levels of proteins (Fig. 4A). In addition, FL and C, but not N1, N2, and N3, were also recognized by the anti-Cx43 antibody (Fig. 4A), because only FL and C contained the amino acid sequence a of Cx43, from which the mouse anti-Cx43 antibody was generated (Fig. 2A).

Then we examined effects of these constructs on the Skp2 expression. Cx43 suppressed expression of Skp2 (Fig. 4A, lanes 1 and 2). Likewise, this inhibitory effect was observed in the FL transiently transfected cells (Fig. 4A, lanes 1–4), indicating that the His6 tag at the C terminus does not obviously disturb the effect of Cx43 on Skp2 expression (also see below, Fig. 4C and Fig. 5). The cells transfected with the mutants N1, N2, and N3 did not show any reduction in the level of Skp2 (Fig. 4A, lanes 1 and 5–10). However, expression of Skp2 was significantly inhibited in the mutant C transfected cells (Fig. 4A, lanes 1, 11, and 12); moreover, the inhibitory effect was quite close to that of the full-length Cx43 (Fig. 4A, lanes 2, 3, 11, and 12), indicating that the C-terminal domain (amino acids 242–382) was not only required but also sufficient for Cx43 to inhibit Skp2 expression. Together with Figs. 1–3, these data suggest that Cx43 did not require the GJ forming domain (amino acids 1–242) to inhibit Skp2 expression. In other words, the inhibitory effect of Cx43 on Skp2 was GJ-independent.

We have previously reported that Cx43 did not change the mRNA level of SKP2, but increased the protein instability (7). Likewise, all these constructs did not show obvious effects on the mRNA level of SKP2 (Fig. 4B). The effects of these constructs on the protein stability of Skp2 were also examined. The results showed that Cx43, FL, and C increased the protein instability of Skp2 in the presence of cycloheximide, whereas N1, N2, and N3 did not show any effect (Fig. 4C). Thus, these data confirmed the negative role of the C terminus of Cx43 on Skp2 expression. They argue again that Cx43 inhibited Skp2...
expression via mechanisms independent of the Cx43-modulated GJs.

The Cytoplasmic C Terminus of Cx43 Inhibited Cell Proliferation—Subsequently, we examined effects of these mutants on cell proliferation by counting the cell number. As reported previously (5, 7), cell proliferation of the Cx43 stably transfected U2OS cells was significantly inhibited compared with the parental cells (Fig. 5). The FL and C constructs also showed strong inhibitory effects on cell proliferation of U2OS cells, although the effects were weaker than that of stably transfected Cx43. However, the N1, N2, and N3 mutants did not inhibit cell proliferation at all (Fig. 5). These data suggest that the C terminus also had the ability to inhibit proliferation of U2OS cells, implying that the GJ-independent role of Cx43 contributed primarily to the Cx43-induced cell proliferation inhibition.

Finally, to preclude the possibility that the inhibition of Cx43 on Skp2 expression were only observed in U2OS cells, we examined the effects of these mutants on other cell lines. As shown in Fig. 6A, the FL and C mutants, but not the N1, N2, and N3 mutants, clearly reduced the level of Skp2 in COS-7 cells. Moreover, the FL and C mutants also significantly slowed the cell proliferation of COS-7 cells, whereas the N1, N2, and N3 mutants did not show any effect on the cell proliferation (Fig. 6B). We noticed that the N3 mutant did not inhibit cell proliferation in both tested cell lines, even though it formed GJs and showed high level of GJIC. However, our previous data showed that the GJ function of Cx43 did play a role in cell proliferation inhibition. One possible explanation may be that the GJs formed by the N3 mutant are not completely the same as those formed by the full-length Cx43. Together, these data suggest a gap junction-independent pathway for Cx43 to inhibit cell proliferation in certain cell lines by down-regulation of Skp2.

DISCUSSION

In the present study, we provided evidence that Cx43 inhibited expression of the human F-box protein Skp2 by mechanisms independent of the Cx43-modulated GJs. First, the specific GJ inhibitors did not affect the inhibitory effect of Cx43 on Skp2 expression. Second, the cytoplasmic C-terminal domain of Cx43 (amino acids 242 to 382 that did not form the GJ channels) exhibited nearly the same effects on the cell proliferation as that of the full-length Cx43 on Skp2 expression, whereas the N-terminal part of Cx43 (amino acids 1 to 242 that contains the four trans-membrane domains of Cx43 and formed the GJ channels) did not. Thus, these data revealed another novel aspect of Cx43 in inhibiting tumor proliferation by negative regulation of Skp2 via GJ-independent mechanisms.

The principal role of the Cx proteins has been thought to be the formation of the GJ channels that mediate the tissue homeostasis. Therefore, the mechanisms by which Cxs inhibit tumor growth were originally proposed to be through the diffusion of putative growth inhibitory factors via the Cx-modulated GJs (10). However, increasing evidence suggests that the GJ-independent roles are also involved in the Cx-induced growth inhibition (5, 6, 11–14). Further, it was reported that the C-terminal domain showed the same growth inhibition effects as the full-length Cx43 in certain cell lines (13, 15) (and the present study). We have previously reported that stable-transfection of Cx43 increased the level of the cyclin-dependent kinase inhibitor p27 (5, 7). This increase was the result of the increased synthesis and the reduced degradation of the p27 proteins. Both the GJ-dependent and the GJ-independent mechanisms contributed to the increase of p27 (5).

We have shown that cAMP was involved in the GJ-dependent pathway (5), whereas our recent study, together with the present data, suggest that a GJ-independent inhibition of Skp2 expression was responsible for the reduced p27 degradation (7).
Although cAMP was involved in the GJ-dependent inhibition of the kinase inhibitor p27 through both the GJ-dependent and the GJ-independent pathway, the level of the cyclin-dependent kinase inhibitor p27 through both the GJ-dependent and the GJ-independent pathway, however, the GJ-independent pathway was the major instigator for the p27 up-regulation (Refs. 5, 7, and this study).

Therefore, we proposed a pathway for Cx43 to inhibit tumor cell proliferation, at least in certain cell lines (Fig. 7). Because we have observed that Skp2 played the major roles in the Cx43-induced p27 increase and cell growth inhibition (7), we suggest that the GJ-independent pathway contributed much more than the GJ-dependent way in the Cx43-induced cell proliferation inhibition.

In comparison with normal cells that have a high level of p27, human tumors often express a low level of p27, and the reduced p27 protein level correlates well with high grade and decreased survival of tumors (reviewed in Refs. 16–18). The reduced level of p27 was reported to be, at least in part, the result of increased expression of Skp2 (19–22). This, then, raises the question of how Skp2 is dysregulated in tumors. Two possibilities may be considered. One is that the normal machinery controlling the level of Skp2 is altered in tumors. It has been reported that the human F-box protein Skp2 (23) and the yeast F-box proteins Cdc4p (24) and Grr1 (25) were themselves subjected to degradation by the ubiquitin-proteasome machinery. Thus, it is possible that the self-regulation mechanisms controlling the level of Skp2 are disturbed in tumors, which consequently results in the elevated level of Skp2. But this hypothesis needs to be tested in human tumors. The other is that Skp2 may be the target of some oncogenes and/or tumor-suppressing genes. Activation of the oncogenes or inactivation of the tumor-suppressing genes increases the level of Skp2 via currently unidentified mechanisms, which may include modulation of the self-ubiquitination system of Skp2. Indeed, recent experimental data supported the later hypothesis. A 2–5-fold increase in the level of Skp2 was observed in lymphomas of TMTV-Nras transgenic mice (19). Transformation of PAX3-FFKR (the oncogenic form of the PAX3 transcription factor) elevated the level of Skp2 accompanied by reduced expression of p27 (26). In contrast, the tumor suppressor PTEN (27) and the anti-tumor agents retinoic acid (28, 29), vitamin D analog (30), and troglitazone (31) caused decreases in Skp2 levels with concomitant increases of p27 proteins in tumor cells. Considering these studies and our observations, we propose that one of the explanations for Cx43 to function as a tumor-suppressing gene may be through targeting the proto-oncogene Skp2, and consequently increasing the level of the cell cycle controller p27.

The mechanisms by which Cx43 promoted the Skp2 degradation remained unclear. Immunoblotting showed that the Cx43 proteins expressed both in the cytoplasm and in the nuclei (data not shown); therefore, it is possible that a protein thought to form the cell-cell interaction channels in the membranes was involved in events happened in the cytoplasm and/or nuclei. Cx43, or the truncated C terminus, may enter the nuclei through the putative nuclear-localization sequence (13) to promote the ubiquitination of Skp2, or they just increase the degradation rate of the Skp2 proteins in the cytoplasm.

Further study is needed to show how Cx43 increased the Skp2 degradation.

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