FEBS Letters 531 (2002) 132–136 FEBS 26622

Identification and functional analysis of novel phosphorylation sites in Cx43 in rat primary granulosa cells

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Received 24 July 2002; revised 10 September 2002; accepted 12 September 2002

First published online 3 October 2002

Edited by Giulio Superti-Furga

Abstract The gap junctional intercellular communication mediated by Cx43 plays indispensable roles in both germ line development and postnatal folliculogenesis. In this study, we focused on the effect of follicle-stimulating hormone (FSH) on the Cx43 protein in rat primary granulosa cells and found that FSH stimulation elevated the phosphorylation in addition to the protein level of Cx43. Serine residues in the carboxyl-terminal region were exclusively phosphorylated in this system and we identified Ser365, Ser368, Ser369 and Ser373 as major phosphorylation sites by FSH stimulation. A Cx43 variant containing mutations at all these serine residues was found to severely reduce dye transfer activity when assayed in HeLa cells. The present study revealed a novel regulatory mechanism of Cx43-mediated gap junctional intercellular communication through phosphorylation in the carboxyl-terminus.

Key words: Connexin 43; Granulosa cell; Follicle-stimulating hormone; Dye-coupling; Gap junction

1. Introduction

Intercellular communications between oocytes and somatic cell components in ovarian follicles are crucial for folliculogenesis and oogenesis [1], and gap junction channels (GJC) might play a principal role in the process [2,3]. Gap junctions are specialized plasma membrane associations containing intercellular channels that are formed by the association of two hemi-channels (connexons), one provided by each of the communicating cells [4,5]. Each connexon is a hexamer subunit, or connexin, which is essential to form a central pore and is a product of members of a multigene family.

We previously detected transcripts of five connexin genes including Cx43 and a newly identified Cx60 in the porcine ovarian follicles [6,7]. Several studies also reported that follicle-stimulating hormone (FSH) could induce Cx43 in ovarian follicles, and the following leuteinizing hormone treatment significantly reduced its expression level [7–10], suggesting that Cx43 was a FSH-responsive gene and also could play an important role for physiological functions granted to FSH. FSH is the central hormone of the female reproductive system [11], and FSH-deficient female mice were infertile due to a block in folliculogenesis prior to antral follicle formation [12]. In a similar fashion, the indispensable roles of gap junctions in the female reproductive process were recently demonstrated using targeted disruption of certain connexin genes including Cx43 [13–15].

Our interest focused on the role of GJC in folliculogenesis, in particular, in clarifying a functional connection between FSH and Cx43. In the present study, we used a FSH-responsive rat primary granulosa cell culture system and analyzed the behavior of Cx43 in terms of its phosphorylation profiles in relation to its function. We found that FSH accelerated the hyperphosphorylation of the Cx43 protein, and identified Ser365, Ser368, Ser369 and Ser373 as major phosphorylation sites. Furthermore, by introducing mutations into these sites, we examined the significance of the phosphorylation for dye transfer activity through Cx43.

2. Materials and methods

2.1. Preparation and culture of granulosa cells

Twenty one-day-old female SD rats (Japan SLC, Shizuoka, Japan) were injected with 2 mg diethylstilbestrol for 4 days, and ovaries were collected after 48 h. Granulosa cells were released by puncturing follicles, and then they were plated in poly-D-lysine-coated dishes at a density of $5\times10^5/cm^2$ with culture medium (F12/Dulbecco's modified Eagle's medium (DMEM), 0.1% bovine serum albumin, 5 μg/ml insulin, 5 μg/ml transferrin, 20 μg/ml gentamicin and 0.5 μg/ml fungizone) supplemented with 5 μg/ml fibronectin. When necessary, granulosa cells thus prepared were stimulated with FSH (100 ng/ml; Vitro Diagnostics Inc.) for the indicated period.

2.2. Cell lysate

Cells were washed with phosphate-buffered saline and lysed in lysis buffer-1 (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 2 mM PMSF, 2 mM Na_3VO_4, 20 mM NaF, 10 mM *N*-ethylmaleimide, 100 kIU/ml aprotinin and 1% Triton X-100) for 10 min at 4°C. Cell lysates were collected in Eppendorf tubes and centrifuged at $17\,000\times g$ for 5 min, and the supernatant was saved.

2.3. Western blot analysis and immunostaining

Proteins were separated by SDS–polyacrylamide gel electrophoresis (PAGE) and transferred to PVDF membranes (Millipore) by electrophoresis. Immune complexes with anti-Cx43 antibody (Sigma; 1:8000) were detected by an enhanced chemiluminescence reagent (Amersham-Pharmacia-Biotech). Dephosphorylation reaction of Cx43 was carried out for 2 h at 37°C by incubating cell extracts in the presence of calf intestine alkaline phosphatase (5 units; Takara, Japan) and when necessary, 20 mM NaF and 1 mM of Na_3VO_4 were added as phosphatase inhibitors [8]. For immunostaining of cells, cells on glass coverslips were fixed and reacted with anti-Cx43 antibody (1:200). After washing the cells, they were incubated with anti-rabbit IgG conjugated with Texas Red (1:100) and mounted in vectashield (Vector Laboratories, CA, USA).

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0014-5793/02/$22.00
PII: S0014-5793(02)03441-5

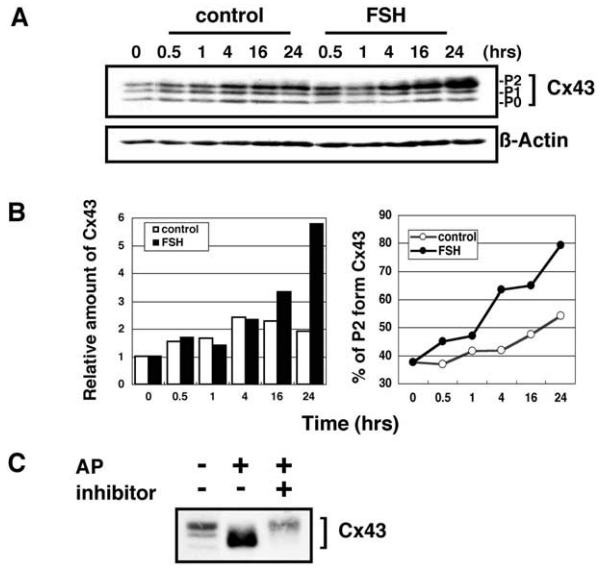

Fig. 1. Expression profiles of Cx43 in FSH-treated and -untreated rat primary granulosa cells. A: Western blot analysis of Cx43 in cells cultured for the indicated periods in the presence (100 ng/ml) or absence of FSH (upper panel). P1 and P2 indicate the hyperphosphorylated forms. The amounts of proteins applied were monitored by probing with anti-β-actin antibody (lower panel). B: Densitometric scanning of the Cx43 protein levels. Total amount of P0, P1 and P2 in each lane of (A) was measured and normalized against that of β-actin (left panel). Percentage of P2 at each time point is shown (right panel). C: Dephosphorylation of Cx43 and mobility shift. Cell extracts were treated with calf intestine alkaline phosphatase in the presence or absence of phosphatase inhibitors as described in Section 2.

2.4. Construction of recombinant Cx43 protein and in vitro phosphorylation reaction

A cDNA encoding a cytoplasmic tail domain of rat Cx43 (Cx43-CT) (amino acid residues from E227 to I382; [16]) was subcloned to 6×His-tagged expression vector pQE31 (Qiagen). Site-directed mutagenesis of each serine residue to alanine was carried out by a PCR mutagenesis method [17]. For the in vitro protein kinase reactions, 2.5 μg of Ni-NTA resin (Qiagen) bound Cx43-CT was mixed with 6 μl of 5×kinase buffer (100 mM Tris–HCl, pH 7.5, 25 mM $MgCl_2$), 1.5 μl of 200 mM $MgCl_2$, 5 μl of [γ-^{32}P]ATP (3000 Ci/mmol), 1.5 μl of 100 μM ATP and adjusted to 25 μl with deionized water. 5 μl of cell lysates was added to the reaction mixture and incubated for 30 min at 30°C. Cell lysates were prepared from granulosa cells that had been stimulated with FSH (100 ng/ml) for 4 h.

2.5. Phosphopeptide mapping and phosphoamino acid analysis

Metabolic labeling, two-dimensional phosphopeptide mapping and phosphoamino acid analysis were performed as previously described [18].

2.6. Expression of Cx43 and dye transfer experiment in HeLa cells

Wild-type (wt) Cx43 and variants were introduced into HeLa cells by lipofectamine plus (Gibco BRL) and expressed. Stably transfected cell lines were selected by G418 and screened by Western blotting. Parental and transfected HeLa cells were maintained in DMEM containing 10% FCS. Cx43-mediated channel activity was measured as described previously [19]; it employed scrape-loading to introduce Lucifer yellow (LY; 0.25%) CH into cells in culture and allowed the monitoring of its transfer into neighboring cells. Images of three different views were captured per one sample and analyzed using the NIH image 1.62 software. Each image was scanned along lines perpendicular to the scrape and at least six lines were scanned. The distance between the internal border of the outmost cell layer and distal edge was measured in micrometers and averaged. In some experiments, rhodamine dextran (RD; 0.75%; molecular weight = 10 kDa), a gap junction-impermeant compound, was administered with LY.

3. Results

3.1. FSH elevates the protein level and induces the hyperphosphorylated form of Cx43

An obvious effect caused by FSH stimulation was the increased expression level of the Cx43 protein. When compared with untreated cells, the difference became significant at 16 h and reached 2.5-fold higher than that in untreated cells at 24 h (Fig. 1A,B). At the same time, three bands (P0, P1 and P2) were constantly observed by Western blotting throughout the incubation period (Fig. 1A), and a characteristic feature seen in FSH-stimulated cells was a significant elevation of the P2 form after 4 h (Fig. 1B). When they were treated with intestine phosphatase, the slowly migrating bands (P1 and P2) disappeared and consequently the intensity of P0 increased (Fig. 1C), implying that the appearance of the slower bands was due to hyperphosphorylation as had been reported previously in other cell systems [8,20]. The FSH-induced increase in the phosphorylation level of Cx43 was further confirmed by metabolic labeling experiments using [^{32}P]orthophosphate (Fig. 2A).

3.2. Phosphorylation profiles of Cx43 and tryptic peptide mapping analysis

Cx43 was reported to be phosphorylated on specific serine or tyrosine residues depending on the cell systems examined [21]. We carried out a phosphoamino acid analysis on Cx43 and revealed that the serine residue was exclusively phosphorylated in the present system with or without FSH stimulation (Fig. 2A). Next, tryptic peptide mapping analysis of metabolically labeled Cx43 in control cells revealed the existence of three major phosphorylated peptides (Fig. 2B, i: a, b and c).

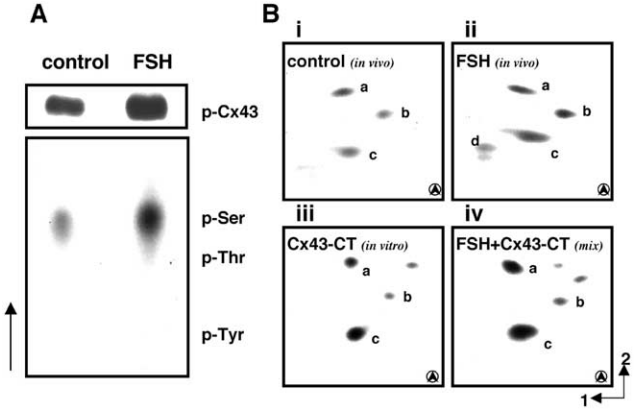

Fig. 2. Phosphorylation profiles of Cx43 in rat granulosa cells. A: Cells were metabolically labeled for 4 h with [^{32}P]orthophosphate in the presence or absence of FSH, and Cx43 was immunoprecipitated and separated on SDS–PAGE (upper panel). Phosphoamino acid analysis of Cx43 (lower panel). The positions of unlabeled ninhydrin-stained phosphoamino acid standards are indicated. B: Two-dimensional tryptic peptide mapping of Cx43 recovered from FSH-treated and -untreated rat primary granulosa cells. Three major phosphopeptide spots (a, b and c) are seen in control (i) and FSH-treated cells (ii). A minor spot d is often seen as discussed in the text. In a similar fashion, the recombinant cytoplasmic tail domain of Cx43 (Cx43-CT) was phosphorylated in vitro using granulosa cell lysate and [γ-^{32}P]ATP (iii). The identities of the spots seen in (ii) and (iii) were confirmed by mixing and developing two samples (iv). The origin is marked by an arrowhead. Arrows 1 and 2 indicate the first dimension of electrophoresis and second dimension of the ascending chromatography, respectively.

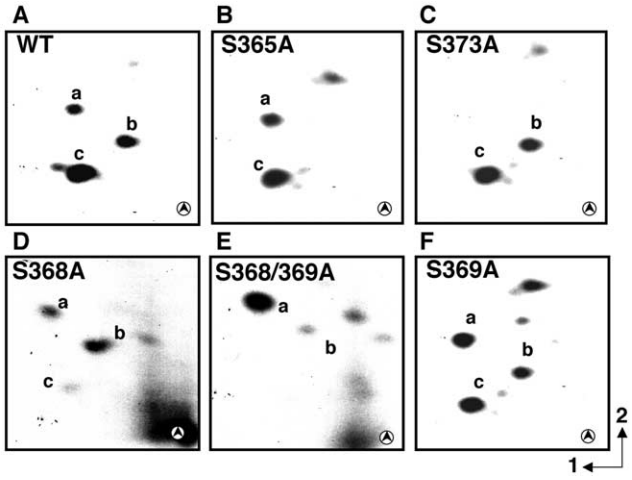

Fig. 3. Two-dimensional peptide mapping of site-directed mutants of Cx43. A variant carrying a substitution from serine to alanine at each residue of Ser365 (S365A), Ser368 (S368A), Ser369 (S369A) or Ser373 (S373A) of Cx43-CT was used as a substrate for in vitro phosphorylation reactions and subjected to tryptic peptide mapping. S368/369A shows a consecutive substitution at Ser368 and Ser369. A: Wt Cx43-CT, B: S365A, C: S373A, D: S368A, E: S368/S369A, and F: S369A. The arrowheads and arrows are as described in Fig. 2.

This profile appeared to be essentially unchanged in FSH-treated cells (Fig. 2B, ii). Faint spots like (d) in Fig. 2B, ii were sometimes observed, but such spots were not seen reproducibly and the same signals were sometimes observed in control cells.

To localize phosphorylation sites on Cx43, we constructed Cx43-CT (amino acid residues from E227 to I382; [16]) and used it as a substrate for an in vitro phosphorylation reaction. It was assumed that the phosphorylation sites on Cx43 seen in the present system would be localized within this cytoplasmic tail, because it contains putative serine phosphorylation sites [22] including several serine residues that had been reported to be targets of various stimuli [21]. In fact, phosphopeptide mapping analysis of the recombinant protein showed essentially identical profiles with those of in vivo labeled Cx43 (Fig. 2B, iii). Mixing of in vivo and in vitro labeled peptides confirmed their identities (Fig. 2B, iv).

3.3. Identification of phosphorylation sites on Cx43

To identify phosphorylation sites precisely, we introduced site-directed mutations (from serine to alanine at each site) into serine residues localized in the Cx43-CT, expressed in *Escherichia coli* and used them as substrates for in vitro labeling reactions. Fig. 3 shows the profiles of two-dimensional tryptic phosphopeptide mapping of the wt and each mutant. The results of the constructs showing identical profiles with that of wt Cx43 are not shown. The S365A and S373A mutants lacked spots b and a, respectively (Fig. 3B,C). A substitution of S368 significantly reduced the density of spot c, but a residual level of the signal remained (Fig. 3D). Since Ser368 is followed by S369 in the same peptide fragment, it was suggested that the existence of consecutive serine residues could affect this profile, and we constructed a variant in which both S368 and S369 were mutated, CT-S368/S369A. Spot c turned out to be completely missing in this variant (Fig. 3E). Since the intensity of spot b became faint in the S368/369A variant, the efficiency of the phosphorylation of Ser365 appeared to be affected by the phosphorylation state of Ser368 and S369. Regarding this, the effect of the mutation at S369

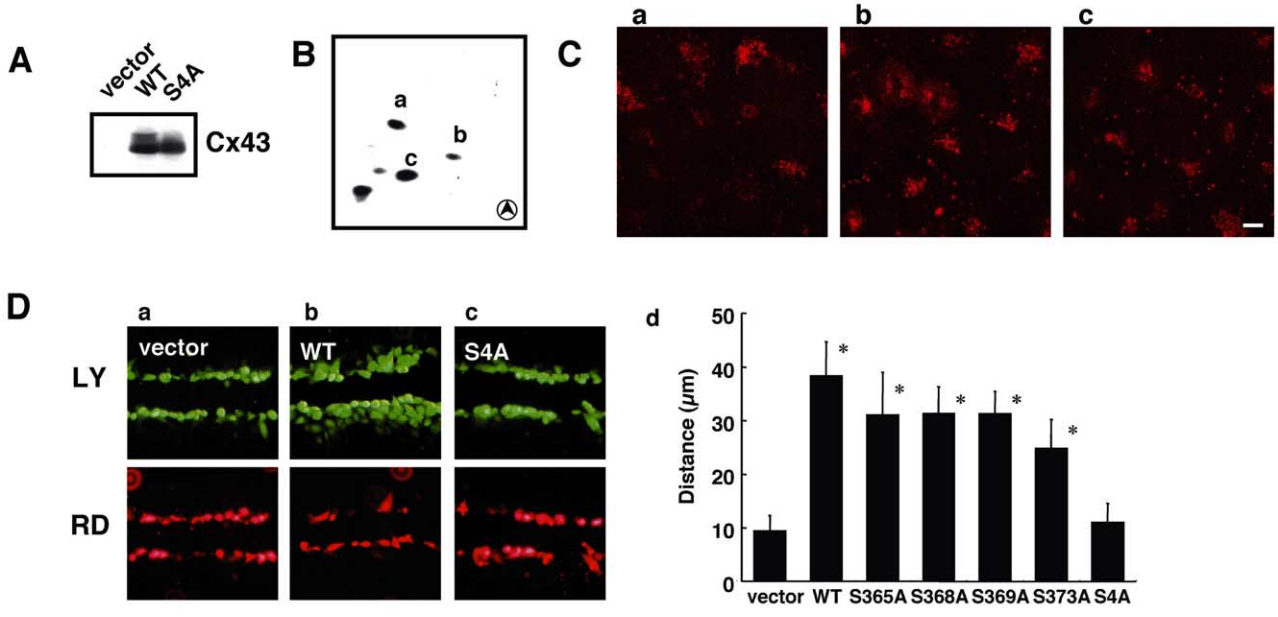

Fig. 4. Expression and dye-coupling of wt Cx43 and variants in HeLa cells. A: Expression profiles in HeLa cells. An empty vector, wt Cx43 or the Cx43-S4A variant was transfected into HeLa cells and the cell extracts were recovered after 24 h. B: Two-dimensional tryptic peptide mapping of wt Cx43 in HeLa cells. Transiently expressed Cx43 in (A) was analyzed as described in Fig. 2. C: Immunostaining of Cx43 in HeLa cells expressing an empty vector (a), wt Cx43 (b) and Cx43-S4A (c). Bar, 10 μm. D: Dye-coupling of wt Cx43 and variants in cloned HeLa cells. Cells were scraped with 27-gauge needles and transmission of LY CH and RD into contiguous cells was monitored. The mock-transfected HeLa cells (a), wt Cx43 (b) and Cx43-S4A (c). Quantification of dye transfer experiments using HeLa cells expressing Cx43 variants (d). The values represent the distance of gap junction-mediated dye transfer (μm). Each value presented is the mean (±S.E.M.) of four experiments ($n=4$); columns with asterisks showed significant differences against vector-transfected cells (Student's t-test, $P<0.05$).

caused little visible effect on the profile (Fig. 3F), suggesting that either Ser368 was more efficient as a target than Ser369 or Ser369 could be a target only when Ser368 was unphosphorylated. Ser365, Ser368, Ser369 and Ser373 were thus identified as major phosphorylation sites and interestingly, these residues consisted of three-double serine sites.

3.4. Mutational and functional analysis of Cx43 in HeLa cells

The above results questioned as to the significance of the phosphorylation of those sites for Cx43-mediated gap junctional intercellular communication. However, since mammalian granulosa cells are known to express at least five kinds of connexin genes including Cx43 [6,7,23], using granulosa cells required caution in performing the functional analyses of Cx43. HeLa cells, on the other hand, are known to lack gap junction-mediated intercellular communication due to undetectable expression of connexin genes and hence, we chose them for the following experiments. First, to examine the phosphorylation states in this cell system, we transiently introduced wt Cx43 and Cx43-S4A, in which Ser365, Ser368, Ser369 and Ser373 were substituted with alanine together, into HeLa cells and analyzed by Western blotting. Although it was not so prominent as in granulosa cells, the slowly migrating form of Cx43 was detected in wt-transfected cells, while no clear band shift was observed in S4A-transfected cells (Fig. 4A). Furthermore, Cx43 was actually found to be phosphorylated in vivo at the same sites detected in granulosa cells (Fig. 4B), which was confirmed by mixing samples from HeLa cells and granulosa cells (data not shown). Spot d shown in Fig. 2B can also be seen in Fig. 4B.

We then made stable cell lines expressing wt Cx43 and variants carrying a single mutation at each residue of Ser365 (S365A), Ser368 (S368A), S369 (Ser369A) and Ser373 (S373A) in addition to the S4A mutant. These lines were confirmed to express the respective Cx43 proteins to comparable levels, and further, their subcellular localization at regions of cell–cell contact was observed (Fig. 4C); only the profiles of wt Cx43 and Cx43-S4A are shown as representative examples. Gap junction-mediated intercellular communication was then assessed and representative results are shown in Fig. 4D. Transfer of LY into contiguous cells, in contrast to RD, was clearly detected in HeLa cell clones expressing wt Cx43, while it was undetectable in cells expressing the empty vector (Fig. 4D, a and b). On the other hand, all the clones expressing the variants with a single mutation (S365A, S368A, S369A and S373A) were found to show the dye-coupling activity at the original or a slightly reduced level compared with wt Cx43, whereas the S4A clone was found to show a level equivalent to that of control cells (vector) (Fig. 4D, c and d). We carried out the same experiments using other clones expressing wt Cx43 and each variant and obtained identical results. Therefore, the results suggested that the phosphorylation of any one of the specific serine residues was not crucial for the dye-coupling under the conditions used, whereas the simultaneous displacement resulted in a significant loss of the activity.

4. Discussion

We newly identified FSH-inducible phosphorylation sites in Cx43 in cultured rat primary granulosa cells. The obvious questions to be answered were how this FSH-induced phosphorylation was accomplished and the significance of the hyperphosphorylation of Cx43. Since at least five connexin genes are known to be expressed in mammalian granulosa cells as described previously, it appeared difficult to specifically examine the effects of FSH on Cx43 in terms of gap junctional communication in primary cells. Therefore, we carried out Triton solubility assay [24] and confirmed that the enhanced phosphorylation of Cx43 by FSH correlated well with the accumulation in gap junctional plaque (data not shown).

Several protein kinases were reported to be directly involved in the phosphorylation of Cx43 and thereby modulated its assembly/disassembly, stability and/or the gating of the gap junctional channel [21]. Since FSH is known to increase the intracellular level of the cAMP concentration, it may be reasonable to suggest that the PKA-dependent pathway somehow plays a role for the hyperphosphorylation of Cx43 in rat granulosa cells. In fact, cAMP was reported to stimulate the gap junction assembly and intercellular communication [21,25–27], and also to elevate phosphorylation levels of Cx43 in certain cell systems [25,28–30], implying the existence of a pathway in which phosphorylation modulates Cx43-mediated intercellular communication positively [21]. Regarding this, the newly identified phosphorylation sites (Ser365, Ser368, Ser369 and Ser373) fit well with putative PKA phosphorylation sites (R-X_{1-2}-S/T-X; [31]), suggesting the possible involvement of PKA for the phosphorylation of these sites. However, the Cx43 protein was shown to be a poor substrate of PKA [27]. Furthermore, evidence has been accumulating that cAMP acts via a PKA-independent mechanism in addition to a PKA-dependent pathway in granulosa cells [32]. Taken together, an elucidation of the role of PKA in the phosphorylation and regulation of the Cx43 protein in the present system may require further investigations.

Since the carboxyl-terminal region of the Cx43 protein was reported to be dispensable for the assembly of functional gap junctions, but crucial for gap junction regulation [27,33], we further examined and revealed the significance of the phosphorylation of these serine residues for Cx43-mediated intercellular communication. FSH-signaling is not an intrinsic property to HeLa cells, making it difficult to examine directly the effects of FSH on Cx43. Instead, we made use of the finding that the same sites in Cx43 were phosphorylated in proliferating HeLa cells and carried out mutational analysis on these sites. Namely, variants containing a single mutation at each of Ser365, Ser368, Ser369 and Ser373 appeared to retain or slightly reduce the dye transfer activity compared with wt Cx43 when assayed in HeLa cells, implying that the phosphorylation of any single serine residue was not crucial for Cx43-mediated dye-coupling. This result further suggested that these serine residues could contribute to the channel activity in a combinatorial manner. In fact, when Cx43-S4A in which Ser365, Ser368, Ser369 and Ser373 were substituted together with alanine was expressed, the dye-coupling was severely reduced despite the localization at cell–cell interfaces. Taken together, it is suggested that the Cx43-CT functions as a negative regulator when it is under the unphosphorylated state, and the accumulation of negative charge in the extreme carboxyl-terminal region could cause a conformational change or a repulsion among each CT, causing the channel to open.

Acknowledgements: This work was supported by a grant from the Japan Livestock Technology Association.

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