Regulation of connexin-43-mediated growth inhibition by a phosphorylatable amino-acid is independent of gap junction-forming ability

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Abstract

The ability of the gap junction phosphoprotein connexin-43 (Cx43) to inhibit DNA synthesis in primary cardiomyocytes is regulated by serine (S) 262, a protein kinase C phosphorylation site that also affects metabolic coupling. We have now examined if the S262-regulated growth suppression is operating in transformed cells and if so whether it depends on gap junction channel forming ability. Serine 262 became phosphorylated in response to protein kinase C stimulation in HEK293 cells transiently expressing either Cx43 or the non-channel-forming carboxy-terminal tail of Cx43 (Cx43CT). Expression of either wild type Cx43 or Cx43CT inhibited DNA synthesis, as did their mutated versions simulating lack of phosphorylation by carrying an S262-to-alanine substitution. The ability to inhibit DNA synthesis was eliminated when expressing mutated versions of either Cx43 or Cx43CT simulating constitutive phosphorylation by carrying an S262-to-aspartate substitution. We conclude that S262 phosphorylation cancels growth inhibition by Cx43 independently of channel-forming ability. (Mol Cell Biochem 289: 201–207, 2006)

Key words: growth regulation, connexins, phosphorylation, structure-function, protein kinase C

Introduction

Connexins are integral membrane proteins that form gap junction intercellular communication (GJIC) channels. Each connexin molecule has three cytosolic, four transmembrane and two extracellular domains [1]. The channel forming ability resides at the N-terminal portion of the molecule, while the cytosolic C-terminal tail (Cx43CT) plays a regulatory role on channel permeability but cannot form channels. GJIC ensures electrical and metabolic coupling of cells, and is important in embryonic development, differentiation and tissue homeostasis [1]. In addition, an inverse relationship has long been known to exist between expression of connexins, GJIC and cell proliferation. In fact, connexin43 (Cx43), has long been viewed as a tumour suppressor [2, 3]. Other connexins, such as Cx32 [4] or Cx26 [5] have also been implicated in suppression of cell proliferation. While earlier studies have implicated the channel forming property of connexins, and GJIC, as necessary for the propagation of growth inhibitory signals [3, 6, 7], several more recent studies have provided evidence for a dissociation of GJIC and ability to suppress growth [8–10]. In addition, while there are some reports that the channel-forming domain of Cx43 can exhibit growth inhibitory activity [11–13], several laboratories have demonstrated that the non-channel forming Cx43CT is capable of inhibiting cell proliferation [14–16]. The mechanism underlying Cx43CT mediated growth inhibition remains largely unknown. It is also not known whether the ability of Cx43CT to inhibit growth can be regulated.
Cx43 is a phosphoprotein, containing several phospho-
rylation sites at its C-tail end [17]. Previously we reported
that S262, a protein kinase C (PKC) target site, affects chan-
nel permeability and is essential for Cx43-mediated growth
inhibition in rat primary cardiomyocytes [18], cells that dis-
play robust endogenous Cx43 expression, GJIC, and are sub-
ject to stringent cell cycle controls. It is not as yet known
whether the S262 site can become phosphorylated and reg-
ulate Cx43-mediated growth inhibition in a transformed and
Cx43-deficient cellular environment, that of HEK293 cells.
It is also not known whether Cx43CT, displaying different
subcellular localization compared to Cx43 [19] can also be-
come phosphorylated at S262 within the cellular milieu, and
whether the S262 site can regulate its ability to suppress
growth. The above questions are addressed in the present
communication.

Materials and methods

Materials

Culture media and fetal bovine serum (FBS) were pur-
chased from Invitrogen, California, or Wisent, Canada, re-
spectively. PMA (4-alpha-phorbol-12-myristate-13-acetate)
was purchased from Sigma Aldrich.

Cell culture

HEK 293 cells (Stratagene, La Jolla, CA) were grown
in Dulbecco’s minimal essential hi-glucose medium supple-
mented with 5% FBS, and 100 μg/ml each of streptomycin
and penicillin. Cells were passaged when the culture reached
around 70% confluence.

Constructs

Construction of plasmids coding for Cx43, Cx43-S262A,
and Cx43-S262D has been described previously [18]. These
plasmids were used as templates for generating, respec-
tively, the N-terminal deletion mutants (aa 243-382) Cx43CT,
Cx43CT(S262A) and Cx43CT(S262D). In brief, the
sense primer (5′-CCGGAATTCAGCATGGATCTCTTCT
ACGTC-3′) containing an EcoRI site and appropri-
ate Kozak sequence and the anti-sense primer (5′-
TTTCGCTCTAGATTAAATCTCCAG-3′) containing a XbaI
site were employed to amplify the C-terminal 139 amino
acids coding sequence. The PCR products were then puri-
ified, digested with both EcoRI and XbaI, and cloned into
pcDNA3.1. The ORF (Open Reading Frame) and coding re-

gion of each construct were sequenced in both directions
(Cortec DNA Service Laboratories Inc., Kingston, ON,
Canada).

Antibodies

Rabbit anti-Cx43 antibodies, raised against a peptide
containing aa 367–382 of Cx43 have been described
and characterized previously [20], were used at 1:5000
dilution for immunofluorescence and 1:20000 for west-
ern blots. Monoclonal anti-Cx43 (aa 250–270) antibod-
ies were from Transduction Laboratories, and polyclonal
anti-P-S262-Cx43 antibodies were from Santa Cruz Biotech-
nology. Both of these anti-Cx43 antibodies are equally ef-

cfective in detecting intact as well as N-terminal truncated
Cx43. Anti-bromodeoxyuridine (BrdU) monoclonal anti-
bodies were from Sigma-Aldrich. Commercially available
antibodies and secondary reagents were used as per manu-
facturer’s instructions. Specificity of all antibodies has been
established in our previous publications [18, 20–22].

Transient gene transfer

HEK 293 cells were seeded at the density of 1 × 10^5 per well
in 6-well (35 mm/well) plates with or without coverslips, one
day before transfection. One μg from each cDNA was added
per well, using the transfection reagent Trans IT 293 (Mirus,
Madison, WI) according to the manufacturer’s instructions.
This procedure resulted consistently in over 60% transfe-
ction efficiency, assessed by immunofluorescence after each
experiment. Cells were allowed to grow for 24–48 hours after
gene transfer, and then processed for immunofluorescence or
protein extraction.

Immunofluorescence

As described previously [18, 22]. Briefly, coverslips were
fixed with 4% paraformaldehyde in phosphate buffered saline
for 15 min, and permeabilized with 0.1% Triton X-100 in
phosphate buffered saline for another 15 min, on ice. Pri-
mary and secondary antibodies were used as described [18,
22]. Cells were counterstained with Hoechst 33342, to vi-

sualize nuclei.

Western blotting

Total cell protein was prepared by lysing cells directly into
denaturing gel electrophoresis buffer as described [19]. Pro-
tein concentration was determined using the BCA assay
(Pierce). Unless otherwise specified, 10 μg of each sample