Mini Review

Mind the Gap; Regulation of Gap Junctional, Intercellular Communication by the Src Oncogene Product and its Effectors

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Abstract. *Gap junctions are channels that connect the interiors of neighboring cells and are formed by the connexin (Cx) proteins. A reduction in gap junctional, intercellular communication (GJIC) often correlates with increased growth and neoplastic transformation. Cx43 is a widely expressed connexin which can be phosphorylated by the Src oncoprotein tyrosine kinase on tyr247 and -265, and this reduces communication. However, Src activates multiple signalling pathways such as the Ras/Raf/Erk and PLCγ/protein kinase C, which can also phosphorylate Cx43 and interrupt communication. In addition, the Src effector Cas, which has an adaptor function, binds Cx43 to suppress gap junctional communication. In sharp contrast, activation of a different Src effector, the cytoplasmic transcription factor Signal transducer and activator of transcription-3 (Stat3) is not required for the Src-mediated, GJIC suppression. In fact, Stat3 is actually required for the maintenance of gap junctional communication in normal cells with high GJIC.*

Contrary to unicellular organisms, cells in multicellular metazoa must divide under strict control. Thus, intercellular communication is crucial in the regulation of cellular functions and it often occurs indirectly through the release of diffusible growth factors by certain cells that initiate the signal through receptors on target cells. Communication between cells can also be achieved directly, through the gap junctions, *i.e.* channels running through the membrane which allow the passage of ions and other molecules between the interiors of adjacent cells. Gap junctions consist of transmembrane proteins, termed connexins, a family of at least 20 members, as described in mammals. They are often designated with a suffix referring to their molecular weight. Gap junctions are formed by the aggregation of two hemi-channels of six connexons each, contributed by the two neighboring cells. This structure forms an aqueous channel through the two plasma membranes, that permits the passage of small-molecules such as ions, nucleotides, aminoacids, short-peptides or even RNA (24), between adjacent cells (46).

Results from a number of labs indicated that an increase in cell proliferation correlates with a reduction in gap junctional, intercellular communication (GJIC). In fact, a number of oncogene products such as the transforming protein of the Rous sarcoma virus, vSrc (29), the polyoma virus middle Tumor antigen (mT (4, 35)), the activated chaperone Hsp90N (18), vRas (3, 8), tumor promotors such as the 12-O-Tetradecanoylphorbol-13-acetate and others, have been shown to interrupt junctional communication.

src is an oncogene with a high clinical relevance and one of the best-studied targets for cancer therapy (reviewed in (1)). src encodes a potent oncoprotein with high tyrosine kinase activity (Src). Src can affect the activity of Cx43 by multiple mechanisms, namely by direct phosphorylation on tyrosine residues, but also by its direct downstream effector kinase pathways, Ras/Raf/Erk and the phosphatidylinositol-3 kinase (PI3k)/Akt which phosphorylate Cx43 on serine residues. In addition, Src may indirectly activate the ser/thr kinase, protein kinase C that can phosphorylate Cx43 and block gap junctions, as well as other kinases (5, 26, 33, 38). Besides activating kinase pathways, Src can make use of the adaptor protein Cas (Crk-associated substrate), that binds Cx43 to suppress gap junctional communication (39). Src is also a potent activator of the cytoplasmic transcription factor, Signal

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transducer and activator of transcription-3 (Stat3). In this short communication we review the prevailing evidence on the role of Src and its effector pathways upon Cx43 and GJIC.

Phosphorylation of Cx43 on tyrosine by the Src kinase

A reduction in gap junctional communication of Src-transformed cells was reported for the first time in 1966 (31). Subsequent cloning of Cx43 enabled a molecular characterisation of the mechanism whereby Src affects Cx43 function, and this led to fundamental studies on Cx43 regulation. A combination of genetic and biochemical evidence indicated that Src can phosphorylate Cx43 directly: At first the SH3 domain of Src binds a proline-rich area between P274 and P284 of Cx43. This brings the Src kinase domain in close proximity to Y265, which is then phosphorylated by Src (Figure 1). The phosphorylated Y265 offers a docking site for the Src, Src-homology-2 (SH2) domain and this enhanced interaction causes the phosphorylation of Y247 of Cx43, which may contribute to GJIC reduction (10, 30, 43). In fact, vSrc co-expression with a Cx43 mutant, where tyr247 and tyr265 were replaced by phenylalanine in *Cx43*-knockout cells, was unable to interrupt communication, indicating that tyr247 and ty265 are important for GJIC suppression by the Src kinase (30). However, expression of the same Cx43 mutants in Xenopus oocytes can result in the formation of gap junctions, but these gap junctions can be disrupted by Src, indicating that the sites of direct phosphorylation by Src are not required for GJIC suppression in this setting (29). This led to the hypothesis that Src effectors may play a role upon GJIC suppression.

Effect of Src effectors upon GJIC suppression

The Ras/Raf/Erk pathway. Prominent among the signalling cascades initiated by the Src kinase to effect neoplastic conversion is the Ras/Raf/Mek/Erk. Src activates the Ras GTPase, which triggers the translocation of the ser/thr kinase Raf to the membrane, leading to Raf activation. Raf then activates Mek, a dual-specificity kinase, which, in turn, activates Erk (37), through phosphorylation on both thr and tyr in a ^{P}TE^{P}Y motif. Activated Ras was shown to suppress GJIC (8), and the Ras function is also required by *mT*, an oncogene which induces neoplastic conversion by binding to and activating cSrc, to reduce gap junctional communication (9).

Erk can phosphorylate Cx43 at S255, S279 and S282 (50). In fact, expression of Cx43 mutants with all Erk phosphorylation sites mutated to alanine, induces gap junction formation, but these gap junctions are not disrupted by Src expression, indicating that phosphorylation by Erk is important for GJIC suppression by vSrc. In addition, pharmacological inhibition of Erk eliminates Src's ability to interrupt gap junctional communication (54) and it was recently shown that ser- residues of Cx43 were phosphorylated in vSrc- transformed cells (40). Taken together, these data suggest that Erk activation by Src may be important in GJIC suppression. However, in addition to the direct phosphorylation of Cx43 by the Erk kinase, Ras may also act through other effectors to down-regulate GJIC, such as RalGDS, p120GAP, AG6 and others (22).

PI3k/Akt. One of the Src downstream effectors is the phosphatidylinositol-3 (PI3) kinase. Work from a number of laboratories has shown that activated-Src activates class I, PI3K, which phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP2) and phosphatidylinositol-4-phosphate (PIP), to generate phosphatidylinositol-3,4,5-trisphosphate (PIP3) and PI(3,4)P2, respectively, in a reaction which can be reversed by the tumor suppressor PIP3 phosphatase, PTEN. The ser/thr kinase Akt binds to PIP3 at the membrane, by virtue of an amino-terminal plekstrin homology domain. Then the PDK1 kinase, also bound to PIP3 at the membrane, phosphorylates the activation loop of Akt at thr308 (Akt1 numbering). Another complex activated by RTK's, the mammalian target of rapamycin complex-2 (mTORC2) phosphorylates Akt1 on the carboxylterminal, hydrophobic domain, at ser473. Akt is thus transiently localised to the plasma membrane during activation and once activated, it phosphorylates substrates throughout the cell to regulate for multiple cellular functions, including growth modulation, survival, proliferation and metabolism. Three isoforms have been described, Akt1, Akt2, Akt3, and studies from knockout mice documented distinct functions for each isoform (reviewed in (16,32)).

The effect of Src-mediated, PI3K/Akt activation upon GJIC is complex. Akt1 was shown to phosphorylate Cx43 at ser373 and ser369 (34). It was recently demonstrated that Akt is essential for the disruption of gap junctional communication by Src, while the expression of a constitutively active Akt1, but not Akt2 or Akt3 was sufficient to suppress GJIC in rat fibroblasts (21). However, results from osteoblasts indicated that PI3K/Akt is necessary for the maintenance of the steady-state expression of Cx43 through an effect on post-transcriptional mRNA stability (6). Given the large variety of substrates of the different Akt isoforms, it is possible that the effect of Akt activation by Src may be different in different settings.

PKC. Protein kinase C (PKC) is a Src- effector serine/threonine kinase (17). Src activates PKC through activation of phospholipase Cγ, but also through direct phosphorylation (19). PKC phosphorylates Cx43 at S368 and S372 (12, 27, 28). These phosphorylation events reduce coupling, since PKC activation with 12-O-Tetradecanoylphorbol-13-Acetate (TPA)

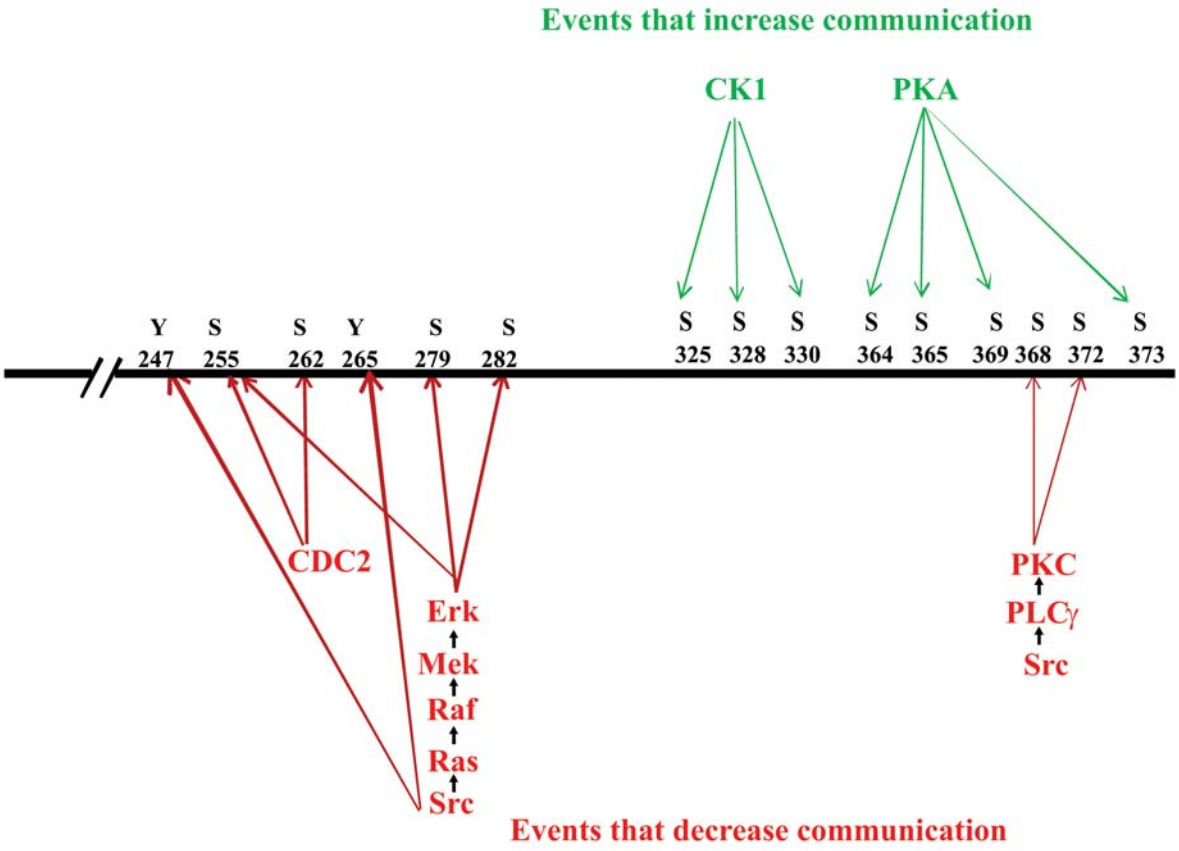

Figure 1. *Cx43 phosphorylations that affect gap junctional communication. Cx43 is shown with the phosphorylation sites targeted by Src and its effector kinases, Ras/Erk, and PKC, by aminoacid number and phosphorylating kinase. Sites phosphorylated by other kinases, such as protein kinase A (PKA) and caseine kinase 1 (Ck1) that increase communication, and the cell division cycle-2 ($p34^{Cdc2}$) kinase that decreases it are also indicated (23, 25, 44). Although the interplay between Src and these kinases has not been firmly established, it is possible that in addition to activating kinases that inhibit GJIC, Src may also suppress the activity of kinases that are required for communication (33). In red are events that decrease communication, in green events that increase it.*

leads to a reduction in GJIC (5, 26). Inhibition experiments indicated that the PKCγ isoform reduces GJIC in lens epithelial cells (49), while PKCα, β or δ can disrupt coupling between fibroblasts (11).

Stat3. The signal transducer and activator of transcription-3 (Stat3), is latent in the cytoplasm in unstimulated cells and is activated by cytokine receptors of the IL6 family, as well as by tyrosine kinase receptors such as EGF-R and PDGF-R (52). Ligand-induced assembly of cell surface receptor complexes causes receptor activation. Subsequent tyrosine phosphorylation of the receptor cytoplasmic tail by the receptor itself or by the associated Jak or Src tyrosine kinases, creates docking sites for recruiting latent, unphosphorylated Stat3 *via* its Src homology 2 (SH2) domain. The receptor-bound Stat3 becomes a substrate for phosphorylation at a critical tyrosine (tyr705). This activates Stat3 by stabilizing the association of two monomers through reciprocal SH2-phosphotyrosine interactions. The Stat3 dimer then binds to specific target sequences in the nucleus, leading to the transcriptional activation of genes which play a role in cell proliferation and survival, such as myc, cyclin D, Bcl-xL, survivin, hepatocyte growth factor (VEGF) (20), Vascular Endothelial Growth Factor and others (13, 52). Activated-Src also activates Stat3 and this is required for Src transformation. It was shown that the process requires the activity of the Jak1 kinase as well, while wild-type or kinase-inactive PDGF-receptor enhances Stat3 activation by vSrc, serving a scaffolding function (53). Stat3 is found to be hyperactive in a number of cancers (15), and the fact that a constitutively active form of Stat3 alone is sufficient to induce neoplastic transformation (7), points to an etiological role for Stat3 in neoplasia.

Stat3 down-regulation does not restore GJIC in Src-transformed cells. Previous results from our lab and others demonstrated that engagement of E-cadherin, as brought

about by confluence of adherent, cultured cells causes a dramatic increase in Stat3, ptyr705 phosphorylation and activity (41, 42, 47, 48), therefore the density must be taken into account when assessing the effect of inhibitors upon Stat3 activity. To examine the effect of Stat3 down-regulation upon GJIC, Stat3 activity was reduced using the pharmacological inhibitor CPA7 (2), or by infection with a retroviral vector carrying a Stat3-specific, siRNA (14). For these experiments, GJIC was examined using an apparatus of electroporation *in situ*, on a partly-conductive slide (36). The fluorescent dye, Lucifer yellow, was electroporated into cells grown on electrically- conductive, optically-transparent, indium-tin oxide, followed by observation of the migration of the dye to the adjacent, non-electroporated cells under fluorescence illumination. The results demonstrated that, contrary to inhibition of the Ras/Erk pathway, Stat3 inhibition in cells expressing activated-Src does not restore GJIC, indicating that Stat3 is not part of a pathway of Src-induced, GJIC suppression (14).

Stat3 is required for the maintenance of gap junctional communication in normal epithelial cells and fibroblasts. Since *Stat3*-knockdown did not restore GJIC in Src-expressing cells, the possibility that *Stat3*-might have a positive role upon GJIC was explored. In fact, Stat3 knockdown in normal rat liver epithelial T51B cells which have extensive GJIC (14), or certain lung cancer lines that retain GJIC (Geletu *et al*., submitted), abolished junctional communication and caused a dramatic reduction in Cx43 levels. That is, rather than increasing communication, Stat3 inhibition eliminates GJIC, indicating that Stat3 activity is, in fact, required for the maintenance of gap junction function in normal cells with extensive GJIC. This could be related to Stat3's ability to prevent apoptosis, since apoptotic death induction through cycloheximide, etoposide or puromycin caused a rapid loss of coupling, due to caspase-3-mediated degradation of Cx43 (45). Whether a similar mechanism might apply to GJIC suppression following Stat3 inhibition remains to be determined. In any event, current evidence demonstrates that Stat3, although it is generally growth-promoting and in an activated form can act as an oncogene, its function is actually required for the maintenance of junctional permeability.

Final remarks. Besides *src*, a variety of oncogenes and growth factors are known to cause gap junction closure. Although the effects of neoplastic transformation upon GJIC are clear, the mechanisms whereby this leads to tumor growth and progression are not well-defined. A better understanding of the relationship between Src and Cx43 will offer useful insights on the paths leading to carcinogenesis. Since Src also plays an important role in other cellular functions, this will also unveil mechanisms involved in processes such as development and homeostasis (51).

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