Interaction of c-Src with Gap Junction Protein Connexin-43

ROLE IN THE REGULATION OF CELL-CELL COMMUNICATION*

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Cell-cell communication via connexin-43 (Cx43)-based gap junctions is transiently inhibited by certain mitogens, but the underlying regulatory mechanisms are incompletely understood. Our previous studies have implicated the c-Src tyrosine kinase in mediating transient closure of Cx43-based gap junctions in normal fibroblasts. Here we show that activated c-Src (c-SrcK) phosphorylates the COOH-terminal tail of Cx43, both in vitro and in intact cells. Coimmunoprecipitation experiments reveal that Cx43 associates with c-SrcK and, to a lesser extent, with wild-type c-Src, but not with kinase-dead c-Src. Mutation of residue Cx43 Tyr265 (Cx43-Y265F mutant) abolishes both tyrosine phosphorylation of Cx43 and its coprecipitation with c-Src. Expression of c-SrcK in Rat-1 cells disrupts gap junctional communication. Strikingly, the communication-defective phenotype is bypassed after coexpression of the Cx43-Y265F mutant or a COOH-terminally truncated version of Cx43 (Cx43Δ263) that lacks residue Tyr265. Our results support a model in which activated c-Src phosphorylates the COOH-terminal tail of Cx43 on residue Tyr265, resulting in a stable interaction between both proteins leading to inhibition of gap junctional communication.

Gap junctions mediate communication between adjacent cells in almost all tissues. Gap junctions are composed of arrays of channel-forming integral membrane proteins, termed connexins (for review, see Refs. 1 and 2). The connexin proteins contain four transmembrane domains with the amino- and COOH-terminal regions located intracellularly. Connexin-43 (Cx43) is the most widespread and best studied member of the connexin family, with much attention being focused on its regulation by physiological and pathophysiological stimuli. Cx43-based gap junctional communication (GJC) is transiently inhibited by certain growth factors (1, 3–6) and permanently reduced or disrupted in most cancer cells and in v-Src-transformed fibroblasts (1, 7–11). The COOH-terminal tail of Cx43 contains several serine/threonine and tyrosine phosphorylation sites that are thought to be important for regulating the opening and closure (“gating”) of Cx43-based channels (1), but little is still known about how phosphorylation of Cx43 may be linked to the regulation of GJC.

We have reported that Cx43-based GJC in normal fibroblasts is rapidly inhibited after activation of certain G protein-coupled receptors, such as those for lysophosphatidic acid (LPA), endothelin, and thrombin (5). We found that agonist-induced gap junction closure is independent of classic second messengers, such as calcium and cyclic nucleotides, and does not require the mitogen-activated protein kinase cascade. Instead, gap junction closure was prevented by tyrosine kinase inhibitors, by dominant-negative c-Src, and in c-Src-deficient cells (5). It thus appears that the C-Src tyrosine kinase is required for disruption of Cx43-based GJC by these G protein-coupled receptor agonists. Although agonist-induced tyrosine phosphorylation of Cx43 could not be detected (5), the possibility remains that active c-Src may directly interact with Cx43 by analogy of what has been reported for oncogenic v-Src, whose effects on Cx43 and Cx43-based GJC have been investigated extensively (1, 7–14). Residue Tyr265 in the COOH-terminal tail of Cx43 has been implicated as a major target for the v-Src kinase, but uncertainty exists as to the relevance of Tyr265 phosphorylation in channel gating (12, 13). In Xenopus oocyte pairs, v-Src-mediated closure of Cx43-based gap junctions was shown to be critically dependent on residue Tyr265, since mutation of Tyr265 in Cx43 prevents gap junction closure (12). In contrast, more recent experiments using the Xenopus system have suggested that v-Src-induced inhibition of Cx43-based GJC occurs via a pathway that is independent of the Tyr265 site but seems to require serine phosphorylation sites for mitogen-activated protein kinase (13). The reason for the discrepancy between both studies is unclear. While the action of oncogenic v-Src on Cx43 has been examined in considerable detail, it remains unknown whether endogenous wild-type c-Src can act similarly on Cx43. Overexpressed c-Src has long been known to mimic v-Src in inhibiting GJC in 3T3 cells (14). However, where tested, an association between wild-type c-Src and Cx43 in vivo could not be detected (11).

In the present study we have examined the possible interaction between c-Src and Cx43, both in vitro and in transfected cells. We find that residue Tyr265 in the COOH-terminal tail of Cx43 is phosphorylated by active c-Src and, furthermore, that Cx43 and active c-Src can be coprecipitated, whereas kinase-dead c-Src fails to stably interact with Cx43. Tyrosine phosphorylation and coprecipitation of Cx43 are abolished after mutation of residue Cx43 Tyr265. The communication-defective phenotype can be bypassed after introduction of mutant versions of Cx43 that cannot be tyrosine-phosphorylated on Tyr265. We conclude that activated c-Src phosphorylates the C-terminal tail of Cx43 on residue Tyr265, resulting in a stable interaction between both proteins that contributes to the inhibition of GJC by extracellular agonists.

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1 The abbreviations used are: Cx43, connexin-43; EtBr, ethidium bromide; GFP, green fluorescent protein; GJC, gap junctional communication; GST, glutathione S-transferase; LPA, lysophosphatidic acid; LY, Lucifer Yellow; PAGE, polyacrylamide gel electrophoresis; wt, wild-type.
Experimental Procedures

Cell Culture—Rat-1 cells, COS-7, A431, and HeLa cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. Prior to experimentation, cells were serum-starved for 16 h.

Construction of Plasmids—Chicken c-SrcY527A (SrcK1), and c-Src in pMT2 were kindly provided by S. Courtneidge (Sugen, Inc., Redwood City, CA). Myc epitope-tagged full-length Cx43 and Cx43Δ263 in pCDNA3 (InVitrogen) were constructed as described previously (15). The Cx43Δ265F and Cx43Δ267F mutants were introduced by site-directed mutagenesis using 5’ oligonucleotides (5’TGC GGA TTC CCA AAA TTC GCC TAC TTC AAT GGC 3’) and the 3’ oligonucleotide (5’GCCGC C AAT CTC CAG GTC ATC AG 3’). Polymerase chain reaction performed according to standard procedures, using rat Cx43 cDNA as template. Polymerase chain reaction products were isolated from gel and BamHI/EcoRI ligated into Cx43Δ263Myc, in which a BamHI site was introduced in the nucleotides encoding amino acids 261 and 262 (silent mutation). Mutations were verified by sequencing. To construct GST-Cx43CT, the COOH-terminal tail of Cx43 was isolated from pMD4Cx43CT (15) with SalI/EcoRI and ligated, in frame with glutathione S-transferase (GST) cDNA, into pGEX-1XsaI/EcoRI. GST and GST-Cx43CT proteins were isolated from DH5α bacteria induced with isopropyl-β-D-thiogalactopyranoside according standard procedures. Proteins were visualized using enhanced chemoluminescence (Amerham Pharmacia Biotech).

cDNA Transfection—Cells were transfected with pCDNA3 plasmid encoding Myc-tagged Cx43 and/or c-Src using standard calcium phosphate precipitation. COS cells were transfected by the standard DEAE-dextran transfection method, using 1 μg of plasmid DNA per 105 cells.

Antibodies—Mouse anti-Cx43 and anti-phosphotyrosine (PY20) monoclonal antibodies were from Transduction Laboratories (Lexington, KY). Mouse anti-Myc was applied as culture supernatant from the 9E10 hybridoma (ATCC). Rabbit anti-human Cx43 (SRC2) was from Santa Cruz Biotechnology (Santa Cruz, CA), and mouse anti v-Src monoclonal antibody was from Calbiochem. Secondary antibodies (rabbit anti-mouse and swine anti-rabbit) conjugated to horseradish peroxidase were obtained from Dako (Glostrup, Denmark), and antibodies conjugated to Texas Red were from Molecular Probes (Eugene, OR). The COOH-terminal tail of Cx43 is a substrate for activated c-Src

RESULTS AND DISCUSSION

The COOH-terminal Tail of Cx43 Is a Substrate for Activated c-Src in Vitro—Previous studies have shown that Cx43 is tyrosine-phosphorylated by oncogenic v-Src, which correlates with permanent disruption of GJC in v-Src-transformed cells (1, 8–12), while endogenous c-Src has been implicated in the transient inhibition of Cx43-based GJC in normal cells (5). We set out to examine whether c-Src can phosphorylate Cx43 on its COOH-terminal tail in vitro. To this end, we used the Cx43 tail (residues 227–382; Fig. 1, upper panel) fused to GST (GST-Cx43CT) and three distinct versions of c-Src: (i) wild-type (wt) c-Src; (ii) constitutively active c-Src (c-SrcK1), in which the inhibitory intramolecular interaction between phosphotyrosine 527 and the SH2 domain is disrupted (Y527A mutation); and...
(iii) kinase-dead c-Src (c-SrcK−, K295M mutation) (Fig. 1, lower panel). Src proteins were immunoprecipitated from transfected COS-7 cells and incubated with GST-Cx43CT. [γ-32P]ATP was then added for in vitro kinase assays. As shown in Fig. 2, the Cx43CT fusion protein is phosphorylated by c-SrcK+ (although radiolabeling is low relative to the in vitro autophosphorylation of c-Src), but not by kinase-dead SrcK−, and only very weakly by wt c-Src. Thus, the COOH-terminal tail of Cx43 can serve as a substrate for active c-Src. For further analysis we turned to intact cell systems.

Active c-Src Phosphorylates Cx43 on Residue Tyr265 in Transfected Cells—Swenson et al. (12) reported that residue Tyr265 in the Cx43 tail is critical for v-Src-induced tyrosine phosphorylation of Cx43 and inhibition of GJC in paired Xenopus oocytes. Coexpression experiments in HEK293 cells have confirmed that oncogenic v-Src can phosphorylate Cx43 on residue Tyr265 (11). However, since v-Src and c-Src show differential association to cellular substrates (16, 17), it remains unclear whether c-Src may act similarly to v-Src in phosphorylating Cx43. To examine the importance of Cx43 residue Tyr265 in the action of c-Src, we generated mutant versions of Cx43 in which tyrosine residues 265 and 267 were substituted by phenylalanine (Cx43-Y265F and Cx43-Y267F, respectively; Fig. 1, upper panel). COS-7 cells were transfected with Myc-tagged Cx43 (wt, Y265F, and Y267F) with or without the various versions of c-Src. Immunoblot analysis of total cell lysates reveals that the three distinct Cx43 proteins are properly expressed to comparable levels (Fig. 3A, middle panels). No tyrosine phosphorylation of any of the Cx43 proteins is detected in control transfectants, nor in cells expressing kinase-dead c-Src (Fig. 3). When c-Src or c-SrcK+ is cotransfected, however, phosphotyrosine is detected in wt Cx43 and Cx43-Y267F, but not in the Cx43-Y265F mutant (Fig. 3A). We also analyzed Cx43 immunoprecipitates for the presence of phosphotyrosine. As shown in Fig. 3B, wt Cx43 and mutant Cx43-Y267F are tyrosine-phosphorylated in c-Src-overexpressing cells, whereas Cx43-Y265F (precipitated at comparable amounts) is not. From these results we conclude that active c-Src phosphorylates Cx43 on residue Tyr265 and that residue Tyr267 is not a Src phosphorylation site.

We note that, at least in some experiments, a faint phosphotyrosine signal was still detectable in the Cx43-Y265F mutant (Fig. 3B). This may represent endogenous Cx43 that is coprecipitated with transfected Myc-tagged Cx43. An alternative or additional possibility is that c-Src may phosphorylate Cx43 on another tyrosine residue with very low stoichiometry. The latter possibility was not investigated in further detail.

Association between Active c-Src and Cx43—We next examined whether c-Src can stably interact with Cx43 in intact cells. COS-7 cells were transfected with Myc-tagged versions of Cx43 (wt, Y265F, and Y267F mutants) together with the various c-Src constructs. We then assayed for the presence of Cx43 in Src precipitates. In SrcK+ precipitates, both wt Cx43 and the Cx43-Y267F mutant are readily detected, whereas the Cx43-Y265F mutant is not (Fig. 4, upper right panel). Fig. 4 further shows that a very weak association is detected between wt Cx43 and wt c-Src and that Cx43 fails to interact with kinase-dead c-Src (SrcK−). These results emphasize the importance of Cx43 residue Tyr265, and they support a model in which phosphorylation of Cx43-Tyr265 is essential for stable interaction between Cx43 and active c-Src.

Our efforts to detect an interaction between endogenous c-Src and Cx43 in agonist-stimulated Rat-1 cells were not successful. This negative result is not entirely unexpected, how-

Fig. 3. Tyrosine 265 of Cx43 is phosphorylated by c-Src. COS-7 cells were cotransfected with c-Src and Myc-tagged versions of Cx43 (wt, Cx43Y265F, or Cx43-Y267F). A, cell lysates were subjected to SDS-PAGE followed by immunoblotting using antibodies to phosphoryrosine (PY20), Myc tag (9E10), and Src, as indicated. Overexpressed c-Src or SrcK+ phosphorylates wt Cx43 and the Cx43-Y267F mutant, but not Cx43-Y265F (upper lanes). Lower panels show Cx43 and Src expression controls. B, Cx43 was immunoprecipitated at equal amounts (second panel, 9E10 blot). Cx43 immunoprecipitates were probed with anti-phosphotyrosine mAb PY20 (upper panel). When c-Src is overexpressed, wt Cx43 and the Y267F mutant Cx43 are phosphorylated, whereas the Y265F mutant shows little or no tyrosine phosphorylation. Lower panel shows Src expression controls.
Fig. 5. Importance of Cx43 residue Tyr^{265} in c-Src-induced inhibition of GJIC in Rat-1 cells. A, phase-contrast and fluorescence photomicrographs of Rat-1 cells in monolayer. SrcK* and Cx43 (wt or mutants) were transiently cotransfected as indicated. Successfully transfected cells were identified by GFP coexpression. GFP-positive cells were micro-injected with a mixture of LY and EtBr as a marker. Cell-to-cell diffusion of LY was measured at 3 min after micro-injection. It is seen that SrcK* expression blocks GJIC. Strikingly, communication is restored after coexpression of either COOH-terminally truncated Cx43-D_{263} or Cx43-Y_{265}F, whereas expression of wt Cx43 or Cx43-Y_{267}F fails to bypass SrcK* action. B, quantitation of the LY micro-injection experiments illustrated in A. The degree of intercellular LY diffusion was determined at 3 min after dye injection into GFP-positive cells (as illustrated in A). GJC was scored as either “positive” or “negative.” In control cells, 90% of gap junctions are open (left bar). SrcK* expression largely blocks GJC. This effect is rescued by cotransfection of Cx43-Y_{265}F or Cx43-D_{263}, but not by wt Cx43 or Cx43-Y_{267}F. That rescue is not 100% complete may be due to less than 100% triple-transfection efficiency per cell. C, expression and localization of Cx43 in SrcK* -transfected Rat-1 cells. Rat-1 cells were transiently transfected with the indicated Cx43 and SrcK* constructs, using the same procedures as in A. GFP was cotransfected to identify transfected cells. Cells were stained for Cx43-Myc, using 9E10 anti-Myc and Texas Red-conjugated goat-anti-mouse antibodies. It is seen that transfected Cx43-Myc is located both at the cell periphery and in intracellular vesicles. Bar: 20 μm.
However, given that: (i) agonist-induced c-Src activation and disruption of GJC are transient events (5, 18, 19); (ii) c-Src and Cx43 are expressed at relatively low levels in normal fibroblasts; and (iii) the high stringency lysis conditions required to solubilize Cx43 from junctional plaques is likely to disrupt protein-protein interactions, particularly when such interactions are labile and of transient nature.

**Cx43-Y265F Rescues the Communication-defective Phenotype in SrcK-expressing Rat-1 Cells**—We next examined the importance of Cx43 residue Tyr265 in Src-mediated closure of gap junctions in fibroblasts. Rat-1 cells are ideally suited for these studies, because they express Cx43 as the sole gap junction protein and Cx43-based communication is tightly regulated (5). Rat-1 cells were transiently transfected with cDNAs encoding c-SrcK- together with different versions of Cx43. In all cases, GFP cDNA was cotransfected to identify successfully transfected cells. At about 40 h after transfection functional cell-cell contacts were formed and GFP-expressing cells were micro-injected with Lucifer Yellow (LY) together with ethidium bromide (EtBr) as a marker to detect injected cells. Cell-to-cell diffusion of LY was measured at 3 min after LY/EtBr injection. LY diffusion from the transfected cell (GFP- and EtBr-positive) to its direct neighbor indicates the open state of Cx43-based gap junctions. When two or more adjacent cells showed LY fluorescence, communication was scored positive. Of note, this assay measures single-cell “all-or-none” responses, since GJC is subject to regulation only in the transfected cell (GFP-positive) in a cell-autonomous manner.

Fig. 5A shows representative results of the LY diffusion experiments, while quantitation of all experiments is summarized in Fig. 5B. In nontransfected Rat-1 cells ("control"), 90% of the micro-injected cells show LY diffusion to their neighbors, indicative of normal gap junctional communication. When active c-SrcK- is introduced, however, communication is strongly inhibited: in about 80% of the transfected cells gap junctional communication is completely lost (Fig. 5B). Cotransfection of wt Cx43 does not restore normal communication, nor does expression of Cx43-Y267F, which both serve as Src substrates (Fig. 5A, middle left). Strikingly, when the Src phosphorylation site Tyr265 in Cx43 is removed either by point mutation (Cx43Y265F) or by COOH-terminal tail truncation (Cx43A463), gap junctional communication is restored (Fig. 5, A and B). Immunofluorescence analysis of transfected cells did not reveal gross differences in expression levels of the Cx43 mutants (Fig. 5C), although we cannot rule out the formal possibility that activated Src may somehow affect Cx43 localization and/or gap junction assembly. Nevertheless, our results suggest that Src-mediated phosphorylation of Cx43 on residue Tyr265 is a key event in closure of Cx43-based gap junctions.

Our findings support a model in which G protein-coupled receptor agonists, such as LPA, endothelin, and thrombin, transiently disrupt GJC through a pathway that involves c-Src-mediated phosphorylation of Cx43 residue Tyr265 as a critical step (5). In an attempt to test this model directly, we stably expressed either wt Cx43 or mutant Cx43-Y265F in communication-defective A431 and HeLa carcinoma cells, and subsequently examined GJC in response to LPA and other growth factors. Unexpectedly, however, while expression of wt Cx43 or Cx43-Y265F did confer GJC to these cells (as determined by dye diffusion and electrophysiological measurements), coupling in the transfectedants could not be inhibited by LPA, thrombin, epidermal growth factor, or phorbol ester. It thus appears that, although transfected Cx43 does restore GJC, its regulation by extracellular agonists is impaired, as if an essential signaling component or Cx43-interacting partner is lacking or nonfunctional in the Cx43 transfectants.

**Concluding Remarks**—We have shown that Tyr265 in Cx43 is a critical site for an interaction between Cx43 and active c-Src and inhibition of GJC. Our results support a model in which activated c-Src phosphorylates the Cx43 tail on residue Tyr265, resulting in a stable interaction between both proteins, most likely via a Src SH2-domain interaction, which then leads to inhibition of GJC. A similar model emphasizing the importance of Cx43 residue Tyr265 has been proposed for oncogenic v-Src in closing Cx43-based gap junctions (Ref. 10; but see also Ref. 13 for a model in which the Cx43-Y265 has no major role). Yet, it should be noted that there are various important differences between v-Src and c-Src. Not only is v-Src constitutively active because it lacks the auto-inhibitory Tyr267 residue, c-Src and v-Src also show differential association to cellular substrates (16, 17). Of particular relevance is the finding that the isolated SH3 domain of v-Src binds to proline-rich motifs in Cx43 in vitro, whereas the c-Src SH3 domain mediates only weak binding to Cx43 under the same conditions (11). Hence, it will be very difficult to detect a pre-existing SH3-mediated association between endogenous c-Src and Cx43 in vivo. Although tyrosine phosphorylation of Cx43 could not be detected in Rat-1 cells following receptor stimulation (for likely reasons outlined above), a recent study showed tyrosine phosphorylation of Cx43 in cardiomyopathic heart tissue (20). This correlated with increased c-Src activity and decreased GJC (20), consistent with a model in which active c-Src interacts with Cx43 and thereby mediates inhibition of GJC.

In conclusion, our results provide further evidence for the direct involvement of c-Src in inhibiting Cx43-based GJC following stimulation of G protein-coupled receptors (5), and they emphasize the importance of residue Tyr265 as a c-Src target in this process. Obviously, a full understanding of how Cx43-based GJC is regulated by physiological and/or pathophysiological stimuli can only be obtained if all Cx43-interacting partners are known. In addition to c-Src, we have identified the ZO-1 protein as an interacting partner of the Cx43 COOH-terminal tail (a PDZ-domain interaction; Ref. 15). One challenge for future studies is to elucidate if and how c-Src and ZO-1 may interact to regulate the function of the Cx43 multiprotein complex and channel gating. Future studies should also elucidate the G protein-effector pathway that is responsible for Src-dependent inhibition of Cx43-based GJC in normal cells.

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