Tyrosine phosphorylation of the gap junction protein connexin43 is required for the pp60^{v-src}-induced inhibition of communication

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Gap junction communication in some cells has been shown to be inhibited by pp60^{v-src}, a protein tyrosine kinase encoded by the viral oncogene *v-src*. The gap junction protein connexin43 (Cx43) has been shown to be phosphorylated on serine in the absence of pp60^{v-src} and on both serine and tyrosine in cells expressing pp60^{v-src}. However, it is not known if the effect of *v-src* expression on communication results directly from tyrosine phosphorylation of the Cx43 or indirectly, for example, by activation of other second-messenger systems. In addition, the effect of *v-src* expression on communication based on other connexins has not been examined. We have used a functional expression system consisting of paired *Xenopus* oocytes to examine the effect of *v-src* expression on the regulation of communication by gap junctions comprised of different connexins. Expression of pp60^{v-src} completely blocked the communication induced by Cx43 but had only a modest effect on communication induced by connexin32 (Cx32). Phosphoamino acid analysis showed that pp60^{v-src} induced tyrosine phosphorylation of Cx43, but not Cx32. A mutation replacing tyrosine 265 of Cx43 with phenylalanine abolished both the inhibition of communication and the tyrosine phosphorylation induced by pp60^{v-src} without affecting the ability of this protein to form gap junctions. These data show that the effect of pp60^{v-src} on gap junctional communication is connexin specific and that the inhibition of Cx43-mediated junctional communication by pp60^{v-src} requires tyrosine phosphorylation of Cx43.

Introduction

Gap junctions are collections of transmembrane channels that allow small molecules to move

directly between the cytoplasm of adjacent cells. Four related rat gap junction structural proteins have been described (Kumar and Gilula, 1986; Paul, 1986; Beyer *et al.*, 1987, 1988; Zhang and Nicholson, 1989). We have generically termed these proteins connexins (Cx) and distinguish them from one another by molecular mass, as calculated by cDNA analysis (Beyer *et al.*, 1987). The four connexins—Cx26, 32, 43, and 46—are expressed in a wide variety of cell types, both singly and in conjunction with one another.

Communication through gap junctions is known to be sensitive to a variety of physiological conditions, including expression of the *v-src* oncogene of Rous sarcoma virus (RSV) (for reviews see Hertzberg and Johnson, 1988). Arising from a transduction of the cellular proto-oncogene *c-src*, *v-src* encodes a membrane-associated tyrosine kinase called pp60^{v-src} (Collet *et al.*, 1980; Hunter and Sefton, 1980; Levinson *et al.*, 1980). Expression of *v-src* alone is sufficient to induce neoplastic transformation of tissue culture cells. It is not yet clear how pp60^{v-src} causes transformation, but both tyrosine kinase activity (Sefton *et al.*, 1980) and membrane association (Cross *et al.*, 1984) are necessary.

The effect of *v-src* gene expression on junctional communication was initially examined by infecting various primary and established cell lines with RSV encoding a temperature-sensitive variant of pp60^{v-src} (Atkinson *et al.*, 1981; Azarnia and Loewenstein, 1984). Rapid and reversible inhibition of communication was observed after a shift to the permissive temperature. Later, transfection studies proved that *v-src* expression alone is sufficient for inhibition of communication (Chang *et al.*, 1985). The cellular proto-oncogene product pp60^{c-src} can also produce inhibition of communication if it is overexpressed or activated either by mutation (Azarnia *et al.*, 1988) or by middle-T antigen transformation (Azarnia and Loewenstein, 1987).

It is not known whether gap junctions in all cells are sensitive to *v-src* expression. Cx43, but not Cx32, is expressed in NIH 3T3 (Crow *et al.*, 1990), normal rat kidney (NRK; Musil *et al.*, 1990b), and fibroblastic primary cultures (Crow *et al.*, 1990). Therefore, it is probable that all of the cell types that have been used to examine the effects of pp60^{v-src} on communication (Atkinson *et al.*, 1981; Azarnia and Loewenstein, 1984; Crow *et al.*, 1990) were joined by gap junctions comprised of Cx43. The effect of pp60^{v-src} on communication between cells expressing connexins other than Cx43 has not been examined.

The mechanism by which pp60^{v-src} inhibits junctional communication is not understood. Recently, it was shown that RSV infection results in tyrosine phosphorylation of Cx43 in NIH 3T3 and vole cell fibroblasts (Crow *et al.*, 1990). Thus, it is possible that tyrosine phosphorylation of Cx43 by pp60^{v-src} could be responsible for the inhibition of communication. Consistent with this idea, significant amounts of pp60^{v-src} are detected at gap junctions in virally transformed NRK cells (Willingham *et al.*, 1979). However, no causal relationship between tyrosine phosphorylation and the inhibition of communication has been experimentally established. In this regard, the effects of pp60^{v-src} on communication can be abridged by pharmacological treatments that do not appear to affect tyrosine kinase activity (Hyrz and Rose, 1990). Therefore, pp60^{v-src} may affect communication by indirect routes, and it is essential that the precise role of tyrosine phosphorylation of gap junction proteins be defined.

The regulation of gap junctions by pp60^{v-src} is of particular interest because of the possible role of communication in the control of cell proliferation. It has been suggested that molecules that influence proliferation may pass between cells through gap junctions (Loewenstein, 1966). Over the years, correlations between the inhibition of communication through gap junctions and increased cell proliferation induced by a number of treatments, including *src* gene product expression, have been reported (Mehta *et al.*, 1986; Azarnia *et al.*, 1988). Part of the transforming effect of *v-src* expression, therefore, may involve inhibition of the normal passage through gap junctions of regulatory molecules that influence cell proliferation.

We have used a functional expression system consisting of paired *Xenopus* oocytes to examine the effect of *v-src* expression on the regulation of communication by gap junctions comprised of different connexins. Previously, we

have shown that injection of connexin RNA produced in vitro from cloned cDNAs is sufficient to induce high levels of communication (Dahl *et al.*, 1987; Swenson *et al.*, 1989). Here, we demonstrate that the specific effect of pp60^{v-src} on communication is restricted to junctions comprised of Cx43. Gap junctions containing Cx32 are only slightly affected by pp60^{v-src}. In addition, we demonstrate by the use of site-directed mutation of Cx43, that tyrosine phosphorylation is required for the specific inhibition of Cx43-based communication.

Results

pp60^{v-src} inhibits communication in oocyte pairs expressing Cx43 but not Cx32

Oocytes were microinjected with connexin and pp60^{v-src} RNAs or with water and paired after manual removal of the vitelline envelope. Pairs were aligned so that centers of vegetal hemispheres, which were the sites of injection, were apposed. The degree of junctional coupling was quantitated by dual cell voltage clamp (Spray *et al.*, 1981) after overnight incubation at 18°C. The conductances observed for various combinations of oocyte pairs are listed in Table 1A.

High levels of junctional conductance were observed in pairs of oocytes expressing either Cx32 or Cx43, as previously reported (Swenson *et al.*, 1989). Water-injected oocyte pairs variably developed very low levels of conductance, presumably because of the presence of an endogenous gap junction protein that we have cloned and characterized (*Xenopus* Cx38) (Ebihara *et al.*, 1989; Gimlich *et al.*, 1990). Most of the water-injected pairs did not develop endogenous conductances. In the cases where endogenous conductance was detected, it was 50–1000 times lower than conductance induced by injection of rat connexin RNA. Endogenous conductance could also be distinguished from conductances induced by Cx32 and 43 because it was strongly inactivated by voltage (see Swenson *et al.*, 1989). Previously, we showed that the oocyte endogenous connexins can sometimes interact with Cx43 to form heteromolecular junctions. However, the characteristic voltage inactivation of the endogenous connexin was maintained in these heteromolecular junctions. This provided a simple test for the presence of endogenous connexin. We did not observe voltage-inactivating conductances after the injection of Cx32 or Cx43 RNA (data not shown). Therefore, potential interactions of endogenous and exogenous connexins were not likely to influence these findings.

Table 1. Conductance developed between paired, injected *Xenopus* oocytes

Oocyte injection, cell 1 cell 2	Mean conductance, uS	SE	No. of experiments (no. of oocyte pairs)
A			
Cx43 Cx43	30.01	4.7	11 (57)
Cx43 + v-src Cx43 + v-src	0.024	0.013	11 (58)
Cx32 Cx32	41.4	6.0	4 (20)
Cx32 + v-src Cx32 + v-src	13.74	2.4	4 (33)
Cx43 Cx43 + v-src	0.33	0.10	2 (15)
water water	0.58	0.25	8 (26)
B			
Cx43 ^{Y265-F} Cx43 ^{Y265-F}	30.6	9.7	4 (22)
Cx43 ^{Y265-F} + v-src Cx43 ^{Y265-F} + v-src	13.1	1.5	4 (24)

Co-injection of v-src RNA dramatically inhibited the conductance normally induced by Cx43 RNA. The mean conductance of these oocytes was 1000-fold less than those injected with Cx43 RNA alone. In contrast, conductance levels induced by Cx32 were only modestly affected by v-src expression, exhibiting a 3-fold reduction in conductance levels compared with oocytes injected with Cx32 RNA alone. Oocytes coexpressing Cx43 and v-src developed 20-fold less conductance than those injected with water alone. This may indicate that v-src expression inhibits endogenous conductance, although other explanations are possible (see Discussion). The effect of v-src expression on endogenous conductance was not directly tested because measurable endogenous conductances were rare and unpredictable.

To determine whether the presence of v-src RNA was required in both oocytes of a pair to affect conductance levels, we created pairs in which both oocytes were injected with Cx43 RNA but only one received v-src RNA. This unilateral v-src injection, in the presence of Cx43, resulted in conductance levels equal to the water-injected pairs, which is a 100-fold reduction relative to pairs expressing Cx43 in the absence of pp60^{v-src}. This reduction is clearly significant,

although 10-fold less than observed for bilateral v-src injection. The conductances observed after unilateral v-src injection were not voltage inactivated, suggesting that they were induced by Cx43 and not endogenous connexins.

pp60^{v-src} induces tyrosine phosphorylation of Cx43

We examined the expression and phosphoamino acid content of Cx43 in the presence or absence of pp60^{v-src} in single oocytes. Extracts of oocytes were immune precipitated by Cx43-specific antisera (Swenson *et al.*, 1989) after metabolic labeling with either ³⁵S-methionine or ³²P-γ-ATP and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE; Figure 1, panel 1). Immune precipitates of ³⁵S-labeled oocytes expressing Cx43 displayed a predominant polypeptide (Figure 11A) with an electrophoretic mobility indistinguishable from that of in vitro synthesized Cx43 (Figure 11, position indicated at arrow). Additionally, minor amounts of poorly resolved, faster migrating polypeptide(s), which may be proteolytic fragments, were detected (Figure 11A, faint band[s] below position of arrow). Immune precipitates made from ³⁵S-labeled oocytes co-in-

Figure 1. Expression and phosphorylation of Cx43 in mRNA-injected *Xenopus* oocytes in the presence and absence of pp60^{v-src}. Stage VI *Xenopus* oocytes were injected with either Cx43 RNA or Cx43 plus pp60^{v-src} RNAs and labeled by injecting either ³⁵S-methionine or ³²P- γ -ATP with incubation at 18°C (see Materials and Methods). Homogenates were made from each oocyte and immune precipitated with anti-Cx43 antisera. (I) Immune precipitates from homogenates of oocytes injected with mRNAs encoding Cx43 only (A and C) or co-injected with mRNAs encoding Cx43 and pp60^{v-src} (B and D) and labeled with ³⁵S-methionine (A and B) or ³²P- γ -ATP (C and D). The arrow to the left indicates the mobility of in vitro synthesized Cx43. The ³²P-labeled polypeptides in gel regions, indicated by numbers 1–4 in C and 5–8 in D, were eluted, digested with trypsin, and then subjected to acid hydrolysis. Phosphoamino acids generated from polypeptides in gel regions 1–8 (I) were then separated by thin-layer electrophoresis and detected by autoradiography, shown in the corresponding lanes 1–8 of Panel II. The positions of phosphoamino acid standards indicated to the left in Panel II were determined using ninhydrin.

jected with RNAs for both Cx43 and *v-src* displayed similarly sized polypeptides in addition to at least one more slowly migrating, well-resolved polypeptide (Figure 1B). No polypeptides were detected from immune precipitates of similarly labeled water-injected oocytes (see Figure 5A).

Immune precipitates from Cx43 RNA injected oocytes labeled with ³²P- γ -ATP displayed polypeptides with electrophoretic mobilities similar to those obtained after ³⁵S-methionine labeling. In addition, a poorly resolved set of more slowly migrating polypeptides were detected as a smear (Figure 1C, region 2–3). Although not visible in Figure 1, on longer exposure lane C, region 1 exhibited a well-resolved, ³²P-labeled band that comigrated with the band displayed

in lane D, region 5. When pp60^{v-src} was present, the ³²P-labeled immunoprecipitates contained one additional well-resolved band (lane D, region 6), as well as increased amounts of poorly resolved polypeptides (Figure 1I, compare C and D). No polypeptides were detected in immune precipitates of ³²P-labeled oocytes injected with water (see Figure 5D).

Comparison of Figure 1I, B and D, reveals that the more slowly migrating forms of Cx43 are more intensely labeled by ³²P- γ -ATP than by ³⁵S-methionine. For example, the ³²P-labeled band 6 was more intensely labeled than band 7 (Figure 1D), whereas the relative intensities of their ³⁵S-labeled counterparts were reversed (Figure 1B). Increases in phosphorylation have been reported to decrease the electrophoretic mo-

Figure 2. Expression and phosphorylation of Cx32 in mRNA-injected *Xenopus* oocytes in the presence and absence of pp60^{v-src}. *Xenopus* oocytes were injected with either Cx32 RNA, Cx32 plus pp60^{v-src} RNAs, or water and labeled using either ³⁵S-methionine or ³²P-γ-ATP with incubation at 18°C. Homogenates were made from each oocyte and immune precipitated with anti-Cx32 antisera. (I) Immune precipitates from homogenates of oocytes injected with water (A and D), mRNA encoding Cx32 only (B and E), or mRNAs encoding Cx32 and pp60^{v-src} (C and F) and labeled with ³⁵S-methionine (A–C) or ³²P-γ-ATP (D–F). The arrow to the left indicates the mobility of in vitro synthesized Cx32. The ³²P-labeled polypeptides in gel regions, indicated by numbers 1 in E and 2 in F, were eluted and subjected to phosphoamino acid analysis, as described for Figure 1. The products are shown in the corresponding lanes 1 and 2 in Panel II.

bility of Cx43 in tissue culture cells (Crow *et al.*, 1990; Musil *et al.*, 1990a,b) and are likely to account for the mobility shifts seen here.

To determine whether the phosphoamino acid content of Cx43-related polypeptides was altered in the presence of pp60^{v-src}, we performed phosphoamino acid analysis on ³²P-labeled polypeptides immune precipitated from oocytes expressing either Cx43 or Cx43 and pp60^{v-src}. Labeled polypeptides produced in the absence (Figure 1C) and in the presence (Figure 1D) of pp60^{v-src} were separated by the use of SDS-PAGE. Gel slices corresponding to four regions (Figure 1I, C1–4 and D5–8) were obtained. Polypeptides eluted from these regions were subjected to acid hydrolysis and the products separated by thin-layer electrophoresis (shown in Figure 1II, 1–8). In the absence of pp60^{v-src}, all four regions of the gel exhibited only phosphoserine-containing polypeptides (Figure 1II, 1–4). In contrast, in the presence of pp60^{v-src}, all four regions exhibited both phosphoserine- and phosphotyrosine-bearing polypeptides (Figure 1II, 5–8). No phosphothreonine was detected in either case.

Pairing of oocytes did not produce gross alterations in the SDS-PAGE profile of Cx43-re-

lated polypeptides (data not shown). More importantly to this study, pairing produced no changes in the phosphoamino acid content of Cx43-related polypeptides in either the presence or absence of pp60^{v-src}.

pp60^{v-src} does not induce tyrosine phosphorylation of Cx32

Extracts of oocytes expressing either Cx32 or Cx32 and pp60^{v-src} were immune precipitated with Cx32-specific antisera after metabolic labeling (Figure 2I, A–F). Immune precipitates of ³⁵S-labeled oocytes expressing Cx32 alone displayed a prominent polypeptide (Figure 2IB) with an apparent mobility indistinguishable from that of Cx32 translated in vitro (Figure 2I, arrow). Also detected were minor levels of presumed proteolytic fragments and aggregations. Aggregation of Cx32 has been shown to occur in SDS solutions (Henderson *et al.*, 1979). Immune precipitates of ³²P-labeled oocytes expressing Cx32 alone displayed a similar protein profile to that of ³⁵S-labeled oocytes (Figure 2I, compare B and E). We observed no effect of pp60^{v-src} on the mobility of either ³⁵S- or ³²P-labeled Cx32 (Figure 2I, C and F). No polypeptides were de-

tected in immune precipitates of water-injected oocytes (Figure 2I, A and D).

Phosphoamino acid analysis of Cx32 produced in the presence (Figure 2IE1) and absence (Figure 2IF2) of pp60^{v-src} was performed as described above. In both cases, only phosphoserine was detected (Figure 2II, 1 and 2).

We determined pp60^{v-src} levels by immunoblot analysis using a monoclonal anti-pp60^{v-src} antibody (EC10, Parsons *et al.*, 1984). This analysis was performed to ensure that an inhibition of pp60^{v-src} expression was not responsible for the observed failure of v-src strongly to inhibit conductance between oocyte pairs expressing Cx32 and to induce Cx32 phosphorylation. We detected pp60^{v-src} only in v-src-injected oocytes (Figure 3, lanes 5, 6, 9, 10; at arrow) and was easily distinguished from nonspecific staining. In this particular experiment, the level of pp60^{v-src} was greater in oocyte pairs expressing Cx32 (lanes 5 and 6) than in those expressing Cx43 (lanes 9 and 10). Therefore, differences in pp60^{v-src} protein levels did not account for the finding that Cx32 is only slightly inhibited by v-src expression. In a number of experiments, slight variations in pp60^{v-src} levels were observed but did not correlate with the type of connexin expressed (data not shown).

pp60^{v-src}-induced inhibition of Cx43-induced gap junctional conductance requires tyrosine phosphorylation of Cx43

To clarify the mechanism by which v-src was affecting conductance, we found it useful to determine whether tyrosine phosphorylation of Cx43 was required for inhibition of conductance. This was investigated by the use of oligonucleotide-directed mutagenesis to produce a Cx43 variant in which a single tyrosine was replaced by phenylalanine.

Cx43 contains 16 tyrosine residues (Figure 4, ★). Studies of the amino acid sequence around the sites of phosphorylation in a number of tyrosine kinase substrates have revealed no consensus context for such phosphorylation. However, a consensus context for tyrosine kinase autophosphorylation has been reported (Patschinsky *et al.*, 1982). Tyrosine 265 of Cx43 fit that context very well and was located in a cytoplasmic domain (Beyer *et al.*, 1989; Yancey *et al.*, 1989), consistent with the hypothesis that it served as a substrate of pp60^{v-src}. Therefore, we chose tyrosine 265 for mutational analysis. To ensure that the site-directed mutation had produced only desired modifications of the cDNA, the entire protein coding region was sequenced. The altered Cx43 protein (Cx43^{Y265-F})

Figure 3. Expression of pp60^{v-src} in mRNA-injected *Xenopus* oocytes. Oocytes were injected with water (1 and 2), mRNAs encoding Cx32 alone (3 and 4), mRNAs encoding Cx32 and pp60^{v-src} (5 and 6), mRNAs encoding Cx43 alone (7 and 8), and mRNAs encoding Cx43 and pp60^{v-src} (9 and 10). Homologous pairs were formed manually, incubated at 18°C for 24 h, and, after electrical measurements, homogenized directly in SDS gel sample buffer. Of each pair, 1/20 was analyzed by immunoblot with anti-pp60^{v-src} antibodies. Each lane represents a different pair. The arrow to the right indicates the position of pp60^{v-src}.

Extracts of oocytes expressing Cx43^{Y265-F}, which had been metabolically labeled with ³⁵S-methionine, were immune precipitated with Cx43-specific antisera and analyzed by SDS-PAGE (Figure 5I, A–C). In the presence or absence of pp60^{v-src}, a predominant polypeptide that comigrated with authentic Cx43 was seen (Figure 5I, arrow). A more quickly migrating polypeptide, presumed to be a proteolytic fragment, was also detected. No more slowly migrating bands were detected. Thus, in contrast to that seen for oocytes expressing wild-type Cx43 (Figure 1I, A and B), the protein profile of

Figure 5. Expression and phosphorylation of Cx43^{Y265-F} in mRNA-injected *Xenopus* oocytes in the presence and absence of pp60^{v-src}. *Xenopus* oocytes were injected with Cx43^{Y265-F} mRNA, Cx43^{Y265-F} plus pp60^{v-src} mRNAs, or water and labeled with ³⁵S-methionine or ³²P- γ -ATP with incubation at 18°C. Homogenates were made from each oocyte and immune precipitated with anti-Cx43 antisera. (I) Immune precipitates from homogenates of oocytes injected with water (A and D), mRNA encoding Cx43^{Y265-F} alone (B and E), or mRNAs encoding Cx43^{Y265-F} and pp60^{v-src} (C and F) and labeled with ³⁵S-methionine (A–C) or ³²P- γ -ATP (D–F). The arrow to the left indicates the mobility of in vitro synthesized Cx43. The ³²P-labeled polypeptides in gel regions, indicated by numbers 1–4 in E and 5–8 in F, were eluted and subjected to phosphoamino acid analysis, as described for Figure 1. The products are shown in the corresponding lanes 1–8 in Panel II.

³⁵S-labeled Cx43^{Y265-F} was not affected by the presence of v-src (Figure 5I, B and C).

The protein profile seen after ³²P-labeling of Cx43^{Y265-F} was strongly influenced by pp60^{v-src}. Expression of pp60^{v-src} resulted in an increase in both the number and intensity of Cx43^{Y265-F}-related bands (Figure 5I, compare E and F), qualitatively similar to its effect on wild-type Cx43 (Figure 1I, compare C and D). We did not determine whether the precise mobilities of the Cx43^{Y265-F} bands were the same as those seen in oocytes expressing Cx43. No polypeptides were detected in immune precipitates of water-injected oocytes labeled with either ³⁵S-methionine or ³²P- γ -ATP (Figure 5I, A and D).

Phosphoamino acid analysis was performed on ³²P-labeled polypeptides immune precipi-

tated from oocytes expressing Cx43^{Y265-F} with or without pp60^{v-src}. Analysis was performed on proteins eluted from slices of the SDS gel corresponding to the same four regions as chosen for Cx43 in the absence (Figure 5IE, 1–4) and in the presence (Figure 5IF, 5–8) of pp60^{v-src}. In both cases, all regions exhibited only phosphoserine (Figure 5II, 1–8), although the amount of label in region 1 (Figure 5II1) was very low compared with the other regions. No phosphotyrosine was detected.

pp60^{v-src} does not prevent accumulation of Cx43 at the appositional region between cells

Earlier experiments indicate that the cellular location of connexins in oocytes are affected by

cell-cell contact (Swenson *et al.*, 1989). In single oocytes, Cx32 and Cx43 are primarily detected in cytoplasm. In contrast, connexins in paired oocytes are detected at or near the cell surface in the appositional region. Here, we examined the effect of *v-src* expression on the distribution of Cx43 in oocyte pairs and on oocyte morphology.

The location of Cx43 in oocyte pairs co-producing pp60^{v-src} was examined by immunocytochemistry. Immunofluorescence microscopy was performed on 10- μ M frozen sections of paired oocytes by the use of a specific anti-Cx43 antibody detected with a rhodamine-labeled secondary antibody (Figure 6A). The micrograph shows a portion of the cortical regions of both oocytes in a pair including some appositional area. Specific Cx43 staining was detected at some (arrow 3) but not all (arrow 2) regions of apposition. Little staining was detected at non-appositional surfaces (arrow 1) or in the cyto-

plasm. No staining above background in preimmune controls was detected (data not shown). The distribution of Cx32 was similar to that of Cx43 (data not shown). These aspects of connexin distribution were similar to those found in oocyte pairs in the absence of pp60^{v-src} (see Figure 6 in Swenson *et al.*, 1989).

We observed only one aspect of connexin distribution that was affected by pp60^{v-src}. There was a marked irregular distribution of Cx43 in the cytoplasm near the injection site. An example is shown in Figure 6A (arrow 4). A similar affect of pp60^{v-src} on Cx32 distribution was seen (data not shown). In the absence of pp60^{v-src}, both Cx32 and Cx43 in paired oocytes show little cytoplasmic staining (see Figure 6, d and e, in Swenson *et al.*, 1989). The non-connexin-specific effects on distribution may result from changes in the organization of the cytoplasm induced by pp60^{v-src} in the vicinity of the injection site. Light microscopic examination showed a

Figure 6. Immunofluorescence distribution of Cx43 in an oocyte pair expressing pp60^{v-src}. Paired oocytes expressing both Cx43 and pp60^{v-src} were frozen and 10- μ M cryostat sections were prepared. (A) the section was stained for immunofluorescence with a specific anti-Cx43 antibody. (B) a phase contrast image of the same section. Cx43 is detected at some (A, arrow 3) but not all (arrow 2) appositional areas and in irregular patches in the cytoplasm (arrow 4) corresponding to regions of pigment granule redistribution (B, arrows) induced by pp60^{v-src}. Little staining is detected at non-appositional surfaces or in the cytoplasm where pigment granule distribution is unaffected. Bar, 25 μ M.

displacement of yolk platelets and a higher-than-usual concentration of cortical pigment granules in this area. An example is shown in the phase contrast micrograph of the same section described above (Figure 6B). The region of cytoplasmic reorganization is indicated by arrows.

Discussion

pp60^{v-src} has specific effects on communication induced by Cx43

We have used coexpression of pp60^{v-src} and the gap junction structural proteins Cx43 or Cx32 in oocyte pairs to examine the mechanism of pp60^{v-src}-induced inhibition of junctional communication. Expression of v-src completely abolished the communication induced by Cx43, but had only a minor effect on communication induced by Cx32, and caused tyrosine phosphorylation of Cx43 but not of Cx32. Both tyrosine phosphorylation and the inhibition of communication by v-src expression were abolished when tyrosine 265 of Cx43 was replaced with phenylalanine. Expression of v-src did not inhibit the ability of Cx43 or Cx32 to move to appositional regions of the oocyte surface in response to cell-cell contact.

The effect of pp60^{v-src} expression on Cx43-induced communication is much more pronounced (>1000-fold reduction) than its effect on communication induced by either Cx32 or Cx43^{Y265-F} (2- to 3-fold reduction). This suggests that pp60^{v-src} has specific effects on Cx43. However, the depression of communication observed for Cx32 and Cx43^{Y265-F} was statistically significant. Several changes in cell physiology, which could indirectly account for this effect, were produced by pp60^{v-src}. For example, v-src expression greatly reduced the ability of oocytes to adhere to one another (data not shown), which could affect their ability to form gap junctions. Several studies correlate increased cell-cell adherence with both increased numbers of gap junctions and increased communication (Keane *et al.*, 1988; Mege *et al.*, 1988; Musil *et al.*, 1990b). Some changes in cytoplasmic organization and connexin distribution were also induced by pp60^{v-src}. These or other changes produced by pp60^{v-src} could indirectly affect the formation of gap junction channels resulting in the slight, non-connexin-specific depression of conductance observed.

Xenopus oocytes have been shown to contain endogenous connexin mRNAs (Ebihara *et al.*, 1989; Gimlich *et al.*, 1990) and to develop low levels of conductance when paired in the ab-

sence of exogenous RNA injection (Swenson *et al.*, 1989). It is possible that endogenous connexins could interact with Cx43 and Cx32, thereby complicating the interpretation of these data. We believe this is unlikely for the following reasons. 1) Endogenous conductance was not detected between the majority of water-injected pairs. 2) The endogenous conductance was always at least 50-fold less, and usually >1000-fold less, than the conductances induced by exogenous RNAs. 3) The endogenous conductance can always be distinguished from RNA-induced conductances because, unlike the conductances induced by Cx32 and Cx43, it is strongly voltage inactivated (Swenson *et al.*, 1989). No significant voltage inactivation was observed in any experiment involving Cx43 and Cx32 expression (data not shown).

Cx43 phosphorylation on serine

When expressed in oocytes, Cx43 and Cx43^{Y265-F} were present in multiple forms. The number and complexity of these forms was much more striking in the ³²P-, as opposed to the ³⁵S-, labeling studies. The pattern of labeling suggests that the more slowly migrating forms contain more phosphate than the faster forms. In tissue culture cells, shifts in mobility, which give rise to more slowly migrating forms of Cx43, have been shown to result from serine phosphorylation (Musil *et al.*, 1990a,b). Together, these observations suggested that changes in serine phosphorylation may account for the different forms of Cx43 and Cx43^{Y265-F} observed in oocytes. In addition, pp60^{v-src} appeared to increase the complexity of SDS-PAGE profiles of Cx43 and Cx43^{Y265-F}. Because the latter protein does not gain phosphotyrosine, it is possible that pp60^{v-src} may indirectly induce serine phosphorylation of Cx43. In support of this notion, pp60^{v-src} was shown to increase general protein phosphoserine content in Xenopus oocytes (Spivack *et al.*, 1984). The biological significance of these complicated changes in SDS-PAGE profile and serine phosphorylation was not clear. However, it was clear that none of the changes in SDS-PAGE profile or inferred changes in serine phosphorylation correlated with changes in communication in our experiments.

Cx43 phosphorylation on tyrosine

There was a precise correlation between the presence of phosphotyrosine in Cx43 and the inhibition of communication by pp60^{v-src}. All forms of Cx43 contained phosphotyrosine in the

presence of pp60^{v-src}, where communication is inhibited; none contained phosphotyrosine in the absence of pp60^{v-src}, where communication is normal. The effect of pp60^{v-src} on phospho-amino acid content of Cx43 in oocytes was very similar to that observed for tissue culture cells (Crow *et al.*, 1990). When tyrosine 265 of Cx43 was replaced by phenylalanine, pp60^{v-src} neither induced tyrosine phosphorylation of the variant connexin nor inhibited junctional communication. Together, these findings indicated that tyrosine phosphorylation of Cx43 was required for inhibition of communication by pp60^{v-src}.

The site-directed mutation experiments suggested that a specific tyrosine, residue 265, is critical for pp60^{v-src}-induced inhibition of communication. However, we did not map the *in vivo* site of Cx43 tyrosine phosphorylation, and, thus, it was possible that different or additional tyrosines may be phosphorylated *in vivo*. The replacement of tyrosine 265 with phenylalanine could have resulted in conformational changes rendering some other tyrosine residue inaccessible to the kinase. Arguments against a major conformational change include the following: 1) The formation of gap junctions was not noticeably affected. Equivalent steady-state concentrations of wild-type and mutated Cx43 resulted in similar conductance levels. 2) The physiological properties of the gap junctions were not grossly affected. Gap junctions formed from Cx43^{Y265-F} displayed reversible sensitivity to cytoplasmic acidification and exhibited no detectable voltage gating (data not shown). The only measured physiological parameter that changed was the response to pp60^{v-src}. Together, these data strongly suggested that phosphorylation of tyrosine 265 was sufficient to account for the inhibitory effect of pp60^{v-src} on communication.

How does tyrosine phosphorylation inhibit communication?

There are a number of possible mechanisms by which phosphorylation of Cx43 could result in the inhibition of communication. The assembly of gap junctions and the establishment of communication between cells must include at least six events, although the temporal sequence of these events is not clear. These steps are 1) connexin synthesis and membrane insertion; 2) formation of the connexon consisting of six connexin monomers; 3) movement of the connexon to the cell surface; 4) accumulation of the connexon at the appositional surface between cells; 5) head-to-head association of

connexons in membranes of adjacent cells to form intercellular channels; and 6) gating, or modulation of the completely assembled junctional channel between open and closed states.

It is likely that pp60^{v-src} affects only some of these steps. The movement of newly synthesized connexin from the cytoplasm to the cell surface in *Xenopus* oocytes is dependent on cell contact (Swenson *et al.*, 1989). This movement was not inhibited by *v-src* expression. The inhibition of communication in cultured cell lines by a temperature-sensitive variant of pp60^{v-src} occurs rapidly (<10 min) after shifting to the permissive temperature (Atkinson *et al.*, 1981; Azarnia and Loewenstein, 1984; Rose *et al.*, 1986). Together, these data suggest that pp60^{v-src} affects either gating of the channel or an aspect of multimeric assembly rather than synthesis or intracellular movement of the connexins. Phosphorylation has been shown to affect gating as well as other functional properties of other ion channels (see Levitan, 1988).

Inhibition of communication and cell proliferation

The finding that several growth-factor receptors and viral oncogene products, including pp60^{v-src}, possess intrinsic tyrosine kinase activity has led to the notion that tyrosine phosphorylation contributes significantly to the transduction of growth-related signals. However, few *in vivo* substrates of pp60^{v-src} in this pathway have been characterized, and in most cases the functional relationship of these proteins to cell proliferation is not clear (e.g., vinculin; Sefton *et al.*, 1981; and Shriver and Rohrschneider, 1981; talin; Pasquale *et al.*, 1986; fibronectin receptor; Hirst *et al.*, 1986). We have shown that gap junctions comprised of Cx43 were altered both biochemically and functionally in the presence of pp60^{v-src}, raising the possibility that Cx43 is a physiologically relevant substrate for *src* gene products. Hypothetically, changes in gap junctional communication could affect the concentration of growth-controlling molecules (see Loewenstein, 1979). Thus, our data support the hypothesis that closing of gap junctional channels could be an important step in the induction of proliferation or oncogenic transformation by pp60^{v-src}. This hypothesis might be tested by restoring communication to *v-src* transformed cells via transfection with Cx32 or Cx43^{Y265-F} cDNAs.

Junctional communication is also known to be inhibited by either platelet-derived growth factor (PDGF) or epidermal growth factor EGF stimu-

lation of NRK and 3T3 cells (Maldonado *et al.*, 1988). Because PDGF stimulation activates a number of proteins involved in transduction of signals related to proliferation, including pp60^{c-src} (Ralston and Bishop, 1985; Gould and Hunter, 1988; see Kaplan *et al.*, 1990 for others), it is possible that the inhibition of communication produced by PDGF may be secondary to the activation of one of these proteins. Alternatively, Cx43 may be directly affected by the tyrosine kinase activity of PDGF receptor. In either case, Cx43 may be a common downstream target for growth factor receptors that are tyrosine kinases, as well as for viral oncogenes. It will be interesting to monitor the phosphotyrosine content of Cx43 on activation of receptor-tyrosine kinases.

Materials and Methods

Preparation of connexin RNAs and microinjection

Cx43 and Cx32 RNAs were prepared as described (Swenson *et al.*, 1989). Collection, microinjection, and pairing of oocytes from *Xenopus laevis* were carried out as described (Swenson *et al.*, 1989).

Preparation of v-src RNA

The v-src coding region of PR(A)-RSV was isolated and subcloned into the transcription vector pSP68-4(RBS) (Piwnicka-Worms *et al.*, 1986) by the following procedure. Plasmid p2.OR1C12 (Gilmer and Erikson, 1981) was restricted with *EcoRI* and the 3.1-kb fragment containing part of the envelope gene and the entire v-src gene was isolated and inserted into the *EcoRI* site of pUC9 to generate plasmid KBS-1. This plasmid was restricted with *NcoI* and *SalI* and the v-src-containing fragment was subcloned into *NcoI* and *SalI* restricted pSP68-4(RBS) to generate plasmid SP6(v-src). For transcription, SP6(v-src) was linearized with *SalI*, and RNA synthesis was accomplished as described for Cx43 in Swenson *et al.* (1989).

Preparation of Cx43^{Y265-F} RNA

Site-directed mutation of Cx43 cDNA was performed by the method of Kunkel (1985) with a Muta-Gene kit (Bio-Rad, Richmond, CA) according to manufacturer's recommendations. Mutagenesis was primed with a 17-base oligonucleotide (TCCAAAATTCGCCTAC) synthesized on an Applied Biosystems (Foster City, CA) Model 391. Base 996 of Cx43 clone G2 (Beyer *et al.*, 1987) was changed from A to T. Subcloning of the mutagenized cDNA into the transcription vector SP64T and RNA synthesis was accomplished as described for Cx43 in Swenson *et al.* (1989).

Antibodies and immunofluorescence

The anti-Cx32 antiserum, produced and characterized as described by Goodenough *et al.* (1988), was made against a synthetic peptide corresponding to residues 98–124 of the deduced amino acid sequence. The anti-Cx43 antiserum was produced against a synthetic peptide corresponding to residues 252–271 and is described by Beyer *et al.* (1989). Monoclonal antibodies to pp60^{c-src} are described by Parsons

et al. (1984). Immunofluorescence microscopy was performed as described in Swenson *et al.* (1989).

Labeling and immune precipitation of connexins

Oocytes were microinjected with water or 10 ng of each RNA in a volume of 40 nl and incubated at 18°C. For metabolic labeling with ³⁵S-methionine, Cx32 or Cx43^{Y265-F} RNAs were co-injected with ³⁵S-methionine (7 µCi; 800 mCi/mmol; New England Nuclear, Boston, MA). To label oocytes expressing Cx43, we injected ³⁵S-methionine (4 µCi; 800 mCi/mmol) 18 h after RNA injection. In all cases, oocytes were incubated for 6 h after ³⁵S-methionine injection. For metabolic labeling with ³²P-γ-ATP (8–40 µCi; 3000 Ci/mmol), label was injected 18 h after water or RNA injection and incubated for 1.5 h. Labeled oocytes were homogenized in 100–300 µl/oocyte of 0.4% SDS, 50 mM tris(hydroxymethyl)aminomethane (Tris; pH 7.4), 100 mM NaCl, 2 mM EDTA, 50 mM NaF, 40 mM β-glycerophosphate, and 1 mM Na₂VO₄ containing 1 mM phenylmethylsulfonyl fluoride and 5 mM diisopropyl fluorophosphate and incubated at 100°C for 3 min. In some cases trasylol (140/ml K.I. Units), chymostatin (1 µg/ml), pepstatin (1 µg/ml), leupeptin (1 µg/ml), and DTT (1 mM) were present to further control proteolysis. The homogenate was brought to 2% with Triton X-100 and cleared by centrifugation in a microcentrifuge at 13 000 RPM for 5 min. Primary antibody (1 µl/oocyte) was added to the supernatant and incubated overnight at 4°C with rotation. Protein A-sepharose CL-4B (Sigma, St. Louis, MO) was added and incubated for 1 h at 4°C. Sepharose was pelleted in a microfuge for 10 s at 13 000 RPM. Pellets were washed two times with 1 ml of 0.5 M LiCl, 100 mM Tris (pH 7.4) and once with 1 ml of 20 mM Tris (pH 7.4), 100 mM NaCl; then solubilized in SDS sample buffer at 100°C for 10 min and analyzed by SDS-PAGE.

Phosphoamino acid analysis

³²P-labeled protein bands were excised from dried polyacrylamide gels; rehydrated; and subjected to trypsin digestion, acid hydrolysis, and thin-layer electrophoretic analysis as described by Musil *et al.* (1990b). The position of ³²P-labeled amino acids was detected by autoradiography; unlabeled standards were detected by ninhydrin staining.

Electrophysiology

A dual-cell voltage-clamp procedure (Spray *et al.*, 1981; see Swenson *et al.*, 1989 for detailed description) was utilized to measure gap junctional conductance. In brief, both cells (initial resting potentials –55 to –90 mV) are clamped at –80 mV to insure zero transjunctional potential. Voltage steps are delivered to cell 1, whereas cell 2 is held constant. Because the potential of cell 2 does not change, there are no changes in nonjunctional currents resulting from voltage gating of other channels. Therefore, the current delivered by the clamp to cell 2 is equal in magnitude but opposite in sign to the current flowing through the junction, and the junctional conductance is equal to the junctional current divided by the transjunctional voltage step. Alternating symmetrical depolarizing pulses of 10–80 mV were imposed to measure junctional conductance and to evaluate possible voltage gating and rectification. Sensitivity of conductance to cytoplasmic acidification was tested by perfusing the bath with Modified Barth's containing 5 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid, pH 7.5, equilibrated with 100% CO₂.

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