The Action of v-src on Gap Junctional Permeability Is Modulated by pH

Krzysztof Hyrc and Birgit Rose
Department of Physiology and Biophysics, University of Miami School of Medicine, Miami, Florida 33101

Abstract. The product of the viral src gene (v-src) is the protein tyrosine kinase pp60v-s'. Among the known consequences of pp60v-src activity is the reduction in permeability of gap junctions, an effect that is counteracted by the calcium antagonist TMB-8 (8-N,N-[diethylamino]octyl-3,4,5-trimethoxybenzoate). We show here that a decrease in intracellular pH (pHi) also counteracts the v-src effect: junctional permeability of cells containing active v-src kinase rose with decreasing pH, in the range 7.15 to 6.75, whereas junctional permeability of cells containing inactive v-src kinase or no v-src at all was insensitive to pH in that range. Low pH also counteracted the known action of diacylglycerol on junction, but only when pp60v-src kinase was inactive. Immunobots of whole-cell lysates using an antibody against phosphotyrosine show that phosphorylation on tyrosine of at least one cellular protein, specific for pp60v-src kinase activity, was reduced by low pH but not by TMB-8. These results suggest that TMB-8 does not inhibit v-src action on junctional permeability by interfering with tyrosine phosphorylation of a protein crucial for closure of gap junction channels, but that the inhibition by low pH may be via this mechanism.

The product of the viral src gene, pp60v-src, is a protein tyrosine kinase (Hunter and Sefton, 1980). Among the various actions attributed to this membrane-bound protein kinase is the inhibition of junctional communication, the intercellular communication provided by the cell-to-cell membrane channels of gap junctions. This inhibition has been inferred from experiments on cells infected with temperature-sensitive mutant avian sarcoma virus (ASV; Atkinson et al., 1981) and Rous sarcoma virus (RSV; Azarnia and Loewenstein, 1984; Yada et al., 1985). The mechanism by which pp60v-src closes cell-to-cell channels is not understood.

During a series of experiments aimed at elucidating this mechanism, we observed that the junctional permeability of v-src-infected cells increased greatly when cells were transferred from medium of normal (pH 7.6) to lower pH (7.0). Because intracellular pH (pHi) is dependent on extracellular pH (pHe) and hence cells at pHe 7.0 are likely to have a lower pHi than cells at pHe 7.6, this was an unexpected result for two reasons: (a) wherever junctional permeability has been found to be pH sensitive, permeability always decreased when pH, was lowered (Spray and Bennett, 1985); (b) the pp60v-src kinase activity is reported to have a maximum at pHi 6.5 (Richert et al., 1982), i.e., kinase activity would be expected to increase, not decrease at lower pH.

We therefore investigated the dependence of junctional permeability on extracellular pH in v-src-infected cells and in the uninfected parent cell line; to relate junctional permeability to pH, we measured the cells' pHi. We also investigated whether low pH interferes with the action of diacylglycerol on junctional permeability. Diacylglycerol, a potent depressant of junctional permeability (Enomoto and Yamasaki, 1985; Gainer and Murray, 1985; Yada et al., 1985) has been suggested as a possible mediator of the v-src effect on junction (Rose et al., 1986). We were also interested whether TMB-8, an inhibitor of the junctional response to diacylglycerol (Yada et al., 1985) and to v-src (Rose et al., 1986), effects this inhibition by lowering pH.

Lastly, we compared the level of tyrosine phosphorylation of proteins from cells at various pHs, and of cells treated or untreated with TMB-8 (8-N,N-[diethylamino]octyl-3,4,5-trimethoxy-benzoate), so as to learn whether v-src-specific tyrosine phosphorylation of any protein(s) correlates with junctional permeability of the cells.

Materials and Methods

Cell Culture
We used mouse Balb/c-3T3 cells uninfected (normal 3T3) or infected with wild-type RSV (Schmidt Ruppin Group D, wv-src), or infected with the temperature-sensitive (Schmidt Ruppin Group D) RSV mutant LA90 (av-src), and NIH 3T3 cells overexpressing c-src (clone NIH-3T3 (pMcsrc)) described by Azarnia et al. [1988] and referred to here as c-src++. Cells were grown on 35-mm plastic dishes (Nunc, Roskilde, Denmark) in DME.
(Gibco Laboratories, Grand Island, NY) with 10% FBS (Hyclone Laboratories, Logan, UT), or 10% calf serum (Gibco Laboratories) for c-src-expressing NIH-3T3 cells, and 50 μg/ml each of penicillin, streptomycin, and kanamycin, in an atmosphere of 5% CO₂/95% air. The tsv-src cells were grown at 34°C; all others were grown at 37°C. The medium was renewed every other day. The density of cells used for experiments was ~8-9 × 10⁴ cells/cm².

**Test Media and Treatments**

For experiments, cells were transferred to the appropriate test medium and temperature ~5 h before measurement. Test medium was DMEM without antibiotics, phenol red and bicarbonate (but with 10% FBS in all cases, including the c-src-expressing cells), and buffered with either Hepes (40 mM) or Pipes (20 mM), and adjusted to the desired pH with 1 M NaOH. At the cell density we used, pH remained stable within 0.05 pH units for at least 5 h. Other media used were a modified PBS, in millimolar: NaCl, 97; KCl, 2.7; KH₂PO₄, 1.5; CaCl₂, 1; MgCl₂, 0.5; Na₂HPO₄, 8; Hepes, 40 (pH 7.55); or Pipes, 20, pH 6.6; propionate-PBS with Hepes (pH 7.6), in which NaCl was substituted (equimolar) by Na-propionate; high-potassium medium, which was composed of, in millimolar: KCl, 90; CaCl₂, 1; MgCl₂, 1; Pipes-K₃, 10; Hepes, 20 (pH 6.6, 7.21, or 7.40). Diacylglycerol analogue 1,2-dioctanoyl-sn-glycerol (diC₈; Molecular Probes, Inc., Eugene, OR) or 3,4,5-trimethoxybenzoic acid 8-(diethylamino)octyl ester (TMB-8, Molecular Probes, Inc.) was added to cells from 20 and 75 mM stock in DMSO to a final concentration of 20 and 75 μM, respectively, by first mixing and sonicating in 1 ml of medium withdrawn from the culture dish to be tested.

**Temperature Control**

Dishes were kept in incubators at the appropriate temperature with an atmosphere of 5% CO₂/95% air, or in the case of cells in Hepes- or Pipes-buffered media, with 100% air. During measurements of pHr or junctional permeability, temperature in the dishes was set by a feedback-regulated heater coil submerged in the medium (Azarnia and Loewenstein, 1984).

**Determination of Junctional Transfer**

To test for junctional permeability, randomly chosen cells in a dish were microinjected with a 7.5% aqueous solution of the lithium salt of the fluorescein dye Lucifer Yellow CH (457 tool wt; Molecular Probes, Inc.) by brief intra-illumination thus allowing high camera gain. Injection experiments were detected in these blots (Fig. 10C).

For immunoblots, cells were grown in 60-mm dishes. The cells were shifted to the appropriate test medium and temperature ~5 h before processing. For preparation of whole-cell lysates, cells in one dish (~3 × 10⁶ cells) were quickly rinsed twice with serum-free test medium at the appropriate temperature, 34 or 40°C. They were then scraped in 1 ml of this medium, transferred to an Eppendorf tube, spun for 15 s in an Eppendorf centrifuge, drained, and boiled in 100 μl lysis buffer (2% SDS, 66 mM Tris pH 7.5, 10 mM EDTA) for 10 min. Samples of equal protein concentration were run on 12% polyacrylamide gels, transferred to nitrocellulose; incubated 6-8 h in blocking buffer (TBS; containing 0.2% NP-40, 10 μM vanadate, 0.5 mg/ml BSA, 5 mg/ml gelatin); incubated overnight at 4°C with affinity-purified polyclonal antibody (1:250 in blocking buffer) against phosphotyrosine (a gift of Dr. William Kincsey, University of Miami); washed three times with blocking buffer; incubated with 125I-protein A (New England Nuclear, Boston, MA) and washed three times. Samples run in parallel were incubated with the antibody in the presence of 5 mM o-phospho-l-tyrosine (Sigma Chemical Co., St. Louis, MO); no bands of significant intensity were detected in these blots (Fig. 10C).

**ImmunobLOTS**

**Extracellular pH and Cells Expressing v-src.** Our measurements of junctional transfer were carried out at room atmosphere. To avoid changes of medium pH (pHr) during our measurements, we used bicarbonate-free medium (bicarbonate-buffered medium rapidly turned alkaline in room air). Cells were therefore transferred 4-5 h before the measurements to Hepes or Pipes-buffered medium (titrated to the desired pH), and to incubators with an atmosphere of 100% air.

**Results**

**Effect of pH on Junctional Transfer**

Fig. 1 A shows that low pHr blocks the usual junctional permeability response to temperature shifts of tsv-src cells, the cells containing the temperature-sensitive v-src mutant: at 40°C, where pp60v-src kinase activity is turned off, junctional transfer was high in cells at both, normal (pHr, 7.6; circles) and low pHr (6.6; triangles). In cells at pHr 7.6, transfer fell upon lowering the temperature to 34°C, where the kinase is active. The response was complete within 30 min of the temperature drop and reversible with a similar time course by raising the temperature back to 40°C (Fig. 1 B). In contrast, in cells at pHr 6.6, junctional transfer remained high after the temperature downshift (Fig. 1 A, trian-
Figure 1. Low extracellular pH inhibits the action of v-src on junctional transfer of Lucifer Yellow in Balb/c-3T3 cells infected with a temperature-sensitive mutant of RSV (tsv-src). (A) A shift from 40°C, a temperature not permissive for v-src kinase activity, to the permissive temperature, 34°C, reduces junctional transfer of cells in medium with pH 7.6 (circles), but not of cells in medium with pH 6.6 (triangles). (B) Junctional transfer of tsv-src cells recovers when the temperature is raised from 34 to 40°C. DME/NaHCO₃ medium, pH 7.6 (open circles), and DME/Hepes medium, pH 7.6 (filled circles). The incidence of junctional transfer is plotted, i.e., the fraction of first order neighbors of the cell injected with Lucifer Yellow that are fluorescent 60 s after the injection, expressed in percent. For this and other similar figures, data from several dishes, pooled for 10-min intervals (e.g., 0-10 min, 10-20 min, etc.) and the mean ± SE was plotted at the mean time for these intervals (i.e., at 5 min, 15 min, etc.). For determination of SE, n = total number of injection trials. Although there was some variation between dishes, this variation (SD, n = number of dishes) always was less than the SD from the mean of injections from an individual or from all dishes. In A, each data set is derived from 3 dishes and each data point is derived from 6-19 injections. In B, data represented by open and filled circles are from 2 dishes, 8-9 injections, and 3 dishes, 5-17 injections, respectively.

Figure 2. Low pH restores junctional communication in v-src transformed cells. Alkaline pH quickly abolishes the restoration. Cells were at 34°C 5 h before and throughout the experiment. (A) After 5 h at pH 7.6 in DME/Hepes (open circles) or DME/NaHCO₃ (filled circles), cells were switched to DME/Pipes buffered medium, pH 6.6. (B) Change from pH 6.6 (cells for 5 h in DME/Pipes medium) to pH 7.6 (DME/Hepes, open circles; DME/NaHCO₃, filled circles). The data for Hepes- or Pipes-buffered media in A and B are from four and three experiments, respectively, each data point is the mean ± SE of 7-20 injections. The data for NaHCO₃-buffered medium are from one experiment each, with four to eight injections per time point.
Extracellular pH and Cells Overexpressing c-src. We tested whether pHc affects junctional transfer in cells (NIH 3T3) overexpressing the cellular src gene (c-src++). These cells overexpress pp60c-src 20 times the endogenous level (Azarnia et al., 1988), but the specific enzyme activity of this kinase (as assayed on enolase) is very much lower than that of pp60v-src. Nonetheless, junctional permeability of c-src++ cells is lower than in the parent cell that expresses only the endogenous pp60c-src. We found that the low junctional transfer of c-src++ cells was not improved by lowering pHc (Fig. 3 D).

Intracellular pH. Because pHi, rather than pHc, most likely is the relevant parameter here (pp60c-src is intracellularly located even though it is membrane-associated [Willingham et al., 1979]) we measured pHi of v-src-infected cells at the permissive (34°C) and nonpermissive (40°C) temperature, and at various pHc.

Steady-state pHi did not vary much locally within any given cell at any pHc. This was so in all cell types and at each pHc.

As seen in Table I, there was no significant difference between the mean pHi of normal, tsv-src, and wtv-src cells at 34 or 40°C. Nor was pHi of normal or of tsv-src cells at 34°C significantly different from pHi at 40°C. Only the pHi of wtv-src cells was significantly higher at 34 than at 40°C. We have no explanation for this difference between wtv-src and tsv-src cells. The relationship of pHi to pHc over the range 6.3 to 7.8 is shown in Fig. 4 for tsv-src cells.

Table I. Intracellular pH of Cells in DME Medium of pH 7.55

| Cell type | 34°C | 40°C | P* |
|-----------|------|------|----|
| Normal    | 7.12 ± 0.07 (28) | 7.09 ± 0.06 (20) | >0.1 |
| tsv-src   | 7.13 ± 0.08 (142) | 7.12 ± 0.08 (101) | >0.1 |
| wtv-src   | 7.15 ± 0.06 (34) | 7.06 ± 0.06 (28) | <0.001 |

* Mean pHi ± SD; in parentheses, number of cells from which pHi was determined.

† Level of significance of difference between pHi values of cells at 34 and 40°C.

Figure 3. Extracellular pH influences junctional transfer only in v-src-infected cells, and only at the temperature permissive for v-src kinase activity. (A) tsv-src; (B) normal, uninfected Balb/c-3T3; (C) Balb/c-3T3 infected with wildtype (temperature-insensitive) RSV (wtv-src); (D) NIH-3T3 cells overexpressing c-src 20-fold compared with normal NIH-3T3 (c-src++). Cells were in DMEM medium at respective temperature and pH for 5 h before junctional transfer was tested (for ~30 min). Data points represent the mean plus SE, in A, from 2-10 dishes, 18-148 injections each point; in B, from 1-3 experiments, 6-38 injections; in C, 1-3 experiments, 7-45 injections; in D, 1-2 experiments, 10-30 injections. Open circles, 34°; filled circles, 40°C. The curves are computer-calculated polynomial regressions of the second order.

Figure 4. Intracellular pH as a function of extracellular pH. Plotted are the mean (steady-state) pHi values ± SD of cells kept for 4-5 h in DME/Hepes or DME/Pipes at various extracellular pH (pHc). Each point represents measurements from 20-54 cells, except at pHc 7.55, where 142 and 105 cells were measured, at 34 and 40°C, respectively.

Figure 5. Time course of pHi change in response to pHc change. The pHi of individual tsv-src cells was measured after a shift in pHc. (A) pHc shift from 7.55 (DME/Hepes) to 6.60 (DME/Pipes) at arrow; data (means ± SD) pooled from two experiments, five cells. (B) pHc shift from 6.60 to 7.55 at arrow. Data from three cells, one experiment. The points before the pHc shifts are the mean pHi of 13 (A) and 14 (B) cells.
Figure 6. Propionate medium reduces pH. Cells were put into modified PBS (pH 7.6) ~10 min before first pH measurements were taken. At arrow, medium was changed to propionate-PBS (containing 97 mM propionate), pH 7.6. Circles: data (mean ± SD) from 2 experiments, 13 cells; temperature was lowered from 40 to 34°C at t = 15 min. Triangles: data from one experiment, six cells. Cells were at room temperature (~25°C) throughout the pH measurements. The lower temperature seems to inhibit pH recovery.

The time course of the pH change in response to steps in pH from 7.6 to 6.6 or in the reverse direction is rather quick. In either case the pH change was complete within 10 min (Fig. 5).

Intracellular, Not Extracellular pH Suppresses pp60- src Action on Junctional Permeability. The question remained of whether, indeed, intra- and not extracellular pH is the relevant determinant of the inhibition of v-src action on junctional permeability. We therefore kept pH of tsv-src cells at 7.55 and lowered pH by exposure to the weak acid propionate. Intracellular pH fell rapidly by ~0.4 units upon medium change from PBS, pH 7.55, to PBS containing propionate, pH 7.55, and remained well below the control pH for ~40 min. With time, however, pH returned to the normal level, even though propionate remained present (Fig. 6). When the temperature of tsv-src cells in such propionate medium was shifted from 40 to 34°C, junctional transfer remained high (Table II), as it did in the low pH, medium. Transfer eventually fell in propionate medium, but with a delay in respect to pH recovery. Synchrony is not expected here, since junctional permeability response lags behind pH change (compare time courses in Figs. 2 and 6) and, moreover, we don't know what effects other than pH change such a high concentration of propionate might have on the cells. The main point to be made here is that it is pH, not pHl, that modulates the v-src effect on junctional permeability.

We can therefore now express junctional transfer as a function of pH, (Fig. 7). Over the rather narrow range of 6.75 to 7.25, transfer in tsv-src cells is steeply dependent on pH, at 34 but very little so at 40°C.

**Table II. Propionate Medium Suppresses pp60- src Action on Junctional Transfer**

| Temperature | Time of test | PBS/Hepes (pH 7.5) | PBS/Propionate/Hepes (pH 7.5) | PBS/Pipes (pH 6.7) |
|-------------|--------------|---------------------|-------------------------------|-------------------|
| °C          | min          |                     |                               |                   |
| 40          | -20 to 0     | 82 ± 9 (15)         | 96 ± 3 (16)                   |                   |
| 34          | 15 to 35     | 18 ± 4 (36)         | 68 ± 6 (28)                   | 74 ± 7 (11)       |
| 34          | 45 to 70     |                     | 67 ± 9 (14)                   |                   |
| 34          | 80 to 90     |                     |                               | 79 ± 6 (8)        |
| 34          | 90 to 110    | 17 ± 7 (11)         | 79 ± 6 (28)                   | 83 ± 5 (17)       |
| 34          | 120 to 155   | 37 ± 8 (16)         | 83 ± 4 (20)                   |                   |

Data from three experiments, each comprising all media, with tests in one to two dishes per medium; data were pooled for the indicated time spans and are given as means ± SE.

Hyrc and Rose pH Modulates v-src Action
Effect of pH on the Junctional Response to Diacylglycerol

Because an increased turnover of phosphoinositides is associated with v-src transformation (Diringer and Friis, 1977; Macara et al., 1984; Sugimoto et al., 1984; Macara, 1985), elevated levels of phosphoinositide breakdown products, namely inositol phosphates and diacylglycerol, may be expected. Because diacylglycerol depresses junctional permeability (Enomoto and Yamasaki, 1985; Gainer and Murray, 1985; Yada et al., 1985) it may play a role in the action of diC8 on junctional permeability (Rose et al., 1986). Would low pH prevent the reduction of junctional permeability by diacylglycerol, too? Fig. 9 A shows that, indeed, at pH 6.6 (closed circles) the diacylglycerol analog diC8 (1,2-dioctanoyl-sn-glycerol) had little effect on junctional transfer of tsv-src cells, whereas it greatly reduced transfer at pH 7.55 (open circles). However, this inhibition of diC8 action temperature drop, but had no significant effect on pHi (Fig. 8). TMB-8 therefore does not inhibit the v-src action by decreasing pHi.

1985, 1986), blocks the junctional response to pp60v-src (Rose et al., 1986). Could TMB-8 lower intracellular pH and thereby inhibit the response to pp60v-src? We treated the cells with 75 μM TMB-8 and monitored pHi of individual cells during a temperature downshift from 40 to 34°C every 3 min for up to 50 min. During this time TMB-8 blocked the junctional response to the activation of pp60v-src by the
Figure 10. Low pH but not TMB-8 inhibits v-src-specific tyrosine phosphorylation. Immunoblots of whole-cell lysates from tsv-src (ts), normal uninfected Balb/C 3T3 (n), c-src+++, and wtv-src (wt) cells that were kept at the indicated temperature and pH, for 5 h before lysis. (Top row) Radiographs of blots immunoreacted with a polyclonal antibody against phosphotyrosine. (Middle row) Corresponding gels stained with Coomassie brilliant blue after blotting. Note equal protein loading (except for C, second lane, which apparently received a slightly higher protein load than the others). Vertical dashes between immunoblots and stained gels indicate corresponding lanes. (A) Effect of low pH and nonpermissive temperature on tyrosine phosphorylation in tsv-src cells. A protein band at ~40-45 kD (arrow) is heavily phosphorylated on tyrosine in cells at 34°C and pH 7.6, but much less so in cells at 40°C, or at low pH. (B) Comparison of tyrosine phosphorylation in the various cell types at normal and low pH. The 40-45-kD band specific for tsv-src at 34°C is also present and pH-dependent in wtv-src cells, but is very weak in c-src+++ and not present in normal, uninfected cells. (C) TMB-8 does not decrease tyrosine phosphorylation in general in any of the cell types, nor does it affect the 40-45-kD band of v-src-containing cells. Lanes marked + are lysates from cells treated with 75 μM TMB-8 for 2 h; lanes marked − are untreated controls. The strong band in c-src+++ cells at about 60 kD, marked with an asterisk in C, most likely is the tyrosine-phosphorylated pp60src overexpressed in these cells. (*ts −* in C was an aliquot of the same lysate as “ts” 34°C, pH 7.6, in A and B.) C (Bottom) Radiograph of immunoblot of the same lysates as in the top and middle, loaded in identical order with the same samples and immunoreacted with the same antiphosphotyrosine antibody solution, but in the presence of 5 mM α-phospho-L-tyrosine. Prestained molecular weight standards (Bio-Rad Laboratories, Richmond, CA) were run in the leftmost lanes of the gels (included in radiographs of B and C). The approximate position of the standards (in kilodaltons) is marked. The 50-kD marker (ovalbumin) shows tyrosine phosphorylation.
by low pH was true only for cells at 40°C, not for cells at 34°C, the temperature permissive for pp60v-src kinase activity. When we exposed cells at 34°C for several hours to pH 6.6 to restore high junctional transfer, diC8 application rapidly reduced transfer (Fig. 9B, filled circles).

**Effect of pH and TMB-8 on Protein Tyrosine Phosphorylation**

Because our pH effect clearly is related to pp60v-src kinase activity, we wondered whether pH and TMB-8 interfere with tyrosine phosphorylation by pp60v-src. We therefore first sought to identify proteins that are phosphorylated on tyrosine in a v-src-specific manner and whose phosphorylation also correlates with junctional permeability, i.e., proteins that are tyrosine phosphorylated in tsv-src cells at 34°C but not or less so at 40°C, phosphorylated in wrv-src cells at both temperatures, but not phosphorylated in uninfected cells. The next question was whether tyrosine phosphorylation of any such protein would be sensitive to pH or TMB-8.

We found that in immunoblots for phosphoryrosine of whole-cell lysates of tsv-src cells a band at ~40–45 kD was heavily phosphorylated on tyrosine in cells at 34°C but much less in cells at 40°C (Fig. 10A, last two lanes). This band was also prominent in wrv-src but, at both 34 and 40°C (Fig. 10, B and C). However, it was very weak in c-src++ cells (Fig. 10, B and C), and it was absent in normal, uninfected cells which, in fact, had no proteins that were significantly phosphorylated on tyrosine (Fig. 11B). Tyrosine phosphorylation of this 40–45 kD protein decreased with decreasing pH, in both wrv-src and tsv-src cells (Fig. 10, A and B). TMB-8, however, did not affect tyrosine phosphorylation of this protein or, for that matter, of any of the other proteins (Fig. 10 C). Three experiments on tyrosine phosphorylation at low pH and two experiments with TMB-8 gave the same results, namely a reduction at low pH and no reduction with TMB-8.

**Discussion**

**Cell-to-Cell Channels and pH**

Intracellular pH is known to affect the cell-to-cell channels of gap junctions in many types of cells (see Turin and Warnier, 1977; Rose and Rick, 1978). Although the pK may vary considerably among the cell types (Spray and Bennett, 1985), in all cases in which a pH sensitivity was found, junctional permeability fell with decreasing pH. This is just in the opposite direction to the effect of pH, we observed here in the cells containing active v-src, where junctional permeability rose when pH was lowered in the range 7.15 to 6.75. Clearly, this pH-sensitivity is related to pp60v-src and is not a property of the cell-to-cell channels per se since in both, uninfected Balb/c 3T3 cells and wrv-src cells at the temperature nonpermissive for the mutant kinase, junctional permeability was not sensitive to pH in this range. In this respect, the cell-to-cell channels of Balb/c-3T3 cells containing no or inactive pp60v-src behave like those in guinea pig heart cells, where junctional conductance is not affected by pH in this range (Noma and Tsuboi, 1987). (In guinea pig heart, junctional conductance begins to fall with pH < 6.5, a value outside of our testing range.) The dominant type of gap junction protein in heart is connexin43 (Beyer et al., 1987; Yancey et al., 1989), and our Balb/c-3T3 cells express this protein, too (J. Brugge, personal communication, and our own observation).

**Tyrosine Phosphorylation and pH**

The block by low pH of the v-src effect on junction was associated with a decrease in v-src specific phosphorylation on tyrosine on at least one protein band, at ~40–45 kD. Several proteins have been identified in various cell types as substrates for pp60v-src, among them proteins of 50 and 42 kD (Hunter and Sefton, 1980; Cooper and Hunter, 1981, 1983; Brugge and Darrow, 1982). The 40–45-kD band was prominent in both wild-type and temperature-sensitive v-src cells at high pH, but greatly reduced in intensity at low pH or at the nonpermissive temperature of the v-src mutant, a result that goes hand in hand with the pH sensitivity of the junctional response in these cells.

Interestingly, this band is only very weakly phosphorylated in c-src++ cells and not at all in uninfected cells, cells both of whose junctional permeability was not affected by pH.

Assuming that junctional permeability is determined by tyrosine phosphorylation and that tyrosine phosphorylation in turn is determined by the interplay of pp60v-src kinase and tyrosine phosphatase in the cells, the simplest explanation here is that either the kinase activity is depressed at low pH or that tyrosine phosphatase activity is enhanced. Our immunoblots cannot distinguish between these two possibilities since they report only on the final phosphorylated state, not on kinase or phosphatase activity per se. However, Leis and Kaplan (1982) have described a tyrosine-specific acid phosphatase (associated with the membrane of human astrocytoma cells) whose pH dependence would be in the right direction, with an optimum at 6–6.5. As for pp60v-src kinase activity, Richert et al. (1982), report a pH optimum of ~6.5 in in vitro tests, an optimum that would not explain our results.

We do not imply that the 40–45-kD protein actually is the protein responsible for the v-src-specific effect on junction, or even is the junctional protein, connexin 43, itself. (Gap junctional protein is such a minor component of whole-cell lysates that one would not expect to detect it in such an immunoblot.) The 40–45-kD protein happens to be a prominent one, and serves to make the point that pH (but not TMB-8) may control the tyrosine phosphorylation by pp60v-src of one or more proteins crucial for the regulation of cell-to-cell channels by this kinase.

**On the Mechanism of v-src Action on Junction**

**Tyrosine Phosphorylation of Cell-to-Cell Channels.** There is evidence that the inhibition of junctional permeability by src product depends on the kinase activity of this protein (Azzam et al., 1988). However, it is not known whether the cell-to-cell channel protein itself is a target substrate for pp60v-src. Tyrosine residues with the appropriate amino acid environment for tyrosine phosphorylation are present in gap junction protein from rat liver (Kumar and Gilula, 1986; Paul, 1986) and heart (Beyer et al., 1987), and, by immunostaining, pp60v-src has been localized in the vicinity of cell junctions (Willingham et al., 1979). It is therefore conceivable that the kinase phosphorylates the cell-to-cell channel directly. However, it seems unlikely that channel phosphory-
loration on tyrosine per se (directly by pp60 v-src kinase or indirectly) is sufficient for channel closure because TMB-8 prevents the junctional permeability reduction by v-src but does not interfere with tyrosine phosphorylation, at least not with that of other proteins. This would suggest that v-src acts on the channel via an intermediary or synergistically with it.

**Diacylglycerol and protein kinase C.** Another possibility to be considered is that v-src affects the channels via the phosphoinositide pathway (Rose et al., 1986). Cells containing activated pp60 v-src display increased phosphatidylinositol turnover (Diringer and Fris, 1977; Sugimoto et al., 1984; Macara et al., 1984; Macara, 1985), which may result in elevated levels of diacylglycerol and inositoltrisphosphates (IP3), and inositoltetraphosphates (IP4). Diacylglycerol is a specific activator of protein kinase C, a Ca2+-and phospholipid-dependent serine/threonine kinase (Nishizuka, 1984). Diacylglycerol reduced junctional permeability in the v-src-infected cells and so does phorbol ester (our unpublished observation), another specific activator of protein kinase C (Nishizuka, 1984) and known inhibitor of junctional communication (Murray and Fitzgerald, 1979; Yotti et al., 1979; Enomoto et al., 1981). Ca2+ may be required for the action of diacylglycerol and phorbol ester on junction because the Ca2+ antagonist TMB-8 inhibits it (Yada et al., 1985).

In this connection it is interesting that the junctional permeability reduction of tsv-src cells by diacylglycerol was blocked at low pH, pointing to pH sensitivity somewhere along the path of diacylglycerol action. But low pH interfered with 

cell and so does phorbol ester (our unpublished observation), another specific activator of protein kinase C (Nishizuka, 1984) and known inhibitor of junctional communication (Murray and Fitzgerald, 1979; Yotti et al., 1979; Enomoto et al., 1981). Ca2+ may be required for the action of diacylglycerol and phorbol ester on junction because the Ca2+ antagonist TMB-8 inhibits it (Yada et al., 1985) and v-src action on junction, is consistent with this possibility.

It is noteworthy here that DAG and phorbol ester have been found to induce tyrosine phosphorylation of the 42-kD protein that is also a substrate for pp60 v-src in chick embryo fibroblasts (Gilmore and Martin, 1983; Cooper et al., 1984).

**Inositolphosphates and Ca2+.** Both IP3 and IP4, are implicated in increasing intracellular Ca2+ (Streb et al., 1982; Berridge et al., 1984; Morris et al., 1987; for a recent review, see Berridge and Irvine, 1989). This ion is known to inhibit junctional permeability (Loewenstein et al., 1967; Rose & Loewenstein, 1976; Dahl & Isenberg, 1980; Noma and Tsuboi, 1987) and so we see a possible explanation of our results along the following line of thought: (a) pp60 v-src activity leads to an increased level of IP3, IP4, and diacylglycerol. Low pH may inhibit this step by interfering with tyrosine phosphorylation. (b) IP3 and IP4, elevate cytoplasmic Ca2+, a step that may be inhibited by TMB-8. (c) Ca2+ in turn is necessary to effect closure of cell-to-cell channels, possibly via diacylglycerol/Ca2+-dependent protein kinase C. This step thus may require phosphorylation on serine/threonine, either of the cell-to-cell channel itself or of another intermediary. For example, the gap junction protein of liver, connexin 32, has been shown to be phosphorylated by protein kinase C in vitro (Takeda et al., 1987), and junctional conductance of pancreatic acinar cells is reduced by cell perfusion with protein kinase C (Somogyi et al., 1989).

(d) The sensitivity of the channels to Ca2+ may be reduced manyfold at pH 6.8 compared with 7.0, as it is in guinea pig cardiac gap junction (Noma and Tsuboi, 1987), offering a second point of control by pH in this scenario of events.

The question remains why the regulation of junctional permeability of pp60 v-src in c-src++ cells is not pH sensitive. We have no answer at this time other than that the pH insensitivity of junctional transfer in c-src++ cells may imply that pp60 v-src acts on the junction via a different mechanism than does pp60 v-src. The very different pattern of tyrosine phosphorylated proteins in c-src++ compared with v-src cells may be indicative of this notion, in particular the lack of phosphorylation of the lower molecular weight bands, such as the 40-45 kDa band, whose tyrosine phosphorylation correlates with low junctional permeability in the v-src cells.

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