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

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Abstract. The product of the viral src gene (v-src) is the protein tyrosine kinase pp60^src. Among the known consequences of pp60^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 pHi, 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 pp60^src kinase was inactive. Immunoblots of whole-cell lysates using an antibody against phosphotyrosine show that phosphorylation on tyrosine of at least one cellular protein, specific for pp60^src kinase activity, was reduced by low pHi 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, pp60^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 pp60^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 (pH_e) and hence cells at pH_7.0 are likely to have a lower pHi than cells at pH_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 pp60^src kinase activity is reported to have a maximum at ~pH 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 pHi, 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 pHi.

Lastly, we compared the level of tyrosine phosphorylation of proteins from cells at various pH_e, 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 (c-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

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Abbreviations used in this paper: ASV, avian sarcoma virus; BCECF, 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein; diC8, 1,2-dioctanoyl-sn-glycerol; TMB-8, 8-N,N-diethylamino)octyl-3,4,5-trimethoxybenzoate; c-src++, NIH-3T3 cells overexpressing c-src (clone NIH-3T3 (pMcsrc).
Test Media and Treatments

For experiments, cells were transferred to the appropriate test medium and temperature ~5 h before measurements. Test medium was DME without antibiotics, phenol red and bicarbonate (but with 10% FBS in all cases, including the c-src++ cells), 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; Na2PO4, 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-K2, 10; Hepes, 20 (pH 6.61, 7.21, or 7.40). Diacylglycerol analogue 1,2-dioctanoyl-sn-glycerol (dilC8; Molecular Probes, Inc., Eugene, OR) or 3,4,5-trimethoxybenzoic acid 8-(diethy lamino)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 medium, with 100% air. During measurements of pH, 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 fluorescent dye Lucifer Yellow CH (457 tool wt; Molecular Probes, Inc.) by brief contact of first-order neighbors of the injected cell that show fluorescence 60 s after injection. The 100-W mercury arc lamp (DAGE 66SIT) equipped with a television camera (DAGE 66SIT). The Lucifer Yellow-containing pipette was brought into contact with the cell chosen for injection under brightfield illumination. Impalement was done under fluorescence illumination, with quick withdrawal of the pipette immediately after injection to remove the bright fluorescence of the pipette from the camera's field of view thus allowing high camera gain. Injection experiments were videotaped together with a continuous time display.

We determined the incidence of junctional transfer, i.e., the percentage of first-order neighbors of the injected cell that show fluorescence 60 s after injection. Cells were used at densities low enough (8-9 x 10⁴ cells/cm²) so that one could easily find areas where there was no cell overlap and where first-order neighbors (3-7) could be clearly identified as such.

Intracellular pH Measurements

For determination of pH, we stained the cells with the fluorescent pH indicator 27-bis-(2-carboxylethyl)-5-(and-6)-carboxyfluorescein (BCECF, Molecular Probes, Inc.) by brief (1-2 s) pneumatic pressure pulses. Cells were observed and microinjected on an inverted fluorescence microscope (Nikon DIAPHOT; 100-W mercury arc lamp) equipped with a television camera (DAGE 66SIT). The Lucifer Yellow-containing pipette was brought into contact with the cell chosen for injection under brightfield illumination. Impalement was done under fluorescence illumination, with quick withdrawal of the pipette immediately after injection to remove the bright fluorescence of the pipette from the camera's field of view thus allowing high camera gain. Injection experiments were videotaped together with a continuous time display.

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Results

Effect of pH on Junctional Transfer

Extracellular pH and Cells Expressing v-src. Our measurements of junctional transfer were carried out at room atmosphere. To avoid changes of medium pH (pHₖ) 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.

Fig. 1 A shows that low pHₖ 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 pp60+tks kinase activity is turned off, junctional transfer was high in cells at both, normal (pHₖ 7.6; circles) and low pHₖ (66; triangles). In cells at pHₖ 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 pHₖ 66, junctional transfer remained high after the temperature downshift (Fig. 1 A, trian-
RESULTS

gles) or, when cells were kept at 34°C, transfer rose to near the level of cells at 40°C (Fig. 2 A). This inhibition of the junctional-permeability response to pp60v-src was quickly reversible by simply raising pH, back to 7.55 (Fig. 2 B).

To test that the effect attributed to pH was not somehow due to the lack of bicarbonate in our media, we performed the same type of reversal experiment in medium buffered with bicarbonate instead of Hepes. Essentially the same results were obtained (compare open and closed circles, Fig. 2; see also Fig. 1 B).

In contrast to the pH sensitivity of the tsv-src cells at 34°C, there was little effect of pH at 40°C, where the mutant pp60v-src tyrosine kinase is turned off (Fig. 3 A). Insensitivity to pH also was displayed, and here at both temperatures, by cells not containing pp60v-src (normal Balb/c, Fig. 3 B), indicating that it is not temperature per se that somehow confers pH sensitivity to cell-to-cell channels. This also was evident from 3T3 cells containing wild-type pp60v-src (wrv-src), where kinase is active at both 34 and 40°C, and whose junctional transfer was pH-sensitive at both temperatures (Fig. 3 C). The junctional transfer/pH relation of wrv-src cells differed from that of tsv-src cells at 34°C: transfer was about maximum between pH 6.6 and 7.2 and then declined very steeply in wrv-src cells, whereas in the tsv-src cells transfer decreased continuously from pH 6.6 to 7.8 (Fig. 3 C).

The changes in junctional transfer are attributable to changes in junctional permeability, not to a change in permeability of the (nonjunctional) cell membrane to Lucifer Yellow. The rate of fluorescence loss from single, neighborless cells injected with Lucifer Yellow was 8-10% over 10 min, whether the cells were at pH 7.5 or 6.5, at 34 or 40°C (three to five cells at each condition; fluorescence intensity here was determined with a video analyzer (Colorado Video, Inc., Boulder, CO) as previously described (Yada et al., 1985).
Extracellular pH and Cells Overexpressing c-src. We tested whether pH, affects junctional transfer in cells (NIH 3T3) overexpressing the cellular src gene (c-src'). These cells overexpress pp60' at least 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 pp60'. Nonetheless, junctional permeability of c-src' cells is lower than in the parent cell that expresses only the endogenous pp60' (Azarnia et al., 1988). We found that the low junctional transfer of c-src' cells was not improved by lowering pH (Fig. 3D).

Intracellular pH. Because pH, rather than pH, is most likely the relevant parameter here (pp60' is intracellularly located even though it is membrane-associated [Willingham et al., 1979]) we measured pH, of v-src-infected cells at the permissive (34°C) and nonpermissive (40°C) temperature, and at various pHc.

Steady-state pH, 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 pH, of normal, tsv-src, and vrc-src cells at 34 or 40°C. Nor was pH, of normal or of tsv-src cells at 34°C significantly different from pH, at 40°C. Only the pH, of vrc-src cells was significantly higher at 34 than at 40°C. We have no explanation for this difference between vrc-src and tsv-src cells. The relationship of pH, 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

<table>
<thead>
<tr>
<th>Cell type</th>
<th>34°C</th>
<th>40°C</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>7.12 ± 0.07 (28)</td>
<td>7.09 ± 0.06 (20)</td>
<td>&gt;0.1</td>
</tr>
<tr>
<td>tsv-src</td>
<td>7.13 ± 0.08 (142)</td>
<td>7.12 ± 0.08 (101)</td>
<td>&gt;0.1</td>
</tr>
<tr>
<td>wrv-src</td>
<td>7.15 ± 0.06 (34)</td>
<td>7.06 ± 0.06 (28)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

* Mean pH, ± SD; in parentheses, number of cells from which pH, was determined.
† Level of significance of difference between pH, values of cells at 34 and 40°C.
Figure 6. Propionate medium reduces pH_i. Cells were put into modified PBS (pH 7.6) ~10 min before first pH_i 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_i measurements. The lower temperature seems to inhibit pH_i recovery.

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

Intracellular, Not Extracellular pH Suppresses pp60
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_E of tsv-src cells at 7.55 and lowered pH_i 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_i for ~40 min. With time, however, pH_i 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_i medium. Transfer eventually fell in propionate medium, but with a delay in respect to pH_i recovery. Synchrony is not expected here, since junctional permeability response lags behind pH_i change (compare time courses in Figs. 2 and 6) and, moreover, we don’t know what effects other than pH_i change such a high concentration of propionate might have on the cells. The main point to be made here is that it is pH_i, not pH_E, that modulates the v-src effect on junctional permeability.

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

TMB-8 and Intracellular pH. TMB-8, a blocker of protein kinase C (Sawamura, 1985) and a Ca^{2+} antagonist (Chiou and Malagodi, 1975; Mix et al., 1984; Kojima et al.,

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

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time of test</th>
<th>PBS/Hepes (pH_i 7.5)</th>
<th>PBS/Propionate/Hepes (pH_i 7.5)</th>
<th>PBS/Pipes (pH_i 6.7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>°C</td>
<td>min</td>
<td>Junctional transfer % ± SE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>−20 to 0</td>
<td>82 ± 9 (15)</td>
<td>96 ± 3 (16)</td>
<td>74 ± 7 (11)</td>
</tr>
<tr>
<td>34</td>
<td>15 to 35</td>
<td>18 ± 4 (36)</td>
<td>68 ± 6 (28)</td>
<td>79 ± 6 (8)</td>
</tr>
<tr>
<td>34</td>
<td>45 to 70</td>
<td></td>
<td>67 ± 9 (14)</td>
<td></td>
</tr>
<tr>
<td>34</td>
<td>80 to 90</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>34</td>
<td>90 to 110</td>
<td>17 ± 7 (11)</td>
<td>79 ± 6 (28)</td>
<td>83 ± 5 (17)</td>
</tr>
<tr>
<td>34</td>
<td>120 to 155</td>
<td></td>
<td>37 ± 8 (16)</td>
<td>83 ± 4 (20)</td>
</tr>
</tbody>
</table>

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.
Figure 8. TMB-8 does not reduce pH. (A) Triangles: pH of tsv-src cells was determined every 2 min after application (at arrow) of 75 μM TMB-8 to cells at 34°C. Circles: pH of untreated cells. In B, TMB-8 was applied to cells at 40°C (at arrow) and temperature was shifted to 34°C at t = 15 min. All data points are means ± S.D. of five cells. Essentially the same results were obtained in two further experiments of this type. (C) TMB-8 inhibits v-src action on junctional transfer. 75 μM TMB-8 was added to cells at arrow. Data pooled from three experiments, 5-10 injections each point. In all experiments, cells were in DME/Hepes, pH 7.55.

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 ppt0 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 (filled 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 by the temperature drop, but had no significant effect on pH, (Fig. 8). TMB-8 therefore does not inhibit the v-src action by decreasing pH.

Figure 9. Low pH inhibits the effect of diC8 on junctional transfer in tsv-src cells at 40 but not at 34°C. (A) Cells were in DME-Hepes pH 7.55 (open circles) or DME-Pipes pH 6.6 (filled circles) at 40°C for 5 h before testing for junctional transfer was begun at t = 0. At t = 30 min, 20 μM diC8 was added. (B) Same as in A, except that cells were at 34°C 5 h before and throughout the experiment. The low pH medium had suppressed the v-src action on the junction and restored high junctional transfer (filled circles); application of diC8 rapidly reduced transfer. Data are means ± SE of 10-20 injections from 6 dishes (filled circles) and 6-11 injections from 3 dishes (open circles) in A; 7-16 injections, 5 dishes (filled circles) and 7-13 injections, 4 dishes (open circles) in B.

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 pH 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 ts v-src (ts), normal uninfected Balb/C 3T3 (n), c-src**, and wt v-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 phosphorylated tyrosine. (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 ts v-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 ts v-src at 34°C is also present and pH-dependent in wt v-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 o-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 pp60^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. 9 B, filled circles).

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

Because our pH effect clearly is related to pp60^src kinase activity, we wondered whether pH and TMB-8 interfere with tyrosine phosphorylation by pp60^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 phosphotyrosine 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. 10 A, last two lanes). This band was also prominent in wrv-src, but at both 34 and 30°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. 11 B). 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 Warner, 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 pp60^src and is not a property of the cell-to-cell channels per se since in both, uninfected Balb/c 3T3 cells and tsv-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 pp60^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 pp60^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 pp60^src protein 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, Les 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 pp60^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, connexin43, 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 pp60^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 (Azbarnia et al., 1988). However, it is not known whether the cell-to-cell channel protein itself is a target substrate for pp60^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, pp60^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-
lation on tyrosine per se (directly by pp60v-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 pp60v-src display increased phosphatidylinositol turnover (Diringer and Friis, 1977; Sugimoto et al., 1984; Macara et al., 1984; Macara, 1985), which may result in elevated levels of diacylglycerol and inositoltrisphosphates (IP₃), and inositoltetrakisphosphates (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 pH₅, pointing to pH sensitivity somewhere along the path of diacylglycerol action. But low pH interfered with diC8 only at 40°C, where pp60v-src kinase activity is low, and not at 34°C, even though low pH had inhibited the junctional response to pp60v-src and restored high transfer (Fig. 9, filled circles). This result would argue against a mediation of the v-src effect solely by diacylglycerol, but leaves open the possibility of a synergistic effect of diacylglycerol and another intermediary (e.g., Ca²⁺, see below) brought into play by pp60v-src kinase activity. The inhibition by TMB-8 of both, the diacylglycerol (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 pp60v-src kinase 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., 1983; 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 Tsuibo, 1987) and so we see a possible explanation of our results along the following line of thought: (a) pp60v-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 Tsuibo, 1987), offering a second point of control by pH in this scenario of events.

The question remains why the regulation of junctional permeability of pp60v-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 pp60v-src acts on the junction via a different mechanism than does pp60v-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-kD band, whose tyrosine phosphorylation correlates with low junctional permeability in the v-src cells.

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