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$^{v\text{-}src}$. Among the known consequences of pp60$^{v\text{-}src}$ 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 (pH_i) also counteracts the v-*src* effect: junctional permeability of cells containing active v-*src* kinase rose with decreasing pH_i 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$^{v\text{-}src}$ 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$^{v\text{-}src}$ 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, pp60$^{v\text{-}src}$, 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;[1] Atkinson et al., 1981) and Rous sarcoma virus (RSV; Azarnia and Loewenstein, 1984; Yada et al., 1985). The mechanism by which pp60$^{v\text{-}src}$ 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 (pH_i) is dependent on extracellular pH (pH_e) and hence cells at pH_e 7.0 are likely to have a lower pH_i than cells at pH_e 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_i was lowered (Spray and Bennett, 1985); (*b*) the pp60$^{v\text{-}src}$ 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 pH_i, we measured the cells' pH_i. We also investigated whether low pH_e 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_i.

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, *wtv-src*), or infected with the temperature-sensitive (Schmidt-Ruppin Group D) RSV mutant LA90 (*tsv-src*), and NIH 3T3 cells overexpressing c-*src* (clone NIH-3T3 (pMcsrc)$_B$ 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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1. *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.

(Gibco Laboratories, Grand Island, NY) with 10% FBS (Hyclone Laboratories, Logan, UT), or 10% calf serum (Gibco Laboratories) for c-src^{++} NIH-3T3 cells, and 50 μg/ml each of penicillin, streptomycin, and kanamycin, in an atmosphere of 5% CO_2/95% 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 × 10^4 cells/cm^2.

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 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_e 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; KH_2PO_4, 1.5; $CaCl_2$, 1; $MgCl_2$, 0.5; Na_2PO_4, 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; $CaCl_2$, 1; $MgCl_2$, 1; PIPES-K_2, 10; Hepes, 20 (pH 6.61, 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% CO_2/95% air or, in the case of cells in Hepes- or Pipes-buffered media, with 100% air. During measurements of pH_i 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 mol wt; 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 vision 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 × 10^4 cells/cm^2) 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_i, we stained the cells with the fluorescent pH indicator 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF; Molecular Probes, Inc.). The 530-nm fluorescence emission of BCECF at 480 (but not at 430) nm excitation wavelength is highly dependent on pH (Rink et al., 1982). For staining, the cells were incubated (at 34° or 40°C, as appropriate) for 10–15 min in 8 μM BCECF-acetoxy methyl ester (Molecular Probes, Inc.). After several washes, cells were visualized through a 530 nm barrier filter with a TV camera (DAGE, Model 66 SIT, at fixed gain and voltage) on a Leitz fluorescence microscope (Ortholux; 50× saltwater immersion objective with numerical aperture 1.0; 75 W Xenon arc lamp). Images at alternating wavelength of illumination (480 and 430 nM, spaced 1-2 sec from one another) were captured and digitized (512 × 480 pixels, 8 bit resolution) by an image processor (model 151; Imaging Technology, Inc., Woburn, MA) and stored on an AT microcomputer. The wavelength was selected by means of an electronically controlled filter changer. A computer program (written for us by Cindy Seiffert, Electronic Imagery, Inc., Delray Beach, FL) automated wavelength selection, shutter open time (0.5–2 s), time interval between 480 and 430 illumination wavelength (1–2 s), and time interval between 480/430 image pairs. For analysis, the program retrieved the experimental images, subtracted the appropriate (i.e., matching wavelength of illumination) averaged background image of unstained cells in the appropriate test medium (cells have no detectable autofluorescence at these wavelengths), divided the resulting subtracted images by one another, and multiplied the ratios by 50, yielding $I_{480/430}$. From the image ratios thus obtained we determined the average pixel intensity of the cells by scanning ~75% of the cell image.

We calibrated this average pixel intensity for pH with $I_{480/430}$ images of 10 μM BCECF in calibration buffers at various pH. (Composition of calibration buffers, in millimolar: KCl, 150; Pipes, 15; Hepes, 15; Mg-succinate, 1; pH adjusted with KOH to values between 6.4 and 7.8.) To test whether this calibration also held for BCECF signals from cells, we determined average intensities of $I_{480/430}$ images of BCECF-loaded cells in media of various pH, captured shortly after application of 0.03% Triton X-100. Triton X-100 disrupts the cell membranes and causes rapid equilibration of intra- with extracellular pH (Paradiso et al., 1984). The pH_i values of Triton-treated cells in pH_e 7.46 or 6.78, calculated with the buffer-based calibration, were 7.45 ± 0.08 (SD; n = 4) and 6.81 ± 0.08 (SD; n = 5), respectively, i.e., the expected pH values. Using 10 μM nigericin to equilibrate extra- and intracellular pH in high-potassium medium (Thomas et al., 1979), we obtained pH_i 6.65, 7.19, and 7.47; for pH_e 6.61, 7.21, and 7.40, respectively.

Immunoblots

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 (~3.8 × 10^6 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 Kinsey, University of Miami); washed three times with blocking buffer; incubated with ^{125}I-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. 10*C*).

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 (pH_e) 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 pH_e 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 $pp60^{v\text{-}src}$ kinase activity is turned off, junctional transfer was high in cells at both, normal (pH_e 7.6; *circles*) and low pH_e (6.6; *triangles*). In cells at pH_e 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 pH_e 6.6, junctional transfer remained high after the temperature downshift (Fig. 1 *A, trian-*

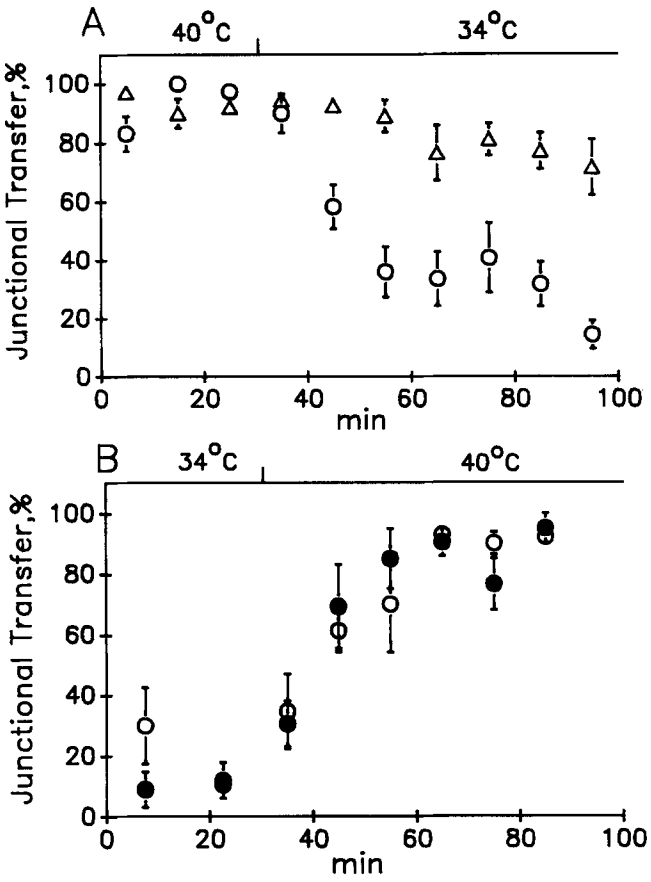

Figure 1. Low extracellular pH inhibits the action of v-*src* on junctional transfer of Lucifer Yellow in Balb/c-3T3 cells infected with a temperature-sensitive mutant of RSV (*tsv-src*). (*A*) A shift from 40°C, a temperature not permissive for v-*src* kinase activity, to the permissive temperature, 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/$NaHCO_3$ 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, probed for junctional transfer for the time span depicted, were 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.

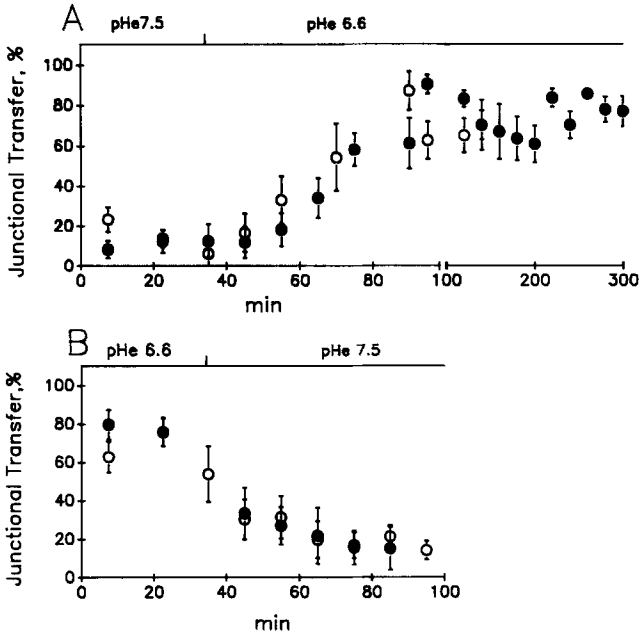

Figure 2. Low pH restores junctional communication in v-*src* transformed cells. Alkaline pH quickly abolishes the restoration. Cells were at 34°C 5 h before and throughout the experiment. (*A*) After 5 h at pH 7.6 in DME/Hepes (*open circles*) or DME/$NAHCO_3$ (*filled circles*), cells were switched 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/$NaHCO_3$, *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 $NaHCO_3$-buffered medium are from one experiment each, with four to eight injections per time point.

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 $pp60^{v\text{-}src}$ was quickly reversible by simply raising pH_e 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_e sensitivity of the *tsv-src* cells at 34°C, there was little effect of pH_e at 40°C, where the mutant $pp60^{v\text{-}src}$ tyrosine kinase is turned off (Fig. 3 *A*). Insensitivity to pH also was displayed, and here at both temperatures, by cells not containing $pp60^{v\text{-}src}$ (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 $pp60^{v\text{-}src}$ (*wtv-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_e relation of *wtv-src* cells differed from that of *tsv-src* cells at 34°C: transfer was about maximum between pH_e 6.6 and 7.2 and then declined very steeply in *wtv-src* cells, whereas in the *tsv-src* cells transfer decreased continuously from pH_e 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).

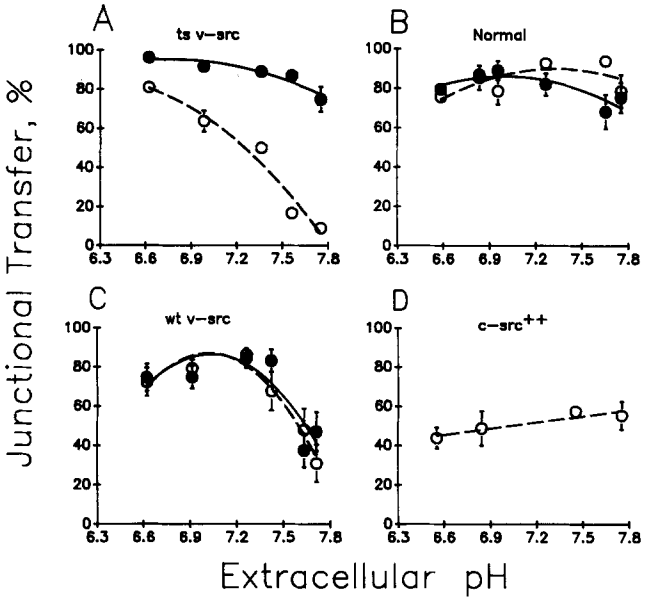

Figure 3. Extracellular pH influences junctional transfer only in v-*src*-infected cells, and only at the temperature permissive for v-*src* kinase activity. (*A*) *tsv-src*; (*B*) normal, uninfected Balb/c-3T3; (*C*) Balb/c-3T3 infected with wildtype (temperature-insensitive) RSV (*wtv-src*); (*D*) 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.

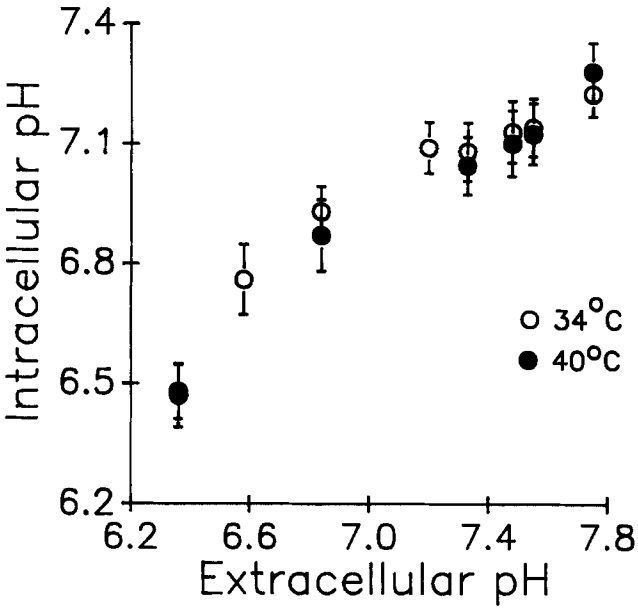

Figure 4. Intracellular pH as a function of extracellular pH. Plotted are the mean (steady-state) pH_i values ± SD of cells kept for 4–5 h in DME/Hepes or DME/Pipes at various extracellular pH (pH_e). Each point represents measurements from 20–54 cells, except at pH_e 7.55, where 142 and 105 cells were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

Extracellular pH and Cells Overexpressing c-src. We tested whether pH_e affects junctional transfer in cells (NIH 3T3) overexpressing the cellular *src* gene (c-src^{++}). These cells overexpress $pp60^{c\text{-}src}$ 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 $pp60^{v\text{-}src}$. Nonetheless, junctional permeability of c-src^{++} cells is lower than in the parent cell that expresses only the endogenous $pp60^{c\text{-}src}$ (Azarnia et al., 1988). We found that the low junctional transfer of c-src^{++} cells was not improved by lowering pH_e (Fig. 3 *D*).

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

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

As seen in Table I, there was no significant difference between the mean pH_i of normal, *tsv-src*, and *wtv-src* cells at 34 or 40°C. Nor was pH_i of normal or of *tsv-src* cells at 34°C significantly different from pH_i at 40°C. Only the pH_i 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 pH_i to pH_e 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	pH_i*		
	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 pH_i ± SD; in parentheses, number of cells from which pH_i was determined.
‡ Level of significance of difference between pH_i values of cells at 34 and 40°C.

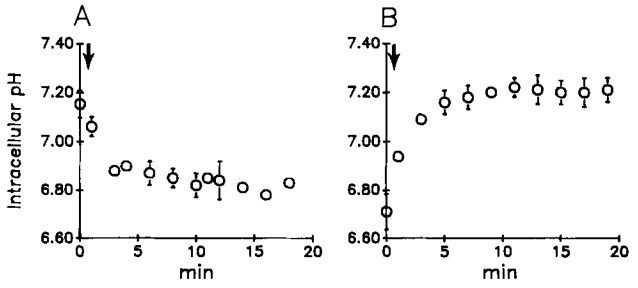

Figure 5. Time course of pH_i change in response to pH_e change. The pH_i of individual *tsv-src* cells was measured after a shift in pH_e. (*A*) pH_e shift from 7.55 (DME/Hepes) to 6.60 (DME/Pipes) at arrow; data (means ± SD) pooled from two experiments, five cells. (*B*) pH_e shift from 6.60 to 7.55 at arrow. Data from three cells, one experiment. The points before the pH_e shifts are the mean pH_i of 13 (*A*) and 14 (*B*) cells.

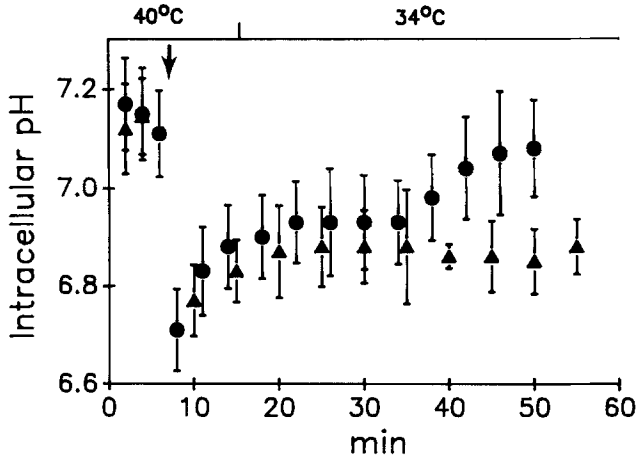

Figure 6. Propionate medium reduces pH_i. Cells were put into modified PBS (pH 7.6) ~10 min before first pH_i measurements were taken. At arrow, medium was changed to propionate-PBS (containing 97 mM propionate), pH 7.6. *Circles*: data (mean ± SD) from 2 experiments, 13 cells; temperature was lowered 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.

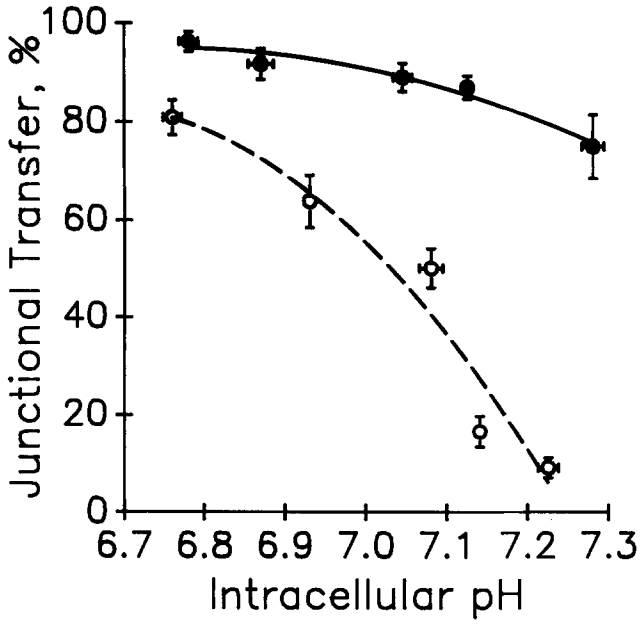

Figure 7. Junctional communication in *tsv-src* cells as a function of pH_i. Replot of junctional transfer, % ± SE, from Fig. 3 *A* vs. mean pH_i ± SE of cells in parallel dishes, at various pH_e and at 34 (*open circles*) or 40°C (filled circles). The pH_i for the leftmost data point at 40°C was calculated here, based on the pH_e/pH_i relation from Fig. 5. The curves are computer-calculated polynomial regressions of the second order.

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$^{v\text{-}src}$ 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_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_e 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$^{v\text{-}src}$ Action on Junctional Transfer

		Medium		
Temperature	Time of test	PBS/Hepes (pH_e 7.5)	PBS/Propionate/Hepes (pH_e 7.5)	PBS/Pipes (pH_e 6.7)
°C	*min*		*Junctional transfer % ± SE*	
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.

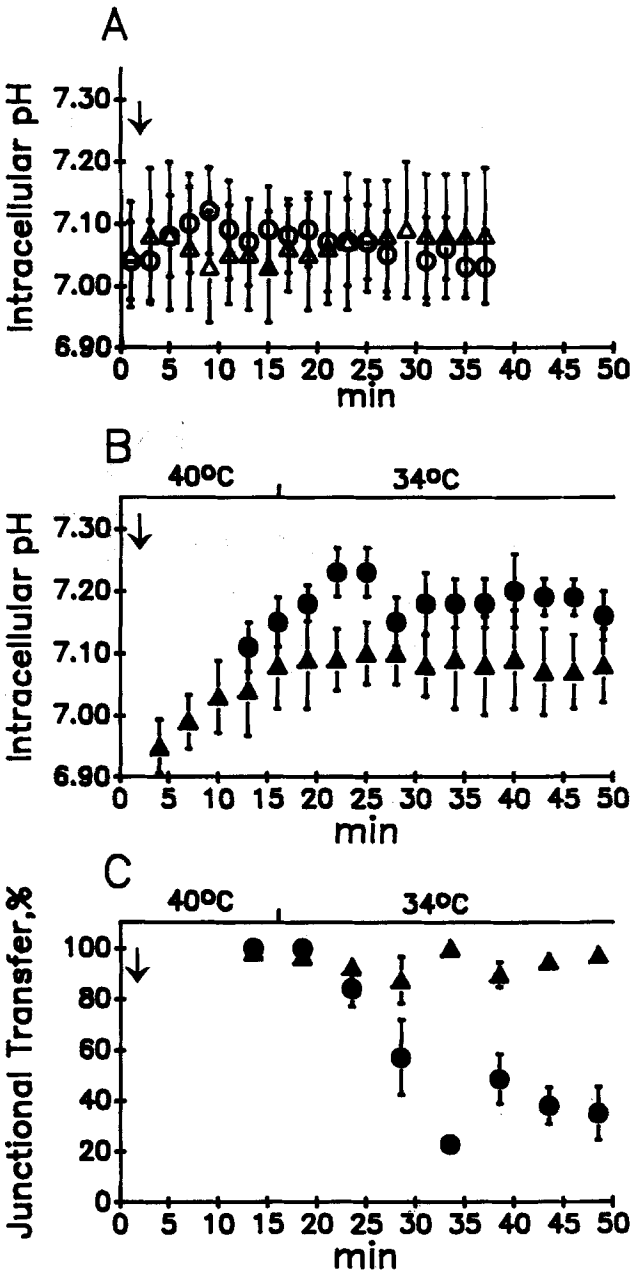

Figure 8. TMB-8 does not reduce pH_i. (*A*) *Triangles*: pH_i of *tsv-src* cells was determined every 2 min after application (*at arrow*) of 75 μM TMB-8 to cells at 34°C. *Circles*: pH_i of untreated cells. In *B*, TMB-8 was applied to cells at 40°C (*at arrow*) and temperature was shifted to 34°C at $t = 15$ 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.

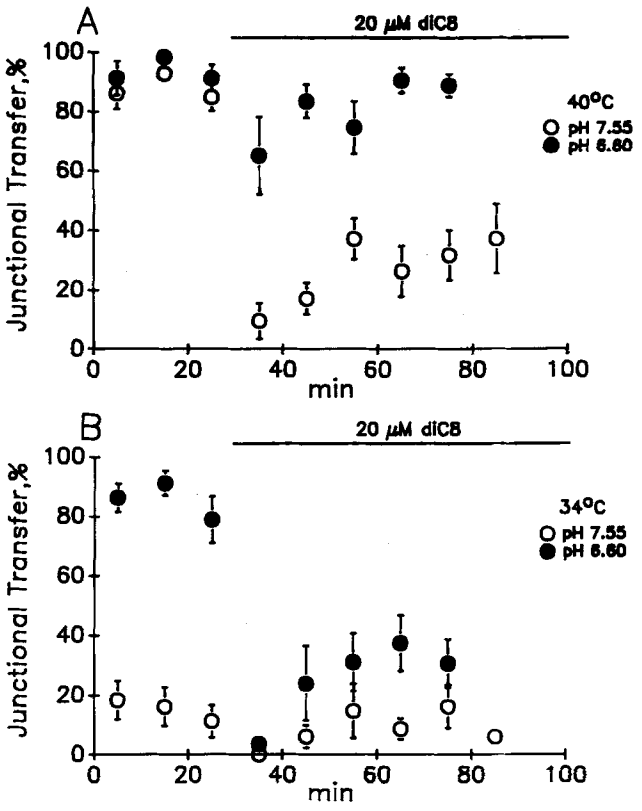

Figure 9. Low pH inhibits the effect of diC8 on junctional transfer in *tsv-src* cells at 40 but not at 34°C. (*A*) Cells were in DME-Hepes pH 7.55 (*open circles*) or DME-Pipes pH 6.6 (*filled circles*) at 40°C for 5 h before testing for junctional transfer was begun at $t = 0$. At $t = 30$ min, 20 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 $pp60^{v\text{-}src}$ (Rose et al., 1986). Could TMB-8 lower intracellular pH and thereby inhibit the response to $pp60^{v\text{-}src}$? We treated the cells with 75 μM TMB-8 and monitored pH_i 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 $pp60^{v\text{-}src}$ by the temperature drop, but had no significant effect on pH_i (Fig. 8). TMB-8 therefore does not inhibit the v-*src* action by decreasing pH_i.

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 $pp60^{v\text{-}src}$ 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_e 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_e 7.55 (*open circles*). However, this inhibition of diC8 action

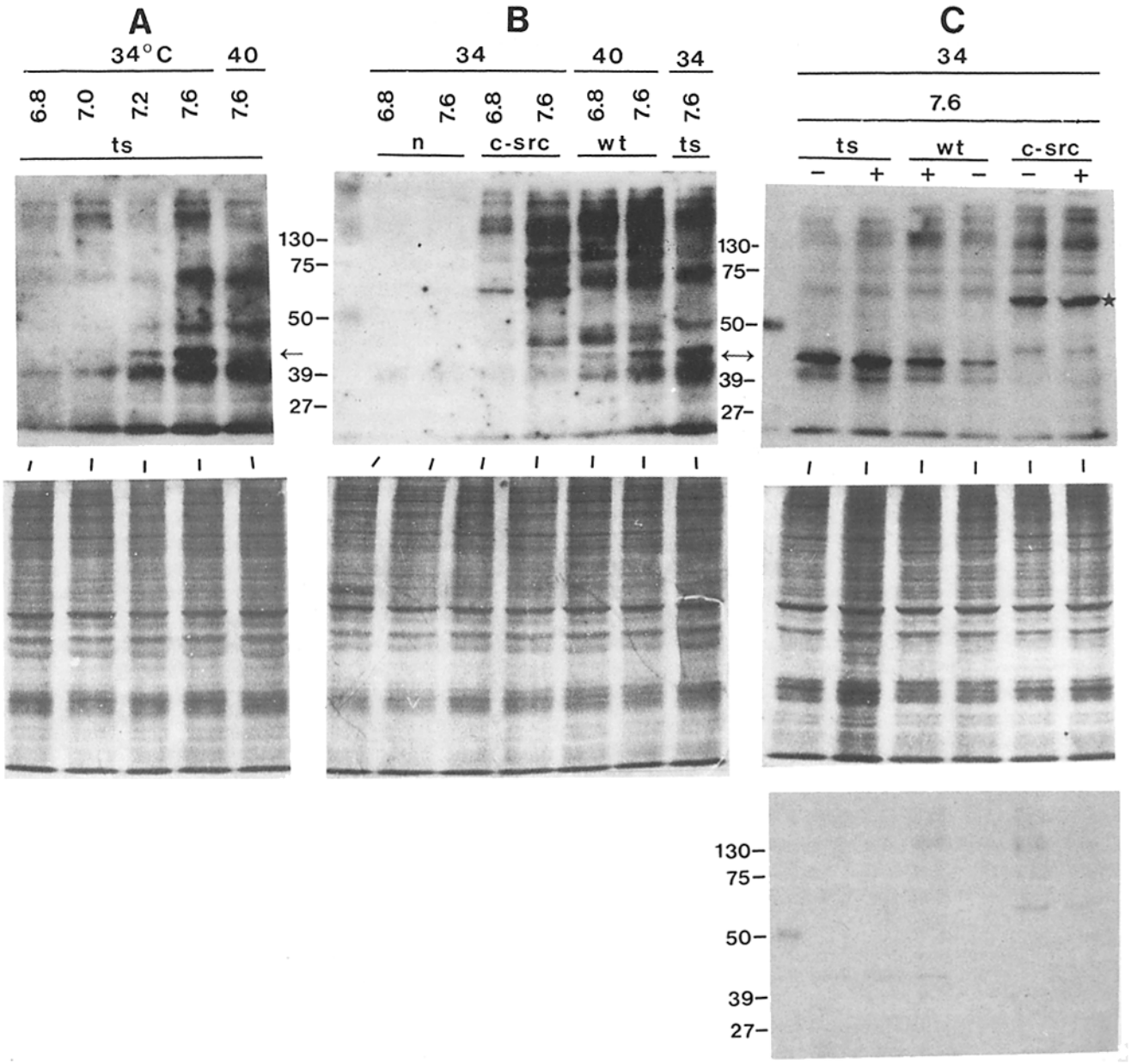

Figure 10. Low pH but not TMB-8 inhibits v-*src*-specific tyrosine phosphorylation. Immunoblots of whole-cell lysates from *ts*v-*src* (*ts*), normal uninfected Balb/C 3T3 (*n*), c-*src*$^{++}$, and *wt*v-*src* (*wt*) cells that were kept at the indicated temperature and pH_e for 5 h before lysis. (*Top 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 *ts*v-*src* cells. A protein band at ~40–45 kD (*arrow*) is heavily phosphorylated on tyrosine in cells at 34°C and pH_e 7.6, but much less so in cells at 40°C, or at low pH_e. (*B*) Comparison of tyrosine phosphorylation in the various cell types at normal and low pH_e. The 40–45-kD band specific for *ts*v-*src* at 34°C is also present and pH_e-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 pp60$^{c\text{-}src}$ 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_e was true only for cells at 40°C, not for cells at 34°C, the temperature permissive for $pp60^{v\text{-}src}$ kinase activity. When we exposed cells at 34°C for several hours to pH_e 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 $pp60^{v\text{-}src}$ kinase activity, we wondered whether pH and TMB-8 interfere with tyrosine phosphorylation by $pp60^{v\text{-}src}$. 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 *ts*v-*src* cells at 34°C but not or less so at 40°C, phosphorylated in *wt*v-*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 *ts*v-*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 *wt*v-*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. 11 *B*). Tyrosine phosphorylation of this 40–45 kD protein decreased with decreasing pH_e in both *wt*v-*src* and *ts*v-*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_i. This is just in the opposite direction to the effect of pH_i we observed here in the cells containing active v-*src*, where junctional permeability rose when pH_i was lowered in the range 7.15 to 6.75. Clearly, this pH-sensitivity is related to $pp60^{v\text{-}src}$ and is not a property of the cell-to-cell channels per se since in both, uninfected Balb/c 3T3 cells and *ts*v-*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 $pp60^{v\text{-}src}$ 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 $pH \leq 6.5$, 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 $pp60^{v\text{-}src}$, 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 $pp60^{v\text{-}src}$ 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, 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 $pp60^{v\text{-}src}$ 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 $pp60^{v\text{-}src}$ 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 (Azarnia 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, $pp60^{v\text{-}src}$ 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 $pp60^{v\text{-}src}$ 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 $pp60^{src}$ 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 (IP_3), and inositoltetraphosphates (IP_4). Diacylglycerol is a specific activator of protein kinase C, a Ca^{2+}- 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). Ca^{2+} may be required for the action of diacylglycerol and phorbol ester on junction because the Ca^{2+} antagonist TMB-8 inhibits it (Yada et al., 1985).

In this connection it is interesting that the junctional permeability reduction of *ts*v-*src* cells by diacylglycerol was blocked at low pH_e, pointing to pH sensitivity somewhere along the path of diacylglycerol action. But low pH_e interfered with diC8 only at 40°C, where $pp60^{v\text{-}src}$ kinase activity is low, and not at 34°C, even though low pH had inhibited the junctional response to $pp60^{v\text{-}src}$ 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., Ca^{2+}, see below) brought into play by $pp60^{v\text{-}src}$ 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 $pp60^{v\text{-}src}$ in chick embryo fibroblasts (Gilmore and Martin, 1983; Cooper et al., 1984).

Inositolphosphates and Ca^{2+}. Both IP_3 and IP_4 are implicated in increasing intracellular Ca^{2+} (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 Tsuboi, 1987) and so we see a possible explanation of our results along the following line of thought: (*a*) $pp60^{v\text{-}src}$ activity leads to an increased level of IP_3, IP_4, and diacylglycerol. Low pH may inhibit this step by interfering with tyrosine phosphorylation. (*b*) IP_3 and IP_4 elevate cytoplasmic Ca^{2+}, a step that may be inhibited by TMB-8. (*c*) Ca^{2+} in turn is necessary to effect closure of cell-to-cell channels, possibly via diacylglycerol/Ca^{2+}-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 Ca^{2+} 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 $pp60^{c\text{-}src}$ 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 $pp60^{c\text{-}src}$ acts on the junction via a different mechanism than does $pp60^{v\text{-}src}$. 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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