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Phosphotyrosine-Containing Proteins and Expression of Transformation Parameters in Cells Infected with Partial Transformation Mutants of Rous Sarcoma Virus

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We have examined the phosphorylation state of five proteins known to become phosphorylated on tyrosine during transformation by Rous sarcoma virus by using cells infected with a panel of partially transforming mutant viruses. Situations of viral mutant and growth temperature were found in which phosphorylation of some proteins occurred more extensively than that of others, indicating that mutations in the *src* gene had affected the specificity of pp60^{src} for some of its substrates as well as affecting the activity of the enzyme. To obtain insight into the biological functions of these phosphorylations, comparisons were made between the degree of phosphorylation of these proteins and the expression of various indicators of the transformed phenotype. The data suggest that phosphorylation of proteins *l*, *p*, and *q* (M_r of 46,000, 39,000 and 28,000, respectively) is not sufficient to induce changes in adhesiveness, hexose transport or morphology. The phosphorylation of protein *p* or *l* or total phosphotyrosine content correlated well with the production of plasminogen activator, and the phosphorylation of proteins *l* and *q* correlated well with increased hexose transport. However, even when good correlations were observed, significant exceptions were sometimes noted. It thus remains possible that some phosphorylations on tyrosine observed in Rous sarcoma virus-transformed cells are not causally related to the expression of the measured parameters of transformation.

When cells become transformed by Rous sarcoma virus (RSV) they display a wide variety of biochemical, morphological, and regulatory alterations, including increases in the rate of glucose transport across the cell membrane and production of a plasminogen-specific protease (plasminogen activator), a decreased adhesiveness, a rounded morphology, and acquisition of the ability to grow in suspension (anchorage-independent growth). This collection of alterations is termed the transformed phenotype (reviewed in reference 18). All of the various manifestations of the transformed phenotype are dependent on the continuous expression of the viral transforming protein, pp60^{src} (18, 35).

pp60^{src} has been shown to be a protein kinase (5, 9, 16, 23, 28) that phosphorylates on tyrosine residues (10, 19, 24, 33). Thus, cellular proteins which become phosphorylated on tyrosine during transformation are candidate substrates for pp60^{src}. Alternatively, some such proteins could be the products of secondary phosphorylations catalyzed by cellular tyrosine-specific kinases activated by pp60^{src}. In any case, the phosphorylations could be important for phenotypic transformation.

It is now clear that many proteins become phosphorylated on tyrosine during transformation by RSV. Martinez et al. (25) found many phosphotyrosine-containing proteins covering a wide span of M_r by electrophoresing a ^{32}P-labeled cell extract in one dimension, slicing the gel into 60 slices, and analyzing the phosphotyrosine content of the protein from each slice; proteins phosphorylated on tyrosine were found in every slice. Beemon et al. (3) came to a similar conclusion, based on experiments in which one-dimensional gels were cut into 10 slices. Radke and Martin (30) identified a 36,000-dalton protein whose phosphorylation was transformation specific; this protein was later found to contain phosphotyrosine (15, 29). Cooper and Hunter have identified seven proteins containing phosphotyrosine by two-dimensional gel electrophoresis (13, 14). In addition, the cytoskeletal protein, vinculin, becomes phosphorylated on tyrosine (31); pp60^{src} is itself phosphorylated on tyrosine (19, 33), as is a 50,000-dalton protein which associates with pp60^{src} (4, 27). The major phosphotyrosine-containing protein of transformed cells is the aforementioned "36,000-dalton" protein (nominal M_r

of 34,000 to 39,000), which constitutes only about 10% of the total phosphotyrosine in the transformed cells (25). This protein is referred to as protein *p* in this communication (13).

Genetic evidence indicates that pp60^{src} has more than one primary cellular target which plays a biologically significant role in generating the transformed phenotype. This evidence is based on the existence of partial transformation mutants of RSV (1, 2, 7, 17, 38). These mutants cause the complete appearance of some transformation parameters, whereas others are induced poorly. For example, the mutant CU2 causes cells to lose their fibronectin, but does not make them fully anchorage independent; in contrast CU12-infected cells retain much of their fibronectin, but are fully anchorage independent in their growth (1). Examples can be found in which each of the measurable parameters is dissociated from most of the others.

We believe that the pp60^{src} proteins coded for by these partially transforming mutants are able to phosphorylate some cellular targets well, but phosphorylate others more poorly resulting in the partially transformed phenotype. This differential phosphorylation could occur either because the mutant pp60^{src} fails to recognize certain targets, or because its intracellular localization is altered so that only certain cellular targets are accessible. The dissociation of biological parameters cannot be explained merely by quantitative variation in pp60^{src} activity, since the mutants cannot be ordered into the same hierarchy for every parameter (1, 38).

Our hypothesis is that when certain transformation parameters are not induced, it is because particular cellular target proteins fail to be phosphorylated. Thus, it should be possible to use a panel of partial transformation mutants to correlate specific intracellular phosphorylations with the appearance of specific aspects of the transformed phenotype, in this way gaining indications as to the biological function of the various proteins which become phosphorylated on tyrosine during transformation. We previously have determined that the phosphorylation of the "36,000-dalton" protein (protein *p*) was neither necessary nor sufficient for loss of surface fibronectin and density-dependent growth inhibition; was not sufficient for loss of adhesiveness and increased glucose transport; and correlated best with the production of plasminogen activator, growth in soft agar, and tumor formation (20, 26). In this communication we examine the phosphorylation of five phosphotyrosine-containing proteins in cells infected with a wide variety of partially transforming mutant viruses. We found that the phosphorylation of protein *q* (M_r of 28,000) could be partially dissociated from the phosphorylation of proteins *p* and *l* (M_r of 39,000 and 46,000), indicating that the mutations in the *src* gene affected the specificity as well as the activity of pp60^{src}. In addition, we confirm and extend our previous biological findings and report that phosphorylation of these proteins is not sufficient for transformation-related changes in adhesiveness and glucose transport or for the appearance of a round morphology. The best correlation was between phosphorylation of proteins *p* or *l* or total phosphotyrosine content and plasminogen activator production and between phosphorylation of proteins *q* or *l* and increased hexose transport.

MATERIALS AND METHODS

Virus and cell growth. Biological and molecular properties of the viruses have been described previously (1, 2, 23, 34), except for CH119 (6). This last mutant was derived by Bryant and Parsons by in vitro mutagenesis at the *Bgl*II site in molecularly cloned Prague A RSV (6). We are grateful to these investigators for making this mutant available to us before publication.

Primary cell cultures were trypsinized and replated at a density of 2×10^6 cells per 100-mm tissue culture dish and infected with virus at a multiplicity of 0.01 to 0.1 CFU per cell. On day 3 after replating, the cells were again trypsinized and replated at a density of 3×10^5 to 5×10^5 cells per 35-mm tissue culture dish and incubated at 36 or 42°C. In all of these cases the cell culture medium was Dulbecco modified Eagle medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% tryptose phosphate broth, 4% calf serum, and 1% heat-inactivated chicken serum. At 36 h after plating, the cells were changed to medium without phosphate or tryptose phosphate broth, but with serum and 3 mCi of carrier-free $^{32}P_i$ per ml. After 12 h of labeling, the cells were harvested for two-dimensional gel analysis as described previously (13) or for assay of transformation parameters (1, 37, 38). The phosphotyrosine content of total cellular protein was estimated as described previously (26). Cultures used for assay of transformation parameters were kept in the same medium as the cells used for gel analysis, but without the $^{32}P_i$. Although no phosphate was added to the medium in this case, there was sufficient phosphate available, primarily from the serum, to allow unhindered growth for 48 h (Weber, unpublished data).

Two-dimensional gel analysis. Labeled cell extracts were adjusted to the same approximate protein concentration and separated by isoelectric focusing for 14,000 V × h by using pH 6 to 8 ampholytes and sodium dodecyl sulfate-gel electrophoresis (13, 22). The gels were incubated in alkali before autoradiography as described previously (13). Since most of the phosphate label is incorporated into nucleic acid, it is difficult to measure the amount of labeled phosphoprotein in each sample. Despite loading of equal amounts of cell protein, the autoradiographs differed in intensity. Since the labeling time was long, we assume that the radioactivity in a spot reflects the absolute amount of alkali-stable phosphate in that phosphoprotein rather than its rate of phosphorylation.

Quantitation of phosphorylation. Radioactivity in

individual ^{32}P-labeled spots was estimated by densitometry. Note that even the most highly labeled alkali-resistant spots contained only about 200 dpm of radioactivity, so quantitation by scintillation counting was not possible. Therefore, we obtained at least two autoradiographs of each alkali-treated gel; one of about 60 h for the weaker spots and one of either 6 or 24 h for the stronger spots. Phosphoproteins *p* and *o* (13) were incompletely resolved in most gels, so their combined intensities were measured.

We scanned through the center of each spot with a fine beam of laser light (high resolution lens; LKB Instruments Inc., Rockville, Md.). Each phosphoprotein has a characteristically shaped spot. By scanning the same phosphoprotein on several different gels, we found that the peak width at half the height was quite constant and presumably depends on physical properties of the phosphoprotein, such as diffusion. Peak height was directly proportional to exposure time, up to film saturation. Thus, peak height is proportional to volume, which is proportional to radioactivity. However, we found that different phosphoproteins have different characteristic peak widths, so the ratio of peak height to radioactivity is different for different phosphoproteins. Peak heights of phosphoproteins *p*, *l*, *q*, *k* and *m* (Fig. 1) on each gel were normalized to the peak height of a phosphoserine-containing protein (*i*) which is unaltered by transformation (13). These normalized peak heights were then calculated as a percentage of the normalized peak height for Schmidt-Ruppin A virus-infected cells at 36°C (actual values: *p*/*i*, 7.3; *l*/*i*, 2.0; *q*/*i*, 0.44; *k*/*i*, 2.25; *m*/*i*, 1.0). We confirmed that phosphorylation of *i* was constant by measuring the intensity of another phosphoprotein (*h*) that contains mostly phosphothreonine and appears not to vary with transformation (10). The ratio *h*/*i* was quite constant (0.66 ± 0.26 [standard deviation]; $n = 22$).

Statistical analysis. Statistical analysis was performed by the University of Illinois Survey Research Laboratory. Data were fitted to each of the following equations: $y = a + bx$; $1/y = a + bx$; $y = a + bx^2$; $y = a + b/x$; $1/y = a + b/x$; $y = a + b/x^2$; $1/y = a + b/x^2$; $y = a \pm b/\sqrt{x}$; $y = a + b\sqrt{x}$; $y = ax^b$; $y = ae^{bx}$; $y = ae^{(bx)^2}$; $y = a + b(\ln x)$; $y = a + bx + cx^2$; $y = \gamma/1 + e^{(a+bx)}$. Correlation coefficients for each equation were calculated by linear regression, and the equation giving the best fit was used to generate the curves shown in Fig. 2 through 7. Points deviating by more than two standard deviations from the expected values were flagged during the computer analysis and are noted below.

RESULTS

Detection and classification of phosphotyrosine-containing proteins. Extracts of cells labeled with ^{32}P were separated by two-dimensional electrophoresis. The gels were dried, treated with 1 M KOH, and then autoradiographed as previously described (13). This procedure releases phosphate predominantly from proteins phosphorylated at serine (8, 13). Direct analysis of spots from such gels has revealed the existence of phosphotyrosine in at least seven proteins in RSV-transformed cells. These proteins are phosphorylated to a lesser degree in uninfected cells (13). We were able to quantitate the degree of phosphorylation of five of these proteins by densitometric scans of autoradiographs. The properties of these five proteins are summarized in Table 1 and Fig. 1A and B. Note that whereas proteins *l* and *p* are essentially undetectable in alkali-treated gels of uninfected cell phosphoproteins, proteins *k*, *m*, and, to a lesser extent, *q* are significantly phosphorylated even before transformation, although not on tyrosine (unpublished data). When purified from the alkali-treated gels all of these proteins contain phosphotyrosine and phosphoserine, and *k* and *m* contain phosphothreonine as well. Thus, estimates of the phosphorylation states of these proteins from autoradiographs of alkali-treated gels include contributions from phosphate linked to serine and threonine besides tyrosine. Examples of autoradiographs of alkali-treated gels of ^{32}P-labeled control and infected chicken cells are shown in Fig. 1, and quantitative estimates of the radioactivity in phosphoproteins *p*, *l*, *q*, *k*, and *m* for cells infected with various RSV strains and mutants are given in Table 2.

If $pp60^{src}$ can directly phosphorylate more than one protein in the cell, partial transformation mutants might induce the phosphorylation of some cell proteins to a greater extent than others. If the mutations alter the activity of the enzyme, but not its specificity per se, a plot of the phosphorylation of one protein against another, or against an estimate of total cellular protein phosphotyrosine content, might be expected to approximate a straight line. On the other hand, if a mutation alters the specificity of $pp60^{src}$, for example by affecting its location or its affinity for a particular substrate, then the relative phosphorylation of one protein may be affected more than that of another. Thus, points deviating from the overall correlation are impor-

TABLE 1. Properties of phosphotyrosine-containing proteins[a]

Phosphoprotein	M_r	pI	Phosphoamino acid content		
			Phosphoserine	Phosphothreonine	Phosphotyrosine
k	46,000	6.95	+++	++	+
l	46,000	7.05	++	+	++
m	43,000	6.80	++++	++	+
p	39,000	7.33	++	±	++++
q	28,000	7.38	+	±	+

[a] The data refer to values in transformed cells. In uninfected cells, *l* and *p* are unphosphorylated; *k*, *m*, and *q* are significantly phosphorylated. Phosphoaminoacid compositions were estimated by electrophoresis of partial acid hydrolysates of phosphoproteins which had been incubated in alkali (13).

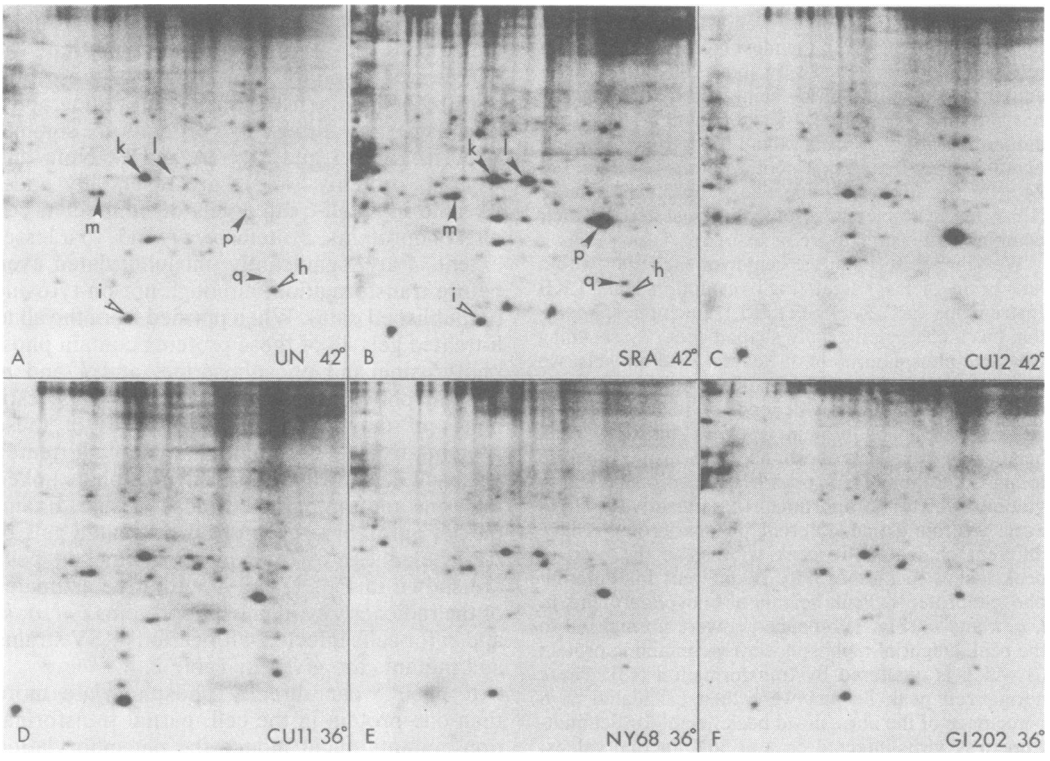

FIG. 1. Autoradiographs of representative two-dimensional gels. (A) uninfected cells, 42°C, 60-h exposure; (B) Schmidt-Ruppin A virus-infected cells, 42°C, 24-h exposure; (C) CU12-infected cells, 42°C, 24-h exposures; (D) CU11-infected cells, 36°C, 50-h exposure; (E) NY68-infected cells, 36°C, 50-h exposure; (F) GI202-infected cells, 36°C, 24-h exposure. In each case, a region from the top of the gel to the position of a 17,000-dalton marker is shown. Protein isoelectric points (measured in 9.2 M urea) range from about pH 6.5 at left to about pH 7.5 at right. Phosphotyrosine-containing proteins *k*, *l*, *m*, *p*, and *q* and reference phosphoproteins *h* and *i* are indicated in panels A and B.

tant for demonstrating that the phosphorylation of two proteins can be dissociated by mutations in pp60^{src}, and these proteins could not be phosphorylated by some common cellular protein kinase activated by pp60^{src}. Failure to find mutants that deviate significantly from the trend

TABLE 2. Protein phosphorylation in cells infected with various strains and mutants of RSV

Virus	Protein (% of Schmidt-Ruppin A virus)[a]										Total phosphotyrosine	
	k		*l*		*m*		*p*		*q*			
	36°C	42°C	36°C	42°C	36°C	42°C	36°C	42°C	36°C	42°C	36°C	42°C
A. Uninfected	63	115	0	2	61	58	0	0	43	18	6	9
B. Schmidt-Ruppin A	100	110	100	97	100	127	100	86	100	80	100	115
C. NY68	79	68	59	3	82	43	66	18	86	34	70	39
D. CU2	93	84	9	9	68	50	4	8	16	7	45	46
E. CU11	91	56	20	2	63	71	35	9	50	14	51	33
F. CU12	120	113	48	77	57	17	81	129	84	68	94	81
G. Prague A	74	71	7	10	81	83	23	20	39	30	48	60
H. GI201	66	56	8	3	89	84	14	2	52	14	37	30
I. GI202	67	99	6	3	66	128	11	3	39	9	41	42
J. GI251	73	37	18	5	20	45	33	10	23	11	71	42
K. CH119	69	54	18	7	93	39	60	24	30	20	60	36

[a] All data are normalized to Schmidt-Ruppin A virus at 36°C.

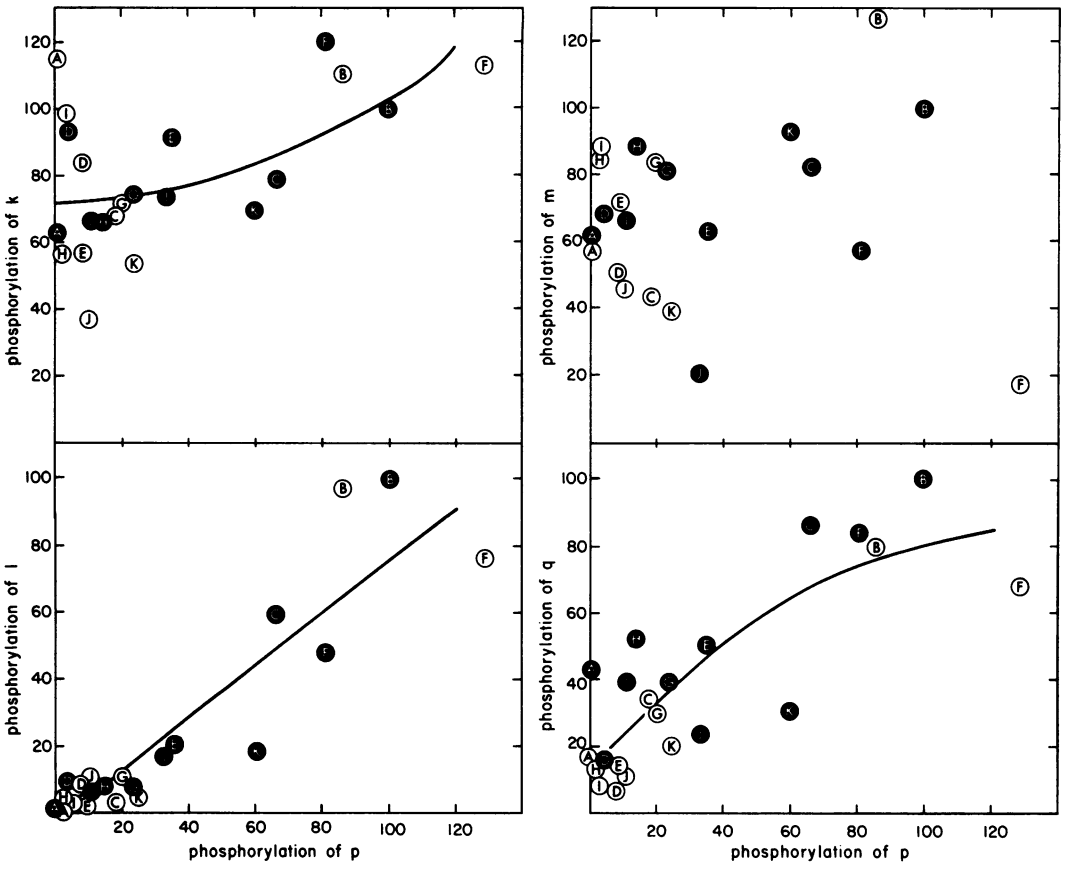

FIG. 2. Phosphorylation of proteins *k*, *l*, *m*, and *q* as a function of the degree of phosphorylation of protein *p*. Capital letters in the symbols refer to the strains and mutants of RSV listed in Tables 2 and 3. Filled symbols, 36°C; open symbols, 42°C.

may indicate the operation of such an intermediary kinase, but could also indicate that pp60^{src} recognizes some common feature of the two proteins compared. Plots of phosphorylation of *p* versus each of the other four proteins under investigation are shown in Fig. 2.

The degree of correlation between the phosphorylation of different proteins was highly variable. Phosphorylation of *p* correlated quite well with phosphorylation of *l* ($r = 0.91$) in three completely independent experiments. There is some scatter in the points, but this may be due to variability in, for example, efficiency of alkaline hydrolysis or in X-ray film processing or densitometry. This correlation may indicate that phosphorylation of *p* is tightly linked to that of *l*. The correlation was not perfect, however, since in cells infected with Schmidt-Ruppin A virus at 42°C, phosphorylation of *p* was more than two standard deviations lower than expected.

Phosphorylation of *q* correlated less well with phosphorylation of *p*. Exceptions included CU12-infected cells at 42°C and CH119-infected cells at 36°C (F and K, Fig. 2), for which *q* was relatively poorly phosphorylated. Our measurements of phosphorylation of *q* are not completely reliable in cases where phosphorylation of this protein was low. Presumably this is because low levels of *q* were too close to background to quantitate accurately. Note that even with Schmidt-Ruppin A virus-infected cells, we estimate that there is about 1/30 as much alkali-stable radioactivity in *q* as in *p* or *l*.

Phosphorylation of *k* showed only a very rough correlation with phosphorylation of *p* ($r = 0.57$), and phosphorylation of *m* showed little, if any, correlation whatever with phosphorylation of *p* ($r < 0.5$). Both *k* and *m* contain mostly phosphoserine and phosphothreonine, even in alkali-treated gels. Thus, it is likely that any variation in the radioactivity in these spots will be due to modulation of serine or threonine phosphorylation and is unlikely to be directly caused by pp60^{src}. Indeed, we now know that *k* is closely related to *l*, and both proteins may be phosphorylation variants of a common precur-

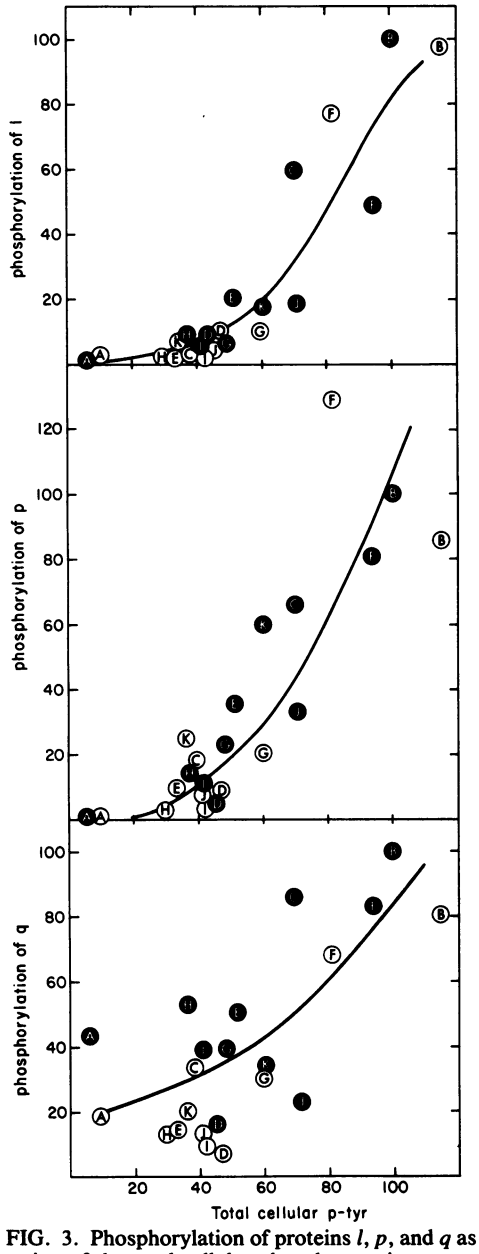

FIG. 3. Phosphorylation of proteins *l*, *p*, and *q* as a function of the total cellular phosphotyrosine content in cells infected with various strains and mutants of RSV. Capital letters in the symbols refer to the strains and mutants of RSV listed in Tables 2 and 3. Filled symbols, 36°C; open symbols, 42°C.

sor (Cooper, unpublished data). It is possible that the phosphotyrosine in *k* results from contamination with *l*. Also, the radioactivity in *k* and *m* only varied over a threefold range. For these reasons, no further analysis of data for these spots will be presented.

One striking feature of the data in Table 2 and Fig. 2 is that the Prague strain of RSV and its derivatives induce phosphorylations to much lower levels than does the Schmidt-Ruppin strain and most of its derivatives. The two strains differ both in overall pp60^{src} kinase activity and in relative specificity for different substrates. It is known that the pp60^{src} protein in cells infected with Prague strain of RSV is less stable than that in Schmidt-Ruppin virus-infected cells (32, 34, 39).

It is also instructive to examine the relationship between the overall cellular phosphotyrosine level and phosphorylation of each of the proteins (Fig. 3). The total cellular phosphotyrosine level, determined as a percentage of total acid-stable protein phosphate, is an estimate of the in vivo activity of pp60^{src} (and cellular tyrosine-specific protein kinases) balanced against phosphatase activity. The contributions from proteins such as *p* and *l* probably each amount to less than 10% of the total phosphotyrosine level (25). There was good correlation between the phosphorylation of *p* and *l* with total phosphotyrosine ($r = 0.94$, for both cases) (Fig. 3). However, these proteins showed significant phosphorylation only under conditions where whole cell phosphotyrosine was high (>50% of control), so that a curve could be fitted more significantly to the data than a straight line. These relationships have been observed in two other experiments, totally independent from the one analyzed in detail here. Phosphorylation of *q* showed a weaker correlation with total phosphotyrosine ($r = 0.77$). Plots of *k* or *m* against total phosphotyrosine showed large scatter, but a trend to higher levels of phosphorylation of the individual proteins at high levels of phosphotyrosine (data not shown).

Relationship to markers of transformation. To correlate the phosphorylation of these proteins with the expression of the transformed phenotype, cells infected with the various mutants were assayed for their adhesiveness, rate of hexose transport, efficiency of colony formation in soft agar, and production of plasminogen activator (Table 3). To control for possible variations in efficiency of infection or reversion of the mutants, these parameters were measured on parallel cultures to those labeled for two-dimensional gel analysis, except for plasminogen activator, which was assayed in separate experiments. The expression of these transformation parameters was plotted as a function of protein phosphorylation (Fig. 4 through 7). Note that the four parameters of transformation measured are all dissociable, and one or another of the mutants surveyed gave levels of one or another parameter which are close to those characteristics of untransformed cells.

TABLE 3. Expression of transformation parameters in cells infected with various strains and mutants of RSV[a]

Virus	Loss of adhesiveness		Glucose transport		Growth in soft agar		Plasminogen activator	
	36°C	42°C	36°C	42°C	36°C	42°C	36°C	42°C
A. Uninfected	4	4	17	17	0.1	0.1	2	2
B. Schmidt-Ruppin A	100	121	100	83	100	133	100	100
C. NY68	125	17	78	16	133	10[b]	100	5
D. CU2	38	6	37	24	10[b]	10[b]	55	45
E. CU11	7	7	36	13	200	<0.1	65	10
F. CU12	69	60	60	57	667	400	85	100
G. Prague A	48	17	58	56	367	400	ND[c]	ND
H. GI201	7	4	54	21	800	<0.1	10	2
I. GI202	99	4	63	20	300	<0.1	85	5
J. GI251	124	7	57	35	33	500	100	15
K. CH119	71	5	43	23	300	133	ND	ND

[a] All data were normalized to Schmidt-Ruppin A virus at 36°C. Adhesiveness, glucose transport, and growth in soft agar were determined on sister cultures of those used for the two-dimensional electrophoretic analyses shown in Table 2. Values for plasminogen activator are from earlier determinations (1, 38).

[b] Numerous tiny soft agar colonies were visible. The efficiency of soft agar colony formation was arbitrarily set at 10% of the wild-type Schmidt-Ruppin A virus value.

[c] ND, Not determined.

Loss of adhesion did not correlate well with phosphorylation of any of the proteins (Fig. 4; Table 4). For *p*, some mutants gave high phosphorylation, but only 50% loss of adhesion (CU12 at 42°C; F in Fig. 4), whereas other mutants caused low phosphorylation, but considerable loss of adhesion (GI202 and GI251 at 36°C; I and J in Fig. 4). Thus, phosphorylation of *p* is certainly not sufficient and may not be necessary for loss of adhesion (23). Similarly for *l*, cells infected with CU12 (F in Fig. 4) showed high phosphorylation with only 50% loss of adhesion, whereas cells infected with GI202 or GI251 at 36°C (I and J in Fig. 4) exhibited large reductions of adhesion with levels of phosphorylation which were more than 2 standard deviations lower than expected. For *q*, two mutants (CU11 and GI201 at 36°C; E and H in Fig. 4) caused 50% of the wild-type level of phosphorylation, but no detectable change in adhesiveness, whereas GI251 at 36°C (J in Fig. 4) caused a large decrease in adhesiveness with little increase in *q* phosphorylation. Thus, for this protein phosphorylation is not sufficient and may not be necessary for loss of adhesiveness. It is possible, however, that some threshold level of total cellular phosphotyrosine is necessary for loss of adhesion since levels of phosphotyrosine of less than 50% of the control correlated with less than 10% loss of adhesion, and greater than 50% levels of phosphotyrosine generally gave greater than 40% loss of adhesion.

In general, the rate of 2-deoxyglucose transport increased with phosphorylation of each of

TABLE 4. Correlation of protein phosphorylations and biological alterations[a]

Biological parameter	Protein			Total phosphotyrosine
	l	*p*	*q*	
Adhesiveness	NN/NS, $r = 0.73$	NN/NS, $r = 0.68$	NN/NS, $r = 0.64$	$r = 0.77$
Hexose transport	NN, $r = 0.83$	NN/NS, $r = 0.75$	$r = 0.81$	$r = 0.82$
Plasminogen activator	NN, $r = 0.85$	$r = 0.83$	NS, $r = 0.68$	$r = 0.85$
Anchorage independence	$r = 0.68$	$r = 0.77$	$r = 0.58$	$r = 0.67$
Round morphology	NN/NS	NN/NS	NN/NS	NS

[a] NN, Not necessary; NS, not sufficient; *r*, correlation coefficient. Conclusions that a phosphorylation is not necessary or not sufficient for the expression of a biological parameter are based on individual data points at least 2 standard deviations from the expected values. Note that it is possible to conclude with much more certainty that a phosphorylation is not sufficient for the induction of a transformation parameter than it is to conclude that the phosphorylation is or is not necessary, since it is possible that only low levels of certain phosphorylations are required to induce biological alterations.

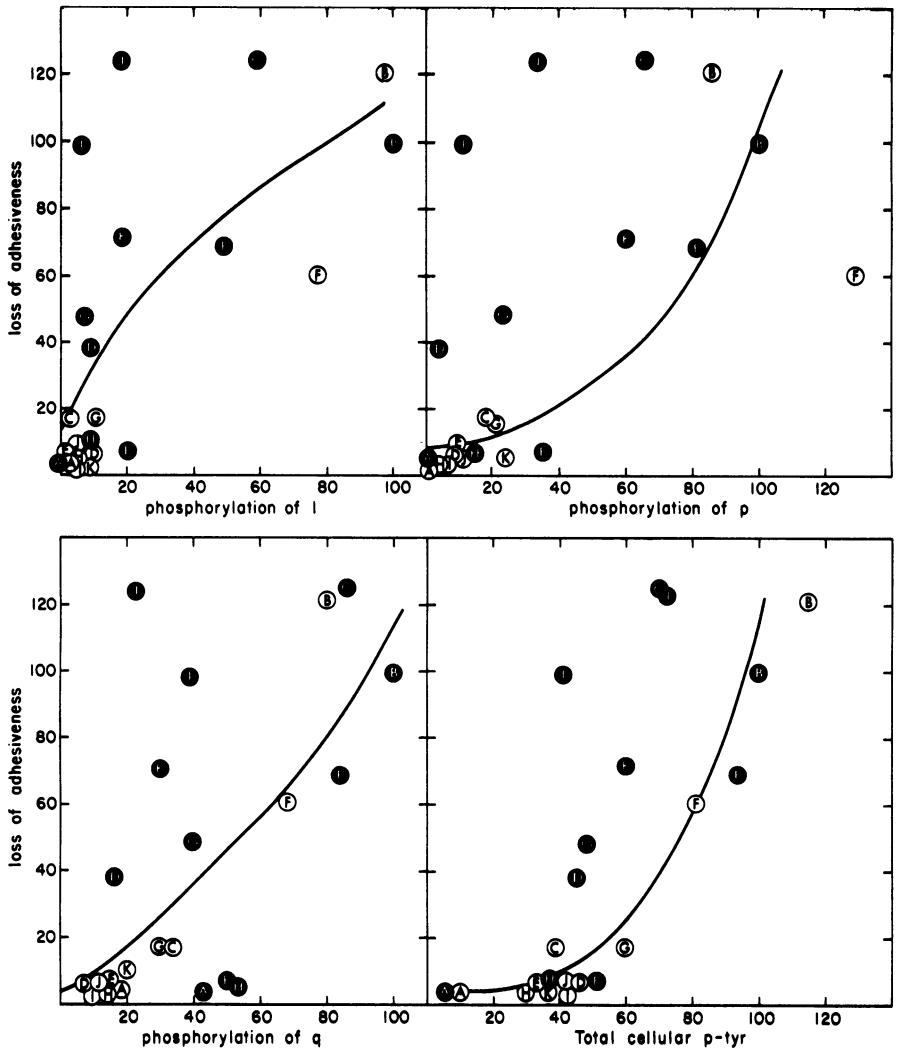

FIG. 4. Loss of adhesiveness as a function of phosphorylation of proteins *l*, *p*, and *q* and the total phosphotyrosine content in cells infected with various strains and mutants of RSV. Capital letters in the symbols refer to the strains and mutants of RSV listed in Tables 2 and 3. Filled symbols, 36°C; open symbols, 42°C.

the proteins and with total phosphotyrosine content. However, CU12-infected cells at 42°C (F in Fig. 5) and GI202-infected cells at 36°C (I in Fig. 5) provided instances of high and low phosphorylation of *p*, respectively, but equal transport rates. A somewhat better correlation for *l* was obtained, but the same exceptions were noted. Phosphorylation of *l* in cells infected with GI202 at 36°C was 2 standard deviations lower than expected. The correlation between *q* phosphorylation and transport rate may be better than is apparent in Fig. 5, since most of the scatter was at low values of *q* which are hard to measure accurately. Nonetheless, CU12 at 36°C provides a case of high phosphorylation of *q* with only intermediate levels of glucose transport. Thus, even though hexose transport showed a better correlation with phosphorylation of proteins *l*, *p*, and *q* than did adhesiveness, it again appears that phosphorylation of these proteins is not sufficient and (in the cases of *p* and *l*) may not be necessary to increase the rate of hexose transport.

As observed before (26), phosphorylation of *p* correlated with plasminogen activator in a nonlinear fashion (Fig. 6). Between 0 and 20% levels of *p* phosphorylation there was a sharp increase in plasminogen activator to the maximal level (Fig. 6). Most conditions giving >20% phosphorylation gave nearly maximal levels of plasminogen activator. The relationship between plasminogen activator and *l* phosphorylation

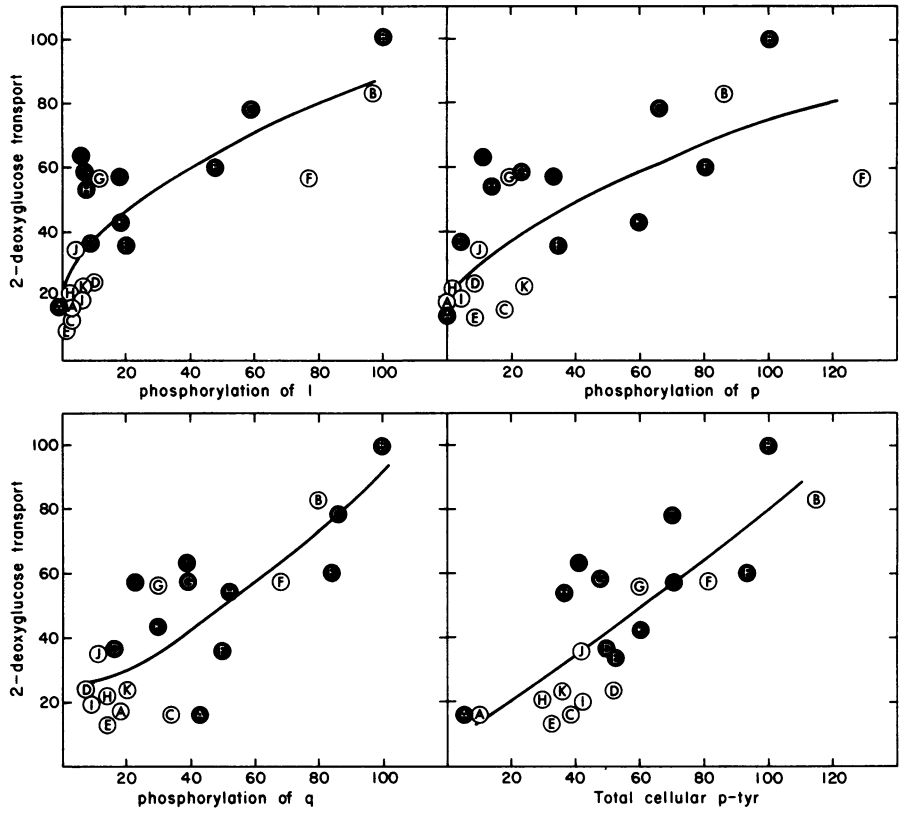

FIG. 5. Rate of 2-deoxyglucose transport as a function of phosphorylation of proteins *l*, *p*, and *q* and the total phosphotyrosine content in cells infected with various strains and mutants of RSV. Capital letters in the symbols refer to the strains and mutants of RSV listed in Tables 2 and 3. Filled symbols, 36°C; open symbols, 42°C.

was about as good as the relationship with *p* phosphorylation. Phosphorylation of *q* showed a poor correlation with plasminogen activator levels. Total phosphotyrosine was related to plasminogen activator in a sigmoidal fashion, with a dramatic increase in plasminogen activator at a threshold level of 50 to 60% of the maximum.

The only growth parameter measured was colony formation in soft agar (Fig. 7). For the GI series of mutants this has been shown to correlate well with other growth parameters such as saturation density in liquid culture (38), but is dissociable in the CU mutants (1). Under three conditions (NY68 at 42°C and CU2 at both 36 and 42°C), numerous tiny colonies grew, suggesting that most of the cells could divide a limited number of times. These points have been arbitrarily given a value 10% of the wild type in Table 2 and Fig. 7. All values less than 0.1% of the wild type have been set at 0.1%, and data are plotted on a logarithmic scale. Conditions giving numbers of colonies from 100 to 800% of control gave values of *p*, *l*, or *q* phosphorylation from 10 to 100% with no clear correlation beyond this point. Thus, if the phosphorylation of these proteins is involved in the acquisition of anchorage-independent growth properties, a very low threshold must exist, beyond which further phosphorylation is redundant. In the exceptional case of mutant GI251 (J in Fig. 7), for which loss of adhesion, hexose transport, and plasminogen activator are all more or less temperature sensitive, growth in soft agar is higher at 42°C than at 36°C (38). Phosphorylation of *p*, *l*, and *q* and total phosphotyrosine level were all reduced at the higher temperature, which would indicate that phosphorylation of these proteins does not correlate with anchorage-independent growth. However, we now know that GI251 is actually cytostatic at 36°C (Weber, unpublished data) rather than being nontransforming. This mutant is thus not suitable for the sort of correlational analysis being attempted here.

Phosphorylation of *l*, *p*, or *q* was not sufficient for acquisition of the round morphology characteristic of wild type-transformed cells, since cells infected with CU12 displayed high levels of phosphorylation, but had only an intermediate,

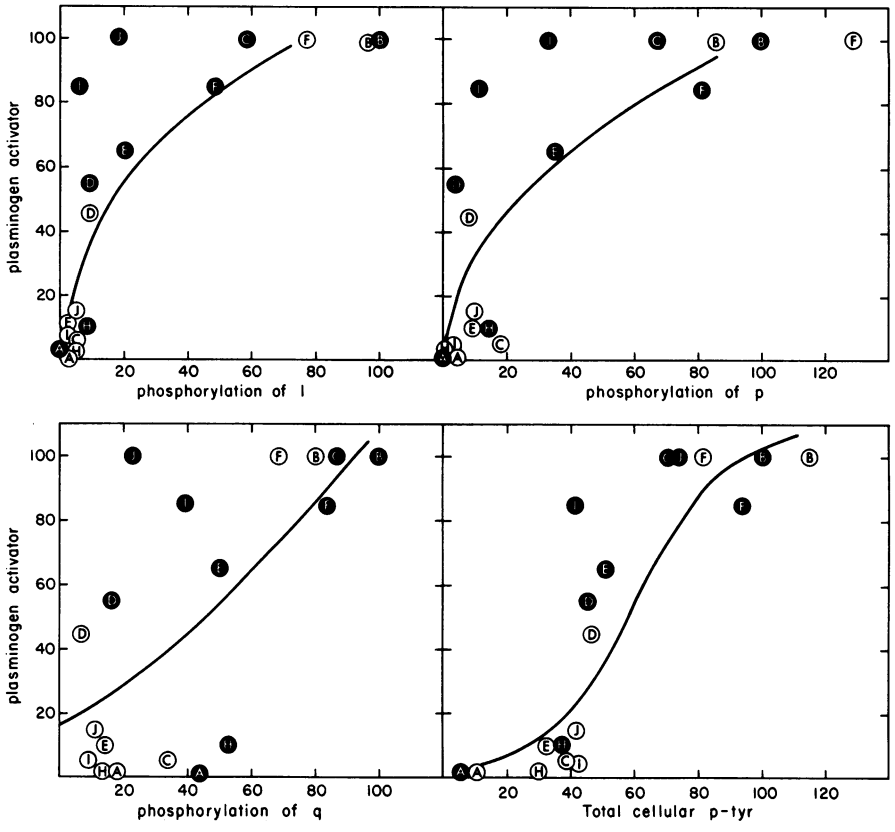

FIG. 6. Plasminogen activator content as a function of phosphorylation of proteins *l*, *p*, and *q* and the total phosphotyrosine content in cells infected with various strains and mutants of RSV. Capital letters in the symbols refer to the strains and mutants of RSV listed in Tables 2 and 3. Filled symbols, 36°C; open symbols, 42°C.

fusiform morphology. Full phosphorylation of proteins *l* or *p* may not be necessary for attaining a round morphology since these proteins were poorly phosphorylated in cells infected with GI202 at 36°C, which were fully transformed morphologically. Protein *q* was phosphorylated weakly in cells infected with GI251 at 36°C, but these cells are completely transformed morphologically (38). Thus, phosphorylation of this protein also may not be necessary for morphological transformation, unless only a low level of phosphorylation is all that is required.

The correlations between the protein phosphorylations and the biological properties of these mutant-infected cells are summarized in Table 4.

DISCUSSION

Genetic evidence indicates that there are several biologically significant targets for pp60^{src} (1, 2, 7, 17, 38), the RSV-coded protein which is ultimately responsible for generating the multiplicity of changes which constitute the transformed phenotype (18, 35). In addition, biochemical experiments indicate that many proteins become phosphorylated on tyrosine during transformation by RSV (3, 4, 13–15, 25, 27, 29–31). In this communication we report experiments in which protein phosphorylation was measured in parallel with biological parameters of transformation, and we attempt to correlate specific phosphoproteins with specific manifestations of the transformed phenotype with a view to determining the function of these proteins.

Specificity of pp60^{src} for cellular targets. We first looked for instances where molecular events could be dissociated from one another in a way comparable to the dissociation of parameters of transformation, which is possible at the cellular level. Could extreme instances be found where phosphorylation of one protein was high relative to another and vice versa, or would the phosphorylations fall into a hierarchy? Several of the cases examined displayed a dissociation of *q* phosphorylation from the phosphorylation

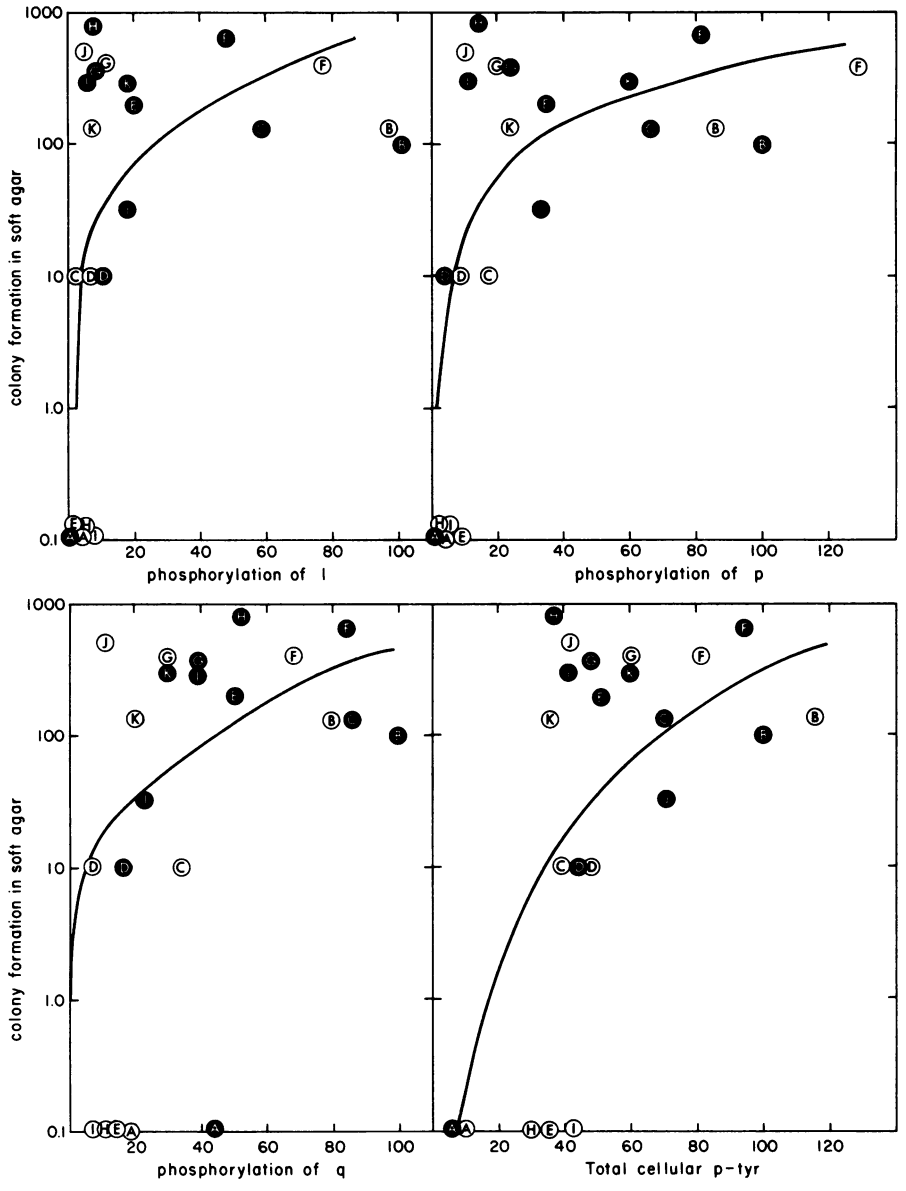

FIG. 7. Efficiency of colony formation in soft agar as a function of phosphorylation of proteins *l*, *p*, and *q* and the total phosphotyrosine content in cells infected with various strains and mutants of RSV. Capital letters in the symbols refer to the strains and mutants of RSV listed in Tables 2 and 3. Filled symbols, 36°C; open symbols, 42°C.

of *p* and *l*. For example, cells infected with CH119 at 36°C or with CU12 at 42°C displayed much less phosphorylation of *q* than of *p*. In addition, there were numerous cases in which the total cellular phosphotyrosine content was greatly elevated whereas proteins *p* and *l* remained poorly phosphorylated. Even in the case of proteins *p* and *l*, which were generally phosphorylated to a similar extent, we found that protein *p* was preferentially underphosphorylated in cells infected with Schmidt-Ruppin A virus and held at 42°C. Thus, it seems highly probable that mutations in the *src* gene can affect the specificity of pp60^{src} as well as its overall kinase activity. This confirms our earlier suggestion (1, 26, 38) that partial transformation mutants of RSV phosphorylate some physiological targets well and others poorly.

It is possible that the specificity of pp60^{src} is determined in part by its subcellular location and

the location of its substrates. However, proteins *p* and *l* may not be localized together in the cell. After breakage in hypotonic buffer, a large fraction of *p* appears to be associated with particulate material, possibly membranes, whereas *l* and *q* are soluble (14a). In nonionic detergents, all three proteins are soluble, although under certain conditions *p* is attached to the detergent-insoluble matrix (8, 14a). However, since at most only a small proportion of each protein becomes phosphorylated, the apparent location of the majority of the protein may not be the same as the location of the molecules which become phosphorylated. L. R. Rorschneider has found that pp60^{src} of CU12 is not concentrated in adhesion plaques, unlike pp60^{src} of other RSVs (personal communication). However, there are only small differences between this mutant and its wild type in the relative phosphorylation of *p*, *l*, and *q* or of vinculin. Chicken embryo cells infected with chimeric RSVs, containing parts of the cellular homolog of the *src* gene and parts of the viral *src* gene, have soluble pp60, but normal levels of protein *p* phosphorylation, and are fully transformed by several criteria, although they are less tumorigenic (21). Thus, the subcellular location of pp60^{src} may not be germane to the relative phosphorylation of proteins *p*, *l*, and *q* and perhaps of other putative targets as well.

Correlation with transformation parameters. A major goal of this work was to correlate the phosphorylation of specific proteins with the appearance of individual parameters of transformation, thus making a first step in determining the biological function of some of these phosphotyrosine-containing proteins. We previously found a good correlation between phosphorylation of *p* and the production of plasminogen activator (26), and this correlation has been confirmed and extended by the work reported here. However, phosphorylation of *l* and the total phosphotyrosine content of the cells both correlated with plasminogen activator levels about as well as phosphorylation of *p*. Phosphorylation of *q*, on the other hand, correlated poorly with plasminogen activator production.

Phosphorylation of *p* displayed a weaker correlation with the rate of glucose transport. As noted previously (26), phosphorylation of *p* is unlikely to be sufficient for induction of increased glucose transport, since cells infected with CU12 display a heavily phosphorylated protein *p*, but possess only half the wild-type level of transport. In addition, we now show that the Prague wild-type virus and GI201 and GI202 at 36°C all display at least 50% of the wild-type level of glucose transport, but only low levels of phosphorylation of *p*. Thus, it is possible that phosphorylation of this protein may not be necessary for induction of the increased transport rate. Phosphorylation of *q* and the rate of hexose transport followed the same trend, but with a fair amount of scatter, possibly due in part to the difficulty of measuring low levels of *q*. Considering this technical problem, the correlation coefficient obtained is surprisingly good and is consistent with the recent finding that protein *q* is phosphoglycerate mutase (Cooper et al., submitted for publication). Protein *l* phosphorylation also correlated well with increased glucose transport, consistent with its identification as the glycolytic enzyme enolase (Cooper et al., submitted for publication). However, phosphorylation of this protein in cells infected with GI202 at 36°C was more than 2 standard deviations lower than predicted on the basis of the glucose transport rate shown by these cells. This finding was obtained in three completely independent experiments. Thus, phosphorylation of *l* to the maximum level is not necessary for increased glucose transport.

None of the individual phosphorylations correlated with loss of adhesiveness. The best fit with adhesiveness was with the total phosphotyrosine content. There was a general, qualitative relationship between all phosphorylations measured and growth in soft agar, but there appeared to be little relationship to published values for saturation density in liquid culture (1, 34). For example, cells infected with GI251 at 42°C or with CU2 both displayed low protein phosphorylation levels, but grew to high saturation densities.

A summary of our correlations between protein phosphorylations and expression of transformation parameters is shown in Table 4. In examining Table 4, one should note that it is possible to conclude with some certainty that a phosphorylation is not sufficient for the expression of a parameter of transformation. However, the conclusion that a phosphorylation is not necessary for the expression of a transformation parameter is weaker, since it is always possible that only very low levels of certain phosphorylations are required to induce biological alterations. The weakest conclusions of all are those cases in which we show a positive correlation between a phosphorylation and a transformation parameter. This is because the phosphorylations examined in this investigation represent only a small minority of the proteins that become phosphorylated on tyrosine during transformation (25). It is possible that the biological effects we observe are the consequences of phosphorylation events that we have not examined. Similarly, the phosphorylations that we have examined may affect cells in ways which were not examined in this investigation.

Finally, we need to consider seriously the

possibility that the proteins phosphorylated on tyrosine which have been examined by us or by others are completely adventitious and unrelated to transformation. The following lines of evidence are consistent with this hypothesis.

(i) Phosphorylation of proteins *p* and *l* only occurs to a significant extent under conditions where the total cellular phosphotyrosine is high (Fig. 3). This may indicate that these are low-affinity phosphorylations.

(ii) Even when the overall correlation between phosphorylation and phenotype appears good, individual cases often are found that render the correlation significantly imperfect. This is what one would expect if the phosphorylations, although catalyzed by pp60^{src}, were adventitious.

(iii) Proteins phosphorylated on tyrosine have displayed a low stoichiometry of phosphorylation in the cases where this has been examined.

(iv) Enzymes that phosphorylate on tyrosine have not shown a great deal of substrate specificity. For example, pp60^{src} will phosphorylate casein, tubulin, and actin in vitro even though these are not normal substrates (10). A431 cells, which have a huge excess of epidermal growth factor receptors, phosphorylate numerous proteins (including protein *p*) when treated with epidermal growth factor (12; Martinez, Nakamura and Weber, unpublished data), whereas cells such as 3T3 cells and chicken embryo cells, which have modest levels of epidermal growth factor receptors, phosphorylate only a few proteins when treated with epidermal growth factor—and protein *p* is not among them (11, 25a). The Abelson virus-transforming protein, when expressed in *Escherichia coli*, phosphorylates numerous *E. coli* proteins, which presumably are not physiological targets (36).

(v) pp60^{src} itself is phosphorylated on tyrosine. However, preventing this phosphorylation by converting the relevant tyrosine to a phenylalanine does not detectably alter the biological or enzymatic activities of pp60^{src} (Snyder et al., Cell, in press). Thus, phosphorylation of pp60^{src} on tyrosine seems likely to be adventitious.

Our overall conclusions concerning the relationship between phosphorylations on tyrosine and the biological manifestations of transformation are thus mixed. On the one hand, we do find some striking correlations, notably the correlations between *p* and *l* phosphorylation and plasminogen activator and the correlations between *q* and *l* phosphorylation and hexose transport. Nonetheless, because of the complexity of the system and the frequent exceptions to the correlations, it is important to interpret these positive results with caution and to consider the possibility that the positive correlations may be coincidental. It will require the isolation of cellular mutants defective in substrates of pp60^{src} or directly mechanistic studies to identify the precise role of tyrosine phosphorylation in malignant transformation.

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