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Phosphorylation of a 36,000 M_r Cellular Protein in Cells Infected with Partial Transformation Mutants of Rous Sarcoma Virus

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We have isolated and characterized mutants of Rous sarcoma virus which induce some parameters of transformation but fail to fully induce other parameters. We believe these mutants code for a pp60^{src} which phosphorylates some targets well but phosphorylates others poorly. Using these mutants, we examined the phosphorylation of a 36,000 M_r protein which is phosphorylated on a tyrosine in cells transformed by Rous sarcoma virus, in an attempt to correlate this phosphorylation with the expression of specific transformation parameters. We found that phosphorylation of the 36,000 M_r protein was neither necessary nor sufficient for loss of fibronectin or for loss of density-dependent inhibition of growth. Phosphorylation of the protein was not sufficient for morphological alterations, increased hexose transport, or loss of adhesiveness. For the parameters measured, the best correlation was with increased plasminogen activator. In addition, it is noteworthy that cells infected with the mutant CU2 displayed low levels of phosphorylation of the 36,000 M_r protein and also were deficient in anchorage-independent growth and tumorigenicity, raising the possibility that the phosphorylation of the 36,000 M_r protein may be required for malignant growth properties.

Malignant transformation by Rous sarcoma virus (RSV) is characterized by a wide variety of biological alterations, including changes in cellular morphology and the cytoskeleton, loss of surface fibronectin, decreased adhesiveness, and increases in hexose transport and plasminogen activator (reviewed in references 11 and 21). Collectively, these changes are termed "the transformed phenotype." The establishment and maintenance of all these transformation parameters require the activity of pp60^{src}, a phosphoprotein which possesses a protein kinase activity (3, 6, 10, 14, 15, 19, 21).

Recently, the kinase activity of pp60^{src} has been shown to have unusual specificity: it phosphorylates tyrosine (7, 12). In addition, it has been found that the level of phosphotyrosine in transformed cells is at least 10-fold greater than that in normal cells (20). In the case of a temperature-conditional transformation mutant of RSV, the phosphorylation of tyrosine is also temperature sensitive and rapidly reversible by temperature shift (20). These results all strongly suggest that this phosphorylation of tyrosine is an important, early event in the transformation process. Moreover, because of the unusual specificity of pp60^{src} kinase for tyrosine, proteins which become phosphorylated on tyrosine during RSV transformation are candidate targets for pp60^{src}.

It has become clear from recent work that at least half a dozen cellular proteins become phosphorylated on tyrosine during transformation (8, 16, 18), and our own work indicates that there are many more (K. D. Nakamura, R. Martinez, and M. J. Weber, manuscript in preparation). In particular, a major cellular protein of 36,000 M_r (36K) which is rapidly phosphorylated on tyrosine after transformation (9, 16, 17) and is also a substrate for pp60^{src} in vitro has been identified (9). Although this 36K protein may be associated with the detergent-insoluble framework of cells (5), nothing is known about the function of this protein in normal cells or about what role, it any, its phosphorylation plays in the process of transformation. The same problem obviously exists for the other phosphotyrosine-containing proteins thus far identified.

To obtain information concerning the possible role of the 36K protein in transformation, we have made use of "partial" transformation mutants of RSV (1, 2, 4, 23). These mutants induce the appearance of various transformation parameters to various degrees. Our interpretation of the biological properties of these mutants is that they code for a pp60^{src} which phosphorylates various primary targets to various degrees. The isolation of these mutants, in fact, provided the first indications that pp60^{src} would interact with more than one primary target. Because we

expect that the phosphorylation of specific cellular proteins is responsible for the appearance of specific manifestations of transformation, it should be possible to correlate the degree of phosphorylation of the 36K protein with the expression of one or more of the transformation parameters, thus providing a first approach to determining the function of the 36K protein in transformation.

MATERIALS AND METHODS

Virus strains. The CU mutants were all derived from the Schmidt-Ruppin strain of RSV, subgroup A, as described previously (1). The stock of the wild-type virus and the temperature-conditional mutant, *ts*NY68, were obtained from H. Hanafusa, Rockefeller University, New York, N.Y. (11, 21). The *ts*GI251 mutant, derived from the Prague strain of RSV, subgroup A, as described by Becker et al. (2), was obtained from R. Friis, Institut, für Virologie, Giessen, W. Germany.

Cell culture. Fibroblast cultures were prepared from 11-day-old chicken embryos (Spafas, Inc., Norwich, Conn.) by standard techniques (22). Secondary cultures were infected with virus and generally used for experiments one or two passages later.

Immunoprecipitation. Cultures were labeled simultaneously with 70 μCi of [^{35}S]methionine per ml (>400 Ci/mmol; New England Nuclear Corp., Boston, Mass.) and 1 mCi of ^{32}P per ml (carrier free; New England Nuclear Corp.) for 12 to 16 h in methionine-free, phosphate-free medium. The Dulbecco modified Eagle medium also contained 4% calf serum, 1% heat-inactivated chicken serum, and 0.04 mM unlabeled methionine (20% that normally present in complete medium). Cells were washed with phosphate-buffered saline and lysed on the dishes in RIPA (3) supplemented with 1% Trasylol (FBA Pharmaceuticals, New York, N.Y.)–1 mM EDTA–40 mM NaF and then clarified at 30,000 × *g* for 30 min. The extracts were incubated for 1 h at 4°C with rabbit anti-chicken 36K serum (a gift from R. Erikson, University of Colorado Medical Center, Denver), after which the immune complex was collected with protein A-Sepharose (Sigma Chemical Co., St. Louis, Mo.). This complex was washed 4 to 5 times with RIPA plus Trasylol and then boiled for 3 min in electrophoresis sample buffer (13). The samples were resolved on sodium dodecyl sulfate-polyacrylamide slab gels, fluorographed (Enhance; New England Nuclear Corp.), and analyzed by autoradiography. ^{35}S radiation was detected on a preflashed film placed directly against the gel. The levels of ^{32}P in the sample made an insignificant contribution to this exposure, a fact which was confirmed by allowing the ^{32}P to decay and then reexposing the gel. Detection of the ^{32}P was aided by the use of intensifying screens (Du Pont Lightning-Plus; Du Pont Co., Wilmington, Del.) and preflashed film. The ^{35}S was prevented from being recorded by placing several sheets of paper between the gel and the film.

Phosphoamino acid analysis of the 36K protein. After immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the ^{32}P-labeled 36K protein was located on the undried gel by autoradiography. The bands were excised from the gel and electroeluted and, after the addition of 100 μg of immunoglobulin G as carrier, the protein was precipitated in 20% trichloroacetic acid. The protein was hydrolyzed under nitrogen in distilled HCl for 2 h at 110°C. After removal of the HCl at reduced pressure, the samples were redissolved in pH 1.9 electrophoresis buffer containing cold phosphoserine, phosphothreonine, and phosphotyrosine (all at 15 mM). Samples were spotted on Whatman 1M chromatography paper and run at pH 1.9 for 1 h at 3.0 kV. The strip containing the phosphoamino acids, located by running appropriate markers, was cut from the first paper, sewn onto a second paper, and run at pH 3.5 for 90 min at 1.5 to 2.0 kV.

Phosphoamino acid analysis of total cells. Cells were labeled with 0.5 to 1.0 mCi of $^{32}P_i$ per ml for 10 to 16 h, lysed in 2% sodium dodecyl sulfate–1.5 mM NaCl–1% sodium deoxycholate–1% Nonidet P-40–1% Trasylol–40 mM NaF, and boiled immediately. After trichloroacetic acid precipitation, the protein was subjected to acid hydrolysis and high-voltage paper electrophoresis as described above but with one modification: the direction of migration in the second run (pH 3.5) was perpendicular to that in the first. This resulted in greater separation of the three phosphoamino acids.

RESULTS

Phosphorylation of the 36K protein. Phosphorylation of the 36K protein was examined with antiserum raised against the purified protein (9). Extracts from cells which were simultaneously labeled with [^{35}S]methionine and $^{32}P_i$ were lysed in buffer containing detergents and immunoprecipitated with the anti-36K protein serum. The immunoprecipitate was then resolved on sodium dodecyl sulfate-polyacrylamide gels. Figure 1 (upper panel) shows the autoradiogram of the [^{35}S]methionine-labeled proteins brought down by this antiserum. The autoradiogram verified the presence of the 36K protein in all extracts,

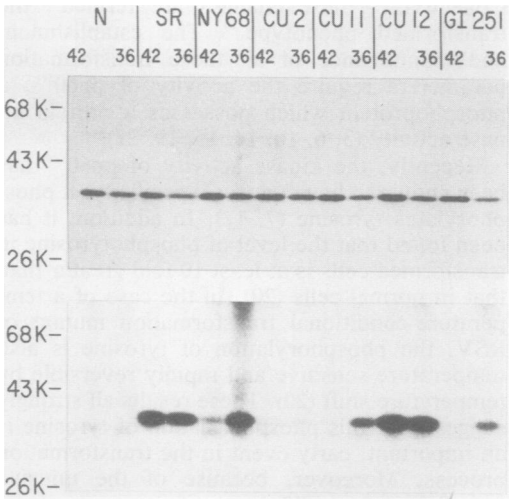

FIG. 1. Phosphorylation of the 36K protein in normal or RSV-infected cells growth at either 36 or 42°C. Upper panel, [^{35}S]methionine; lower panel, $^{32}P_i$. SR, Schmidt-Ruppin cells; N, normal cells.

TABLE 1. Phosphorylation by partial transformation mutants

Strain	Temp (°C)	Phosphorylation of ^{32}P-labeled 36K protein[a]	Total cell phosphotyrosine content[b]
Normal	42	0.03	0.06
	36	0.04	0.04
*ts*NY68	42	0.16	0.36
	36	0.78	0.78
CU2	42	0.09	0.40
	36	0.07	0.69
*ts*CU11	42	0.07	0.25
	36	0.51	0.62
CU12	42	0.64	1.13
	36	1.09	1.32
*ts*GI251	42	0.11	0.56
	36	0.59	0.74
Schmidt-Ruppin	42	1.0	1.02
	36	1.0	1.15

[a] Expressed as a fraction of Schmidt-Ruppin strain phosphorylation.
[b] Expressed as a percentage of the total phosphoamino acid content.

whether they were from normal or from virus-infected chicken embryo fibroblasts. The autoradiogram in the lower panel of Fig. 1 recorded the level of phosphorylation of these same immunoprecipitated proteins. A quantitative analysis of these data is presented in Table 1. As previously reported, there was little, if any, phosphorylation of the 36K protein in uninfected chicken embryo fibroblasts, whereas in cells infected with the wild-type Schmidt-Ruppin strain of RSV, the 36K protein was clearly phosphorylated both at 36 and 42°C. The data further confirm that in cells infected with the temperature-conditional mutant, *ts*NY68, phosphorylation of the 36K protein was temperature sensitive. Figure 1 and Table 1 also show a similar temperature sensitivity in the phosphorylation of the 36K protein in cells infected with two other temperature-conditional mutants, *ts*CU11 and *ts*GI251. Cells infected with the fusiform mutant, CU12, showed the same degree of phosphorylation of the 36K protein as did cells infected with the wild-type virus, at both temperatures. A most interesting finding was the apparent low degree of phosphorylation of the 36K protein in cells infected with the CU2 mutant at both 36 and 42°C.

To determine whether there was a qualitative difference in phosphorylation of the 36K protein among cells infected with the mutants, we analyzed the phosphoamino acids in the 36K protein. As has been previously reported for the wild-type virus (9, 16), the 36K protein was phosphorylated on both tyrosine and serine (Fig. 2). The same two amino acids were phosphorylated in the virus mutants. In all cases, phosphotyrosine was by far the predominant phosphoamino acid. Phosphorylation of both amino acids was detectable even in the CU2-infected cells, as well as in cells infected with the various temperature-sensitive mutants grown at the nonpermissive temperature.

Whole-cell phosphotyrosine. The phosphotyrosine content of uninfected and infected chicken embryo firbroblasts was determined (Fig. 3 and Table 1). As has been reported by others, the phosphotyrosine content in normal, uninfected cells was less than 5% that found in cells infected with the wild-type virus SR-A. All of the cells infected with the mutants, with the exception of CU12, contained lower levels of phosphotyrosine than did wild-type virus-infected cells, but even these levels were considerably greater than the levels in normal, uninfected cells. Although the phosphotyrosine content in all of the virus-infected cells (including the wild-type virus) showed some degree of temperature sensitivity, the most pronounced effect was seen with the temperature-conditional mutants *ts*NY68 and *ts*CU11. However, even with these mutants, the phosphotyrosine level at the nonpermissive temperature was significantly higher than the level found in the normal, uninfected cells. These results are remarkably consistent with our previous determinations of the pp60^{src} kinase activity in cells infected with these mutants. These determinations indicated substantial residual pp60^{src} kinase activity in the immune complex

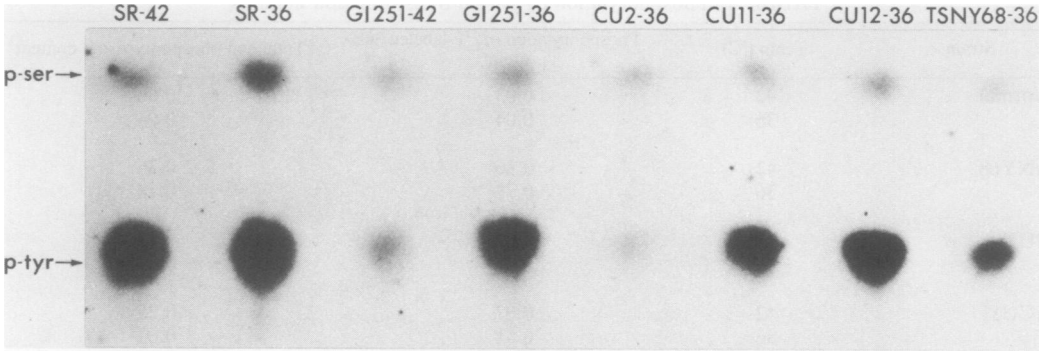

FIG. 2. Phosphoamino acid content of the 36K protein at 42 or 36°C in cells infected with RSV mutants. The 36K protein was excised from a gel, acid hydrolyzed, and electrophoresed at pH 1.9 and then at pH 3.5, as described in the text. p-ser, Phosphoserine; p-tyr, phosphotyrosine. SR, Schmidt-Ruppin cells. Numbers after each mutant represent the temperature (°C).

assay (6) at the nonpermissive temperature (1).

Comparison of the degree of phosphorylation of the 36K protein with the total phosphotyrosine levels revealed that there was less than a perfect correspondence between phosphorylation of the 36K protein and total phosphotyrosine content. Cells infected with *ts*GI251 and held at 42°C and cells infected with CU2 and held at either temperature displayed levels of 36K protein phosphorylation 10% those in the wild-type virus, whereas the total phosphotyrosine content of the cells was approximately 50% that in the wild-type virus. The other mutant-infected cells showed a rough correlation between total phosphotyrosine content and 36K protein phosphorylation, although *ts*NY68-infected cells held at 42°C may have been somewhat "leakier" with respect to total phosphotyrosine than they were for 36K protein phosphorylation.

Correlation with transformation parameters. In the hope of obtaining some insight into the possible function of the 36K protein in malignant transformation, we attempted to correlate phosphorylation with the expression of various manifestations of the transformed phenotype. It is clear from simple inspection of Fig. 1 and Table 1 that phosphorylation of the 36K protein was not sufficient for morphological transformation, because in cells infected with *ts*CU11 and held at 36°C the morphology remained near normal (1), but the 36K protein became reasonably well phosphorylated. Cells infected with *ts*GI251 and held at 36°C became fully transformed morphologically and had a comparable level of 36K protein phosphorylation. Cells infected with CU2 did not become fully rounded but did show substantial morphological changes (notably, surface blebs). These cells displayed a very low level of 36K protein phosphorylation. Thus, the phosphorylation of the 36K protein does not correlate with morphological transformation.

For those parameters of transformation which can be measured quantitatively, it is possible to test the degree of correlation with 36K protein phosphorylation by plotting the extent of 36K protein phosphorylation as a function of the extent of transformation. This was done with a Hewlett-Packard 9825 computer with a curve-fitting program. The best-fit curves, the equations they were fit to, and the correlation coefficients calculated are shown in Fig. 4. We wish to

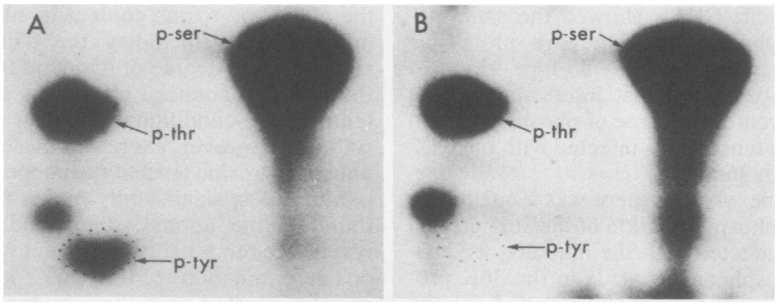

FIG. 3. Phosphoamino acid analysis of whole cells, either transformed by wild-type RSV (A) or uninfected (B). The dotted circle shows the outline of the unlabeled phosphotyrosine (p-tyr) standard, detected with ninhydrin. p-ser, Phosphoserine; p-thr, phosphothreonine.

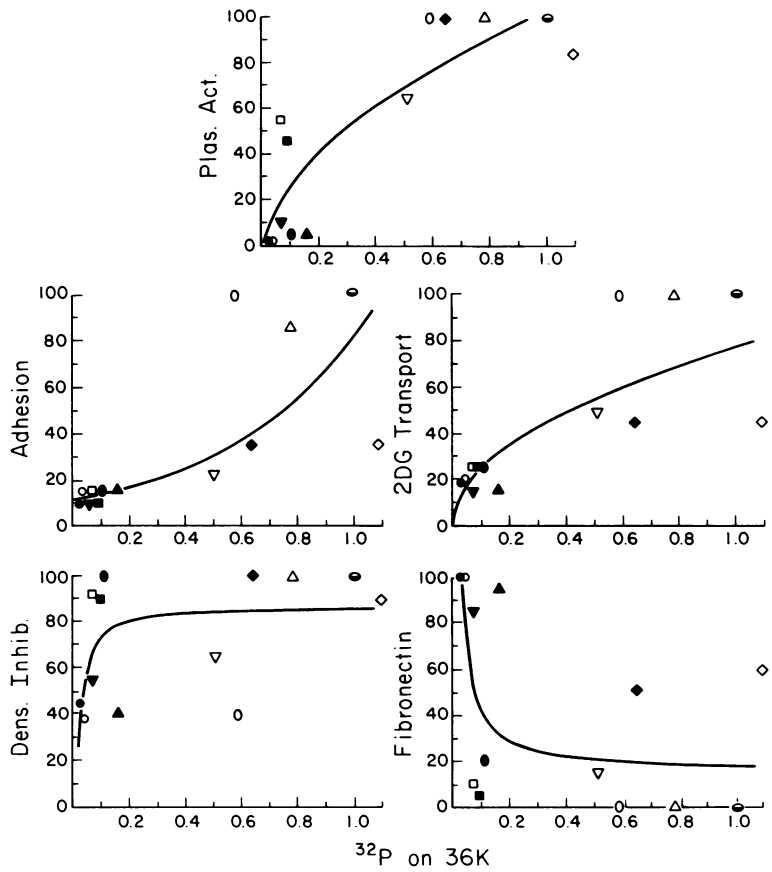

FIG. 4. Relationship between expression of transformation parameters and 36K protein phosphorylation. Data on transformation parameters are taken from previous publications (1, 23). Although the transformation parameters of *ts*GI251 were measured with a growth medium containing only calf serum, whereas all of our other work was performed with media containing calf and chicken sera, we found that 36K protein phosphorylation was not affected by this difference (data not shown). Closed symbols, Cells grown at 42°C; open symbols, cells grown at 36°C. Symbols: ○ and ●, normal cells; △ and ▲, *ts*NY68 cells; □ and ■, CU2 cells; ▽ and ▼, *ts*CU11 cells; ◇ and ◆, CU12 cells; 0 and ⧫, *ts*GI251 cells; ⬭ and ⬬, Schmidt-Ruppin wild-type cells. The equations describing the curves displayed and the calculated correlation coefficients are as follows: plasminogen activator (Plas. Act.), $y = a + b\sqrt{x}$ and $r = 0.89$; adhesion, $y = ae^{bx}$ and $r = 0.86$; 2-deoxyglucose (2DG) transport, $y = ax^b$ and $r = 0.86$; density-dependent inhibition of growth (Dens. Inhib.), $y = a + b/x$ and $r = 0.51$; and fibronectin, $y = a + b/x$ and $r = 0.63$.

emphasize that the presentation of these computer-generated curves is not meant to convey any implications concerning the quantitative relationship between 36K protein phosphorylation and transformation. These curves are meant to be used solely as a means of simplifying inspection of the data and detecting cases in which 36K protein phosphorylation does not correlate with a particular manifestation of transformation. It is clear that there was only a poor correlation between 36K protein phosphorylation and loss of surface fibronectin. In particular, cells infected with CU12 retained substantial amounts of fibronectin but showed good 36K protein phosphorylation, whereas cells infected with CU2, which has very poorly phosphorylated 36K protein, lost essentially all of their surface fibronectin.

The loss of density-dependent growth inhibition also correlated poorly with 36K protein phosphorylation. Cells infected with CU2 grew to very high densities in cell culture (1) even though the 36K protein was barely phosphorylated in these cells. Cells infected with *ts*GI251 grew to high cell densities at 42°C but not at 36°C, whereas phosphorylation in these cells was higher at 36°C than at 42°C.

There was somewhat better correlation between 36K protein phosphorylation and both 2-deoxyglucose uptake and loss of adhesiveness.

However, although cells infected with CU12 displayed heavily phosphorylated 36K protein, they had neither a fully transformed level of glucose transport nor a fully transformed loss of adhesiveness.

The best correlation was between 36K protein phosphorylation and the production of plasminogen activator. Note, however, that in CU2-infected cells, substantial amounts of plasminogen activator were produced.

DISCUSSION

The discovery that pp60^{src} phosphorylates tyrosine residues in proteins provides a way to identify candidate targets of pp60^{src} activity. Proteins which become phosphorylated on tyrosine during transformation could be physiologically significant targets for pp60^{src}, whose phosphorylation plays a role in transformation; substrates of pp60^{src}, which might be phosphorylated nonspecifically or adventitiously and which play no role in transformation; or the products of secondary phosphorylations, catalyzed by tyrosine-specific protein kinases which are activated by pp60^{src}. To determine what role, if any, a phosphotyrosine-containing protein plays in transformation, one must begin to associate the phosphorylation of that protein with the appearance of a biological alteration.

We have made use of partial transformation mutants of RSV in an investigation of the possible function of a 36K protein during transformation. This protein was first reported by Radke and Martin (17) to be rapidly phosphorylated in RSV-transformed cells. Subsequently, it was found that the protein is phosphorylated on tyrosine (9, 16), is a substrate in vitro for purified pp60^{src} (9), and is found (at least in its phosphorylated form) in the insoluble residue of detergent-extracted cells (presumably the cytoskeleton) (5). We have found this protein to be very poorly phosphorylated in cells infected with *ts*GI251 and held at 42°C and in cells infected with CU2 and held at either 36 or 42°C. In both of these cases, the level of 36K protein phosphorylation was 10% that seen in wild-type virus-infected cells, even though the total phosphotyrosine content of these cells was 50% that in wild-type virus-infected cells. Thus, the 36K protein is preferentially underphosphorylated in these cells. This finding provides the first biochemical evidence in support of our suggestion that the biological properties of these mutants could best be explained by a multiple-target model of transformation in which the mutant pp60^{src} proteins are phosphorylating some cellular targets well but phosphorylating others poorly.

We found that phosphorylation of the 36K protein is neither necessary nor sufficient for the loss of surface fibronectin, because cells which were infected with CU2 and which did not possess detectable surface fibronectin displayed very low levels of 36K protein phosphorylation, whereas cells which were infected with CU12 and which had lost only half of their fibronectin had a well-phosphorylated 36K protein. Because the 36K protein has been reported to be associated with the Triton X-100-insoluble framework of the cell (5), a fraction which also contains fibronectin and the cytoskeleton, one might be tempted to speculate that the 36K protein is somehow involved in controlling surface fibronectin expression or in linking it to the cytoskeleton. However, our data argue against this possibility.

Phosphorylation of the 36K protein also is neither necessary nor sufficient for the loss of density-dependent inhibition of growth, because cells which were infected with CU2 grew to very high densities in culture, whereas cells which were infected with *ts*GI251 and which were thermosensitive for 36K protein phosphorylation were cold sensitive for growth at high densities. However, it is important to note that we so far have been unable to determine unambiguously whether the cold-sensitive growth properties of *ts*GI251 are due to the restoration of normal growth at low temperature or to the presence of virus-induced cytoxicity at this temperature.

Phosphorylation of the 36K protein is not sufficient for morphological alterations, because cells infected with *ts*CU11 at 36°C had a morphology similar to that seen with uninfected cells but also had a moderately well-phosphorylated 36K protein. It is possible, however, that phosphorylation of this protein, although not sufficient, may be necessary for the acquisition of a rounded morphology, in conjunction with some other pp60^{src}-induced modification which is defective in *ts*CU11.

Phosphorylation of the 36K protein is also not sufficient for loss of adhesiveness or a full increase in hexose transport, because both of these transformation parameters were expressed at only an intermediate level in CU12-infected cells, even though the 36K protein was well phosphorylated.

Of the transformation parameters measured, phosphorylation of the 36K protein correlated best with the increase in plasminogen activator. However, the correlation here was also not perfect, because cells infected with CU2 had intermediate levels of plasminogen activator, although they had low levels of 36K protein phosphorylation. However, it is possible that expression of this transformation parameter could be extremely sensitive to 36K protein phosphorylation. In any event, it is important to

note that this positive correlation is not nearly as significant as the negative correlations discussed above, because this correlation could be adventitious, and 36K protein phosphorylation could well be causally involved in generating some manifestations of transformation which we have not measured.

The fact that the 36K protein is poorly phosphorylated in cells infected with CU2 is particularly interesting in view of the biological properties of cells infected with this mutant. Cells infected with CU2 are only partially anchorage independent and form "mini-colonies" in soft agar (1). In addition, this mutant is the least tumorigenic of all the mutants tested (P. Kahn, S. Shin, R. Smith, and M. J. Weber, J. Virol., in press). Thus, this mutant extends the well-known correlation between anchorage independence and tumorigenicity and may indicate a relationship between these parameters of transformation and the phosphorylation of the 36K protein.

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