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Proteins antigenically related to the human erythrocyte glucose transporter in normal and Rous sarcoma virus-transformed chicken embryo fibroblasts

(membrane alterations/malignant transformation)

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ABSTRACT **Antibody raised against the purified human erythrocyte glucose transporter specifically precipitated four proteins from normal and Rous sarcoma virus-transformed chicken embryo cells: a major protein of M_r 41,000 and minor proteins of M_r 68,000, 73,000, and 82,000. The M_r 41,000 and 82,000 proteins were found only in a membrane fraction, not in the soluble fraction, and displayed a heterogeneous mobility on NaDodSO$_4$/polyacrylamide gel electrophoresis, suggesting glycosylation. The M_r 41,000 and 82,000 proteins were increased in amount after malignant transformation in direct proportion to the increase in hexose transport rate, and the increase was dependent on the expression of the *src* gene product, as revealed with a temperature-conditional *src* mutant. We suggest that the M_r 41,000 and 82,000 proteins are the glucose transporter of chicken embryo fibroblasts, or a component of the glucose transporter. These experiments provide direct evidence that malignant transformation increases the rate of glucose transport by increasing the number of transporters in the membrane.**

Malignant transformation of chicken embryo cells by Rous sarcoma virus (RSV) causes a striking increase in the rate at which these cells transport glucose across the cell membrane (1–4). Despite much research, little is known about the molecular basis for this alteration. It is clear that the activity of the viral transforming protein pp60^{src} is essential for the increase in glucose transport because viral mutants coding for a temperature-sensitive pp60^{src} are temperature-sensitive in their ability to increase the transport rate (5, 6). Inhibitor data suggest that the increased transport rate requires new protein synthesis but not new RNA synthesis (6, 7). Induction of the increased transport requires a functional nucleus (8). The mechanism by which pp60^{src} causes this increase in transport rate is completely unknown.

In theory, it seems possible that pp60^{src} could act (*i*) to increase the number of normal cellular transporters, (*ii*) to induce the synthesis of a new class of transporters, or (*iii*) to increase the activity of preexisting transporters. The fact that transformation results in an increase in the maximal velocity for transport but does not detectably change the half-saturation constant (2, 3) has been used to argue for the first hypothesis—namely, that transformation causes an increase in the number of normal carriers. However, this argument is a weak one because cases are known in which allosteric regulation causes an increase in the maximal velocity without affecting the half-saturation constant for substrate (9). Recently, Salter and Weber (10) demonstrated that a portion of the binding of cytochalasin B to chicken embryo cells could be stereospecifically inhibited by D-glucose, and this "glucose-specific" cytochalasin B binding was increased in transformed cells which displayed a high hexose transport rate. Because it had been shown that the purified glucose transporter from human erythrocytes bound cytochalasin B (11, 12), this result provided strong, but still indirect, evidence for the suggestion that transformed cells contain increased numbers of glucose transporters in their membranes.

In addition to their cytochalasin binding properties, the chicken embryo cell glucose transporter and the human erythrocyte glucose transporter share the following features: both are facilitated diffusion systems (2, 13) and, to the extent that substrate specificity has been determined, both have similar specificities (2, 14). These similarities suggested to us the possibility that the two proteins might have some antigenic similarities. Since Baldwin and Lienhard (14) recently characterized an antiserum prepared against the purified human erythrocyte transporter, it became possible to determine whether immunologically crossreactive proteins could be found in chicken embryo cell membranes and whether these proteins increased in amount during malignant transformation. While this work was in progress, Sogin and Hinkle (15) reported immunological crossreactivity between the human erythrocyte transporter and proteins of cultured mammalian cells. We have found proteins in chicken embryo cells that appear to be antigenically related to the human erythrocyte glucose transporter. Two of these proteins are probably glycosylated, are found in a crude membrane fraction, and increase in approximately direct proportion to the glucose transport rate upon transformation.

MATERIALS AND METHODS

Virus Strains. The Schmidt–Ruppin strain of RSV subgroup A (SR-RSV) and its temperature-conditional mutant counterpart, tsNY68, were obtained originally from S. Kawai and H. Hanafusa (Rockefeller University) (6).

Sera. The preparation of preimmune serum (about 6 mg of IgG per ml) and antiserum against the human erythrocyte glucose transporter (about 13 mg of IgG per ml) has been described (14). Normal rabbit serum was prepared from an unrelated, unimmunized rabbit.

Other Materials. [^{35}S]Methionine, [^{32}P]phosphate, and EN^{3}HANCE were obtained from New England Nuclear. Protein A-Sepharose was obtained from Sigma. The human erythrocyte glucose transporter was purified in the presence of the detergent octaethylene glycol *n*-dodecyl ether and reconstituted with additional dioleoylphosphatidylcholine as described (16). The preparations contained 118 μg of protein and 3.5 μg of

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Abbreviations: RSV, Rous sarcoma virus; SR, Schmidt–Ruppin strain.

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phospholipid per ml. All other chemicals were of reagent grade or higher quality.

Cells and Cell Culture. Fibroblasts were obtained from 11-day $gs^-/chf^\pm$ chicken embryos (Spafas, Norwich, CT) and cultured as described (2). Secondary cell cultures were infected with SR-RSV (subgroup A) or tsNY68 and maintained at 40°C or 42°C, respectively; uninfected cells were maintained at 40°C. Prior to radiolabeling, tertiary cell cultures were plated at 1.8×10^6, 1.4×10^6, and 1.1×10^6 cells per 100-mm dish (Corning) for SR-RSV, tsNY68, and normal cells, respectively, and incubated at the temperature indicated above. Because of differences in the efficiency of attachment, this provides cell cultures with similar densities. In addition, the use of medium with high glucose and the protocol of medium changes (see below) were sufficient to prevent glucose depletion.

Radiolabeling and Preparation of Cell Fractions. For [^{35}S]methionine labeling, 30 hr after subculture, the medium was changed to medium containing 4% calf serum, 1% heat-inactivated chicken serum, and only 0.01 mM nonradioactive L-methionine plus [^{35}S]methionine (150–300 μCi/100-mm dish; 1 Ci = 3.7×10^{10} becquerels). For [^{32}P]phosphate labeling, the medium was changed to phosphate-free medium containing 4% calf serum, 1% heat-inactivated chicken serum, and isotope at 500 μCi/100-mm dish. One tsNY68-infected culture was then shifted to the permissive temperature (36°C), and the cultures were then incubated for approximately 24 hr. Particulate and soluble fractions were prepared from the radiolabeled cells by the method of Esko *et al.* (17). In a typical cell fractionation, 22% and 27% of the total cell protein was found in the particulate fraction from transformed and normal cells, respectively. Similar values were obtained with tsNY68-infected cells labeled at the permissive or nonpermissive temperature.

The trichloroacetic acid-insoluble [^{35}S]methionine-labeled proteins of the soluble and particulate fractions had approximately the same specific activities, 1×10^5 and 1.2×10^5 cpm/μg of protein, respectively.

Preparation of Extracts. The particulate fraction was extracted with radioimmunoprecipitation assay lysis buffer (18) (50 mM Tris·HCl, pH 7.5/150 mM NaCl/1% Nonidet P-40/1% sodium deoxycholate/0.1% sodium dodecyl sulfate) containing 100 units of Trasylol (FBA Pharmaceuticals) at a protein concentation of approximately 0.18 μg/μl. Samples were incubated for 2 hr at 4°C with intermittent mixing and then centrifuged for 15 min in an Eppendorf Microfuge at 12,500 × *g*; the pellet was discarded. An extract of purified glucose transporter was similarly prepared. In a typical experiment, 44%, 75%, 74%, and 75% of the [^{35}S]methionine-labeled proteins were extracted from the particulate fractions of normal cells, SR-RSV-infected cells, tsNY68-infected cells at 36°C, and tsNY68-infected cells at 42°C, respectively. The apparent low value for normal cells appeared to be due to a larger amount of radiolabeled fibronectin present in the particulate fraction of normal cells in comparison to the other cells. Polyacrylamide gel electrophoresis revealed that little or no fibronectin was solubilized under these conditions but that there was no preferential extraction of any other major proteins between the cell types (data not shown).

Immunoprecipitation. Extracts were incubated with antiserum and the antigen–antibody complexes formed were collected by binding to protein A-Sepharose. In a typical experiment, 125–150 μl of radiolabeled extract, containing 18–22 μg of particulate proteins, was incubated with either 5 or 10 μl of unblocked or blocked antiserum or 10 μl of preimmune serum. Control experiments demonstrated that antiserum was in excess. Blocked antiserum was prepared by preincubating 30 μl of solubilized, purified glucose transporter (1.8 μg of protein and 53 μg of phospholipid) with the antiserum for 15 min prior to the addition of labeled extracts. Protein A-Sepharose was prepared by rehydrating 70 mg in 1 ml of water overnight and resuspending in 1 ml of 0.05% Nonidet P-40/150 mM NaCl/5 mM EDTA/50 mM Tris, pH 7.5. An excess (50 μl) of this slurry was then added to collect the antigen–antibody complexes. Incubation was continued for 1 hr at 4°C with intermittent mixing. The protein A-Sepharose–antibody–antigen complex was centrifuged for 2 min in the Microfuge, and the pellet was washed seven times with 600 μl of lysis buffer and once with 10 mM Tris·HCl (pH 7.5) at 4°C. Samples were resuspended and then boiled to dissociate the antigen–antibody complex.

Samples were then electrophoresed (19) and, for [^{35}S]methionine-labeled extracts, fluorographed with EN^{3}HANCE (20). Under these conditions (with preflashed film), the amount of radioactivity in a band is approximately proportional to band intensity. Thus, quantification was performed by scanning autoradiograms with an Ortec model 4310 scanning densitometer. Electropherograms of [^{32}P]phosphate-labeled extracts were autoradiographed with an intensifying screen (Dupont Lightning Plus) and exposure at −70°C for 5 days.

RESULTS

Crossreactive Proteins in Normal and Transformed Cells. Fig. 1 shows results obtained when the [^{35}S]methionine-labeled particulate fraction from transformed and normal chicken fibroblasts was extracted with lysis buffer and the extract was immunoprecipitated with antiserum prepared against the purified glucose transporter from human erythrocytes. In both transformed and normal cells, there were three proteins—M_r

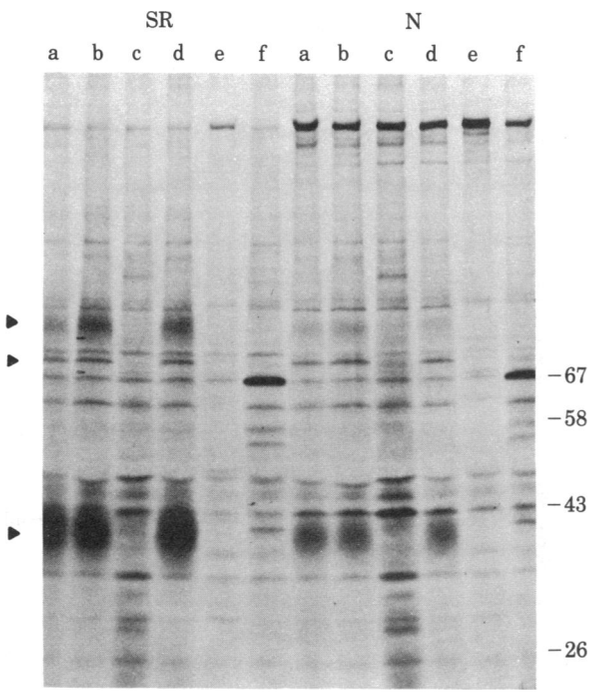

FIG. 1. Gel electropherogram of immunoprecipitates of particulate fractions. SR, transformed cells; N, normal cells. Lanes: a, immune serum, 5 μl; b, immune serum, 10 μl; c, immune serum, 10 μl, plus purified erythrocyte glucose transporter; d, immune serum, 10 μl, plus bovine serum albumin; e, normal rabbit serum, 10 μl; f, preimmune rabbit serum, 10 μl. The large number of nonspecific bands is often obtained in immunoprecipitates from the particulate fraction. M_r of standards are shown $\times 10^{-3}$.

41,000, 73,000, and 82,000—that were immunoprecipitated by the immune serum (lanes a, b, and d) but not by normal rabbit serum (lane e) or preimmune serum (lane f). In addition, the immunoprecipitation of these three bands was blocked by purified glucose transporter plus phospholipid (lane c) but not by an equivalent amount of bovine serum albumin and phospholipid (lane d). Two of the proteins (M_r 41,000 and 82,000) ran as broad bands in the gels; this behavior indicates heterogeneity, which in other cases has been found to be due to heterogeneous glycosylation (21, 22). The M_r 73,000 protein electrophoresed as a sharper band. Visual inspection of the chromatogram revealed that the M_r 41,000 and 82,000 bands were in much higher amount in transformed cells than in normal cells (compare lanes a, b, and d within each cell type).

Cells Infected with a Temperature-Conditional *src* Mutant. Infection of chicken embryo cells by RSV results not only in malignant transformation but also in virus production. It is thus conceivable that the data shown in Fig. 1, which indicate a virus-induced increase in the production of proteins antigenically related to the human erythrocyte glucose transporter, are the consequence of virus replication rather than transformation. To distinguish between these possibilities, we made use of tsNY68, a mutant coding for a temperature-sensitive $pp60^{src}$. Cells infected with tsNY68 produce high titers of infectious virus at both 36°C and at 42°C but display the transformed phenotype only at 36°C (the permissive temperature) (6).

Fig. 2 demonstrates that the increased levels of crossreactive proteins depend on the activity of $pp60^{src}$. As in Fig. 1, proteins of M_r 41,000, 73,000, and 82,000 were immunoprecipitated by immune serum (lanes a, b, and d) but not by normal rabbit serum (lane e) or preimmune serum (lane f). Immunoprecipitation of these three bands was blocked when purified glucose

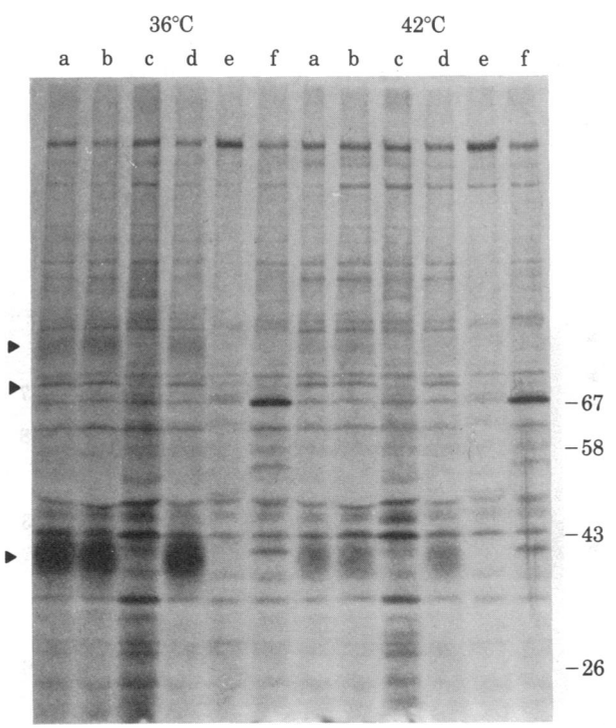

FIG. 2. Gel electropherogram of immunoprecipitates of particulate fractions from cells infected with the temperature-conditional mutant tsNY68 and held at either the permissive (36°C) or nonpermissive (42°C) temperature. Lanes: a, immune serum, 5 μl; b, immune serum, 10 μl; c, immune serum, 10 μl, plus purified erythrocyte transporter; d, immune serum, 10 μl, plus bovine serum albumin; e, normal rabbit serum, 10 μl; f, preimmune serum, 10 μl.

transporter and phospholipid were included in the reaction mixture (lane c); an equivalent amount of bovine serum albumin and phospholipid had little effect on the immunoprecipitation of these three proteins (lane d). Visual inspection reveals increased amounts of the crossreactive proteins in cells grown at 36°C (the permissive temperature) compared to cells grown at 42°C (the restrictive temperature).

Quantitation of Crossreactive Proteins. Table 1 summarizes the results of the quantitation of the amount of each of these three bands in the four different cell types. The data have been normalized to the value found in normal cells. The amounts of the M_r 41,000 and 82,000 proteins were significantly increased in cells transformed by the wild-type SR-RSV and in tsNY68-infected cells labeled at the permissive temperature; tsNY68-infected cells labeled at the nonpermissive temperature had the amount of these two protein bands characteristic of normal, uninfected cells. The M_r 73,000 protein band appeared to be only slightly increased in the transformed cells; the significance of this difference is not known.

The transformation-specific difference in the levels of the M_r 41,000 and 82,000 proteins was comparable to the differences in transport rate generally shown by these cell types when the cells were cultured under identical conditions. The uptake of 2-deoxyglucose (nmol/mg of protein per 15 min) was: normal cells, 11; transformed cells, 32; tsNY68-infected cells, 5 and 25 at 42°C and 36°C, respectively.

Crossreactive Proteins in the Soluble Fraction. We also examined the soluble protein fraction for proteins specifically precipitated by the immune serum (Fig. 3). The M_r 41,000, 73,000, and 82,000 proteins were not specifically immunoprecipitated from the soluble portion of the lysed cells. However, small amounts of a protein of M_r ≈65,000 were precipitated by the immune serum, blocked by purified glucose transporter, and not precipitated with either preimmune serum or normal rabbit serum. It appeared that the amount of this protein was not greatly increased in transformed cells and it was present in much lesser amount than the three protein bands associated with the particulate fraction.

It is noteworthy that the preimmune serum precipitated several chicken proteins not precipitated by normal rabbit serum (serum from an unrelated, unimmunized rabbit) (compare lanes e and f in Figs. 1 and 2). In particular, a protein of M_r 68,000 was specifically precipitated by the preimmune serum. Immune serum from the same rabbit did not precipitate this protein as strongly as did the preimmune serum at the same or higher IgG concentration (lanes a and b, Figs. 1 and 2). This protein band was not blocked with erythrocyte membrane extracts (data not shown) and thus appears to be antigenically unrelated to the glucose transporter. The amount of this protein did not change with transformation. A protein with a similar M_r was precipitated from the soluble protein fraction by preimmune serum (Fig. 3). These surprising data may be a consequence of serendipitous crossreaction between certain chicken cell proteins and the natural antibodies of the rabbit used for

Table 1. Relative amounts of proteins immunoprecipitated by anti-erythrocyte glucose transporter

Protein band, M_r	Ratio: transformed/ normal	tsNY68-infected cells	
		42°C	36°C
41,000	3.7 ± 1.0	0.8 ± 0.1	2.7 ± 1.0
73,000	1.5 ± 0.7	0.8 ± 0.3	1.4 ± 0.7
82,000	5.6 ± 2.5	0.9 ± 0.2	3.8 ± 1.5

Results are shown as mean ± SD of five independent determinations on the same extract.

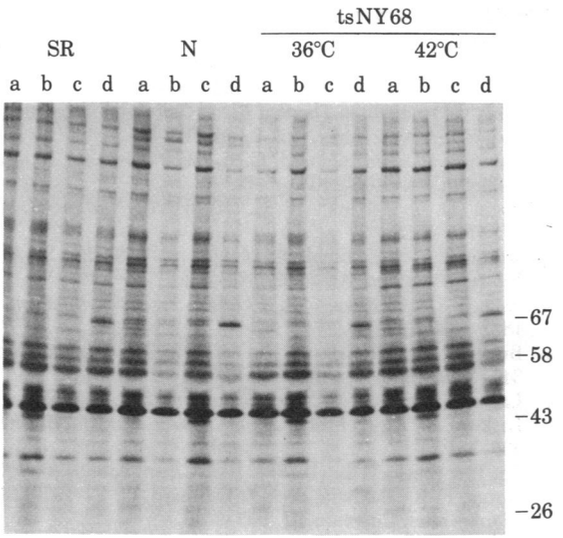

FIG. 3. Gel electropherogram of immunoprecipitates of soluble fractions from normal and transformed cells. SR, wild-type transformed cells; N, normal cells; tsNY68, infected cells at either permissive (36°C) or restrictive (42°C) temperature. Lanes: a, immune serum, 10 μl; b, immune serum plus purified erythrocyte transporter; c, preimmune serum; d, normal rabbit serum.

immunization. We recently prepared another antiserum against a different preparation of the human erythrocyte transporter. This antiserum also precipitated proteins of M_r ≈41,000 and ≈82,000 but the preimmune serum from this rabbit did not precipitate the M_r 68,000 protein.

Crossreactive Proteins Are Not Phosphorylated. Because pp60^{src} is a protein kinase (23, 24), we wished to investigate the possible phosphorylation of the three particulate proteins specifically precipitated by antiserum against the human erythrocyte transporter. ^{32}P-Labeled particulate fractions from normal and transformed cells were extracted with lysis buffer and precipitated with immune serum. No additional phosphorylated proteins were immunoprecipitated with immune serum when compared to preimmune serum under conditions such that the phosphorylated pp60^{src} protein could easily be detected (25) (data not shown).

DISCUSSION

We have used antisera raised against the purified human erythrocyte transporter to detect immunologically crossreactive proteins in normal and transformed chicken embryo fibroblasts. When we began these experiments, we were concerned that, even though the human and the chicken transporter were functionally similar, the evolutionary distance between the chicken and the human was so great that we would not detect immunological crossreaction. Our results presented the opposite problem: not one but four immunologically crossreactive bands. Because one protein has an apparent M_r double that of the M_r 41,000 protein and because both are coregulated, found in crude membranes, and appear to be glycosylated, we suspect that the M_r 82,000 protein is a dimer of the M_r 41,000 protein. About 20% of the protein in preparations of purified erythrocyte glucose transporter [which has a M_r of about 45,000 for the monomer (11, 22)] often runs in the dimer region upon NaDodSO$_4$/polyacrylamide gel electrophoresis (ref. 11; unpublished data). The M_r 65,000 protein found in the soluble fraction of the cell, and the M_r 73,000 protein found with the membrane fraction could be precursors to, proteolytic fragments of, or unrelated to the M_r 41,000 and 82,000 proteins. The fact that the amount of these proteins appears not to vary greatly with transformation argues (albeit, weakly) against the first two possibilities.

We believe that the M_r 41,000 and 82,000 protein bands represent the chicken fibroblast glucose transporter or a component of the transporter, for the following reasons. (*a*) These proteins could be precipitated by antisera raised against the human erythrocyte transporter but not by preimmune or normal sera. (*b*) The immunoprecipitation was blocked by purified erythrocyte transporter or by human erythrocyte ghosts; the precipitation was not blocked by bovine erythrocyte ghosts, which do not possess a cytochalasin-sensitive glucose transport system (unpublished data). (*c*) The proteins were found in the membrane fraction of the cells and appeared to be glycosylated. (*d*) The amount of M_r 41,000 and 82,000 proteins varied upon transformation in direct proportion to the hexose transport rate of the cells. (*e*) The amount of these proteins is comparable to the amount of glucose-specific cytochalasin B binding displayed by these cells (see below).

The following calculation indicates that the M_r 41,000 and 82,000 proteins are present in transformed cell membranes in amounts consistent with their being the glucose transporter. We previously found (10) that, when cytochalasin B was incubated with the particulate fraction from transformed cells at a concentration near its apparent dissociation constant, 31.6 pmol of cytochalasin B was bound per mg of protein to a glucose-inhibitable site. This value converts to approximately 60 pmol of "glucose-specific" cytochalasin B sites per mg of protein at saturation. In the experiments reported here, we obtained approximately 1800 cpm in the M_r 41,000 plus M_r 82,000 bands from the particulate fraction of transformed cells. The specific activity of the total cellular proteins was 81,500 cpm/μg of protein in these experiments, which corresponds to 0.02 μg of protein in the putative transporters. Because 18.5 μg of protein was used in the immunoprecipitation, there were approximately 1.1 μg of the M_r 41,000 and 82,000 proteins per mg of total protein in the particulate fraction. Taking 41,000 as the monomer M_r and assuming a methionine content in the transporter equivalent to that found in the total cell protein, we calculate that there were 27 pmol of transporter per mg of protein in the particulate fraction. Considering the assumptions involved in these calculations, and the possibilities for experimental error, we think that this value is remarkably close to the value, 60 pmol/mg, obtained from the cytochalasin B measurements.

Although we believe that our antisera recognize the glucose transporter of chicken embryo cells, we have been unsuccessful in attempts to immunoprecipitate D-glucose-inhibitable cytochalasin B binding activity from an octyl glucoside extract of transformed cells. We suspect that this is due to inefficient precipitation of the crossreactive proteins from the detergent extract. Nonetheless, these negative results point out both the difficulty and the importance of determining unambiguously the functional identity of the crossreactive proteins.

The transformation-related increase in the M_r 41,000 and 82,000 proteins is dependent on the activity of pp60^{src} because cells infected with a temperature-conditional mutant of RSV displayed the increase only at the permissive temperature. Because pp60^{src} has been shown to be a protein kinase, it was of interest to determine whether the putative transporters were phosphorylated. We were unable to detect phosphorylation of any of the proteins specifically precipitated by our antiserum, under conditions in which we can readily detect phosphorylation of pp60^{src} itself (25). Because the transporter and pp60^{src} are present in cells in similar amounts (see above and ref. 26) we think that this negative result is significant. Thus, it seems unlikely that the glucose transporter is a primary target of pp60^{src}.

Our data strongly suggest that the transformation-related increase in glucose transport rate is due to an increased number of normal cellular transporters. Because the M_r of the putative transporter is the same in normal and transformed cells and because our previous studies have not revealed changes in transport kinetics, specificity, or sensitivity to inhibitors (2, 10), neither a new transport system (27) nor allosteric effects are likely to play major roles in increasing the glucose transport rate in transformed cells.

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