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The *src* Oncogene Can Regulate a Human Glucose Transporter Expressed in Chicken Embryo Fibroblasts

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When fibroblasts are transformed by the *src* oncogene, there is a two- to fivefold increase in glucose transport and in the level of immunoprecipitable glucose transporter protein. In chicken embryo fibroblasts (CEFs), this increase is correlated with a comparable reduction in the rate at which the glucose transporter protein is turned over. In contrast, in mammalian fibroblasts glucose transporter biosynthesis is increased by *src*, but there is little or no change in its turnover. To further understand the action of *src* on transporter turnover, we investigated whether a mammalian transporter can be stabilized by *src* in a chicken cell environment. The human type 1 glucose transporter protein (hGT), originally cloned from HepG2 cells, was expressed in CEFs or Rat-1 fibroblasts by using a retroviral vector. In CEFs transformed by a temperature-sensitive *src* mutant, *ts*NY68, turnover of hGT was lower at the permissive temperature (36°C) than at the nonpermissive temperature (42°C). When this protein was expressed in CEFs transformed by wild-type *src*, no difference in turnover was observed at the two temperatures. In the case of Rat-1 cells transformed by the temperature-sensitive *src* mutant *ts*LA29, turnover of hGT was the same at the permissive temperature (35°C) as at the nonpermissive temperature (39.5°C). These data demonstrate that a heterologous glucose transporter behaves in the same way in chicken and rat cells as the respective endogenous transporter, i.e., when *src* is active, the protein is stabilized against turnover in chicken cells but not in rat cells.

Glucose transport into mammalian cells is mediated by a family of proteins that differ in tissue distribution and are encoded by discrete genes. There are at least four different types of glucose transporter that have been cloned from cDNA libraries. Type 1 transporters include the HepG2/erythrocyte transporter (human glucose transporter [hGT]) (17), which was used in this study, and the rat brain transporter (4), which appears to be the rat equivalent and is 98% identical at the amino acid level. This is the type of transporter that is expressed in rodent fibroblasts (5, 9, 23). The second type is that found in the liver, which has been cloned from rat (21) and human (11) cells, the third type has been cloned from human fetal skeletal tissue (15), and the fourth is found exclusively in tissues that show an insulin sensitivity in glucose transport and has been named the insulin-regulatable glucose transporter (3, 6, 10, 13, 14).

Both chicken embryo fibroblasts (CEFs) and mammalian fibroblasts show an increase in their rates of glucose transport when they are transformed by the *src* oncogene; this increase is due to an increase in the amount of glucose transporter protein (18–20). However, we have previously shown that CEFs differ from rodent fibroblasts in the mechanism by which this increase is induced (23). In CEFs, *src* reduces the rate at which the glucose transporter protein is degraded (20, 23), whereas in rat cells it is the rate of biosynthesis that is regulated, with little or no effect on degradation (23). Regulation of biosynthesis was also observed in primary rat embryo fibroblast cultures, and thus the difference in regulation is not a function of cell immortality (23). Also, we have observed control of transporter degradation in CEFs transformed by either *ts*NY68 or *ts*LA29 and in *ts*NY68-transformed CEFs labeled in normal Dulbecco modified Eagle medium, showing that this difference is not dependent on the type of temperature-sensitive *src* mutant used, nor is it due to some effect of the low level of methionine that is routinely used in cell-labeling experiments (M. K. White and M. J. Weber, unpublished data).

Recently, the molecular basis of the induction of rodent cell glucose transporter biosynthesis by tyrosine kinase oncogenes has been clarified: the level of transporter mRNA is elevated in response to *src* (9, 23) or *fps* (5) transformation, and at least in the case of *fps*, the rate of transporter gene transcription is enhanced (5, 25). A promoter region has been identified (in genomic clones of the rat glucose transporter gene that have been characterized and sequenced) from which transcription is increased after *fps* transformation or serum stimulation of rat fibroblasts (25), and it seems likely that *src* also acts at this site.

In contrast, the mechanism by which *src* regulates transporter turnover in CEFs is not known. There are two possible but not exclusive reasons why the effect is observed in chicken cells but not in rat cells. (i) There is a difference in the regulatory environment of the chicken cell as compared with the mammalian cell. For example, chicken cellular factors that mediate the interaction of $pp60^{v\text{-}src}$ with the transporter may be absent or different in mammalian cells. (ii) There is a difference in the chicken glucose transporter itself that allows it to be regulated at the level of turnover. For example, CEFs may express a different type of transporter that contains a domain not found in the isoform expressed in mammalian cells, and this domain might mediate its interaction with proteins that regulate its turnover or with $pp60^{v\text{-}src}$ or with both.

To distinguish between these possibilities, hGT was expressed in chicken and rat cells. In CEFs transformed by a temperature-sensitive *src* mutant, hGT behaved in the same way as endogenous chick transporters; i.e., it was stabilized at the permissive temperature. In control experiments, no effect of temperature on total protein turnover was seen, nor was there any effect of temperature on hGT turnover in

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CEFs transformed by wild-type *src*. By contrast, in rat fibroblasts transformed by a temperature-sensitive *src* mutant, hGT turnover was not affected by temperature.

MATERIALS AND METHODS

Cells and cell culture. Experiments on chicken and rat embryo fibroblasts were performed with passage 3 or 4 cells prepared by standard techniques. Rat-1 cells were obtained from John Wyke (Imperial Cancer Research Fund, London, United Kingdom). Cells were grown in high-glucose Dulbecco modified Eagle medium (GIBCO Laboratories, Grand Island, N.Y.) plus 10% fetal calf serum.

CEFs transformed by the Schmidt-Ruppin strain of Rous sarcoma virus (RSV) or with the temperature-conditional derivative of this virus, *ts*NY68 (designated *ts*NY68-CEFs), and Rat-1 cells carrying the temperature-conditional *src*, *ts*LA29 (designated Rat-1/*ts*LA29 cells), have been described in an earlier paper (23). All cultures were subconfluent or just confluent at the time of experimentation.

To label cells with [^{35}S]methionine, the culture medium was aspirated and replaced with methionine-free medium plus 150 μCi of [^{35}S]methionine (Dupont, NEN Research Products, Boston, Mass.) per ml supplemented with 10% fetal calf serum (which also supplies nonradioactive methionine to a level roughly equivalent to 5 to 10% of the normal growth medium).

Immunoprecipitation. Labeled cultures of cells were lysed in ice-cold RIPA buffer (1% sodium deoxycholate, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate, 0.15 M NaCl, 50 mM Tris [pH 7.2]) and stored at −20°C. Lysates were cleared by centrifugation at 100,000 × *g* for 30 min and then further clarified by incubating the supernatant with Pansorbin (Calbiochem-Behring, La Jolla, Calif.) for 30 min, followed by centrifugation at 12,000 × *g* in an Eppendorf microfuge for 1 min.

The supernatant was incubated for 30 min with excess monoclonal antibody (MAb) G3, which is species specific for the HepG2/erythrocyte type of hGT (1). This antibody was obtained from Gustav Lienhard (Dartmouth Medical School, Hanover, N.H.). Excess Pansorbin was then added, followed by a second 30-min incubation. The immune complexes were collected by microcentrifugation (12,000 × *g*, 1 min) and washed three times with RIPA buffer containing 1 M NaCl and then three times with RIPA buffer.

The immune complexes were released from the Pansorbin by boiling for 5 min in 2× Laemmli sample buffer without 2-mercaptoethanol. After the Pansorbin was centrifuged out, the samples were electrophoresed on a sodium dodecyl sulfate-polyacrylamide gel. 2-Mercaptoethanol was omitted, since unreduced immunoglobulin G runs near the top of the gel and thus does not interfere with the transporter band.

After immunoprecipitation of the hGT from cell lysates under these conditions, there remained no hGT in the lysate supernatants that could be immunoprecipitated by addition of more MAb G3. These supernatants could therefore be used to quantitate the amount of endogenous glucose transporter by immunoprecipitation with polyclonal anti-glucose transporter antiserum as we have previously described (M. K. White and M. J. Weber, J. Cell Biol. **107**:486a, 1988).

Introduction of the HepG2 glucose transporter into chicken cells. Plasmid pGTDOL contains the HepG2 glucose transporter cDNA insert cloned into the *Bam*HI site of the retroviral expression vector pDOL (12). Transcription of the inserted cDNA is driven by the left-hand long terminal repeat derived from Moloney leukemia virus (MLV). Downstream of the cDNA insert is the neomycin resistance gene, the transcription of which is driven by the simian virus 40 promoter. Transcription is terminated at the right-hand MLV long terminal repeat. This plasmid was transfected into the psi-CRIP cell line (obtained from Ricardo Martinez, Whitehead Institute, Cambridge, Mass.) by calcium phosphate-mediated transfection, and transfectants were selected and cloned in G418 (750 μg/ml). The psi-CRIP cell line is an NIH 3T3 derivative containing two defective plasmids, one producing MLV *gag* protein and the other producing MLV *env* protein, allowing pGTDOL to be packaged into amphotropic virions (8).

Virus was harvested from the transfected clones by incubating the cells overnight in Dulbecco modified Eagle medium and removing and filtering the medium through a 0.22-μm-pore-size filter. The virus was added together with Polybrene (2 μg/ml) to CEFs that had previously been transformed with either *ts*NY68 RSV or wild-type RSV. Confluent cells were split 1:3 immediately before infection and 2 days later were split 1:3 into medium containing 200 μg of G418 per ml.

G418-resistant cells were grown up and tested for expression of hGT by immunoprecipitation with MAb G3, which is species specific for the human HepG2/erythrocyte type of transporter. *ts*NY68-CEFs were also tested for temperature sensitivity of the transformed phenotype by examination of cellular morphology and measurement of 2-deoxyglucose uptake rate. hGT was introduced into Rat-1/*ts*LA29 cells by the same method except that 500 μg of G418 per ml was used for selection. The cultures investigated resulted from mass infections and were not clonal.

TABLE 1. [^{3}H]2-deoxyglucose uptake rates

Cell type and treatment	Relative rate[a]
*ts*NY68-CEF	
41°C	1
41°C + hGT	1.4
35°C	2.6
35°C + hGT	3.0
Rat-1/*ts*LA29	
39.5°C	1
39.5°C + hGT	2.2
35°C	2.0
35°C + hGT	2.9

[a] Normalized to the basal, untransformed level (not expressing exogenous glucose transporter). Average of duplicate samples, with less than 15% error.

RESULTS

hGT was introduced into *ts*NY68-CEFs by infection with an amphotropic murine retroviral vector containing the neomycin resistance gene and the HepG2 cDNA clone. The former was expressed from the simian virus 40 promoter, and the latter was expressed from the MLV long terminal repeat. The infected cells were resistant to G418 (200 to 400 μg/ml), exhibited a 1.5- to 2-fold-higher rate of 2-deoxyglucose uptake than uninfected control cells, and expressed immunoprecipitable hGT. Thus, the murine promoters of the retroviral vector function adequately in these avian cells. Also, the infected cells retained their temperature sensitivity for morphology and 2-deoxyglucose uptake (Table 1).

Figure 1 shows the turnover of hGT in these cells. The cells were labeled with [^{35}S]methionine at the permissive temperature (36°C) and then chased with cold methionine at 36°C or at the restrictive temperature (42°C), and hGT was

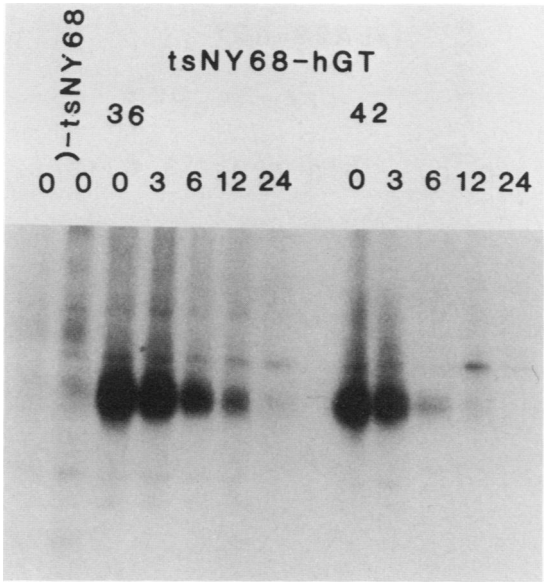

FIG. 1. Turnover of hGT in *ts*NY68-CEFs. *ts*NY68-CEFs expressing hGT were grown for 12 h at 36°C in labeling medium containing [^{35}S]methionine, and fluid was changed at time zero to chase medium containing cold methionine. At time zero, half of the cultures were shifted to 42°C. Cells were lysed at 0, 3, 6, 12, and 24 h, as indicated, and hGT was immunoprecipitated with MAb G3. The first two lanes are immunoprecipitations from lysates of regular *ts*NY68-CEFs not expressing hGT, which were labeled and lysed in the same way and used as controls for antibody specificity.

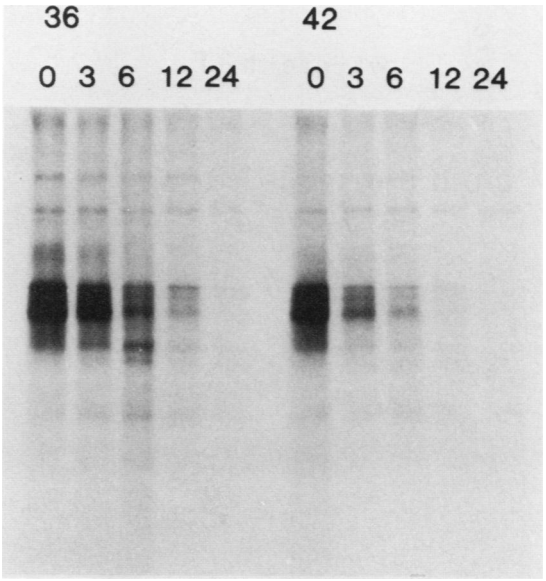

FIG. 2. Turnover of endogenous transporter in *ts*NY68-CEFs expressing hGT. After immunoprecipitation of hGT from *ts*NY68-CEF lysates as described in the legend to Fig. 1, the supernatants were immunoprecipitated with a rabbit polyclonal anti-human erythrocyte glucose transporter antiserum, which allows quantitation of the endogenous chicken glucose transporter.

immunoprecipitated with MAb G3. It is clear that hGT was turned over faster at the restrictive temperature. When the logarithms of the intensities of the hGT bands, measured densitometrically, were plotted against time, approximate half-lives of 4 and 8 h were obtained at 42 and 36°C, respectively, a difference sufficient to account for the observed differences in transport (Table 1). The first two lanes of Fig. 1 show MAb G3 immunoprecipitates from parallel labeled cultures of *ts*NY68-CEFs that were not infected with the hGT vector; it is clear that MAb G3 did not recognize the endogenous CEF glucose transporter. Similar temperature dependence of the hGT in CEFs was found not only for the cultures arising from mass infection (Fig. 1) but also from a culture expanded from a single neomycin-resistant cell clone (data not shown).

The supernatants of lysates of *ts*NY68-CEFs expressing hGT were cleared of all hGT by immunoprecipitating with MAb G3 at antibody excess. Thus, the supernatants could be used to quantitate the endogenous CEF glucose transporter by immunoprecipitation with a polyclonal anti-glucose transporter antiserum (White and Weber, J. Cell Biol. **107**:486a, 1988). The turnover of endogenous glucose transporters in these cells was temperature sensitive, as was the case for regular *ts*NY68-CEFs (20, 23) (Fig. 2). The rate of turnover of total cell protein was not obviously altered by transformation in these cells (Fig. 3) as we have previously reported for regular *ts*NY68-CEFs (20). The exposure times for autoradiography of gels of human and endogenous transporter immunoprecipitations from the same lysates were similar, indicating roughly comparable expression of the two proteins.

The hGT protein was also introduced by the same method into CEFs transformed by wild-type RSV. Turnover of hGT in these cells at 36 and 42°C is shown in Fig. 4. Turnover was the same at both temperatures and had an approximate half-life of 8 h. Total protein turnover was also not affected by temperature (data not shown).

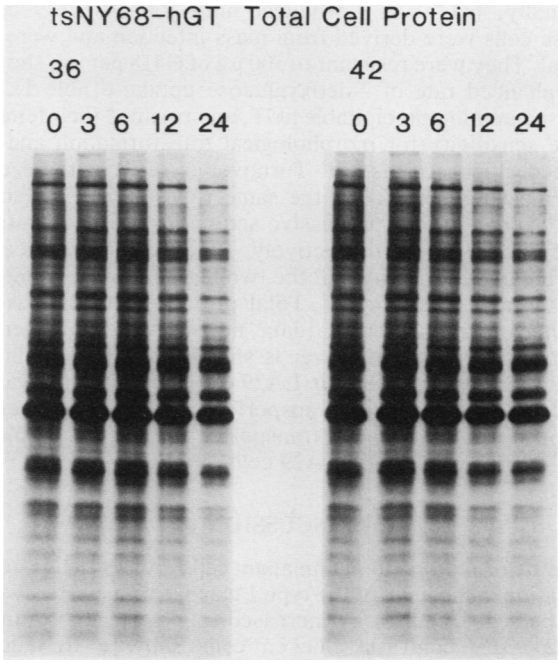

FIG. 3. Turnover of total cell protein in *ts*NY68-CEFs expressing hGT. Lysates from [^{35}S]methionine-labeled *ts*NY68-CEFs were prepared as described in the legend to Fig. 1. After clearing of the lysates by high-speed centrifugation (see Materials and Methods), small volumes were added to Laemmli sample buffer and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

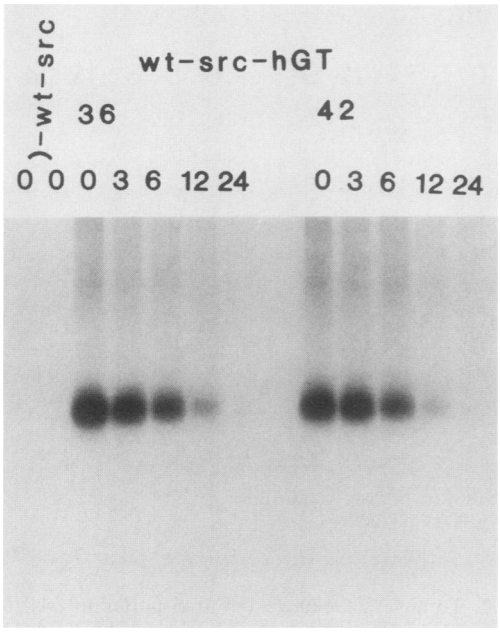

FIG. 4. Turnover of hGT in wild-type RSV-transformed CEFs. CEFs transformed by wild-type (wt) *src* and expressing hGT were grown for 12 h at 36°C in labeling medium containing [^{35}S]methionine, and fluid was changed at time zero to chase medium containing cold methionine. At time zero, half of the cultures were shifted to 42°C. Cells were lysed at 0, 3, 6, 12, and 24 h, as indicated, and hGT was immunoprecipitated with MAb G3. The first two lanes are immunoprecipitations from lysates of uninfected wild-type *src*-transformed CEFs not expressing hGT, which were labeled and lysed in the same way and used as controls for antibody specificity.

Finally, hGT was introduced into Rat-1/*ts*LA29 cells. These cells were derived from mass infection and were not clonal. They were resistant to 500 μg of G418 per ml, showed an enhanced rate of 2-deoxyglucose uptake (Table 1), expressed immunoprecipitable hGT, and retained their temperature sensitivity for morphological transformation and enhanced glucose transport. Turnover of hGT in these cells was measured in exactly the same way as for the chicken cells except that the permissive and restrictive temperatures were 35 and 39.5°C, respectively. No difference was seen in the rate of hGT turnover at the two temperatures, being 6 to 7 h in each case (Fig. 5). Total protein turnover was not affected by temperature (data not shown). Endogenous glucose transporter turnover is shown in Fig. 6. As in the case of the parental Rat-1/*ts*LA29 cell line (23) there was no effect of temperature on transporter turnover. The levels of endogenous and human transporter were comparable in hGT-expressing Rat-1/*ts*LA29 cells.

DISCUSSION

Transformation of mammalian cells by *src* leads to an elevation in the level of the type 1 glucose transporter, which can be accounted for by increased transporter biosynthesis (9, 23). By contrast, chicken cells show a *src*-induced elevation of immunoprecipitable glucose transport protein associated with decreased turnover of the protein (20, 23). In an earlier paper, we compared chicken and rodent fibroblasts with respect to the effect of *src* transformation on glucose transporter mRNA level, glucose transporter protein biosynthesis, and glucose transporter protein turnover.

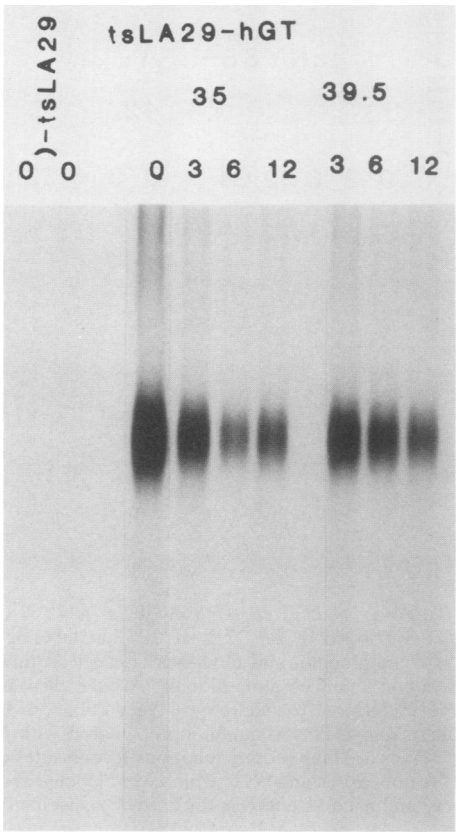

FIG. 5. Turnover of hGT in Rat-1/*ts*LA29 cells. Rat-1/*ts*LA29 cells were grown for 12 h at 35°C in labeling medium containing [^{35}S]methionine, and fluid was changed at time zero to chase medium containing cold methionine. At time zero, half of the cultures were shifted to 39.5°C. Cells were lysed at 0, 3, 6, 12, and 24 h, as indicated, and hGT was immunoprecipitated with MAb G3. The first two lanes are immunoprecipitations from lysates of uninfected Rat-1/*ts*LA29 cells not expressing hGT, which were labeled and lysed in the same way and used as controls for antibody specificity.

These data clearly demonstrated that there was indeed a species difference in the mechanism of transporter elevation (23).

The mechanism of *src*-mediated induction of transporter

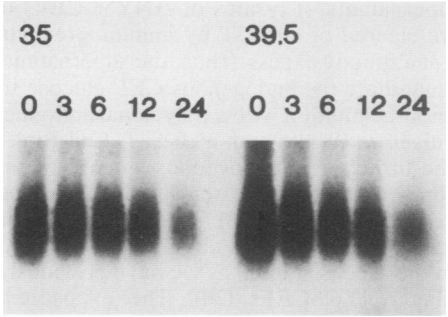

FIG. 6. Turnover of endogenous transporter in Rat-1/*ts*LA29 cells expressing hGT. After immunoprecipitation of hGT from Rat-1/*ts*LA29 lysates as described in the legend to Fig. 1, the supernatants were immunoprecipitated with a rabbit polyclonal anti-human erythrocyte glucose transporter antiserum, which allows quantitation of the endogenous rat glucose transporter.

biosynthesis in rat cells appears to be at the level of initiation of transcription (as was discussed in the introduction), but the mechanism whereby *src* stabilizes the glucose transporter in chicken cells is unknown. Here we report that the introduction of a mammalian type 1 glucose transporter (hGT) into chicken cells allows it to be regulated in the same manner as the endogenous transporter: in chicken cells, hGT is stabilized when *src* is active.

The stabilization of hGT by *src* contrasts with findings for the rat glucose transporter, which is not significantly stabilized by *src* in Rat-1/*ts*LA29 cells (23). This is unlikely to be due to a difference between the rat and human transporters for the following reasons: (i) the predicted amino acid sequences of the two transporters are 98% identical (4, 17), and (ii) the hGT is also not stabilized by *src* when it is introduced into the same Rat-1/*ts*LA29 cells (Fig. 5).

Thus, these data indicate that *src* can stabilize hGT in chicken cells but not in rat cells, and hence the ability of *src* to stabilize glucose transporters is a feature of the chicken cell environment. Therefore, we conclude that one or more factors present in CEFs but not Rat-1 cells allow $pp60^{v\text{-}src}$ to interact with glucose transporters. What might this factor(s) be?

We are attracted to the hypothesis that it is the endogenous chicken glucose transporter itself. In this model, the endogenous transporter would have a signal sequence for *src* stabilization and would also be able to interact with heterologous transporters. The heterologous transporter, hGT, would then be affected by *src* via a piggyback mechanism.

This model is attractive for a number of reasons. (i) The model implies that the glucose transporter exists as a dimer or larger complex. There is other biochemical and physical evidence that this is the case (7, 16). (ii) The endogenous CEF transporter and hGT both have highly conserved leucine zippers, which might mediate dimerization (24; M. K. White and M. J. Weber, manuscript in preparation). (iii) In experiments analogous to the ones reported here, type 1 transporters have been introduced into insulin-sensitive cells. When insulin is added to these cells, the heterologous transporters are translocated to the plasma membrane, as are the endogenous transporters (2, 12). Insulin-sensitive cells express type 4 insulin-regulatable glucose transporters (3, 6, 10, 13, 14), and therefore a similar piggyback mechanism could explain these findings.

The transporter expressed in CEFs is more homologous to the type 3 glucose transporter (15), first found in human fetal skeletal muscle, than to other types of mammalian glucose transporters (White and Weber, in preparation). The C-terminal 32 amino acids of the CEF transporter have homology with the human type 3, but not type 1, C-terminal sequence. This could be an isotype-specific signal sequence involved in stabilization (23). This peptide is predicted to lie on the cytoplasmic face of the plasma membrane, as does $pp60^{v\text{-}src}$.

The availability of different transporter clones, expression vectors, and specific antibodies provides a powerful approach to studying the regulation of glucose transporter in different cell types. Chimera construction and site-directed mutagenesis may allow the dissection of the domains of the transporter involved in regulation.

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