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Degradation and Biosynthesis of the Glucose Transporter Protein in Chicken Embryo Fibroblasts Transformed by the *src* Oncogene

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The rate of glucose transport in cultured fibroblasts is regulated in response to a number of physiological variables, including malignant transformation by *src*, glucose starvation, and stimulation with mitogens. Much of this transport regulation can be accounted for by variations in the amount of transporter protein in the cells. To determine the mechanisms by which levels of the transporter are regulated, we measured the rates of synthesis and degradation of the transporter by pulse-chase experiments and immunoprecipitation of the transporter. We found that transformation by the *src* oncogene results in a large decrease in the rate at which the transporter protein is degraded but that it does not appreciably increase the rate of transporter biosynthesis. On the other hand, glucose starvation and mitogen stimulation increase the rate of transporter biosynthesis, although a role for control of degradation is possible in these circumstances also. Variations in the rate of glucose transport or the amount of the transporter are not associated with phosphorylation of the transporter protein.

D-Glucose is transported into fibroblasts by a stereospecific carrier-mediated facilitated transport system (for a review, see reference 47). The rate at which glucose enters these cells is regulated by a number of factors, including malignant transformation (2, 3, 17, 18, 21, 23, 26, 27, 33, 45, 48), glucose starvation (9–12, 16, 25), and stimulation by mitogens (4, 14, 24, 32, 35, 40, 45; for a review, see reference 19). The following several lines of evidence indicate that the four- to fivefold-increased transport rate observed in glucose-starved cells or in cells transformed by the *src* oncogene is due in large part to an increase in the number of transporters in the cell membrane. (i) Increased transport is associated with an increased V_{max} for transport with little or no change in K_m (45, 48). (ii) Quantitation of the number of transporters by using binding of [^{3}H]cytochalasin B, a potent inhibitor of facilitated glucose transport, reveals an increase in the amount of ligand binding in direct proportion to the increase in transport rate (8, 37, 39, 41, 42). (iii) Antisera raised against the human erythrocyte glucose transporter immunoprecipitate a membrane glycoprotein the amount of which varies directly with the transport rate (16, 38, 46).

The use of the anti-transporter antisera and the use of [^{3}H]cytochalasin B as a photoactivatable probe also identified the transporter in fibroblasts as being, like the erythrocyte transporter, a heterogeneously glycosylated protein with a molecular mass of approximately 50 to 60 kilodaltons. Some controversy still exists as to whether the 50,000-kilodalton protein is solely responsible for facilitated transport of glucose into cells (43). However, it is generally believed to be the predominant glucose transporter or a component of a glucose transport system.

Although it is clear that the amount of glucose transporter protein is under physiological control, little evidence is available concerning the molecular basis for that control. In theory, transporter levels could be controlled by varying the rate of biosynthesis, the rate of degradation, or both. Indeed, various experiments, mostly using metabolic inhibitors, suggest that both synthesis and degradation of the transporter can be varied. However, these results are not entirely consistent with each other. For example, several investigators have reported that the increased transport rate induced by *src* can occur even in the presence of actinomycin D, suggesting the involvement of a posttranscriptional control mechanism (21, 23, 26, 27). However, Bader (2) and Bader et al. (3) found that *src*-induced transport is blocked by actinomycin D. Some investigators have proposed that increased transport in glucose-starved cells is dependent on new transcription, whereas others have provided evidence for control of protein degradation (11, 12, 16, 25, 52).

Because of the intrinsic uncertainty involved in interpreting the effects of various metabolic inhibitors, we chose to measure directly the rates of biosynthesis and degradation of the glucose transporter by pulse-chase experiments, followed by immunoprecipitation of the transporter with antiserum raised against the purified human erythrocyte glucose transport protein. In this communication, we report that the increased amount of glucose transporter observed in *src*-transformed cells can be explained largely as being due to a decrease in the protein degradation rate in the transformed cells. On the other hand, we found that increased glucose transport in mitogen-stimulated cells and in glucose-starved normal cells is associated with an increase in the biosynthesis of the transporter (although some role for variations in protein degradation rate are possible in these circumstances, also). Phosphorylation of the transporter was barely detectable, and variations in transporter phosphorylation were not observed under any of these conditions; thus, phosphorylation is not likely to account for differences in the stability of the protein. However, phosphorylation of the transporter could be detected in response to a phorbol ester tumor promoter.

MATERIALS AND METHODS

Cells and cell culture. All experiments were performed with third- or fourth-passage chicken embryo fibroblasts

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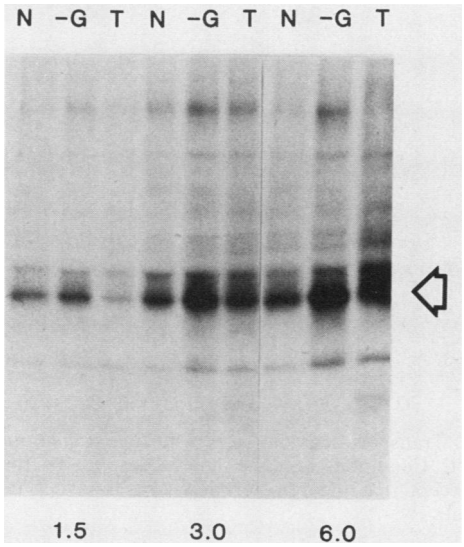

FIG. 1. Differential rates of glucose transporter biosynthesis in normal, glucose-starved, and transformed cells. Cultures of chicken embryo fibroblasts, normally growing (N), starved for glucose (−G), or transformed by *src* (T), were labeled with [^{35}S]methionine for 1.5, 3.0, or 6.0 h. At the end of the labeling period, cells were lysed in RIPA buffer. Before immunoprecipitation, lysates of the N, −G, and T cultures were adjusted so that, at any given time point, the same number of total trichloroacetic acid-precipitable counts per minute were used for immunoprecipitation of the transporter from cultures grown under all three conditions. This adjustment compensated for the small variations in rates of [^{35}S]methionine uptake and incorporation observed between the different culture types and provided a more accurate determination of the differential rates of transporter biosynthesis; in any event, all three culture types displayed specific radioactivities in protein which were within ±30% of each other. In the experiment shown, the *src*-transformed (T) culture at 1.5 h shows a lower rate of [^{35}S]methionine incorporation into the transporter than does the normally growing (N) culture; this result has not been reproduced. The higher-molecular-mass material seen at later times may represent aggregates or isoforms of the transporter (15).

prepared by standard techniques. Cells were grown in high-glucose Dulbecco modified Eagle medium (GIBCO Laboratories, Grand Island, N.Y.)–10% fetal calf serum. Transformed cultures were infected with the Schmidt-Ruppin strain of Rous sarcoma virus, Subgroup A, or with the temperature-conditional derivative of this virus, tsNY68 (21). Glucose starvation was performed by placing cells in glucose-free Dulbecco modified Eagle medium–10% dialyzed fetal calf serum–5 mM uridine (49) for 16 to 18 h. Serum starvation was for 12 h. Except for experiments involving quiescent cells, all cultures were subconfluent or just confluent. Because the labeling protocols involved transferring cells to fresh serum-containing medium (see below), all of the cultures except those designed to be quiescent were actively growing.

To label cells with [^{35}S]methionine, the culture medium was aspirated from the culture dish and was replaced with medium containing 5 to 10% of the normal amount of methionine, either 150 μCi of [^{35}S]methionine per ml for the turnover experiments or 500 μCi/ml for the biosynthesis experiments (New England Nuclear Corp., Boston, Mass.), and 10% dialyzed fetal calf serum.

Immunoprecipitation. Cultures were lysed in radioimmunoprecipitation (RIPA) buffer (1% sodium deoxycholate, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate [SDS], 0.15 M NaCl, 50 mM Tris [pH 7.2]) and frozen at −20°C. The lysates were then thawed, clarified by incubation with a suspension of *Staphylococcus aureus* Cowan I or with Pansorbin (Calbiochem-Behring, La Jolla, Calif.), and centrifuged at 50,000 × *g* for 30 min. The supernatant was then incubated with excess antiserum raised against the human erythrocyte glucose transporter (see below) for 30 min, and the immune complexes were collected by incubation with excess Pansorbin for 30 min and by centrifugation of the suspension for 30 s at 12,000 × *g* in a Microfuge (Beckman Instruments, Inc., Fullerton, Calif.). The pellet was then washed as follows: two washes with RIPA buffer–1 M urea; two washes with RIPA buffer–1 M NaCl, and two washes with RIPA buffer. After being washed, the complex was released from the Pansorbin by boiling in electrophoresis sample buffer (30) without 2-mercaptoethanol, and the sample was electrophoresed on an SDS-polyacrylamide gel. 2-Mercaptoethanol was omitted so that the immunoglobulin G (IgG) would run near the top of the gel and thus not interfere with the electrophoresis of the transporter.

Characterization of the antiserum. Because this antiserum was raised against a preparation of the human erythrocyte transporter which was only 90 to 95% pure, because it was being used in a heterologous system (38), and because of the controversy over the identity of the transporter (43), considerable effort was expended on characterizing the antiserum thoroughly prior to the initiation of these experiments. The results of this characterization are summarized as follows. First, because the nucleoside transporter is found with the glucose transporter in the band 4.5 region of SDS gels (51), we felt that the nucleoside transporter would be the most likely contaminating antigen. However, our antibody removed the cytochalasin B binding activity from a lysate of human erythrocyte ghosts without removing the binding activity for NBMPR which binds to the nucleoside transporter (53). Thus, our antiserum has minimal reactivity with the nucleoside transporter. Second, immunoprecipitation of the 50,000-kilodalton protein from lysates of chicken embryo fibroblasts was blocked by purified human erythrocyte transporter or by human erythrocyte ghosts but was not blocked by bovine or porcine ghosts, which do not have a cytochalasin-sensitive glucose transport system. This suggests the similarity of the chicken embryo fibroblast protein to the transporter protein from human erythrocytes. Third, this antiserum was used to clone a gene from a lambda gt11 library of human tumor cells (34), the putative glucose transporter gene. The product coded by this gene also reacts with monoclonal antibodies against the human erythrocyte transporter (1), consistent with both the identity of the gene and the specificity of the antiserum.

RESULTS

Transporter biosynthesis. Biosynthesis rates for the glucose transporter were determined by labeling cells cultured under various conditions for increasing periods of time, lysing the cells in RIPA buffer, and then immunoprecipitating the glucose transporter. Radioactivity in the transporter was determined by electrophoresing the immunoprecipitate on SDS-polyacrylamide gels, followed by autoradiography of the gel. The data are displayed in Fig. 1 and demonstrate that, at short labeling times (1.5 to 3 h), no increase in incorporation of radioactivity in the transporter was apparent in the *src*-transformed cells relative to the normal cells. However, at longer labeling times (6 h), increased radioac-

tivity accumulated in the transporter in transformed cells. These data indicate that the biosynthesis of the transporter is not greater in the transformed cells than in the normal growing cells but that the synthesized transporter is more stable in the transformed cells and thus accumulates. In contrast, labeling of the transporter occurred more rapidly in the glucose-starved cultures, even at the earliest labeling periods. This indicates that the rate of transporter biosynthesis is faster in the glucose-starved cells. The antiserum used in these experiments recognizes both SDS-denatured transporter and transporter from tunicamycin-treated cells (data not shown). Thus, these results are not likely to be caused by physiological differences in posttranslational modification but rather reflect the rates of biosynthesis and degradation of the protein itself.

Transporter degradation. To examine directly the question of whether the physical stability of the transporter is regulated by *src* transformation, cell cultures were infected with a temperature-conditional *src* mutant tsNY68 (20), and labeled at the permissive temperature (36°C) with [^{35}S]methionine. After the labeling period, the cultures were washed and chased with an excess of nonradioactive methionine, and one half of the cultures were shifted to the restrictive temperature (42°C). Degradation of the transporter occurred much more rapidly at the restrictive temperature, at which the cells become phenotypically normal and have a low transport rate, than at the permissive temperature (Fig. 2). The half-life of the transporter was in the range of 12 to 24 h in the transformed cells, whereas it was only 3 to 6 h in the cultures shifted to 42°C. This approximately three- to fourfold difference is sufficient to account for most of the difference in transport rate observed between the two cell types (45, 46).

A similar experiment, performed with cells infected with the parental, non-temperature-sensitive Rous sarcoma virus, revealed very little effect of temperature itself on the stability of the transporter (Fig. 3); at both 36 and 42°C, the rate of transporter degradation was similar to that observed in tsNY68-infected cells at 36°C.

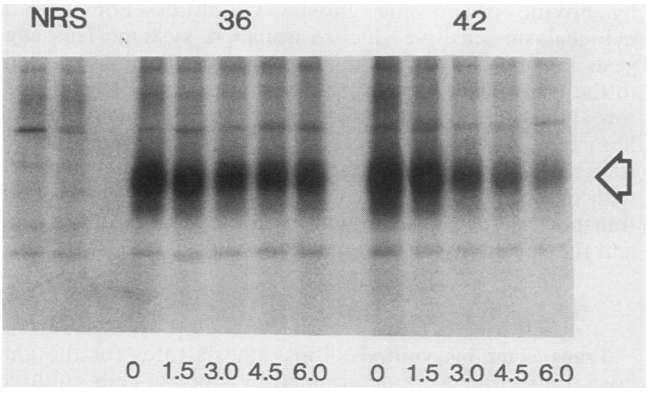

FIG. 2. Increased rate of transporter degradation in transformed cells. Cultures infected with tsNY68, a temperature-conditional *src* mutant, were labeled with [^{35}S]methionine for 10 h at 36°C, the permissive temperature for transformation. After the labeling period, cultures were rinsed and transferred to Dulbecco modified Eagle medium containing unlabeled methionine, and one half of the cultures were shifted to 42°C, the restrictive temperature. Cultures were lysed 0, 1.5, 3.0, 4.5, or 6.0 h after the beginning of the chase, and the glucose transporter was immunoprecipitated. Additional samples at 0 and 6 h were immunoprecipitated with normal rabbit serum (NRS).

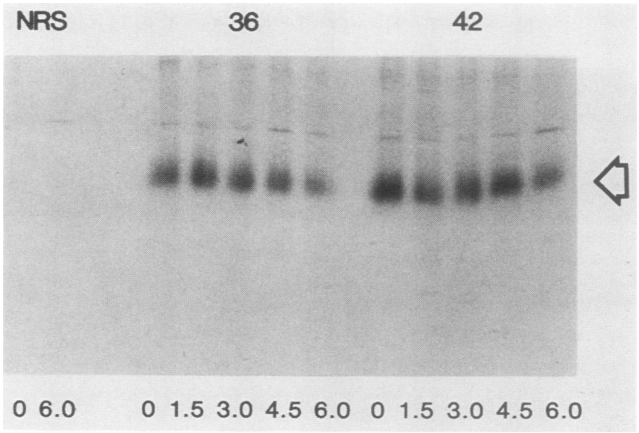

FIG. 3. Transport degradation in wild-type transformed cells at 36 and 42°C. Conditions were the same as described in the legend to Fig. 2, except that the transforming virus was not temperature conditional.

The effect of transformation on transporter stability was not a consequence of global changes in protein stability in the transformed cells, since no changes in total protein turnover were evident in the transformed cells whether those changes were measured by trichloroacetic acid precipitation of total cell protein or SDS-polyacrylamide gel electrophoresis of total cell lysates (data not shown).

When glucose-starved or nonstarved cells were labeled with [^{35}S]methionine and then chased in the presence or absence of glucose, the effect of glucose on the stability of the transporter was slight (4). Thus, the major factor responsible for the increased amounts of transporter in the glucose-starved cells is enhanced biosynthesis rather than decreased degradation.

Synthesis and degradation in serum-stimulated cells. To study biosynthesis of the transporter in response to mitogenic stimulation, confluent cultures of cells were deprived of serum for 12 h, and 20% dialyzed serum was then added in the presence of [^{35}S]methionine for 2 h (Fig. 5). This time period included the early, protein synthesis-independent increase in transport rate, as well as much of the portion of the increased transport rate which required new protein synthesis (24) (Fig. 6). The results (Fig. 5) indicate that a modest (slightly less than twofold) increase in biosynthesis of transporters occurs in response to serum factors.

To determine whether serum might also stabilize transporters against degradation, cultures were labeled with [^{35}S]methionine in the presence of serum, and then the cultures were chased with unlabeled methionine for up to 6 h in the presence or absence of serum. A small increase in transporter degradation rate was observed in the absence of serum (Fig. 5). With or without serum, the rate of transporter degradation was closer to that observed in tsNY68-infected cells held at the restrictive temperature than to that observed in transformed cultures (Fig. 2).

Transporter phosphorylation. To determine whether changes in the stability, biosynthesis, or activity of the transporter were associated with phosphorylation of the transporter protein, cultures were labeled with inorganic $^{32}PO_4$, and the transporter was immunoprecipitated from the cell lysates (Fig. 7). As a control for our ability to detect the phosphorylated transporter, cultures were also treated with the tumor promoter tetradecanoyl phorbol-13-acetate (TPA) (50). The transporter became heavily phosphorylated in

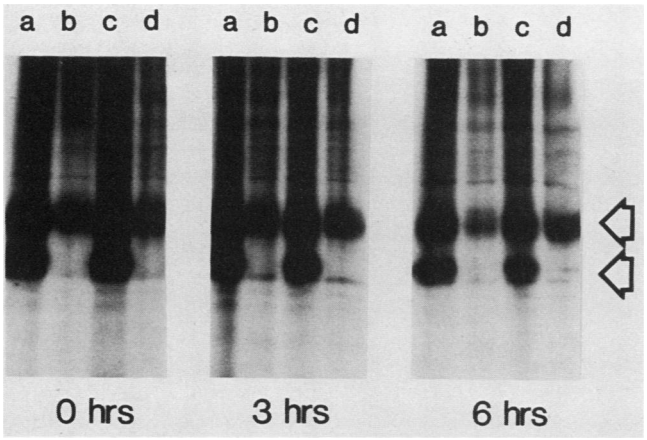

FIG. 4. Degradation of the glucose transporter in glucose-starved cells. Cells were either starved for glucose (lanes a and c) or left in complete medium (lanes b and d) and were then labeled with [^{35}S]methionine as described in Materials and Methods. The cultures were then rinsed and chased either with (lanes b and c) or without (lanes a and d) glucose. Cells were lysed at 0, 3, or 6 h after the start of the chase, and the glucose transporter was immunoprecipitated and electrophoresed as described in Materials and Methods.

response to TPA. The time course of this phosphorylation roughly paralleled the increase in transport rate induced by TPA (14, 32, 35; our unpublished data). However, very little phosphorylation was evident in either the glucose-starved or the transformed cultures in the absence of TPA. Incubating the transformed cultures with vanadate did not increase the level of phosphorylation. All of the detectable phosphorylation in all of the cultures was lost upon treatment with KOH and thus was probably not phosphotyrosine. Similarly, phosphorylation of the transporter was barely detectable in quiescent cultures treated with serum or in glucose-starved cultures to which glucose was added (data not shown).

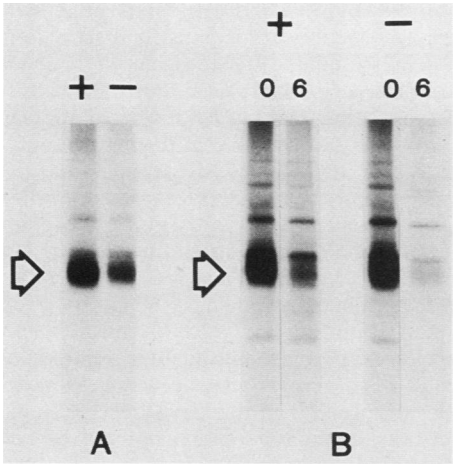

FIG. 5. Biosynthesis and degradation of the glucose transporter in serum-stimulated cells. (A) Biosynthesis. Confluent cells deprived of serum for 12 h were labeled for 2 h with [^{35}S]methionine either in the presence (+) or absence (−) of 20% dialyzed fetal calf serum. (B) Degradation. Confluent cultures were labeled with [^{35}S]methionine in the presence of serum, and the cultures were then chased with cold methionine for 0 or 6 h in the presence (+) or absence (−) of 10% fetal calf serum.

DISCUSSION

Both glucose starvation and malignant transformation by *src* increase the levels of glucose transporter in cultured chicken embryo fibroblasts. We have shown here, however, that different mechanisms underlie this common effect. Transformation by *src* affects predominantly the transporter protein degradation rate, whereas glucose starvation affects transporter biosynthesis (although it may affect degradation as well). We also found that mitogenic stimulation increases the rate of transporter biosynthesis.

Our finding that transformation by *src* affects predominantly the transporter degradation rate is consistent with the many earlier reports that the *src*-induced increase in transport rate in chicken embryo fibroblasts does not require new RNA synthesis (21, 23, 25, 26). Our results were obtained directly, by performing pulse-chase experiments in normal and transformed cells to measure transporter synthesis and breakdown. Since cells infected with a temperature-conditional mutant were used for these experiments and since the labeling of all of the cells was performed at the permissive temperature for transformation, physiological or structural differences in the transporter which might have arisen during the biosynthesis cannot explain these results. Rather, the stabilization of the synthesized transporter in the transformed cells appears to be an early effect of the action of pp60^{src}. This effect of *src* is specific for the transporter or for a small group of cellular proteins, since no transformation-specific difference in the degradation rates of total protein could be detected whether the measurements were made on total trichloroacetic acid-precipitable protein or by electrophoresing total cell lysates.

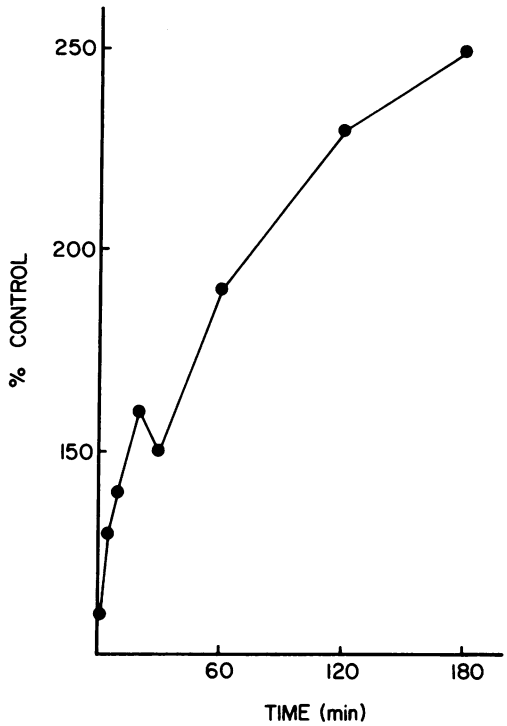

FIG. 6. Glucose transport rate in serum-stimulated cells. Confluent cultures were deprived of serum for 12 h. At various times after addition of 20% fetal calf serum, the uptake rate of [^{3}H]2-deoxyglucose was determined as described previously (44).

The results reported here are the first, to our knowledge, in which the stability of a specific protein has been shown to be affected by the action of a cytoplasmic oncogene product. It has been known for some time, however, that the nuclear transforming protein, simian virus 40 large T antigen, stabilizes the cellular p53 protein by binding to it (36). Although it is clear that the glucose transporter is not a substrate for in vivo tyrosine phosphorylation by pp60^{src}, it is not known whether pp60^{src} binds to the glucose transporter. However, pp60^{src} does bind to another glucose-regulated protein; prior to its attachment to the inner face of the plasma membrane, newly synthesized pp60^{src} forms a complex with a 50-kilodalton protein and an 80-kilodalton glucose-regulated protein (7, 13, 31). The 80-kilodalton protein also is not phosphorylated by pp60^{src}. It is conceivable that pp60^{src} forms an analogous complex with the glucose transporter upon reaching the plasma membrane and that the transporter is stabilized in this complex. Estimates of 3×10^6 molecules of pp60^{src} and 2×10^6 molecules of transporter per cell are consistent with this possibility (7, 13, 39). Another possibility is that pp60^{src} alters the intracellular location of the transporter; or it might regulate a specific protein degradation system. In any event, we currently are trying to identify the molecular basis and metabolic requirements for this specific control of protein degradation induced by pp60^{src}.

Our finding that glucose starvation affects biosynthesis of the transporter in chicken embryo fibroblasts differs from that of Haspel et al. (16). These workers could not detect an increase in transporter biosynthesis or in translatable mRNA levels in glucose-starved 3T3-C2 cells and surmised on that basis that glucose starvation affected transporter degradation. Yamada et al. (52) and Christopher et al. (9–12) have also suggested a glucose-regulated control of transporter degradation on the basis of indirect inhibitor data. There are several possible explanations for this discrepancy. First, the cell types used in this other work were rodent cell lines, whereas in our work we used primary chicken embryo fibroblasts. It is possible that different cells use different mechanisms for regulating transporter levels in response to physiological changes. Indeed, there may even be different transporter species in different tissue types. In fact, we have obtained preliminary data that the glucose transporter mRNA level is dramatically induced by *src* in Rat-1 cells but not in chicken embryo fibroblasts (M. K. White and M. J. Weber, unpublished data). However, another point worth noting is that all of our glucose starvation experiments were carried out in the presence of 5 mM uridine, which provides a source of pentose (49). This allows unimpaired growth of the cells, even in the absence of glucose. Haspel et al. (16) did not use uridine in their medium, and thus their cells may have been starved for nucleotides as well as for glucose. Regardless of how these discrepancies are resolved, the important point is that, depending on the cell and the physiological effector, the levels of functional transporter can be regulated not only by controlling translocation to the membrane (20, 22, 28, 29) but also by regulating both the rate of synthesis and the rate of degradation. Thus, the central role this protein plays in cellular metabolism is reflected in the complexity of its regulation.

Although protein phosphorylation could be a possible mechanism for regulating the susceptibility of a protein to degradation, only very low levels of transporter phosphorylation were detectable in normal, glucose-starved, or transformed cells. In addition, incubation of the *src*-transformed cells with vanadate, which inhibits phosphotyrosine phosphatases, did not increase the level of phosphorylation of the transporter. Lysis of cells in boiling SDS, which inactivates phosphatases and proteases, also did not increase the amount of phosphorylation detected in the immunoprecipitated transporter (data not shown), indicating that the low levels of phosphorylation are not likely to be due to phosphatases acting after lysis. All of the transporter phosphorylation which was detectable could be removed by treatment with KOH, suggesting that there was no phosphotyrosine on the transporter. We conclude that the transporter is not phosphorylated on tyrosine and that it is phosphorylated to only a small extent on other amino acids under physiological conditions. On the other hand, treatment of cells with the tumor promoter TPA induced a dramatic increase in transporter phosphorylation, in agreement with the results of Witters et al. (50). It is possible that this increased phosphorylation is adventitious and that it reflects simply the massive, acute activation of protein kinase C by TPA. Alternatively, it is conceivable that a similar reaction occurs transiently and to a more modest extent in cells stimulated by mitogens or oncogenes which increase the rate of phosphatidyl inositol turnover and that this phosphorylation plays a role in regulating transporter function (22). It is not possible to distinguish between these possibilities with the available evidence.

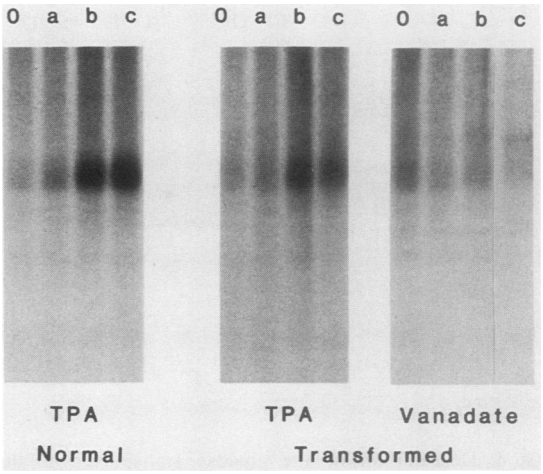

FIG. 7. Phosphorylation of the glucose transporter. Normal or transformed cells were labeled for 12 h with 3 mCi of $^{32}PO_4$ per ml and then treated with TPA (50 ng/ml) or with $NaVO_4$ (50 μM) for 0, 5 (lane a), 60 (lane b), or 240 (lane c) min. Cultures were lysed in RIPA buffer, and the glucose transporter was immunoprecipitated.

The time course of transporter phosphorylation in response to treatment of cells with TPA paralleled the TPA-induced increase in transport rate, increasing slowly over a period of 2 h or more. This was surprising, since we generally find that TPA-induced phosphorylations are extremely rapid, requiring only a few minutes (5, 6). Thus, the steady, prolonged increase in transporter phosphorylation in response to TPA may reflect not only the activation of protein kinase C but also the biosynthesis of new transporters or the translocation of transporters to a site where they are susceptible to phosphorylation by protein kinase C (22).

In conclusion, we find that the level of glucose transporter protein found in chicken embryo fibroblasts is regulated at the level of both biosynthesis and degradation, with malignant transformation by the *src* oncogene affecting predominantly degradation. The biochemical bases for these changes in synthesis and degradation are currently under investigation.

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