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Identification of the stereospecific hexose transporter from starved and fed chicken embryo fibroblasts

(cytochalasin B/photoaffinity labeling/plasma membrane)

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ABSTRACT **When deprived of D-glucose for 24 hr, chicken embryo fibroblasts exhibit a marked increase in hexose transport activity compared with that of control cells. Scatchard analysis of [^{3}H]cytochalasin B binding to starved cell plasma membranes (46 pmol/mg) indicated a six-fold increase compared with fed cell plasma membranes (7.5 pmol/mg). Irradiation of starved cell plasma membranes with high-intensity UV light in the presence of 0.5 μM [^{3}H]cytochalasin B resulted in covalent labeling of polypeptides of M_r 52,000 and 46,000. In fed cell plasma membranes irradiated under the same conditions, both polypeptides were labeled but at greatly decreased levels. In fact, labeling of the M_r 52,000 polypeptide was barely detectable. The amount of D-glucose-sensitive [^{3}H]cytochalasin B covalent insertion into these membrane components was increased 11 ± 2 (n = 4)-fold in starved versus fed cell plasma membranes. Photoaffinity labeling of both polypeptides in starved cell plasma membranes was inhibited by D-glucose, 3-*O*-methylglucose, 2-deoxyglucose, cytochalasin B, and cytochalasin A but not by D-sorbitol, L-glucose, or cytochalasin E. Half-maximal inhibition of labeling of the M_r 52,000 polypeptide occurred at 8 mM D-glucose whereas, for the M_r 46,000 polypeptide, half-maximal inhibition occurred at 40 mM D-glucose. It is concluded that (*i*) two hexose transport proteins, one of M_r 46,000 and one of M_r 52,000, have been identified in chicken embryo fibroblasts and (*ii*) the increased affinity labeling of these transporter components after cell starvation may reflect increased numbers of transporters in the plasma membrane.**

The facilitated D-glucose transport systems found in adipose tissue, skeletal and heart muscle, and various cultured cells can be regulated in response to hormones and cellular nutritional states. Hamster and chicken embryo fibroblasts maintained in D-glucose-deprived culture medium for 24 hr have greatly elevated levels of hexose transport activity compared with that of normally fed cells (1–6). This increase in transport activity due to D-glucose starvation can be prevented by protein synthesis inhibitors (1–4, 7–10). Addition of D-glucose to the starved cell culture medium results in a gradual decrease in the elevated transport activity back to the level found in normally maintained D-glucose-fed cultures (4, 10). When cycloheximide and D-glucose are added simultaneously to the starved cell culture medium, the elevated transport activity decreases at a much slower rate than observed in the presence of D-glucose alone (10). These observations suggest that the nutritional state of the cell regulates the relative rates of synthesis and degradation of the transport system component(s).

Further clarification of transport regulatory mechanisms requires a method to specifically label or quantitate (or both) the hexose transporters. One such method is to use cytochalasin B, which is a potent reversible inhibitor of hexose transport in a number of animal cells (11) and appears to be a competitive antagonist (12, 13). Saturating concentrations of cytochalasin B inhibit the hexose transport activity of both starved and fed cultures to the same level (2, 10). This increased hexose transport activity due to D-glucose starvation of intact cells is preserved in isolated plasma membrane vesicles and can be completely inhibited by cytochalasin B (ref. 14; unpublished observations). D-Glucose-specific cytochalasin B binding has been used to estimate the number of hexose transporters in the plasma membranes of normal and Rous sarcoma virus-transformed chicken embryo fibroblasts (15). These data support the hypothesis that the increased hexose transport activity in transformed cells is due to an increase in the number of hexose transporters. Similar results have been reported for insulin activation of D-glucose transport in the rat adipocyte (16).

To identify and study metabolic regulation of the hexose transporter, we have developed a method to label membrane component(s) of the hexose transport system. The method involves photoactivation by high-intensity UV light of [^{3}H]-cytochalasin B, which can then specifically and covalently label the hexose transport polypeptide(s). Identification of the hexose transporter is assessed by the ability of D-glucose to inhibit the covalent labeling. Photoaffinity labeling of the D-glucose transporter in the human erythrocyte by this method has established its validity (17). This D-glucose transporter has previously been identified and purified as a M_r 55,000 polypeptide (18–20). Photoaffinity labeling of erythrocyte ghosts by [^{3}H]cytochalasin B specifically labeled the M_r 55,000 polypeptide, with 90% of the labeling inhibited by D-glucose. In the present work, photoaffinity labeling by [^{3}H]cytochalasin B is used to identify and monitor the metabolic regulation of the hexose transporter from D-glucose-starved and -fed chicken embryo fibroblasts.

MATERIALS AND METHODS

Materials. [^{3}H]cytochalasin B (8.3 Ci/mmol; 1 Ci = 3.7 × 10^{10} becquerels), [^{14}C]sucrose (673 mCi/mmol), and Protosol were from New England Nuclear. Cytochalasin B, cytochalasin A, and cytochalasin E were from Aldrich. Tris, Hepes, EDTA, D-fructose, D-glucose, and D-glucose analogues were from Sigma. Instagel and aqueous counting scintillant were from Packard and Amersham. Plastic roller bottles (850 cm^2), tryptose/phosphate broth, fetal calf serum, and Dulbecco's modified Eagle's medium were from Corning Glass Works, Difco, M. A. Bioproducts, and GIBCO, respectively. All other reagents were of the highest purity commercially available.

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Cell Culture. Chicken embryo fibroblasts were prepared from 10-day-old embryos by modification of described methods (21). The secondary cultures were seeded at 3×10^7 cells per roller bottle in 150 ml of Dulbecco's modified Eagle's medium supplemented with D-glucose at 4.5 g/liter, 10% tryptose/phosphate broth, and 2% irradiated fetal calf serum. The cells reached confluence after incubation at 41°C for 3 days and, at that time, medium was changed for all cells. The medium change, which was 20–24 hr prior to harvesting, involved substituting D-fructose and dialyzed fetal calf serum for D-glucose and irradiated fetal calf serum to obtain starved cells and fresh medium identical to the starting medium for fed cells.

Plasma Membrane Isolation. Crude membranes were prepared as described (21). The crude membranes were suspended in 1 mM Tris·HCl/1 mM Hepes, pH 8.2 (buffer A) and centrifuged at 100,000 × *g* for 60 min. The pellets were suspended in buffer A/0.5 mM $MgCl_2$ and layered onto a discontinuous step gradient of 10% (wt/vol) and 20% (wt/vol) dextran in buffer A/0.5 mM $MgCl_2$. The 10% dextran top interface fraction was collected, diluted with 50 mM K_2SO_4/1 mM EDTA/1 mM Tris·HCl, pH 7.5 (buffer B/1 mM EDTA), and centrifuged at 100,000 × *g* for 60 min. The pellet was suspended in buffer B (without EDTA) to a final protein concentration of 2 to 3 mg/ml. This membrane preparation was 7- to 10-fold enriched for 5′-nucleotidase activity compared with that of the cell homogenate.

Cytochalasin B Binding. Cytochalasin B binding was measured as described by Cushman and Wardzala (22). In brief, the membrane suspension was mixed with 2 μM cytochalasin E/buffer B containing 500 mM D-sorbitol or D-glucose. Aliquots of this mixture were incubated for 10 min at room temperature with various concentrations of [^{3}H]cytochalasin B and [^{14}C]sucrose in buffer B/6 mM $MgCl_2$/6 mM $CaCl_2$. The samples were centrifuged at 150,000 × *g* for 30 min at 4°C. The pellets were dissolved in 300 μl of 0.1 M NaOH/0.1% $NaDodSO_4$ and mixed with Instagel scintillation cocktail. Corrections for trapped [^{3}H]cytochalasin B in membrane pellets were determined by subtracting the amount of [^{14}C]sucrose associated with each sample. The data were analyzed as described by Scatchard (23). D-Glucose-sensitive cytochalasin B binding was determined from a plot of cytochalasin B bound versus free cytochalasin B concentration. The curve obtained in the presence of 500 mM D-glucose was subtracted from the D-sorbitol curve. The resultant values were analyzed by linear regression.

Photoaffinity Crosslinking. [^{3}H]Cytochalasin B in 100% ethanol was dried under nitrogen and suspended in 5 mM NaH_2PO_4/1.0 mM EDTA/250 mM sucrose, pH 7.4, to a concentration of 10 μM. This stock cytochalasin B solution was prepared daily, immediately prior to use. Sugar solutions were prepared as 2.7 M stocks in the same buffer. Cytochalasin B analogue solutions were 2 mM in 100% ethanol. In the experiments using the cytochalasin B analogues, the concentration of ethanol in the labeling mixture never exceeded 0.5%. Plasma membrane protein solutions (500 μg) were 500 mM in sugar or 10 μM in each cytochalasin analogue or both. [^{3}H]Cytochalasin B was then added to a final concentration of 0.1–2.0 μM, depending on the experiment. This mixture, at a final protein concentration of 1 mg/ml, was placed on ice in the dark, and allowed to equilibrate for 30 min in 1-cm path-length quartz cuvettes. The samples were then irradiated at 4°C with a 140-W mercury arc lamp (Conrad-Hanovia model 30600) at a distance of 10 cm for 10 min. The reaction was stopped by removal of the samples from light and dilution with ice-cold buffer. The samples were then centrifuged at 150,000 × *g* for 1 hr and the pellets suspended in distilled water. Protein was determined by the method of Lowry *et al.* (24) using Sigma protein standard.

$NaDodSO_4$/Polyacrylamide Gel Electrophoresis. The membrane suspensions were adjusted to 1% in $NaDodSO_4$ and 0.5% dithiothreitol and then heated for 1 min at 100°C. They were subjected to electrophoresis on 3-mm-thick 9% polyacrylamide slab gels as described by Laemmli (25). Radioactivity in the various protein bands was determined by cutting the lanes into 3-mm slices with a gel slicer, shaking the slices for several hours in 0.5 ml of Protosol, and then assaying in 3.5 ml of aqueous counting scintillation fluid.

RESULTS

The amount of cytochalasin B bound in starved and fed cell plasma membrane vesicles was determined by Scatchard analysis (Fig. 1). Starved cell plasma membranes exhibit a much larger amount of D-glucose-inhibited cytochalasin B binding compared with that of fed cell plasma membranes. Both types of membranes displayed typical curvilinear Scatchard plots. D-Glucose-sensitive cytochalasin B binding (*Inset*) is determined as the difference between binding in the presence of D-sorbitol (a nontransportable sugar alcohol) and binding in the presence of D-glucose. The apparent K_d values of cytochalasin B binding to starved and fed cell plasma membranes were 0.1 μM and 0.07

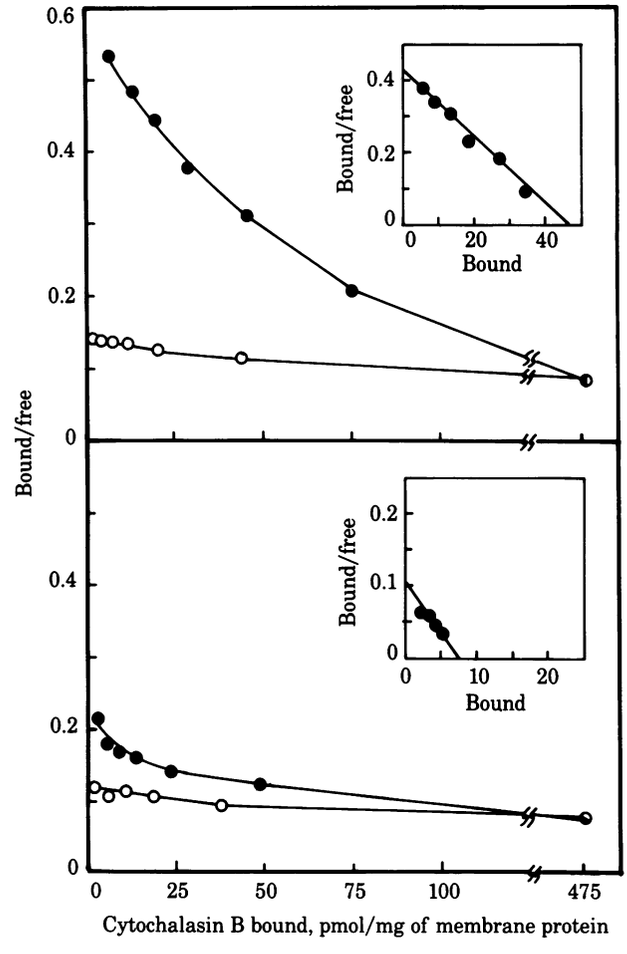

FIG. 1. Scatchard analysis of [^{3}H]cytochalasin B binding to plasma membranes isolated from chicken embryo fibroblasts. Plasma membrane proteins (115 μg) from starved (*Upper*) and fed (*Lower*) cells were incubated with 2 μM cytochalasin E/20 nM [^{3}H]cytochalasin B. Unlabeled cytochalasin B was added to give final concentrations of 20, 40, 62, 102, 187, 437, and 10,020 nM. Cytochalasin B binding was determined in the presence of 500 mM D-sorbitol (●) or 500 mM D-glucose (○). (*Insets*) D-Glucose-sensitive cytochalasin B binding. Data represent the means of duplicate determinations.

μM, respectively. This is similar to the K_i for hexose transport inhibition in human erythrocytes, rat adipocytes, and chicken embryo fibroblasts (13, 26, 27). The amount of D-glucose-sensitive cytochalasin B bound in starved cell membranes was 46 pmol/mg while, in fed cell membranes, it was 7.5 pmol/mg. This indicates an apparent 6-fold enrichment in the amount of cytochalasin B specifically bound to hexose transporters. Similar results have been reported for Rous sarcoma virus-transformed and normal chicken embryo fibroblasts (15).

Isolated plasma membranes from D-glucose-starved and -fed cells were incubated with 0.5 μM [^{3}H]cytochalasin B and irradiated, and the membrane polypeptides were separated on a 9% NaDodSO$_4$/polyacrylamide gel (Fig. 2). Photoaffinity labeling of [^{3}H]cytochalasin B to fed cell plasma membranes covalently labeled a polypeptide of M_r 46,000 (*Right*). The covalent labeling was inhibited ≈15% in the presence of 500 mM D-glucose. The small amount of labeling of the M_r 52,000 polypeptide in fed cell membranes was not always observed. However, when starved cell plasma membranes were photoaffinity labeled, two covalently labeled polypeptides were clearly resolved (*Left*). Covalent labeling of [^{3}H]cytochalasin B to polypeptides of M_r 52,000 and 46,000 was highly sensitive to the presence of 500 mM D-glucose (Fig. 2 and Table 1). The lower molecule weight polypeptide (M_r 46,000) was less D-glucose sensitive than the M_r 52,000 polypeptide (Figs. 2 and 3 and Table 1). A significant amount of [^{3}H]cytochalasin B was found at the gel dye front. This labeling was not D-glucose sensitive and probably represents [^{3}H]cytochalasin B covalently bound to phospholipids, since the same amount of labeled material was found at the dye front of 15% gels. The D-glucose-sensitive cytochalasin B covalent labeling in starved cell plasma membranes was increased ≈11-fold in this experiment and had a range of 11 ± 2 (n = 4)-fold compared with that in fed cell plasma membranes.

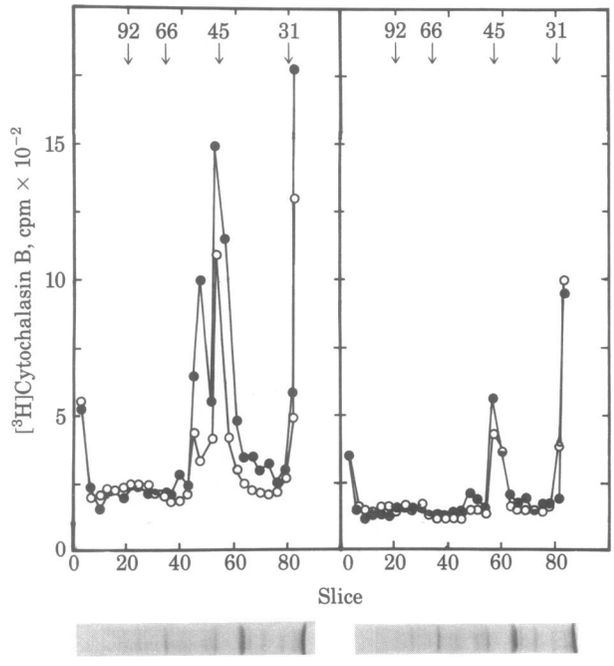

FIG. 2. Photoaffinity labeling of chicken embryo fibroblast plasma membranes with [^{3}H]cytochalasin B. Starved (*Upper left*) and fed (*Upper right*) cell plasma membranes were incubated with 0.5 μM [^{3}H]cytochalasin B in the presence of 500 mM D-sorbitol (●) or 500 mM D-glucose (○). In each experiment, 500 μg of plasma membrane protein (final concentration, 1 mg/ml) was used. After equilibrium binding was reached, samples were irradiated, washed, subjected to electrophoresis on 9% polyacrylamide/NaDodSO$_4$ gels. Result is representative of four separate determinations. ↓, Marker proteins (M_r × 10^{-3}). (*Lower*) Coomassie blue-stained gel profiles representative of experiments in which 200 μg of membrane proteins were separated on a 1.5-mm-thick 9% slab gel.

Table 1. Effects of various sugars and cytochalasins on incorporation of [^{3}H]cytochalasin B into D-glucose-sensitive polypeptides in starved chicken embryo fibroblasts

Agent	[^{3}H]Cytochalasin B incorporated, %	
	M_r 52,000	M_r 46,000
None	102	103
D-Sorbitol	100	100
L-Glucose	104	105
D-Glucose	32	46
3-*O*-Methylglucose	31	41
2-Deoxyglucose	25	31
Cytochalasin A	45	28
Cytochalasin B	0	4
Cytochalasin E	92	83

Percent labeling is relative to that observed in the presence of D-sorbitol. Data are means of two separate experiments. Final sugar concentrations were 500 mM. Final cytochalasin concentrations were 10 μM.

The [^{3}H]cytochalasin B concentration dependence of covalent labeling in starved (total of both polypeptides) and fed cell plasma membranes was also determined (data not shown). Photoaffinity labeling of starved cell plasma membranes appeared to be linear up to 0.2 μM [^{3}H]cytochalasin B. However, due to the small amount of labeling at low cytochalasin B concentrations, 0.5–1.0 μM [^{3}H]cytochalasin B was routinely used. At all concentrations tested, there was substantial inhibition of covalent labeling by 500 mM D-glucose. D-Glucose inhibited ≈40% of labeling at 2.0 μM [^{3}H]cytochalasin B but 80% at 0.1 μM. The amount of covalent labeling in fed cell plasma membranes was relatively small compared with that of starved cell plasma membranes.

To further characterize the D-glucose sensitivity of starved

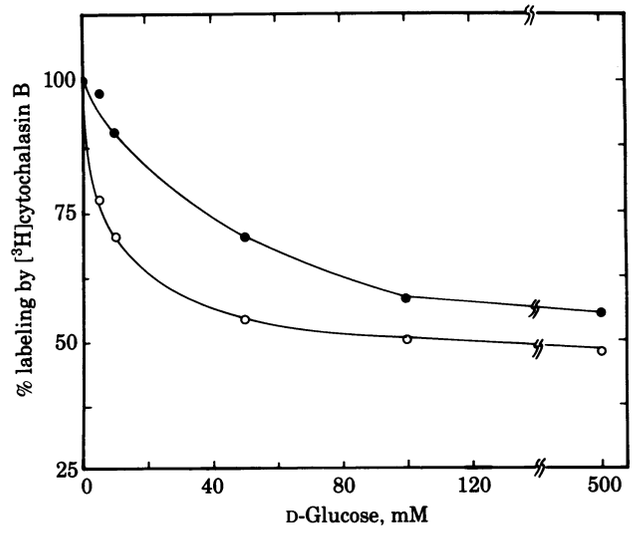

FIG. 3. Effects of various D-glucose concentrations on photoaffinity labeling of the M_r 52,000 (○) and 46,000 (●) polypeptides in starved cell plasma membranes. Percent inhibition of covalent labeling by 1.0 μM [^{3}H]cytochalasin B was determined as a function of D-glucose concentration. Total monosaccharide concentrations were adjusted to 500 mM by addition of appropriate amounts of D-sorbitol. The experiment was carried out twice with essentially identical results.

cell plasma membranes, the D-glucose concentration dependence of covalent labeling by 1.0 μM [^{3}H]cytochalasin B is shown in Fig. 3. These experiments indicate the greater inhibition of [^{3}H]cytochalasin B labeling by D-glucose in the M_r 52,000 polypeptide relative to that in the M_r 46,000 polypeptide. Half-maximal inhibition of covalent labeling by 1.0 μM [^{3}H]cytochalasin B occurred at $\approx$8 mM D-glucose in the M_r 52,000 polypeptide. This was similar to the inhibition of covalent labeling found for the human erythrocyte transporter (17). Half-maximal inhibition of labeling for the M_r 46,000 polypeptide occurred at $\approx$40 mM D-glucose. This analysis was not carried out with fed cell plasma membranes due to the small amount of D-glucose-sensitive covalent labeling in these membranes.

D-Glucose and cytochalasin B analogues were tested for ability to inhibit covalent labeling by 1.0 μM [^{3}H]cytochalasin B of both polypeptides in starved cell plasma membranes (Table 1). D-Sorbitol or L-glucose (500 mM) had no effect on covalent labeling of either polypeptide. D-Glucose, 3-*O*-methylglucose, or 2-deoxyglucose inhibited covalent labeling of the M_r 52,000 polypeptide $\approx$70% and of the M_r 46,000 polypeptide $\approx$60%. Unlabeled cytochalasin B (10 μM) completely inhibited labeling of both polypeptides by 1.0 μM [^{3}H]cytochalasin B. Cytochalasin A decreased labeling of the M_r 52,000 polypeptide 55% but inhibited labeling of the M_r 46,000 polypeptide 72%. Cytochalasin E had little or no effect on covalent labeling of either polypeptide.

DISCUSSION

Identification of the cytochalasin B binding component(s) in chicken embryo fibroblast plasma membranes was achieved by taking advantage of an intrinsic property of cytochalasin B, that, on high-intensity UV irradiation, this molecule becomes highly reactive. Irradiation of chicken embryo fibroblast plasma membranes in the presence of [^{3}H]cytochalasin B resulted in covalent labeling of polypeptides of M_r 52,000 and 46,000 in starved cell plasma membranes, while the M_r 46,000 polypeptide was predominantly labeled in fed cell plasma membranes (Fig. 2). The covalent labeling of both polypeptides in starved cell plasma membranes was highly sensitive to the presence of D-glucose; 40% of the labeling was inhibited by 500 mM D-glucose at 2 μM [^{3}H]cytochalasin B and, at lower concentrations of [^{3}H]cytochalasin B, up to 80% of the total covalent labeling was inhibited by D-glucose. In contrast, covalent labeling of the M_r 46,000 polypeptide in fed cell plasma membranes was much less sensitive to the presence of 500 mM D-glucose. Half-maximal labeling occurred at $\approx$0.3 μM for both membranes, similar to the K_d of 0.1 μM found by Scatchard analysis. This is in good agreement with K_i values of 0.1–0.7 μM found for hexose transport inhibition in chicken embryo fibroblasts (13), human erythrocytes (26), and rat adipocytes (27).

The photolabeling characteristics of the two polypeptides in starved cell plasma membranes were consistent with the expected properties of the stereospecific hexose transporter. Covalent labeling in both polypeptides was inhibited when the plasma membranes were incubated with 500 mM D-glucose, 3-*O*-methylglucose, or 2-deoxyglucose (Table 1); these sugars have similar affinities for the transport system with K_d values of $\approx$10 mM (28). D-Sorbitol or L-glucose (500 mM) had no significant effect on labeling by [^{3}H]cytochalasin B, consistent with their K_d values of >3 M in the human erythrocyte (28). Excess unlabeled cytochalasin B (10 μM) abolished labeling of both polypeptides. Cytochalasin E, which has a K_i for hexose transport inhibition of >100 μM (29), had little or no effect on the amount of [^{3}H]cytochalasin B incorporated into either polypeptide. Cytochalasin A was partially effective, as expected from its relative affinity for the cytochalasin B binding site and ability to inhibit transport activity (29).

The possibility existed that the labeled M_r 46,000 polypeptide in both membranes was actually chicken actin (M_r 45,000). However, this appears to be highly improbable, because we found that rabbit muscle actin can covalently bind only 20 cpm of [^{3}H]cytochalasin B per μg of actin under the conditions of our experiments (17). Further, covalent labeling to actin was completely D-glucose insensitive. These observations suggest that actin is probably not the cytochalasin B-photolabeled M_r 46,000 polypeptide.

The D-glucose transporter purified from human erythrocytes is a M_r 55,000 polypeptide (17–20). This hexose transporter, when treated with *endo*-β-galactosidase, behaves as a nonglycosylated polypeptide of M_r 46,000 (30). This could be analogous to the situation observed in the labeling of starved cell plasma membranes (Fig. 2) and suggests that the two labeled polypeptides may represent the same polypeptide with different degrees of glycosylation. The possibility that these two polypeptides may be disulfide linked was ruled out by electrophoresis of labeled membranes in an oxidized state. No difference in the electrophoretic profile was found in the presence or absence of the reductant dithiothreitol (unpublished results).

Enhanced hexose transport in intact cells that have been activated by starvation, transformation, or insulin treatment has been correlated with an increase in the V_{max} of transport activity without any change in the K_m (2, 8, 12, 21, 31–33). Kinetic analysis of D-glucose transport by plasma membrane vesicles derived from starved or fed chicken embryo fibroblasts showed that both types have at least two transport systems, a low-affinity (K_m, 12 mM) system and a higher affinity (K_m, 2–3 mM) system (unpublished results). The V_{max} values for starved cell plasma membranes at both the low- and higher affinity states were enhanced 4- to 5-fold over the V_{max} values for fed cell plasma membranes. Similar results have been reported for transport of D-glucose by intact cells (3). From the gel profile (Fig. 2), we can calculate a 7-fold increase in D-glucose-sensitive labeling of the M_r 52,000 polypeptide and a 4-fold increase in labeling of the M_r 46,000 polypeptide of starved cell plasma membranes. Comparison of these photolabeling results with the transport kinetic analysis suggests the possiblity that the two labeled polypeptides may be discrete transporters with different functional characteristics.

Based on the D-glucose-specific cytochalasin B binding data (Fig. 1), we estimate $\approx$110,000 hexose transporters per cell under starved conditions. This corresponds to 0.25% of starved cell plasma membrane protein and 0.04% of fed cell plasma membrane protein (wt/wt). This was based on assumptions of one cytochalasin B bound per hexose transporter (13), an average M_r of 50,000, and 2.5×10^8 cells per mg of membrane protein. The photolabeling method in the human erythrocyte has been shown to have an efficiency of 5% at saturating concentrations of [^{3}H]cytochalasin B (17). Assuming a similar efficiency in chicken embryo fibroblast plasma membranes, we estimate 60,000 hexose transporters per cell under starved conditions and 5,500 hexose transporters per cell in fed cells.

In summary, the present results show reasonable agreement between Scatchard analysis of cytochalasin B binding, photoaffinity labeling by [^{3}H]cytochalasin B, and kinetic analysis of hexose transport activity. It has not been rigorously determined whether the covalent photoaffinity labeling of hexose transporters by [^{3}H]cytochalasin B is a quantitative measure of the number of hexose transporters in the plasma membrane. This issue has important implications with regard to regulation of hexose transport activity during starvation of chicken embryo

fibroblasts. This methodology should have wide application to the study of metabolic regulation of hexose transport system components in chicken embryo fibroblasts and many other systems.

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