Effect of Glucocorticoids on Hexose Transport in Rat Adipocytes

EVIDENCE FOR DECREASED TRANSPORTERS IN THE PLASMA MEMBRANE*

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Glucocorticoids are known to rapidly inhibit glucose transport when added to isolated rat adipocytes. To determine whether this inhibition of transport persists following isolation of the plasma membranes, adipocytes were incubated in the absence or presence of a maximally inhibitory concentration of dexamethasone, a synthetic glucocorticoid, and plasma membrane vesicles were prepared. D-Glucose uptake into vesicles from steroid-treated cells was inhibited by an average of 40%. The ability of dexamethasone to inhibit transport depended upon pretreatment of cells with hormone prior to membrane isolation. Furthermore, the decreased rate of transport was prevented by the simultaneous addition of actinomycin D or cycloheximide with dexamethasone, indicating a requirement for RNA and protein synthesis.

The effect of dexamethasone on glucose transport was further investigated using our recently developed cytochalasin B affinity-labeling protocol to identify the transporter on sodium dodecyl sulfate-polyacrylamide gels. A peak of radioactivity having $M_r = 54,000$ was identified which exhibited the properties expected for the glucose transporter, in that label incorporation was prevented by D-glucose and unlabeled cytochalasin B, but not by D-sorbitol or unlabeled cytochalasins A, D, or E. Dexamethasone was found to cause a significant (average 33%) decrease in the amount of labeled transporter in the plasma membrane fraction which was prevented by the simultaneous addition of actinomycin D with dexamethasone to the cells. A similar percentage decrease was not found in a microsomal membrane fraction nor in a total cellular membrane fraction. These results suggest that glucocorticoids may decrease glucose transport in rat adipocytes by selectively decreasing the number of transporters in the plasma membrane.

Glucocorticoids added in vitro have been shown to inhibit hexose transport in rat adipocytes (1–4), rat thymocytes (5, 6), murine fibroblasts (7), lymphosarcoma cells (8), 3T3-L1 cultured adipose cells (9), and rat hepatoma tissue culture cells (10). Adrenal corticosteroids have also been shown to inhibit at least three amino acid transport systems in hepatoma tissue culture cells (including system A (11), system L (12), and a distinct system for glycine (13)); amino acid (system A) and uridine transport in thymocytes (14, 15) are also inhibited. Thus, inhibition of nutrient transport systems by glucocorticoids contributes to a decrease in cellular metabolism in a number of cell types.

The glucose transport system in rat adipocytes provides one of the best model systems for studying the ability of glucocorticoids to inhibit nutrient transport. The glucose transport system in adipocytes has been well characterized in both intact cells and in isolated plasma membranes (see review, Ref. 16). Furthermore, the ability of dexamethasone to inhibit transport into rat adipocytes represents one of the more rapid actions described for glucocorticoids, with transport being maximally inhibited within 60-90 min (1, 3). Several lines of evidence suggest that glucocorticoids stimulate the synthesis of a protein (or proteins) that, either directly or indirectly, inhibits glucose transport (1–4). However, the exact mechanism by which the glucocorticoid-induced protein brings about this inhibition in rat adipocytes or any other cell type remains completely unknown. We report that the inhibition of glucose transport caused by dexamethasone added to intact cells persists throughout membrane isolation procedures. The decreased rate of uptake in the isolated membranes is not observed if the intact cells are also treated with actinomycin D or cycloheximide or if dexamethasone is added directly to the isolated plasma membranes.

We also have been able to apply our recently developed affinity-labeling procedure (17, 18) to identify the glucose transport protein in cellular membrane fractions from rat adipocytes and to study the ability of dexamethasone to alter the amount of label associated with the glucose transporter. The labeling method entails incubating plasma membranes with [3H]cytochalasin B and exposing them to intense UV light, which causes the covalent attachment of [3H]cytochalasin B to the transporter. The effective wavelength is 280 nm (19) suggesting that the attachment is via a tryptophan or tyrosine on the glucose transporter. We and others have now shown that the glucose transporter in human erythrocytes (17, 19, 20), chick embryo fibroblasts (18, 21), cultured skeletal muscle cells (22), human placenta (23, 24), and rat adipocytes (21, 25) can be covalently labeled with [3H]cytochalasin B using this methodology. In this study, we show that dexamethasone causes a decrease in the amount of affinity-labeled transporter in the plasma membrane. A similar decrease in the total cellular pool of glucose transport proteins, or a microsomal membrane fraction, was not observed. Both the transport studies and affinity-labeling studies of isolated plasma membranes suggest that glucocorticoids may decrease transport in target cells by selectively decreasing the number of transporters in the plasma membrane.

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Experimental Procedures

Materials—The deoxymethasone and actinomycin D were kind gifts of Merck Sharpe and Dohme. Crystalline pork insulin (285 units/mg) was purchased from Lilly. BSA, ovalbumin, carbonic anhydrase, phosphorylase b, catalase, and glucose-6-phosphate dehydrogenase were obtained from Sigma or CRG-7 from Armour, and the collagenase was crude Clostridium histolyticum, Type I, from Worthington. The cytochalasin was obtained from Aldrich. [4-3H]Cytochalasin B (13–15 Ci/mmol), L-[3H]glucose (10.7 Ci/mmol), d-[3H]glucose (346 Ci/mmol), tissue solubilizer (Protosol), Omnifluor, and Atomlight liquid scintillation fluid were obtained from New England Nuclear. Sodium lauryl sulfate was obtained from Pierce Chemical Co. and cycloheximide from Sigma. Molecular weight standards were also obtained from Sigma and consisted of myosin (Mr = 205,000), β-galactosidase (116,000), phosphorylase b (97,400), BSA (66,000), catalase (58,000), ovalbumin (45,000), and carbonic anhydrase (29,000). The remaining chemicals were of the highest grade available.

Preparation of Adipocytes—Adipocytes were isolated from epididymal adipose tissue of 150–200-g male Sprague-Dawley rats (Charles River) by collagenase digestion according to the method of Rodbell (26) as described previously (1). The isolated adipocytes were incubated at 37 °C for 30–60 min as designated. When added, deoxymethasone (final concentration 10–7 M), actinomycin D (2 μg/ml), and cycloheximide (10 μg/ml) were present in the collagenase digestion buffer, the wash buffers, and the subsequent 30–60-min incubation buffer. The cells and all buffers were maintained at 37 °C. Insulin (1 milliunit/ml), when present, was added only during the final 30 min of the incubation period. To terminate the incubation, cells were centrifuged and washed with 0.25 M sucrose, 1 mM EDTA, and 10 mM Tris-HCl, pH 7.4 (sucrose-EDTA buffer) at room temperature. The cells were resuspended in sucrose-Tris buffer at 16–20 °C.

Preparation of Plasma Membranes—Cell membranes were prepared as described by Simpson et al. (27), with some modification. Briefly, adipocytes were disrupted by 10 strokes of a Teflon pestle in a glass homogenizing tube. Cell homogenates were centrifuged at 15,900 × g for 15 min at 0–1 °C. All subsequent steps were carried out at 4 °C. The fat cake and supernatant were removed, and the pellet was resuspended a second time in the same buffer and centrifuged at 193,000 × g. The supernatant of the 39,800 × g fraction was centrifuged at 290,000 × g for 60 min. The resultant pellet was resuspended in sucrose-phosphate buffer and centrifuged at 278,000 × g for 90 min. The supernatant of the 39,800 × g centrifugation was further centrifuged at 290,000 × g for 75 min. The pellet was resuspended in sucrose-phosphate buffer and maintained at room temperature unless noted otherwise.

Preparation of Microsomal Membranes—The supernatant of the first centrifugation (15,900 × g) was centrifuged at 39,800 × g for 15 min. The pellet was resuspended in sucrose-phosphate buffer and is referred to as the low density microsomal fraction (LDMF). The LDMF had levels of 5′ nucleotidase specific activity that were between 3.2 and 4.6 times the plasma membrane fraction.

The supernatant of the 39,800 × g centrifugation was further centrifuged at 290,000 × g for 75 min. The pellet was resuspended in sucrose-phosphate buffer and centrifuged at 305,000 × g for 90 min. The resultant pellet was resuspended in the same buffer and is referred to as the low density microsomal fraction (LDMF). The LDMF exhibited as much as a 13-fold increase in the specific activity of UDP-galactose:N-acetylglucosamine galactosyltransferase as compared to the homogenate, 9.5 times greater activity than the plasma membrane fraction and 3.8 times greater activity than the plasma membrane. The LDMF also had as little as one-seventh the specific activity of 5′ nucleotidase as the plasma membrane.

Preparation of a Combined Membrane Fraction—Cell homogenates were centrifuged at 3,440 × g for 5 min at 0–1 °C. After removal of the fat cake, the pellet was resuspended into the supernatant, and the mixture was centrifuged at 302,000 × g for 90 min. The resulting pellet was resuspended in ice-cold sucrose-phosphate buffer.

All samples were quick frozen using a dry ice-acetone bath and stored at −70 °C. Note that for both the transport and affinity-labeling experiments described below, equal amounts of membrane protein from each experimental treatment (based on a Lowry protein assay, Ref. 28) were used, regardless of the total protein yield. No experimental treatment reproducibly altered the protein yield (i.e. deoxymethasone did not cause a reproducible increase or decrease in the yield of plasma membranes, LDMF, LDMF, or total membranes).

We routinely obtained 2–5 mg of protein in the LDMF and HDMF in the plasma membranes.

Measurement of Glucose Transport into Plasma Membranes—Plasma membrane fractions (1–1.5 mg protein/ml) were sonicated for 10 s in a Branson 220 bath sonicator. Membranes were aliquoted (40 μl) and maintained at room temperature unless noted otherwise. Glucose uptake was initiated by the addition of 10 μl of sucrose-Tris buffer containing d-[3H]glucose (0.75 μCi/μl, 250 μM final concentration) and L-[3H]glucose (1.9 μCi/μl, 250 μM final concentration). At various intervals, transport was terminated by addition of 2 ml of ice-cold Krebs-Ringer phosphate buffer, the mixture was rapidly filtered through a 0.45-μm filter (Millipore, HAWP-0025) in a Gelman filter holder attached to a vacuum pump. The filter was washed twice with 2 ml of ice-cold buffer, air dried, and immersed in 4 ml of Atolmig liquid scintillation fluid. Nonspecific trapping and binding were accounted for by subtraction of a “zero” value determined by adding the radioactive and ice-cold stopping buffer simultaneously and filtering immediately. The validity of this approach is supported by the finding that the “zero” value in picomoles of glucose was the same for both D- and L-glucose and for membranes of all experimental conditions, including membranes from cells treated with deoxymethasone or insulin. No treatment appeared to alter the intravesicular content of d-[3H]glucose. The effect of D- and L-glucose was determined from the ratio of d-glucose uptake to L-glucose uptake. The remaining vesicles after 60–120 min of incubation. Representative experiments are shown in which each transport value was determined in triplicate. The error bars indicate the standard error of the mean.

Photoaffinity Labeling of the Glucose Transporter—The hexose transporter was affinity-labeled with [3H]cytochalasin B using modifications of the technique we developed to label the hexose transporter in isolated human erythrocytes (17). Briefly, [3H]cytochalasin B in 100% ethanol was dried under nitrogen and resuspended in sucrose-phosphate buffer at a final concentration of 2.69 μM, immediately prior to use. Solutions of D- and L-glucose at concentrations of 2.5 mM in 10 mM NaH2PO4, 1.0 mM EDTA, pH 7.4. Solutions of unlabelled cytochalasins A, B, D, or E were prepared in sucrose-phosphate buffer at a concentration of 80 μM. Membrane fractions (300–500 μg of protein) were incubated in glass culture tubes (12 × 75 mm) in the presence of 500 mM D-glucose or D-sorbitol for 60 min at room temperature unless noted otherwise. [3H]Cytochalasin B (0.135 μM) was then added, and the mixture sat on ice in the dark for 10 min. Where designated, cytochalasins A, B, D, or E were added simultaneously with [3H]cytochalasin B to give a final concentration of 10 μM. The total volume of the mixture was 200 μl, and the final membrane protein concentration was 1.5–2.5 mg/ml. In some samples the membrane suspensions containing the labeled transporter were added to the reaction mixture after a 1000-watt Portacure lamp (American Ultraviolet Co.) at a distance of 17 cm. Up to 12 samples were irradiated simultaneously. [3H]Cytochalasin B was added to the mixture 2 more times to give concentrations of 0.270 and 0.405 μM, respectively, and the membrane suspensions were incubated and irradiated each time as described above.

The use of three exposure periods was adapted from the method of Kip et al. (22) to label the hexose transporter in cultured muscle cells. Leupeptin (final concentration, 0.1 μg/ml) in 5 mM NaH2PO4 and phenylmethylsulfonyl fluoride (final concentration, 100 μM) in dimethyl sulfoxide were routinely added to the samples after irradiation to protect the labelled vessels prior to electron microscopy, as a precaution, although whether the inhibitors were present or absent did not affect significantly the results of the affinity-labeling experiments.

Several modifications of the labeling procedure were used in an attempt to improve the efficiency and specificity of labeling. These included addition of [3H]cytochalasin B at 0.625 μM followed by only space as energy source to UV light, changing the incubation times with sugar or [3H]cytochalasin B, and addition of dithiothreitol to the membranes prior to the first addition of [3H]cytochalasin B. None of the modifications significantly improved the labeling nor did they alter the relationship between labeling of the transporter in membranes from control versus deoxymethasone-treated cells.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis—The membrane suspensions containing the labeled transporter were adjusted to 1% in sodium lauryl sulfate and 10 mM dithiothreitol and heated at 37 °C for 10 min. The samples were electrophoresed on 9% polyacrylamide slab gels as described by Laemmli (29). Gels were

1 The abbreviations used are: BSA, bovine serum albumin; HDMF, high density microsomal fraction; LDMF, low density microsomal fraction.
stained with Coomassie Blue, destained, and individual lanes were sliced into 1-mm slices using a Hoefer gel slicer. 3 slices were placed in mini-scintillation vials, solubilized overnight with 0.5 ml of Protosol, and 3.5 ml of toluene containing Omnifluor was added for counting.

Enzyme Assays—Protein was determined by the method of Lowry et al. (28) using BSA as a standard. 5’ nucleotidase activity was determined by the method of Avruch and Wallach (30). UDP-galactose:N-acetylglucosamine galactosyltransferase was determined by the method of Bergeron et al. (31).

RESULTS

Hexose Transport in Plasma Membranes Isolated from Dexamethasone-treated Cells—We and others have previously reported that adipocytes treated with dexamethasone exhibit significantly decreased rates of hexose transport (1-4). In order to determine whether this inhibition of transport persists following isolation of the plasma membranes from hormone-treated cells, rat adipocytes were incubated in the absence or presence of a maximally inhibitory concentration of dexamethasone (10⁻⁷ M) (1). Cells were exposed to dexamethasone during the incubation period with collagenase (1 h), during the subsequent washes (20-30 min), and during a 30-60 min incubation period prior to membrane preparation. In this way, cells were exposed to dexamethasone for a total of 110-150 min, a period of time sufficient for dexamethasone to maximally inhibit 3-O-methyl glucose uptake in intact cells (1).

Hexose transport activity into plasma membrane vesicles prepared from hormone-treated cells was assessed by comparing the simultaneous uptake of D-[¹⁴C]glucose (250 μM) and L-[¹⁴H]glucose (250 μM). L-Glucose is a poor substrate for the transporter (32). Its uptake was monitored in order to provide an estimate of the contribution of passive diffusion to the total uptake of D-glucose into these vesicles. The experiment shown in Fig. 1 illustrates that D-glucose transport into vesicles prepared from cells treated with dexamethasone (curve marked DEX) is reduced compared to transport into vesicles prepared from control cells (curve marked CON). The transport of L-glucose into the vesicles was unaltered. When the rate of uptake of D-[¹⁴C]glucose was adjusted to reflect only carrier-mediated transport by subtracting the uptake of L-glucose, the degree of inhibition of D-glucose uptake brought about by pretreatment of cells with dexamethasone was found to be roughly 40% in this experiment. The average degree of transport inhibition in 8 different experiments was found to be 40 ± 7% (S.E.), with a range of inhibition between 20 and 80%. These findings agree very well with our finding that dexamethasone inhibits 3-O-methyl glucose uptake in intact cells between 20 and 80%, with a usual value between 40 and 50%. The ability of dexamethasone to inhibit transport depends upon pretreatment of cells with dexamethasone prior to membrane isolation. When dexamethasone (10⁻⁷ M) was added directly to isolated plasma membranes (curves marked +DEX₎ₐ) instead of the intact cell (curves marked DEX), the rate of glucose transport was not altered (Fig. 1).

The ability of dexamethasone to inhibit hexose transport in intact cells is blocked by the addition of actinomycin D at concentrations equal to or greater than 0.5 μg/ml and by cycloheximide at concentrations equal to or greater than 1 μg/ml (1). In order to test whether the decreased rate of transport observed in isolated plasma membranes reflects the action of dexamethasone via the same mechanism, cells were treated with actinomycin D (2 μg/ml) or cycloheximide (10 μg/ml) alone or in combination with dexamethasone prior to membrane preparation. Fig. 2 illustrates that exposure of cells to actinomycin D with dexamethasone prevents the decreased rate of D-glucose transport found in plasma membrane vesicles from cells treated with dexamethasone alone. Fig. 3 illustrates a similar finding for cycloheximide. Exposure of cells to cycloheximide with dexamethasone also prevents the decreased rate of glucose transport due to dexamethasone.

Identification of the Glucose Transporter in Plasma Membranes by Cytochalasin B Affinity Labeling—To characterize the effect of dexamethasone on glucose transporters in the plasma membrane, we used cytochalasin B affinity labeling...
to identify the glucose transporter. We first tested the feasibility of using this technique to identify the glucose transporter in rat adipocyte plasma membranes by labeling plasma membranes isolated from insulin-treated cells. Insulin is thought to increase the number of glucose transporters in the plasma membrane (25, 27, 33-37), and an increased number of transporters should enhance our ability to detect incorporation of label into the transporter. As can be seen in the curve marked SORB in Fig. 4A, labeling membrane proteins with [3H]cytochalasin B resulted in incorporation of radioactivity into several peaks of protein. Sorbitol was included as a control for osmolarity because it is not transported by the glucose transporter and was not expected to interfere with cytochalasin B binding to, or affinity labeling of, the transporter (data not shown). Replacing the sorbitol with glucose (Fig. 4A, curve marked GLU) or including unlabeled cytochalasins A, B, D, or E in the incubation buffer prior to UV irradiation (Fig. 4B) enabled us to identify the peak associated with the glucose transport protein. D-Glucose, a physiological substrate for the transport protein, prevents the binding of cytochalasin B to the transport protein (34) and would be expected to prevent [3H]cytochalasin B labeling of the transporter. D-Glucose prevented [3H]cytochalasin B labeling of protein primarily in peak C, corresponding to Mr = 54,000 (Fig. 4A). The smaller displacement of peak D was not routinely observed. Unlabeled cytochalasin B added at a concentration between 25- and 70-fold excess over labeled cytochalasin B also prevented incorporation of radioactivity into the protein comprising Peak C (Fig. 4A, curve + CYTO B), as would be predicted from the relatively high affinity of the transporter for cytochalasin B (Kd = 1 x 10^-7 M) (27, 34). The unlabeled cytochalasin B also caused a significant reduction in radioactivity incorporated into peak D but not in the radioactivity associated with other protein peaks, including the top of the separating gel (Peak A) and the dye front (TD) (data not shown).

Cytochalasins A, D, and E are analogues of cytochalasin B which bind with considerably less affinity for the transport protein than cytochalasin B (Ref. 34 and data not shown) and would not be expected to prevent [3H]cytochalasin B labeling of the transport protein. When used at the concentration used for cytochalasin B in Fig. 4A (10 μM), the other cytochalasins had no effect on [3H]cytochalasin B labeling of Peak C (Fig. 4B). However, they did prevent labeling of the protein in Peak D. In order to enhance our ability to identify [3H] cytochalasin B associated with protein in Peak C, the presumed glucose transporter, we blocked labeling of the proteins in Peak D by inclusion of 10 μM cytochalasin A during the [3H]cytochalasin B binding step in subsequent experiments.

To estimate the amount of label incorporated into Peak C, the counts/min in the fractions comprising Peak C were summed, and a correction for background (radioactivity associated with nontransport proteins) was made. Because affinity labeling of the glucose transporter was not significantly
pattern more closely resembled that of the untreated plasma membranes. The d-glucose displacable [\(^{3}H\)]cytochalasin B-labeled protein migrated with the same apparent molecular weight and incorporated the same counts/min/\(\mu\)g of protein (data not shown) in these membranes as in the membranes labeled using the standard protocol, despite the differences in Coomassie Blue staining pattern. This suggests that the apparent molecular size of the glucose transporter is not affected by the irradiation process to the same extent as that of other membrane proteins.

**Affinity Labeling of the Hexose Transporter in Plasma Membranes from Cells Treated with Dexamethasone**—In order to test the effect of dexamethasone on the glucose transporters in the plasma membrane, adipocytes were incubated in the absence or presence of dexamethasone, and plasma membranes were prepared. The membranes were then incubated with sorbitol or glucose and subjected to affinity labeling with [\(^{3}H\)]cytochalasin B. The affinity-labeled transport protein was identified by the sensitivity of the labeling to D-glucose and its location in the gel. When the counts/min incorporated into the transport protein in membranes from control cells were compared to the counts/min in membranes from dexamethasone-treated cells, dexamethasone was found to reduce significantly the radioactivity associated with the transport protein (Table I). While the actual difference in counts/min represented a small number of counts (e.g. 200 cpm/380 \(\mu\)g protein, Table II), the decrease was reproducible. In nine different experiments, the radioactivity in the transport peak of dexamethasone-treated cells was 67 \(\pm\) 4\% of the radioactivity in control cells, representing a 33\% reduction in radioactivity (Method 1, Table I). This decrease is similar in magnitude to the decrease in glucose transport observed in intact cells (1) and isolated plasma membranes (Figs. 1–3).

The above calculations were based on the assumption that all the label in the 'sorbitol' peak is associated with the glucose transporter. This assumption was based on the concept that complete protection by glucose of photolabeling of the transporter would not be expected, because the light-induced covalent reaction of the transporter with [\(^{3}H\)]cytochalasin B is irreversible while the interaction of the transporter with the sugar is reversible. If this assumption is not made and only the counts/min in the transport peak displaceable by D-glucose are summed, dexamethasone was found to decrease the amount of radioactivity in the transport peak to an even greater extent (Method 2, Table I).

**Table I**

<table>
<thead>
<tr>
<th>Membrane fraction</th>
<th>Per cent of control*</th>
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<tbody>
<tr>
<td></td>
<td>Method 1(^{b})</td>
</tr>
<tr>
<td>Plasma membranes</td>
<td>67 (\pm) 4 (n = 9)</td>
</tr>
<tr>
<td>LDMF</td>
<td>100 (\pm) 5 (n = 16)</td>
</tr>
<tr>
<td>Total cellular membranes</td>
<td>98 (\pm) 1 (n = 2)</td>
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\(^{a}\) (cpm, dexamethasone-treated cells)/(cpm, control cells) \(\times\) 100.

\(^{b}\) The counts/min in the glucose transporter peak were calculated by subtracting background counts/min from the counts/min in the peak labeled in the presence of sorbitol. The average % of control \(\pm\) S.E. for \(n\) different experiments is shown.

\(^{c}\) The counts/min in the glucose transporter peak were calculated by subtracting the counts/min in the peak in the presence of D-glucose (corrected for background) from the counts/min in the peak in the presence of D-sorbitol (corrected for background).
Actinomycin D prevents the dexamethasone-induced inhibition of \( ^{3}H \)cytochalasin B incorporation into the glucose transporter in plasma membranes

<table>
<thead>
<tr>
<th></th>
<th>Counts/min*</th>
<th>Per cent of control*</th>
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<tbody>
<tr>
<td>Control</td>
<td>503</td>
<td>100</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>308</td>
<td>61 ± 2</td>
</tr>
<tr>
<td>Actinomycin D</td>
<td>533</td>
<td>89 ± 13</td>
</tr>
<tr>
<td>Dexamethasone + actinomycin D</td>
<td>538</td>
<td>107 ± 16</td>
</tr>
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* Portions (350 µg) of the plasma membranes used in Fig. 2 were subjected to affinity labeling as described under "Experimental Procedures." The counts/min in the transporter peak were calculated using Method 1 as described under "Results" and in Table I. The results are given for a representative experiment.

The mean per cent of control ± S.E. is given for three separate experiments, including the representative experiment. Two of the experiments were performed as described in Footnote a. The third experiment used a higher concentration of actinomycin D (5 µg/ml), 30 min less incubation with reagents, and a modified affinity-labeling technique (10-min incubation with sugars followed by 30-min incubation with 0.625 µM \( ^{3}H \)cytochalasin B, and one 10-s exposure to UV light).

If the decrease in affinity labeling in plasma membranes in response to dexamethasone relates to the decreased rate of transport observed in intact cells (1) and isolated plasma membranes (Fig. 2), then addition of actinomycin D to the cells with dexamethasone would be expected to prevent this decrease. Table II shows the calculated cpm in the \( d \)-glucose transporter peak in the plasma membrane fractions from cells treated in the presence or absence of dexamethasone and actinomycin D. Plasma membranes from the same preparation were tested for transport capability as shown previously in Fig. 2. In this representative experiment, labeling of the transporter in membranes from dexamethasone-treated cells was 61% of the labeling in membranes from control cells. This decreased labeling was prevented by actinomycin D. Similar results were obtained in two other experiments as shown in Table II.

It should be noted that the affinity-labeled transporter in membranes from both control and dexamethasone-treated cells migrated to the identical position on sodium dodecyl sulfate-polyacrylamide gels and spanned the same number of gel slices (data not shown). Thus, if dexamethasone changes the structure of the transport protein, it either changes the structure in such a way that differences in its migration properties cannot be detected in our gel system and/or it changes the ability of the transporter to bind to and/or be labeled with \( ^{3}H \)cytochalasin B.

Identification of the Glucose Transporter in LDMF and the Effect of Dexamethasone—Numerous groups, including our own, have isolated a cellular membrane fraction from rat adipocytes which contains a larger number of transport proteins than the plasma membrane (25, 27, 33-37). Transport proteins in this fraction were originally identified using cytochalasin B binding (19, 33, 36) and reconstitution assays (27, 34, 37). This membrane fraction, which we refer to as the LDMF, contains both a larger total number of transport proteins and a larger number of transporters per mg of membrane protein than does the plasma membrane. It has been hypothesized that insulin increases glucose transport in rat adipocytes by "recruiting" transporters from this cellular LDMF pool of transporters to the plasma membrane (27, 34-37). Furthermore, in several cases of insulin resistance, the cause of the reduced number of transporters in the plasma membrane in insulin-treated rat adipocytes appears to be a greatly reduced number of transporters in the LDMF (38, 39). Therefore, it was of interest both to identify the glucose transporter in the LDMF fraction using the affinity-labeling technique and to see if dexamethasone treatment substantially decreased the amount of affinity-labeled transporter in this fraction.

To verify that the glucose transporter in the LDMF migrated with the same molecular weight as the transporter in plasma membranes, proteins in the LDMF were affinity labeled with \( ^{3}H \)cytochalasin B. The LDMF used for the experiment shown in Fig. 6 was isolated from the same cells as the plasma membranes used for the experiment shown in Fig. 4. Only one peak of radioactivity (Peak C) was displaced by both \( d \)-glucose and 10 µM unlabeled cytochalasin B (Fig. 6A) but not by 10 µM cytochalasin A, D, or E (Fig. 6B). This peak migrated with the same apparent molecular weight as the

![Fig. 6. Identification of the glucose transporter in LDMF using \( ^{3}H \)cytochalasin B affinity labeling. Following isolation and washing, rat adipocytes were incubated for 15 min before addition of 1 milliunit/ml insulin for an additional 30 min, as described for Fig. 4. LDMF were isolated and 500 µg of membrane protein in a total volume of 200 µl was incubated in: panel A, 500 mM \( d \)-glucose (C) or 500 mM D-sorbitol in the absence (C) or presence (X) of 10 µM unlabeled cytochalasin B; or panel B, 500 mM D-sorbitol in the absence (C) or presence of 10 µM unlabeled cytochalasin A (A), cytochalasin D (V), or cytochalasin E (E). LDMF were then subjected to affinity labeling with \( ^{3}H \)cytochalasin B as described under "Experimental Procedures," and the samples were electrophoresed. The first two fractions are the stacking gel, and the dye front is denoted by TD. The migration pattern of molecular weight markers is noted on the top of A.
protein identified as the transport protein in the plasma membranes isolated from the same cells.

To test whether dexamethasone causes the amount of affinity-labeled transporter to increase in the LDMF, LDMFs were isolated from both control and dexamethasone-treated adipocytes. The membranes were affinity labeled with \[ ^3H \] cytochalasin B in the identical manner as the plasma membranes. Dexmethasone was found to cause no significant change in the amount of radioactivity associated with the transporter (Table I). The average ratio of labeled transporter in the LDMF of dexamethasone-treated cells compared to control cells from 16 different experiments was 1.00 ± 0.05. It should be noted that the amount of radioactivity incorporated into the glucose transport peak in the LDMF from control cells was significantly greater than that incorporated into the glucose transport peak in plasma membranes on a per mg of protein basis (5.40 ± 0.40, n = 14, for LDMF compared to 2.06 ± 0.29, n = 9, for plasma membranes). The amount of radioactivity associated with the transporter was even greater based on total membrane protein because the protein yield of LDMF was 1.5–2.5 times greater than that of plasma membranes.

Affinity Labeling of the Hexose Transporter in a Total Cellular Membrane Fraction—To further verify that the decrease in affinity labeling of the transporter in the plasma membranes of dexamethasone-treated cells is not a reflection of a similar decrease in the total cellular pool of transporters, a membrane fraction that contained essentially all cellular membranes was prepared from control and dexamethasone-treated cells as described under “Experimental Procedures.” Aliquots of each “total” membrane fraction were incubated with either D-glucose or D-sorbitol and subjected to affinity labeling with \[ ^3H \] cytochalasin B. As was observed with the LDMF, the amount of radioactivity associated with the transport protein peak in this total membrane fraction was similar in membranes prepared from control or dexamethasone-treated cells (Table I). Thus, the decrease in the amount of \[ ^3H \] cytochalasin B incorporated into glucose transporters in plasma membranes isolated from dexamethasone-treated cells was not accompanied by a similar percentage decrease in the overall amount of \[ ^3H \] cytochalasin B labeling of transporters in the cell.

In a similar series of affinity-labeling experiments, the remaining microsomal fraction which is enriched in marker enzymes for the endoplasmic reticulum (Refs. 27 and 37 and data not shown) and is referred to as the HDMF, was tested for the presence of the transporter as well as the influence of dexamethasone on the degree of affinity labeling. The degree of labeling was relatively small in this fraction, and in two out of three experiments, dexamethasone was found to slightly decrease the amount of affinity-labeled transporter (data not shown). This decrease is believed to be associated with plasma membrane present in this fraction as judged by the presence of 5’ nucleotidase activity.

DISCUSSION

Identity of the Cytochalasin B Affinity-labeled Proteins—When we affinity labeled protein in rat adipocyte membranes using \[ ^3H \] cytochalasin B, only one peak of radioactivity (\( M_r = 54,000 \)) was identified as the glucose transport protein, based on competition by a high concentration of D-glucose and unlabeled cytochalasin B. The fact that D-sorbitol or unlabeled cytochalasins A, D, or E did not affect labeling of the glucose transport protein verifies the identity of the glucose transporter, since sorbitol is not a substrate for the transporter and the cytochalasins have very low affinity for the transporter. Further support for the identity of the glucose transporter is provided by our finding that the amount of labeling is increased in plasma membranes of insulin-treated cells, whereas the amount of labeling is decreased in the LDMF fraction of insulin-treated cells.\(^ 3 \) Oka and Czech (25) recently reported a similar finding using a similar labeling protocol. Our value of \( M_r = 54,000 \) for the transporter compares to a value of 45,000–50,000 daltons reported by Shanhavan et al. (21) and Oka and Czech (25) using similar affinity-labeling techniques and Wheeler et al. (40) and Lienhard et al. (41) using Western blotting with antibodies produced in rabbits immunized with greatly purified human erythrocyte glucose transporter. In contrast, Troper and Levy (42) and Malchoff et al. (43) have synthesized affinity probes that identify glucose transporters of larger molecular weight (80,000–100,000). The explanation for these different molecular weights is not readily apparent. The suggestion has been made that the \( M_r = 45,000–55,000 \) form of the glucose transporter identified in human erythrocyte membranes is a proteolytic product of the higher molecular weight form of the glucose transporter (44). Although we cannot rule out this possibility in our affinity-labeling experiments, we have never observed significant glucose-sensitive \[ ^3H \] cytochalasin B labeling of any protein having an apparent molecular weight greater than 55,000, even when membranes were labeled 1 day after being prepared.

Proteins other than the glucose transporter are clearly labeled in rat adipocyte membranes by \[ ^3H \] cytochalasin B. The labeling properties of the protein comprising the peak to the left of the transporter (Peak B, Figs. 4A and 5A) and its migration in the gel are consistent with those of serum albumin, suggesting it may be labeled rat albumin or BSA that was added to the cell isolation and incubation buffers. The labeling properties of Peak D in Figs. 4A and 5A are consistent with the properties of actin. Like the protein in Peak D, actin has a higher affinity for cytochalasins A, D, and E than for cytochalasin B (45) and is capable of being affinity labeled by \[ ^3H \] cytochalasin B in a non-D-glucose-displaceable manner (17, 20). Peak D incorporated more radioactivity in the plasma membranes than in the LDMF, as would be expected for a protein associated with the cytoskeleton, such as actin.

Effect of Dexamethasone on Glucose Transport and Labeling of the Transporter—These studies show that dexamethasone, when added to isolated rat adipocytes, causes a significant decrease in glucose transport activity in plasma membranes subsequently isolated. This suggests that a stable change in the number or activity of transporters in the plasma membrane has occurred. This decrease in glucose transport caused by dexamethasone was found to be associated with a significant decrease in the amount of affinity-labeled transporter in the plasma membrane. Because cytochalasin B-binding experiments have identified numerous instances in which the number of cytochalasin B-binding sites have been found to be altered in rat adipocyte membranes (34, 38, 39), we would predict that the decrease in labeling results from a decreased number of transporters. However, because \[ ^3H \] cytochalasin B incorporation was measured using only one concentration of \[ ^3H \] cytochalasin B, the decrease in counts/min can be used only to indicate that decreased labeling occurs and cannot be used to determine an absolute decrease in the number of transporters. Furthermore, our data cannot rule out the possibility that dexamethasone causes a conformational change in the glucose transporter which results in its having a greatly reduced affinity for \[ ^3H \] cytochalasin B or reduced ability for

\(^ 3 \) C. Carter-Su and K. Okamoto, manuscript in preparation.
UV light to cause its covalent labeling by [3H]cytochalasin B. Although we would predict it to be difficult to determine shifts in the K_{d} using this methodology because of the low level of labeling of the transporter in plasma membranes imposed upon a rather high background, our experience indicates that the present labeling procedure is more sensitive to small changes cytochalasin B binding than a binding assay per se because it physically separates the glucose transporter from other proteins that bind cytochalasin B. The difficulty in monitoring changes in the number or affinity of transporters in the plasma membrane using a cytochalasin B-binding assay has been noted by Smith et al. (46). These investigators suggested that a lack of sensitivity of the assay rather than an absence of change accounted for the fact that they saw a decrease in the number of cytochalasin B-binding sites in microsomal membranes but not in the plasma membranes of adipocytes treated with isoproterenol in combination with adenosine deaminase and dibutyryl cAMP (46).

The decrease in labeled transporter in the plasma membrane was not accompanied by a similar percentage change in the labeled transporter in either the LDMF or a total cellular membrane fraction. The initial rate of glucose transporter labeling in the plasma membrane may be distinct to glucocorticoid-induced inhibition of transport. In other situations in which glucose transport is decreased in rat adipocytes, such as by addition of isoproterenol in combination with adenosine deaminase and dibutyryl cAMP (46) or by fasting (47), the decrease in transport appears to be accompanied by a decrease in transporters in the LDMF.

Using the proposed mechanism of action of insulin as a model system for how cells can rather rapidly alter rates of glucose transport (34-37), it is intriguing to speculate that dexamethasone acts by causing the synthesis of some protein which in turn causes transporters to shift from the plasma membrane to the LDMF. Thus, a decrease in transport appears to be accompanied by a decrease in transporters in the LDMF. When one would predict to occur. This calculation results in the observation that the number of transporters in the plasma membrane represents approximately 10% of the transporters in the LDMF. Thus, a 40% decrease in the number of transporters in the plasma membrane would represent an increase of less than 5% of the transporters in the LDMF, which would probably not be detectable in our system.