Biochemical and Functional Heterogeneity of Rat Adipocyte Glucose Transporters*

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We have studied the biochemical mechanism of insulin action on glucose transport in the rat adipocyte. Plasma membranes and low-density microsomes were prepared by differential ultracentrifugation of basal and insulin-stimulated cells. The photochemical cross-linking agent hydroxyxysuccinimidyl-4-azidobenzoate was used to covalently bind [3H]cytochalasin B to the glucose transporter which migrated as a 45–50-kDa protein on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Isoelectric focusing of the eluted 40–55-kDa proteins revealed two peaks of D-glucose-inhibitable [3H]cytochalasin B radioactivity focusing at pH 6.4 and 5.6 when low-density microsomes were used as the starting material. In contrast, only one D-glucose inhibitable peak, focusing at pH 5.6, was found in plasma membranes. Pretreatment of the cells with insulin led to a marked redistribution of the pH 5.6 form of the glucose transporter from low-density microsomes to plasma membranes with no effect on the pH 6.4 form of the glucose transporter. Following isolation from the isoelectric focusing and sodium dodecyl sulfate-polyacrylamide gels, both glucose transporter isoforms were shown to cross-react with an antiserum raised against the purified human erythrocyte glucose transporter. Following incubation of [3H]cytochalasin B-labeled low-density microsomal and plasma membranes with neuraminidase, the pH 5.6 transporter isoform was shifted on isoelectric focusing to a more basic pH, while the pH 6.4 isoform was not affected.

These data demonstrate that: 1) there is a heterogeneity of glucose transporter species in the intracellular pool while the plasma membrane transporters are more uniform in structure. 2) The pH 5.6 glucose transporter isoform is translocated by insulin from the low-density microsome to the plasma membrane but the pH 6.4 isoform is not sensitive to insulin. 3) Differential sensitivity of the glucose transporter isoforms to neuraminidase suggests that the heterogeneity is at least partially due to differences in glycosylation state.

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There is now a considerable body of evidence in support of the notion that insulin stimulates glucose transport in isolated rat adipocytes by inducing a rapid and reversible translocation of glucose transporters from an intracellular membrane pool to the plasma membrane (1–5). This insulin-induced translocation of hexose carriers occurs via an energy-dependent process (6, 7) probably involving divalent metal cations (8) and sulfhydryl groups (9, 10). Although the studies described above have greatly aided our understanding of the cellular mechanism of insulin action on glucose transport, the biochemical events that accompany these processes are still largely unknown. Major impediments in this area of research have been the relative lack of information on the molecular properties of the glucose transporter due mainly to the unavailability of a suitable probe of sufficient affinity and specificity with which to label the protein. Recently, some of these problems have been alleviated with the discovery that cytochalasin B, a reversible competitive inhibitor of glucose transport (11), can be covalently attached to the glucose transporter either by direct or indirect photochemical reactions (12–14). Using these techniques several groups have shown that the glucose transporter migrates in a relatively broad band on SDS-PAGE with a molecular mass of 45–50 kDa (12–14).

In an earlier report (14) we showed that [3H]cytochalasin B could be chemically cross-linked to adipose cell membrane fractions and specifically label the glucose transporter. Using this approach, the current studies reveal that the glucose transporters in the intracellular pool exist in at least two molecular forms with different isoelectric points, whereas plasma membranes contain only one of these forms. Furthermore, only one of the glucose transporter isoforms in low density microsomes is translocated by insulin. Finally, these studies reveal significant differences in the glycoprotein nature of these glucose transporter isoforms.

EXPERIMENTAL PROCEDURES

Materials—Male Sprague-Dawley rats (140–170 g) were obtained from Charles River Laboratories. Animals were fed laboratory chow ad libitum and were killed by cervical dislocation. [3H]Cytochalasin B (specific activity 10–15 Ci/mmol) was obtained from New England Nuclear. Carrier ampholytes and all other reagents for isoelectric focusing and electrophoresis were from Bio-Rad. Crude collagenase (Type I) was from Worthington, bovine serum albumin, fraction V, was from Reheis Chemical Co. Tris and all other reagent grade chemicals were from Sigma.

Isolation of Adipocytes, Preparation of Subcellular Fractions, and Determination of the Number of Glucose Transporters—Isolated adipocytes were obtained by enzymic digestion of epididymal fat pads.
from rats as described by Rodbell (15) and modified by Cushman and Wardzala (2). Subcellular fractions from isolated adipocytes were prepared by differential ultracentrifugation as outlined by Karnieli et al. (7). Protein was determined by the method of Lowry et al. (16), as modified by Peterson (17). Marker enzyme assays were as previously described (20). The number of D-glucose-inhibitable cytochalasin B binding sites in the subcellular fractions was determined as previously described (7).

**Photochemical Cross-linking of Membranes and SDS-PAGE—** Membrane fractions prepared from rat adipocytes were resuspended in 50 mM phosphate buffer (pH 7.4), to a final concentration of 1–5 mg/ml membrane protein/ml. After incubation for 10 min at 4°C with [3H]cytochalasin B (10 μM) the membranes were photochemically cross-linked with hydroxy-succinimidyl-4-azidobenzoate (400 μM) as previously described (14). Experiments involving D- or L-glucose were carried out as described above except that the sugar (500 μM) was incubated with the membranes for 30 min prior to the addition of cytochalasin B.

Membranes covalently cross-linked to [3H]cytochalasin B were solubilized by boiling for 5 min in Laemmli sample buffer (18). The solubilized membranes were then centrifuged (100,000 × g for 15 min) and the supernatant was analyzed by SDS-PAGE on 5-mm (internal diameter) cylindrical gels or on 1.5-mm slab gels. Resolving gels of 9 or 10% polyacrylamide were used. Electrophoresis was carried out at 3 mA/gel for the cylindrical gels and at 30 mA/gel for the slab gels. After electrophoresis, the cylindrical gels were serially sliced with a Hoefer vibrating gel slicer. The slices were incubated in 20-ml scintillation vials with 1 ml of NCS (Amersham Corp.) overnight at 60°C. The solubilized gel slices were cooled, 10 ml of scintillation fluid 3a20 (Research Products International) and 50 μl of acetic acid were added, and the vials counted in a scintillation counter. The radioactive profile obtained was used as a guide to locate the position of the glucose transporter in duplicate gels. Similar procedures were carried out in slab gels. Prestained molecular weight marker proteins (Bethesda Research Laboratories) were used to estimate molecular weight of the [3H]-labeled membrane proteins. Proteins corresponding to a molecular mass of 40–55 kDa were excised from the slab gels and electrophoretically extracted as described by Hunkapiller et al. (19).

**Isoelectric Focusing—** The extracted and concentrated SDS-solubilized glucose transporter was subjected to isoelectric focusing on cylindrical polyacrylamide gels. Gel composition and isoelectric focusing conditions were as described previously (20). Duplicate gels were run; one was processed for scintillation counting as above, while the other was used for SDS-PAGE. Gel composition and isoelectric focusing were as described above except that the sugar (500 mM) was present in the buffer. Membrane fractions prepared low-density microsomes and plasma membranes were analyzed by isoelectric focusing. The first dimension was focused with 50 mM phosphate buffer (pH 7.4), while the second dimension focused at pH 6.4, 5.6, respectively, were discernible in the low-density microsomes but only one peak focusing at pH 5.6 was observed in the plasma membranes. Preincubation of these membranes with 500 mM D-glucose reduced the labeling of these peaks by 70–80%. This preferential inhibition of cytochalasin B binding of the protein is consistent with the stereoselectivity of the glucose transporter for D-glucose (24). These data thus strongly suggest the presence of at least two glucose transporters in low-density microsomes but only one in plasma membranes.

In order to test whether insulin had any effect on the structure or distribution of the glucose transporter isoforms described above, we incubated adipose cells with insulin and prepared low-density microsomes and plasma membranes from these cells. The low-density microsomes and plasma membranes from insulin-treated cells were then photolabeled with [3H]cytochalasin B and analyzed by two-dimensional electrophoresis. As shown in Fig. 3, A and B, when the 40–55-kDa radiolabeled membrane proteins eluted from the SDS gel were analyzed by isoelectric focusing, two major peaks of radioactivity, focusing at pH 6.4 and 5.6, respectively, were found in the low-density microsomes. Only one peak, focusing at pH 5.6 was observed in the plasma membranes. This pattern of glucose transporter isoforms observed in membranes from insulin-treated cells is identical to that from membranes derived from basal cells; thus, insulin treatment

**RESULTS**

Rat adipocyte subcellular membrane fractions were covalently labeled with [3H]cytochalasin B and the radiolabeled membrane proteins in each of these fractions were separated and analyzed by two-dimensional electrophoresis. The first phase of this separation involved SDS-PAGE. A typical SDS-PAGE profile for [3H]cytochalasin B-labeled plasma membranes is shown in Fig. 1. Here we see that the rat adipocyte glucose transporter resolves on SDS-PAGE as a protein of molecular mass 45–55 kDa, as assessed by D-glucose-inhibitable [3H]cytochalasin B radioactivity. Following SDS-PAGE, proteins of molecular mass 40–55 kDa (a molecular mass range which encompasses the glucose transporter) were excised and electrophoretically eluted from the gel slices. Recovery of [3H]cytochalasin B counts from the gel slices was routinely monitored by addition of a known amount of 125I-labeled membrane protein. Typically, recoveries of around 70–75% were obtained.

As shown in Fig. 2, A and B, when the 40–55-kDa radiolabeled membrane proteins eluted from basal low-density microsomes and plasma membranes were analyzed by isoelectric focusing, two major peaks of radioactivity, focusing at pH 6.4 and 5.6, respectively, were discernible in the low-density microsomes but only one peak focusing at pH 5.6 was observed in the plasma membranes. Preincubation of these membranes with 500 mM D-glucose reduced the labeling of these peaks by 70–80%. This preferential inhibition of cytochalasin B binding of the protein is consistent with the stereoselectivity of the glucose transporter for D-glucose (24). These data thus strongly suggest the presence of at least two glucose transporters in low-density microsomes but only one in plasma membranes.

**Neuraminidase Treatment of Membranes—** [3H]Cytochalasin B-labeled low-density microsomes and plasma membranes (300–500 μg) were incubated with 1 unit of neuraminidase (Sigma) in 1 ml of 100 mM acetate buffer (pH 6.0) containing the protease inhibitors pepstatin (5 μg/ml), leupeptin (5 μg/ml), aprotinin (6 μg/ml), and 1:10 diluted soybean trypsin inhibitor (1 mg/ml). After 2 h at 37°C, samples were prepared for SDS-PAGE as already described.

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of adipose cells does not appear to structurally modify the glucose transporter in a way that can be detected by isoelectric focusing. Although insulin did not appear to influence the heterogeneity of glucose transporter isoforms the hormone did have an effect on their subcellular distribution. For example insulin induced an increase in the pH 5.6 isoform in the plasma membranes together with a concomitant decrease in the pH 5.6 isoform in the low-density microsomes; however, insulin did not alter the distribution of the pH 6.4 isoform in the low-density microsomes (compare Figs. 2 and 3). This is made clearer in Fig. 4 which shows the D-glucose inhibitable \[^{[H]}\text{cytochalasin B}\] covalently incorporated into the glucose transporter isoforms. There is a 65% reduction in the incorporation of \[^{[H]}\text{cytochalasin B}\] into the pH 5.6 isoform of the glucose transporter in low-density microsomes prepared from insulin-treated compared to basal cells (Fig. 4). In contrast, the distribution of the pH 6.4 glucose transporter isoform in low density microsomes is not affected by insulin pretreatment of adipocytes. Taken together, insulin induced a 41% decrease in the total number of glucose transporters in low-density microsomes as assessed by combining the D-glucose-inhibitable \[^{[H]}\text{cytochalasin B}\] counts in the pH 6.4 and 5.6 isoforms. This decrease in glucose transporter number correlates well with the 45% decrease in the number of glucose transporters in the same membrane fractions as assessed by the more conventional cytochalasin B binding assay (7), Fig. 5). Furthermore, insulin pretreatment of adipocytes resulted in a 4.8-fold increase in the incorporation of \[^{[H]}\text{cytochalasin B}\] in the pH 5.6 isoform.
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B into the pH 5.6 glucose transporter isoform in insulin plasma membranes compared to basal plasma membranes (Fig. 4). This increase in glucose transporter number in plasma membranes from insulin-treated cells correlates well with a similar increase (4.5-fold) measured in the same membranes by the D-glucose-inhibitable cytochalasin B binding assay (2).

As a further means of testing for glucose transporter heterogeneity in rat adipocyte membrane fractions we analyzed the subcellular fractions with an antiserum prepared against the purified human erythrocyte glucose transporter which is cross-reactive with SDS-treated adipocyte glucose transporter (23). Basal low-density microsomes and insulin plasma membranes were used for these studies since they contain the largest number of glucose transporters (2). First we repeated the isoelectric focusing of [3H]cytochalasin B-labeled low-density microsomes and plasma membranes on a preparative scale as described under "Experimental Procedures." Next we isolated proteins from each of the fractions across the entire pH spectrum of the isoelectric focusing gel. When the eluted proteins from these fractions are rerun on SDS-PAGE, transferred via electrophoretic blotting to nitrocellulose, and reacted with antibodies raised against the human erythrocyte glucose transporter (Fig. 6A and B), the pH 6.4 and 5.6 peaks are readily identified from low-density microsomes, whereas the 5.6 (but not 6.4) peak is identified in plasma membranes.

These different charge properties could correspond to covalent modification of the glucose transporter such as differences in glycosylation and/or phosphorylation. To assess the possibility of differential sialylation of the glucose transporters we incubated photolabeled low-density microsomes and plasma membranes with or without neuraminidase as described under "Experimental Procedures." In a preliminary experiment we ran these membranes on an SDS gel and observed a 2–3 kDa decrease in molecular mass of the glucose transporter in the neuraminidase-treated compared to untreated membranes (data not shown). The membranes were then resolved by two-dimensional electrophoresis. As shown in Fig. 7 treatment of photolabeled membranes with neuraminidase altered the isoelectric focusing profile of the two dimensionally separated [3H]cytochalasin B-labeled proteins of low-density microsomes (panel A) and plasma membranes (panel B). In both cases neuraminidase cleavage of membranes generated a new glucose transporter isoform focusing at pH 6.0. At the same time the amount of pH 5.6 transporter isoform was considerably reduced while the pH 6.4 isoform was not affected by the neuraminidase digestion. This neuraminidase-induced alkaline pH shift in the photolabeled pH 5.6 glucose transporter isoform is consistent with the removal of charged sialic acid residues from the transporter by the enzyme. Thus the charge heterogeneity observed in the two isoforms can at least be partially ascribed to differences in sialylation. Neuraminidase had no effect on the D-glucose-uninhibitable [3H]cytochalasin B-labeled protein profile and for reasons of clarity these data were omitted from Fig. 7.

To eliminate the possibility that low-density microsomal membranes might contain a specific protease capable of enzymatically converting the pH 5.6 isoform to the pH 6.4 isoform, or that plasma membranes might contain a specific protease capable of degrading the pH 6.4 isoform we incubated [3H]cytochalasin B-labeled low-density microsomes with unlabeled plasma membranes and [3H]cytochalasin B-labeled plasma membranes with unlabeled low-density microsomes, respectively. Since we were unable to detect differences in the [3H]cytochalasin B profile of the labeled membranes under either condition (data not shown) we ruled out the possibility that the two glucose transporter isoforms were generated by nonspecific proteolysis.

**FIG. 4.** D-Glucose-inhibitable [3H]cytochalasin B incorporated into low-density microsome and plasma membrane glucose transporter isoforms after isoelectric focusing. Cytochalasin B counts refer to specific incorporation of [3H]cytochalasin B into the pH 5.6 and 6.4 glucose transporter isoforms (see Figs. 3 and 6) obtained by subtracting the D-glucose-insensitive counts from the total counts under each pH peak.

**FIG. 5.** Distribution of glucose transporters in low-density microsomes and plasma membranes from rat adipocytes incubated with and without insulin. Binding sites have been assessed by D-glucose-inhibitable cytochalasin B binding assay (2).

**FIG. 6.** Immunological detection of the rat adipocyte glucose transporter. Top, basal low-density microsomal (LDM) membrane proteins and bottom, insulin plasma membrane (PM) proteins were fractionated by preparative isoelectric focusing and eluted as described previously (14). The eluted proteins were analyzed by SDS-PAGE on 9% resolving gels, and transformed to nitrocellulose. The D-glucose-insensitive counts from the total counts under each pH peak. Binding sites have been assessed by D-glucose-inhibitable cytochalasin B binding assay (2).
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**FIG. 7.** Isoelectric focusing of the neuraminidase-treated and untreated SDS-PAGE-purified photoaffinity-labeled glucose transporters. [3H]Cytochalasin B labeled (A) basal low-density microsomes and (B) insulin plasma membranes were incubated with (○) and without (●) neuraminidase as described under “Experimental Procedures.” The membranes were solubilized and subjected to SDS-PAGE, proteins migrating at 40-55 kDa were excised from the gel and after elution, were subjected to isoelectric focusing as described previously (14). Arrows indicate the isoelectric points of the focused protein peaks. ▲, pH.

**DISCUSSION**

Our major goal in initiating these studies was to gain insight into the mechanism of insulin activation of glucose transport at the biochemical level. Thus, we have identified and characterized glucose transporter isoforms in the rat adipocyte plasma membrane and low-density microsomal fractions using a combination of [3H]cytochalasin B photochemical cross-linking followed by two-dimensional electrophoresis. The results show that the basal low-density microsome glucose transporter is a structurally diverse protein existing as a mixed population of conformers with isoelectric points of pH 6.4 and 5.6 (Figs. 2 and 6). In contrast, the plasma membrane glucose transporter is structurally homogeneous on isoelectric focusing and only a single isoform focusing at pH 5.6 was detected.

We consider it extremely unlikely that the glucose transporter isoforms observed here (Fig. 2) could have been generated by partial proteolysis of the molecule, particularly since only one isoform is found in plasma membranes and partial proteolysis would have been expected to generate multiple forms of transporter in this membrane fraction also. Furthermore, when low-density microsomes were mixed with plasma membranes or plasma membranes with low-density microsomes the radioactive profiles were the same, indicating no protease activity in the low-density microsomes which generates pH 6.4 isoform from pH 5.6 and no protease activity in the plasma membranes which destroys the pH 6.4 isoform. Since fat cell plasma membranes prepared by the subcellular fractionation scheme used here (25) are contaminated with small amounts of low-density microsomes (less than 10%), one might expect that some pH 6.4 isoform would be detected in the plasma membranes after isoelectric focusing. However, even 10% contamination of plasma membranes with low-density microsomes would only yield [3H]cytochalasin B radioactivity in the pH 6.4 peak below background and therefore would not be detected.

More likely the existence of glucose transporter isoforms is explained by covalent modification of the molecule such as differences in the glycosylation state and/or phosphorylation.

It is known, for example that the human erythrocyte glucose transporter is a glycoprotein containing some 5%, by weight, sialic acid (26). This fact, together with the well documented evidence in the literature for charge heterogeneity in proteins involving sialic acid (27-29), led us to consider the possibility that the glucose transporter isoforms observed above could correspond to a non-uniform distribution of sialic acid residues on the transporter. To test for this possibility we incubated photolabeled low-density microsomes and plasma membranes with neuraminidase. Digestion of these fractions with neuraminidase did not affect the pH 6.4 isoform (Fig. 7) suggesting either that this isolectric variant did not contain sialic acid residues or that if it did they were not accessible to the enzyme. It might be argued that neuraminidase does indeed convert both 5.6 and 6.4 glucose transporter isoforms; however, the almost identical recovery of [3H]cytochalasin B counts in the treated versus untreated membranes together with the absence of any pH shifting of the pH 6.4 isoform militates against this view. As reported for the insulin receptor and other glycoproteins (30, 31) some sialic acid moieties are resistant to cleavage with neuraminidase due to their inaccessibility. In contrast to the pH 6.4 isoform, neuraminidase treatment almost totally converted the pH 5.6 glucose transporter isoform to a more basic form focusing at pH 6.0 (Fig. 7). This pH shift is consistent with the cleavage of acidic sialic acid moieties from the molecule. It is extremely unlikely that the pH shift induced by neuraminidase-treatment of photolabeled membranes is due to cleavage by contaminating proteases in the neuraminidase, since the reactions were carried out in the presence of several protease inhibitors (see “Experimental Procedures”). Furthermore, only the pH 5.6 peak is affected by enzymatic treatment (the pH 6.4 isoform is resistant to enzymatic treatment) and contaminating proteases would be expected to degrade both isoforms equally. The fact that neuraminidase treatment did not shift the pH 5.6 isoform all
the way to pH 6.4 indicates either that there are still some neuraminidase inaccessible sialic acid residues present on the molecule or that the charge differences remaining can be ascribed to other post-translational modifications, such as phosphorylation or acetylation.

Based on these studies, we speculate that the pH 6.4 glucose transporter isoform represents an immature species which is converted to the mature pH 5.6 form by differential glycosylation probably involving terminal capping with sialic acid residues. The functional significance of this differential sialylation is unclear. It seems unlikely that sialylation affects glucose transport activity, since preliminary data (not shown) suggests that neuraminidase treatment of plasma membranes did not affect 3-O-methylglucose uptake by membrane vesicles. More likely the differences in glycosylation might play an important role in glucose transporter translocation or intracellular processing, or both. However, whether those post-translational modifications are critical to glucose transporter activity and structure will have to remain a subject for further research.

Of particular significance is the finding that insulin alters the distribution of glucose transporter isoforms in the adipose cell. We have shown that pretreatment of adipose cells with insulin leads to the translocation of the pH 5.6 glucose transporter isoform from low-density microsomes to the plasma membrane (Figs. 2 and 3). The magnitude of this effect of fully equivalent to that detected by the cytochalasin B binding assay in the same membranes (Fig. 5), which presumably is a measure of total transporter number. The pH 6.4 glucose transporter isoform does not appear to be translocated by insulin. It is possible, however, that insulin mediates the conversion of the pH 6.4 glucose transporter to the pH 5.6 glucose transporter which can then be translocated to the plasma membrane in this form. Whether these pH variants are all active transporters capable of mediating D-glucose transport into the cell remains to be answered.

The ability of insulin to cause recruitment of the pH 5.6 isoform but not the pH 6.4 isoform is an important finding and one that merits further investigation. It is tempting to speculate that the pH 6.4 isoform could be in a separate intracellular pool from its pH 5.6 counterpart and thus not be available for export to the cell surface. The movement of hexose carriers from an inert intracellular pool to a more active one involved in the mainstream of mediating glucose transport could in part be dictated by the differences in glycosylation of the isoforms. The biochemical events involved in mediating the conversion of the pH 6.4 isoform to the pH 5.6 isoform in the cell needs further investigation since it offers potential insight into the mechanism of insulin action on glucose transport.

In conclusion these studies have revealed important differences in the molecular properties of the rat adipocyte glucose transporter. The main findings are that 1) the intracellular pool contains two distinct glucose transporter charge isomers that can be distinguished by ionic focusing. 2) The plasma membrane glucose carriers are more uniform in molecular properties and display no charge heterogeneity on ionic focusing. 3) Only the pH 5.6 glucose transporter isoform is translocated by insulin from the intracellular pool to the plasma membrane and 4) the pH 6.4 isoform is probably a precursor of the pH 5.6 isoform differing from it mostly in its degree of sialylation.

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