Radiation Inactivation Target Size of Rat Adipocyte Glucose Transporters in the Plasma Membrane and Intracellular Pools*

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The in situ assembly states of the glucose transport carrier protein in the plasma membrane and in the intracellular (microsomal) storage pool of rat adipocytes were assessed by studying radiation-induced inactivation of the D-glucose-sensitive cytochalasin B binding activities. High energy radiation inactivated the glucose-sensitive cytochalasin B binding of each of these membrane preparations by reducing the total number of the binding sites without affecting the dissociation constant. The reduction in total number of binding sites was analyzed as a function of radiation dose based on target theory, from which a radiation-sensitive mass (target size) was calculated. When the plasma membranes of insulin-treated adipocytes were used, a target size of approximately 58,000 daltons was obtained. For adipocyte microsomal membranes, we obtained target sizes of approximately 112,000 and 109,000 daltons prior to and after insulin treatment, respectively. In the case of microsomal membranes, however, inactivation data showed anomalously low radiation sensitivities at low radiation doses, which may be interpreted as indicating the presence of a radiation-sensitive inhibitor. These results suggest that the adipocyte glucose transporter occurs as a monomer in the plasma membrane while existing in the intracellular reserve pool either as a homodimer or as a stoichiometric complex with a protein of an approximately equal size.

It is well established that the glucose transporters in insulin-responsive cells, such as adipocytes and muscle cells, occur not only in the plasma membrane but also in an intracellular storage pool of microsomal membranes, and insulin stimulates glucose transport in these cells mainly by shifting the distribution of the carrier between these two pools such that more transporter molecules are available at the plasma membrane (1–7). Many membrane receptors and transporters are now known to recycle via endosomes between the plasma membrane and intracellular pools (8–10), and it is likely that the glucose transporter may also be translocated through the same pathway (11–12). The molecular mechanisms by which insulin modulates such a net translocation, however, are not understood. We (13) have previously shown that sulfonylureas potentiate the insulin-induced redistribution of the glucose transporter in rat adipocytes, and this accounts for most of their well-known augmentation of the insulin-induced stimulation of glucose transport in this cell type. More recently, we have identified several membrane-associated sulfonylurea binding proteins in the adipocyte and have suggested that one of these proteins may regulate the insulin-induced translocation of the glucose transporter by direct molecular interaction. The association of the glucose transporter with the putative carrier that we have postulated above may be related to the suggested molecular heterogeneity in the glucose transporter of the adipocyte (5, 6, 14–16). Gel electrophoresis of transporter labeled with cytochalasin B by chemical cross-linking has revealed two forms of peptide of M, 45,000 and 54,000 (14). Isoelectric focusing studies (15) have detected two major components with isoelectric points at pH 5.6 and 6.4 in the intracellular pool, whereas only the pH 5.6 component was detected in the plasma membrane. The insulin-induced maximum depletion of the glucose transporter in the intracellular pool was found to be less than 50% (6). These observations suggest the existence of two different forms of the transporter in the storage pool, only one of which (with isoelectric point of pH 5.6) is translocated to the plasma membrane. Similarly, two forms of the transporter have been suggested to explain the observed time lag between the insulin-induced increase in the cytochalasin B binding activity of the plasma membrane and the stimulation of transport activity (5).

The radiation inactivation target size measurement (17) is a noninvasive technique which has been used successfully in studying the in situ quaternary or multimolecular assembly states of many membrane proteins (18). In the present report, we have used this technique to detect differences in the form of the glucose transporter in adipocytes and to correlate them with protein interaction possibly related to insulin action. The present study shows that the target size of the glucose transporter in the plasma membrane is approximately 58,000 daltons, indicating that it occurs as a monomer. The observed target size of the transporter in the intracellular reserve pool, on the other hand, is approximately 110,000 daltons for both insulin-stimulated and nonstimulated cells. This would indicate that the transporter in the reserve pool occurs as either a homodimer or as a tight complex with a distinct peptide. These findings suggest that the microsomal transporter may undergo a structural change of either monomerization or loss of an associated polypeptide upon translocation to the plasma membrane.

**EXPERIMENTAL PROCEDURES**

Materials—Collagenase was purchased from Worthington. Bovine albumin powder (CRG-7) was from Armour Pharmaceuticals (Kankakee, Ill.).

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kakee, IL). D-Glucose, L-glucose, and cytochalasin B were purchased from Sigma. [3H]Cytochalasin B was obtained from Du Pont-New England Nuclear. Insulin (porcine crystalline) was a gift from Lilly. All other chemicals were standard commercial products of reagent grade quality.

Isolation of Adipocytes—Adipocytes were isolated from the epididymal fat pads of Sprague-Dawley male rats (150-200 g of body weight) given free access to lab chow. Fat cells were prepared by the method described by Rodbell (19) and modified as follows. Minced fat pads were put in several 25-ml polyethylene scintillation vials with 15 ml of Krebs-Ringer bicarbonate buffer containing collagenase (1.5 mg/ml), bovine serum albumin (40 mg/ml), and D-glucose (3 mM) as described (13). The vials were then gassed briefly (10-20 s) with a 95% O2, 5% CO2 mixture, sealed, and incubated with gentle shaking at 37°C for 1 h, and the suspension was filtered through a nylon mesh to remove all extraneous tissue. The fat cells were then washed four times in a Krebs-Ringer buffer containing bovine serum albumin (40 mg/ml) and D-glucose (3 mM) (incubation buffer) by centrifugation (900 × g for 1 min).

Insulin Treatment—Immediately following isolation 12-ml aliquots of adipocyte suspension (40-50% cytocr) were distributed in 25-ml polyethylene scintillation vials containing 10 ml of the incubation buffer and enough insulin to achieve final concentrations at either 0 or 7.0 nM. The cells were then incubated for 45 min at 37°C with gentle shaking.

Preparation of Plasma Membrane and Microsome Fractions—Plasmas and microsomal membrane fractions were prepared by the differential centrifugation method described by McKeel and Jarett (20) and modified by Cushman and Wardzala (21), using a medium (medium I) composed of 10 mM Tris HCl, pH 7.4, EDTA (1 mM), and sucrose (0.25 M). Where insulin-treated cells were used, insulin was added to medium I. The suspension was then transferred in 30-ml aliquots to a glass homogenizer (Arthur H. Thomas Co., Philadelphia) and homogenized with the pestle rotating at 2,000 rpm and kept on ice. The homogenate was then centrifuged at 16,000 × g for 15 min, and the resulting fat cake was discarded. To isolate the microsomal membranes, the 16,000 × g supernatant was spun for 90 min at 200,000 × g, and its pellet was resuspended in the hypotonic buffer and stored in liquid nitrogen.

To isolate plasma membranes, the 16,000 × g pellet of homogenized cells was resuspended in medium I and centrifuged for 10 min at 1,000 × g. The resulting supernatant was centrifuged at 16,000 × g for 20 min, and the pellet was resuspended in 2 ml of medium I. The suspension was layered on 15 ml of medium (medium II) composed of 1.12 M sucrose, 20 mM Tris, and 1 mM EDTA, pH 7.4, and centrifuged at 110,000 × g for 60 min following the method of Karniell et al. (22). This procedure gave a single band of plasma membrane that was visible on the upper portion of medium II. This portion was removed and suspended in 20 volumes of medium I and spun for 20 min at 17,000 × g. The pellet was resuspended in medium I and stored in liquid nitrogen.

Cytochalasin B Binding Assays—Cytochalasin B binding was measured by a centrifugation method as described previously (23), with slight modification. The binding assay mixture contained membrane preparation of up to 200 μg of protein in medium I as described above, 0.01 μCi of [3H]cytochalasin B with a given concentration of unlabeled cytochalasin B, and 500 mM L- or D-glucose, made up to a final volume of 200 μl. The mixtures were incubated in 250-μl cellulose propionate centrifuge tubes for 30 min at 4°C and then centrifuged at 200,000 × g for 60 min at that temperature. 50 μl of resulting supernatant was removed and used for radioactivity measurement of the free [3H]cytochalasin B. The remaining supernatant was aspirated completely, and the inner walls of the tubes were scrubbed with cotton swabs to remove any residual medium. Pellets thus obtained were resuspended in 2% sodium dodecyl sulfate overnight and used for radioactivity measurement of bound ligands. Radioactivities were measured in a liquid scintillation spectrophotometer (Nuclear Chicago, Mark II, Chicago) using Hydrofluor.

Irradiation Procedure—Samples were irradiated in an oxygen-free closed chamber at −45 to −50°C maintained by a stream of liquid nitrogen as detailed elsewhere (18). A Van de Graaff-generated 1.5-MeV electron beam was used as a source of radiation. The radiation dose was measured at sample irradiation temperature using a blue cellophane (Du Pont, MSC 3000) film whose radiation sensitivity was calibrated against a chemical dosimeter (18).

RESULTS

Irradiation of isolated subcellular membrane preparations of adipocytes with the 1.5-MeV electron beam at −45°C produced a dose-dependent reduction in the cytochalasin B binding. This is illustrated in Fig. 1 using typical results obtained with the microsomal membrane of insulin-treated adipocytes. Radiation reduced the ligand binding in the presence of a 500 mM excess of either L- or D-glucose; the difference between them was taken as the glucose-sensitive binding (1, 5, 13). Scatchard analyses of this glucose-sensitive binding revealed a single component for nonirradiated as well as irradiated samples (Fig. 1, inset). Radiation did not affect the dissociation constant (KD) of the glucose-sensitive binding significantly but reduced the total capacity (BT)2 of the binding sites (Fig. 1). This was found to be true also for the plasma membrane of adipocytes that were not treated with insulin as well as for the plasma membrane of insulin-treated adipocytes (data not illustrated). The sets of adipocyte preparations used in this study possessed glucose-sensitive cytochalasin B binding activities and the insulin responsiveness that are comparable to those previously reported (1, 13). Nonirradiated microsomes showed BT andKD (average of three independent preparations with S.E.) of 42.6 ± 2.6 pmol/mg protein and 1.51 ± 0.01 × 10−7 M, respectively, for adipocytes without insulin treatment and 21.4 ± 1.8 pmol/mg protein and 1.46 ± 0.28 × 10−7 M, respectively, after insulin treatment. Nonirradiated plasma membranes prepared from insulin-treated adipocytes showed BT andKD of 24.1 ± 2.3 pmol/mg protein and 1.48 ± 0.28 × 10−7 M, respectively. The glucose-sensitive binding in the plasma membranes of adipocytes that were not treated with insulin was typically small even without irradiation, showing BT and KD of 6.2 ± 1.8 pmol/mg protein and 1.28 ± 0.04 × 10−7 M, respectively, the BT of which was further reduced by irradiation (data not illustrated). Because of the sparsity, quantitative measurements of the radiation-induced reduction in BT of the plasma membrane in the absence of insulin treatment was found to be not reliable, and they were

FIG. 1. Effects of high energy irradiation on cytochalasin B binding of microsomal membrane preparation. Cells were treated with 7 nM insulin for 30 min at 37°C, and the membranes were isolated from these cells and immediately frozen and irradiated at 2.07 (triangles) and 7.78 (circles) Mrad. Nonirradiated control is represented by squares. Cytochalasin B binding was measured after thawing at 21°C using ligand concentrations ranging from 10−9 to 10−5 M in the presence of 500 mM D-glucose (closed symbols) or L-glucose (open symbols). Each binding assay mixture contained 450 μg of protein of microsomal membranes/ml. Inset, Scatchard plots of the glucose-sensitive portion of the cytochalasin B binding as estimated by the difference in the ligand bindings observed in the presence of L- and D-glucose at the same concentration of free cytochalasin B in the assay mixture. B/F is the ratio of bound-to-free ligand, and B is bound ligand in pmo1/450 μg of protein.

The abbreviation used is: BT, total number of binding sites.
not analyzed for the target size estimation in the present study.

Survival of the glucose-sensitive cytochalasin B binding capacity \( B_x \) after irradiation at an increasing radiation dose was analyzed based on the classic single hit-single target theory (17, 18). The log survival against dose plots are shown in Fig. 2 for the plasma membranes of insulin-treated adipocytes (panel A), the microosomal membranes of adipocytes without insulin treatment (panel B), and the microsomal membranes of insulin-treated adipocytes (panel C). Each plot showed an asymptotic slope at higher doses. From the asymptotic slope, the target sizes of glucose-sensitive cytochalasin B binding protein was calculated to be 58,000, 112,000, and 109,000 daltons for the plasma membrane of insulin-treated cells, microsomes without insulin treatment, and microsomes with insulin treatment, respectively. A shoulder is apparent (panel C) at low radiation doses in the case of the microsomal membranes of the insulin-treated adipocytes. A slight but definite shoulder is also seen in the plot for the microsomal fraction in the absence of insulin stimulation (panel B). The plasma membrane, on the other hand, evidences only the slightest, probably insignificant hint of such a shoulder (panel A).

DISCUSSION

The observed target size for the adipocyte glucose transporter in the plasma membrane after insulin treatment (55,000 daltons) is practically identical to the monomeric size of the human erythrocyte glucose transporter, which is a 55,000-dalton peptide (24). There are many experimental evidences that the adipocyte transporter is similar if not identical to the human erythrocyte glucose transporter (16). The target size thus suggests that the adipocyte glucose transporter exists as a monomer in the plasma membrane. It is interesting to recall that the target size of the human erythrocyte glucose transporter in purified preparation is approximately 120,000 daltons, suggesting that it occurs as a dimer (25).3

The target sizes of the glucose transporter in the microsomal pool measured at the basal state (112,000 daltons) and the insulin-stimulated state (109,000 daltons) are not significantly different. Both of these sizes, on the other hand, are almost twice as large as the size in the plasma membrane. The results of our target size measurements provide further support to the suggested heterogeneity of the glucose transporter in adipocytes. The heterogeneity reported here, however, is in terms of assembly state of the transporter and thus is of a different nature from those (5, 6, 14, 15) discussed in the Introduction. Because of the incomplete understanding of the nature of energy transfer in protein quaternary structure and supramolecular assemblies, interpretation of measured target size at the molecular level should be considered tentative. Recognizing this limitation, however, we would propose the following molecular model, which may be useful for further elucidation of the nature of the observed heterogeneity and its functional significance. The adipocyte glucose transporter, a 55,000-dalton peptide, exists as a monomer (T) in the plasma membrane. In the intracellular storage pool, however, it occurs in a complex either as a homodimer \( (T_x) \) or with a protein, X, of equal size forming TX. It is a tempting speculation, based upon this model, that either monomerization of putative transporter-dimers or dissociation of the postulated protein X from the glucose transporter may play a key role in insulin-mediated redistributions of the glucose transporter from the storage pool to the plasma membrane. We have recently identified several sulfonylurea binding peptides in adipocyte microsomal membranes whose apparent \( M_x \) ranges from 30,000 to 120,000. It is a possibility that one of these sulfonyl binding peptides may be the protein X postulated above. Definitive biochemical identification of X postulated here remains to be accomplished.

The significance of the observed shoulder in the semilog plot of the inactivation data for the microsomal membranes is not clear. It may indicate a molecular association of the glucose transporter with a large inhibitor molecule (26-28), should such association not allow intermolecular energy transfer, where measurements made following radiation would reflect not only the loss resulting from radiative destruction of transporter but also the counter effect of transporter being unmasked by the destruction of the inhibitor molecule.

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