Insulin Regulation of Protein Traffic in Rat Adipose Cells*

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Rat adipocytes were biotinylated with cell-impermeable reagents, sulfo-N-hydroxysuccinimide-biotin and sulfo-N-hydroxysuccinimide-S-s-biotin in the absence and presence of insulin. Biotinylated and nonbiotinylated populations of the insulin-like growth factor-II/mannose 6-phosphate receptor, the transferrin receptor, and insulin-responsive aminopeptidase were separated by adsorption to streptavidin-agarose to determine the percentage of the biotinylated protein molecules versus their total amount in different subcellular compartments. Results indicate that adipose cells possess at least two distinct cell surface recycling pathways for insulin-like growth factor-II/mannose 6-phosphate receptor (MPR) and transferrin receptor (TfR): one which is mediated by glucose transporter isoform 4 (Glut4)-vesicles and another that bypasses this compartment. Under basal conditions, the first pathway is not active, and cell surface recycling of TfR and, to a lesser extent, MPR proceeds via the second pathway. Insulin dramatically stimulates recycling through the first pathway and has little effect on the second.

Within the Glut4-containing compartment, insulin has profoundly different effects on intracellular trafficking of insulin-responsive aminopeptidase on one hand and MPR and TfR on the other. After insulin administration, insulin-responsive aminopeptidase is redistributed from Glut4-containing vesicles to the plasma membrane and stays there for at least 30 min with minimal detectable internalization and recycling, whereas MPR and TfR rapidly shuttle between Glut4 vesicles and the plasma membrane in such a way that after 30 min of insulin treatment, virtually every receptor molecule in this compartment completes at least one trafficking cycle to the cell surface. Thus, different recycling proteins, which compose Glut4-containing vesicles, are internalized into this compartment at their own distinctive rates.

In fat and skeletal muscle cells, intracellular traffic of several proteins is controlled by insulin (1). Thus, insulin causes redistribution of the glucose transporter Glut4 (2, 3) and the recently discovered insulin-responsive aminopeptidase, IRAP1 (4, 5), from their intracellular pool(s) to the cell surface. Plasma membrane recycling of the receptors for IGF-II/mannose 6-phosphate (6–8) and transferrin (9–11) in fat cells is also sensitive to insulin. However, the magnitude of the insulin effect on the overall translocation of the receptor proteins is different. The amount of Glut4 and IRAP at the plasma membrane increases dramatically within minutes after insulin stimulation, whereas translocation of the IGF-II/mannose 6-phosphate receptor (MPR) and the transferrin receptor (TfR) under the same conditions is hardly noticeable by Western blot analysis of the plasma membrane preparations but is clearly detectable by cell surface biotinylation or with the help of radioactive ligands (6–11). Because insulin is known to have a small stimulatory effect on the recycling of TfR and MPR in other cells, such as fibroblasts (12–14), it is not clear whether or not intracellular pathways of MPR and TfR in insulin-sensitive cells are significantly different from the recycling of these receptors in nonspecialized cells.

Several lines of evidence, including the results of sucrose gradient centrifugation and immunoadsorption, demonstrate that in adipocytes, IRAP and partially MPR and TfR are co-localized in the same compartment with Glut4 (“Glut4-containing vesicles”) (4, 8, 11, 15). It was shown in these experiments that IRAP is completely co-localized with Glut4 (4, 16–18), whereas only about 50% of intracellular TfR and 10% of intracellular MPR is present in Glut4-containing vesicles (8, 11, 15). It has been suggested that Glut4 and IRAP co-localize with MPR and TfR only in sorting and/or recycling endosomes (19), whereas the major pool of Glut4 and IRAP may be sorted out into a specialized “insulin-sensitive compartment” that does not contain recycling receptors (15, 20). This hypothesis has been based mainly on the “ablation experiments,” which demonstrate that only 60% of Glut4 can be ablated with transferrin-HRP conjugate and diaminobenzidine (15, 20). However, given that in the adipocyte the number of Glut4 molecules may exceed the number of TfRs by 1,000-fold (2, 9), it may not be possible to quantitatively ablate Glut4 with the TfR ligand even if Glut4 and TfR are present in the same vesicular compartment, because not every Glut4-containing vesicle may also possess a TfR molecule (21).

In a previously done studies, I and others (22–24) have applied cell surface biotinylation of fat cells to study insulin-regulated plasma membrane recycling of various intracellular proteins. Neither of these studies, however, addressed the question of what part of the total population of the individual proteins is biotinylated at the cell surface, or in other words, what is the efficiency of such recycling. Here, I describe an experimental approach by which biotinylated and nonbiotinylated individual proteins are separated by adsorption to streptavidin-agarose, and the percentage of the biotinylated protein molecules versus their total amount may readily be determined.

The results of these experiments clearly demonstrate that the fraction of MPR and TfR that is associated with Glut4, traffics quite differently from these same receptors that reside

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The abbreviations used are: Glut4, glucose transporter isoform 4; IRAP, insulin-responsive aminopeptidase; IGF-II, insulin-like growth factor II; MPR, IGF-II/mannose 6-phosphate receptor; TfR, transferrin receptor; IRV, insulin-responsive vesicles; HRP, horseradish peroxidase; NHS, N-hydroxysuccinimide; PBS, phosphate-buffered saline; LM, light microsomes.

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outside of the Glu4 compartment. In the absence of insulin, there is virtually no significant plasma membrane recycling of the Glut4-associated receptors, whereas MPR and TIR not present in Glut4 vesicles do recycle to the cell surface. Insulin dramatically stimulates plasma membrane recycling of MPR and TIR, which are co-localized with Glut4, and only minimally affects receptor trafficking outside of the Glut4 compartment. These results suggest that a part of the population of MPR and TIR in adipocytes is incorporated, along with Glut4 and IRAP, in the “insulin-responsive compartment.”

Interestingly, within the Glut4-containing compartment(s), insulin has profoundly different effects on intracellular trafficking of IRAP on one hand and MPR and TIR on the other. After insulin administration, IRAP is redistributed from Glut4-containing vesicles to the plasma membrane and stays there for at least 30 min with minimal detectable internalization and recycling, whereas MPR and TIR rapidly shuttle between Glut4 vesicles and the plasma membrane in such a way that after 30 min of insulin treatment virtually every receptor molecule in this compartment completes at least one trafficking cycle to the cell surface. These differences in intracellular trafficking of receptor proteins and IRAP may reflect their distinct and specific biological functions in the cell and may unmask previously unknown aspects of insulin action.

**EXPERIMENTAL PROCEDURES**

Antibodies—In this study, we used the monoclonal anti-Glut4 antibody 1F8 (25), monoclonal anti-TR antibody (Zymed Laboratories Inc.), polyclonal anti-IRAP antibodies (4), and DEAE-cellulose purified anti-IGF-II mannose 6-phosphate receptor polyclonal antibodies (a kind gift of Dr. M. Czech, University of Massachusetts Medical School, Worcester, MA).

Cell Biotinylation and Fractionation—Adipocytes were isolated from the epididymal fat pads of male Harlan Sprague Dawley rats (150–175 g) by collagenase digestion (26) and transferred to KRP buffer (12.5 mM NaCl, 6 mM KCl, 1.2 mM MgSO4, 1 mM CaCl2, 0.6 mM Na2HPO4, 20 nM. Sulfo-NHS-biotin or sulfo-NHS-S-S-biotin (both from Pierce) was added to cells 2 min after insulin to a final concentration of 0.5 mg/ml. Biotinylation was usually performed at 37 °C, then 1 M Tris, pH 7.4, and 0.2 μM insulin were added to final concentrations 50 mM and 0.2 μM, respectively, for 2 min. For “0” time point, insulin, sulfo-NHS-biotin, KRN, and Tris were added immediately one after another. After that, cells were washed 3 times with HES buffer (20 mM HEPES, 250 mM sucrose, 1 mM EDTA, 5 mM benzamidine, 1 mM phenylmethanesulfonyl fluoride, 1 μM pepstatin, 1 μM aprotinin, 1 μM leupeptin, pH 7.4) cooled to 14–16 °C, homogenized with a Potter-Elvehjem Teflon pestle, and subcellular fractions were prepared as described previously (27). Isolated fractions were resuspended in PBS, which contained all of the protease inhibitors listed above.

**RESULTS**

Isolated rat adipocytes were incubated with sulfo-NHS-biotin in the absence and presence of insulin for 5, 15, and 30 min and fractionated into subcellular fractions as described under “Experimental Procedures.” Glut4-containing vesicles were isolated from LM by immunoadsorption on 1F8 beads, and their component proteins were eluted with 1% Triton X-100 in PBS. As we have previously reported (11, 22), incubation with insulin rapidly increases biotinylation of the major component proteins of Glut4-containing vesicles, such as MPR and IRAP as determined by streptavidin-alkaline phosphatase staining (compare lanes 1–4 and 5–8). These data are also consistent with the results of Ross et al. (31) who have recently determined by streptavidin-HRP staining that the amount of biotinylated IRAP is rapidly increased in the cell after insulin stimulation. TIR in Glut4 vesicles were also detectable by these experiments, although its total biotinylation is much less than that of the first two proteins, probably because of the small amount of TIR present in adipocytes (11).

The problem with these earlier experiments including the one shown in Fig. 1, however, is that they provide only a very general illustration of the insulin-dependent recycling of Glut4
Fig. 2. Intracellular traffic of the individual component proteins of Glut4-containing vesicles. Rat adipocytes were biotinylated with sulfo-NHS-biotin in the absence and the presence of insulin as described under “Experimental Procedures” for 0 min (lanes 1 and 5), 5 min (lanes 2 and 6), and 30 min (lanes 3 and 7) in the absence (lanes 1, 3, and 5) and in the presence (lanes 2, 4, 6, and 8) of insulin. (A) Western blot analysis of total and non-biotinylated protein levels in IgG and TFR eluates. (B) Quantitative analysis of MPR, TFR, IRAP, and Glut4 levels.
FIG. 3. **Plasma membrane content of the translocatable proteins.** Rat adipocytes were biotinylated with sulfo-NHS-biotin in the absence and presence of insulin for 0 min (lanes 1 and 5), 5 min (lanes 2 and 6), 15 min (lanes 3 and 7), and 30 min (lanes 4 and 8). Plasma membrane fractions were isolated and analyzed by Western blotting (40 μg/lane) using HRP-conjugated secondary antibodies and enhanced chemiluminescent kit. An aliquot of each plasma membrane preparation (40 μg) was solubilized in 100 μl of PBS with 1% Triton (final volume), passed over 40 μl of streptavidin-agarose for 5 h at 4°C to remove biotinylated proteins, and electrophoresed simultaneously with the total fractions. All proteins were visualized by consecutive staining of the same membrane. Panel B shows the mean ± S.E. of three independent experiments.

2 and 6), 15 min (lanes 3 and 7), and 30 min (lanes 4, 8, and the lane marked IgG). Glut4-containing vesicles were immunoadsorbed from 400 μg of LM with 100 μl of 1F8- or IgG-coupled beads and solubilized in 300 μl of 1% Triton X-100 in PBS. Eluted material was divided into two, and the first half (lanes 1–4) was electrophoresed without further treatment. The second half was passed over 20 μl of streptavidin-agarose for 5 h at 4°C to remove biotinylated proteins and electrophoresed simultaneously with the first half. To elute Glut4, immunobeads were treated with an equal volume of 2× Laemmli sample buffer after Triton elution. Panel A represents Western blot analysis of the individual proteins obtained by consecutive staining of the same membrane using HRP-conjugated secondary antibodies and enhanced chemiluminescent kit. Panel B shows the mean ± S.E. of three independent experiments. Because no changes were detected in any of the three experiments with basal cells (top panel of panel A), quantification is provided only for samples obtained from insulin-treated cells (bottom panel of panel A).
vesicles. For example, it is not clear from Fig. 1 what fraction of the individual proteins in Glut4-containing vesicles is biotinylated at the cell surface. One can imagine, for example, that 100% of MPR and only 1% of IRAP in Glut4 vesicles is biotinylated after a 30-min exposure of cells to insulin and sulfo-NHS-biotin, whereas the streptavidin-alkaline phosphatase-generated signal from these two proteins may still be the same because of their different content in the vesicles, number of the biotinylation sites they have, etc. This possibility seems real, because the amount of IRAP in Glut4 vesicles was estimated to exceed the amount of MPR in this compartment at least 8-fold (22). Thus, to monitor protein trafficking through Glut4-containing vesicles more closely, I used the following approach.

Material eluted from 1F8 beads was divided into two and the first half was electrophoresed with no further treatment, whereas the second half was passed over streptavidin-agarose beads for 5 h. Material not bound to streptavidin-agarose (lanes 9–12) was electrophoresed together with the first half (lanes 5–8), transferred to a polyvinylidene fluoride membrane, and stained with alkaline phosphatase conjugated with streptavidin to visualize biotinylated proteins (Fig. 1). Lanes 9–12 of Fig. 1 show that incubation with streptavidin-agarose completely removes biotinylated proteins from the preparation thus allowing one to determine by regular Western blot what fraction of the total pool of the individual protein does not contain biotin. Unfortunately, in these experiments, it is not possible to elute biotinylated proteins from streptavidin-agarose for quantitative analysis because of the SDS-stable nature of biotin-streptavidin binding.

When this approach was applied to basal cells, it was shown that incubation of adipocytes without insulin for up to 30 min does not lead to biotinylation of any significant fraction of MPR, TfR, or IRAP in Glut4-containing vesicles (Fig. 2, top panel), which is also consistent with Fig. 1, lanes 1–4. Thus, MPR, TfR, and IRAP practically do not cycle between the cell surface and intracellular Glut4 vesicles in the absence of insulin. Stimulation of adipocytes with insulin drastically changes the pattern of trafficking of these component proteins of Glut4-containing vesicles (Fig. 2, bottom panel, and B). With time, increasing amounts of MPR and TfR molecules in Glut4-containing vesicles.

**Fig. 4.** Identification of the internalized fraction of IRAP by cell surface biotinylation with cleavable sulfo-NHS-S-S-biotin. Rat adipocytes were biotinylated with sulfo-NHS-S-S-biotin in the absence and presence of insulin as described under “Experimental Procedures” for 30 min. LM (300 μg) were solubilized in 1% Triton X-100 in PBS (final volume, 200 μl) and passed over 50 μl of streptavidin-agarose overnight at 4 °C. Streptavidin-agarose was washed 4 times with 1 ml of 1% Triton X-100 in PBS, and the bound material was eluted with Laemmli sample buffer with freshly added 10% β-mercaptoethanol for 1.5 h at 90 °C. This preparation was analyzed for IRAP by Western blotting along with the total fractions of plasma membrane (PM) and LM (50 μg each) and the material not bound to streptavidin-agarose (40 μg). The figure shows a representative result of three independent experiments.

**Fig. 5.** Plasma membrane recycling of the transferrin receptor not associated with Glut4 vesicles. Rat adipocytes were biotinylated with sulfo-NHS-biotin in the absence and presence of insulin as described under “Experimental Procedures” for 0 min (lanes 1 and 5), 5 min (lanes 2 and 6), 15 min (lanes 3 and 7), and 30 min (lanes 4 and 8). Glut4-containing vesicles were removed from the LM fraction by immunoadsorption as described in the legends to Figs. 1 and 2. Glut4-depleted material was solubilized in 1% Triton X-100 (final volume, 600 μl), divided into two, and the first half (lanes 1–4) was electrophoresed without further treatment. The second half (lanes 5–8) was passed over 20 μl of streptavidin-agarose for 5 h at 4 °C to remove biotinylated proteins and electrophoresed simultaneously with the first half. TfR was analyzed in the samples by Western blotting using HRP-conjugated secondary antibodies and enhanced chemiluminescent kit. The bottom panel shows the mean ± S.E. of three independent experiments.
icles pick up biotin tags at the cell surface and are removed from the preparation by streptavidin-agarose binding. According to these data, in 5 min 50% of the receptor molecules have already gone through at least one round of turnover to the cell surface, and in 30 min practically every receptor molecule in intracellular Glut4 vesicles becomes biotinylated and, thus, completes one or more cycles to and from the cell surface. Insulin-induced acceleration of the receptor recycling does not lead to any significant decrease in the total amount of these proteins in intracellular Glut4 vesicles (Fig. 2A, bottom panel, lanes 1–4), nor does it substantially increase the concentration of these proteins at the plasma membrane (Fig. 3A and B, lanes 1–4), which suggest that MPR and TfR indeed rapidly recycle between these two compartments without any substantial retention in either of them.

IRAP demonstrates quite a different response to insulin. Unlike the case for MPR and TfR, the amount of this protein in intracellular Glut4-containing vesicles is decreased 3-fold after incubation with insulin for 30 min. This decrease is accompanied by a corresponding augmentation in the plasma membrane IRAP content (Fig. 3, A and B, lanes 1–4), which suggest that MPR and TfR indeed rapidly recycle between these two compartments without any substantial retention in either of them.

The amount of Glut4 in intracellular membrane fractions rapidly decreases after insulin administration (Fig. 2, A, bottom panel, and B). Thus, only a minimal fraction of the cell surface IRAP is internalized to the intracellular Glut4-containing vesicles which is, however, sufficient to generate a strong signal on streptavidin-alkaline phosphatase-stained Western blots (Fig. 1).

To confirm this last result and to also check the possibility that IRAP may be internalized into another intracellular compartment different from Glut4-containing vesicles, I carried out experiments with the total fraction of intracellular LM using the cleavable reagent sulfo-NHS-S-S-biotin. Cells were biotinylated for 30 min in the absence and presence of insulin and were separated into subcellular fractions by differential centrifugation. LM fraction (300 μg) in 1% Triton X-100 was incubated with streptavidin-agarose beads, which resulted in a complete binding of biotinylated proteins to the resin (not shown). Beads were washed 4 times with 1% Triton X-100 in PBS, and the bound material was eluted with Laemmli sample buffer containing 10% β-mercaptoethanol. Eluted proteins were electrophoresed in parallel with the total preparations of plasma membrane and LM (50 μg each) as well as the material not bound to streptavidin-agarose (40 μg) and blotted for IRAP (Fig. 4). The results were quantitated in a computing densitometer and normalized to the total amount of plasma membrane and LM recovered in the experiment. It turns out that the amount of biotinylated IRAP in LM of cells treated with insulin for 30 min represents only 1.4% of its total amount in this fraction and only 2.2% of the IRAP content in the plasma membrane. In basal cells, the amount of biotinylated IRAP in the LM fraction was below the detection limit. Unfortunately, heating of protein samples in the presence of β-mercaptoethanol completely destroys antigenicity of TfR and MPR, so I was unable to perform the same type of analysis on these two proteins.

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tion. It has been determined by independent approaches, however, that Glut4 recycles between its intracellular compartments and the cell surface both in the absence and presence of insulin (32–36). Although \( t_{1/2} \) values for Glut4 recycling vary in different studies (32, 33), all estimations indicate that Glut4 in insulin-treated cells may recycle faster than IRAP (summarized in Ref. 35). This does not necessarily mean that Glut4 and IRAP do not co-localize in the cell, because these two proteins may, nevertheless, share the same intracellular compartments (see “Discussion”).

To exclude the input of the Glut4 vesicle-mediated pathway in receptor recycling, I used material not bound to 1F8 beads and performed virtually the same experiments as those described above and shown in Fig. 2. Fig. 5 demonstrates that the population of TIR excluded from Glut4 vesicles recycles to the cell surface both in the absence and presence of insulin, and insulin has little effect on TIR trafficking outside this compartment. Basal recycling of MPR is not as pronounced as that of TIR but is still statistically significant (Fig. 6). This may be explained by the fact that the majority of MPR molecules traffics between the Golgi apparatus and late endosomes (37) explained by the fact that the majority of MPR molecules TfR but is still statistically significant (Fig. 6). This may be considered above and shown in Fig. 2. Fig. 5 demonstrates that the and performed virtually the same experiments as those described above and shown in Fig. 2. Fig. 5 demonstrates that the recycling block is released, presummably at its own distinctive rates.

According to this model, Glut4 vesicles (i.e. the material that is obtained by immunoabsorption with anti-Glut4 antibody) represent, in fact, a mixture of two different compartments: sorting/recycling endosomes and insulin-responsive vesicles, IRV, that have been postulated in fat cells (1, 38–40) but never identified. MPR and TIR, on the other hand, have different biological functions that are facilitated not by augmentation of the plasma membrane concentration of these proteins, as is the case with IRAP, but rather by intensification of their recycling, and this is exactly what has been determined in these experiments. This suggests that there must be a stringent sorting step at the level of the plasma membrane and that individual component proteins of Glut4 vesicles are internalized into this compartment at their own distinctive rates.

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