**Translocation of Small Preformed Vesicles Is Responsible for the Insulin Activation of Glucose Transport in Adipose Cells**

**EVIDENCE FROM THE IN VITRO RECONSTITUTION ASSAY***

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Insulin stimulates translocation of the glucose transporter isoform 4 (Glut4) from an intracellular storage compartment to the plasma membrane in fat and skeletal muscle cells. At present, the nature of the Glut4 storage compartment is unclear. According to one model, this compartment represents a population of preformed small vesicles that fuse with the plasma membrane in response to insulin stimulation. Alternatively, Glut4 may be retained in large donor membranes, and insulin stimulates the formation of transport vesicles that deliver Glut4 to the cell surface. Finally, insulin can induce plasma membrane fusion of the preformed vesicles and, also, stimulate the formation of new vesicles. In extracts of fat and skeletal muscle cells, Glut4 is predominantly found in small insulin-sensitive 60–70 S membrane vesicles that may or may not artificially derive from large donor membranes during cell homogenization. Here, we use a cell-free reconstitution assay to demonstrate that small Glut4-containing vesicles are formed from large rapidly sedimenting donor membranes in a cytosol-, ATP-, time-, and temperature-dependent fashion and, therefore, do not represent an artifact of homogenization. Thus, small insulin-responsive vesicles represent the major form of Glut4 storage in the living adipocyte cell. Fusion of these vesicles with the plasma membrane may be largely responsible for the primary effect of insulin on glucose transport in fat tissue. In addition, our results suggest that insulin may also stimulate the formation of Glut4 vesicles and accelerate Glut4 recycling to the plasma membrane.

Insulin regulates postprandial blood glucose levels by reversible translocation of the glucose transporter isoform 4 (Glut4)

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The abbreviations used are: Glut4, glucose transporter isoform 4; to the plasma membrane in fat and skeletal muscle cells (1). The molecular mechanism of this process is not completely understood. It is not known, in particular, whether insulin stimulates plasma membrane fusion of the pre-existing Glut4-containing vesicles, formation of new transport vesicles from an intracellular storage compartment, or both.

Previously, we reported that, in extracts of adipose cells, the majority of Glut4 is present in two distinct populations of small membrane vesicles, one of which contains cellugyrin, whereas another lacks this protein (2). Subsequent analysis suggested that cellugyrin-positive vesicles transport Glut4 from early to recycling endosomes, whereas cellugyrin-negative vesicles may represent a specialized insulin-responsive population of vesicles that derive from recycling endosomes (3). These data (see also Refs. 4–6) indicate that insulin-responsive 60–70 S vesicles may represent the major form of Glut4 storage in vitro. It is possible, however, that large tubular membrane structures, such as endosomes, may be artificially shattered during cell homogenization into rather homogeneous vesicular population. Therefore, it is unclear whether the Glut4 storage compartment in the living cell is represented by small vesicles or by large fragile membrane structures. To address this question, we broke adipocytes open without homogenization by centrifugation only. We found that endosomes were not visibly affected by homogenization and that the major pool of Glut4 was still compartmentalized in small cellugyrin-positive and -negative vesicles, which, therefore, were not likely to represent an artifact of homogenization (3).

We have now established a cell-free reconstitution assay for cellugyrin-positive and -negative Glut4-containing vesicles, and we show here that these vesicles can be formed in vitro in a physiological process. This result supports our previous conclusion and suggests that small 60–70 S insulin-responsive vesicles and not large donor membranes represent the major form of Glut4 storage in the living adipose cell. In addition, we found that insulin stimulated formation of Glut4 vesicles in vitro by ~50%. Thus, although fusion of the preformed vesicles with the plasma membrane should mainly account for the initial effect of insulin on glucose transport in adipocytes, Glut4 recycling through intracellular compartments may also be expedited by insulin.

**EXPERIMENTAL PROCEDURES**

**Materials**—In the present study we used monoclonal anti-Glut4 antibody 1F8 (7) and rabbit polyclonal antibody against cellugyrin (Ac-CQVETTEGYQPPPY-OH) that had been raised and affinity-purified by BioSource International (Camarillo, CA). Dexamethasone, 3-isobutyl-1-methylxanthine, insulin, benzamidine, potassium aspartate, potassium glutamate, potassium gluconate, MOPS, sodium carbonate, magnesium sulfate, glutathione (reduced form), ATP, creatine phosphate, creatine phosphokinase, fetal bovine serum, brefeldin A (BFA), wortmannin, guanosine 5′-O-(thio)triphosphate (GTP[S]) and neomycin were purchased from Sigma. Aprotinin, leupeptin, pepstatin A, and phenylmethylsulfonyl fluoride were obtained from American Bioanalytical (Natick, MA). Calf bovine serum and Dulbecco’s modified Eagle’s medium (DMEM) were purchased from Invitrogen.

**Cell Culture**—Murine 3T3-L1 preadipocytes were cultured, differentiated, and maintained as described previously (8). Briefly, cells were grown in DMEM supplemented with 10% calf bovine serum until confluence. Two days later, the cells were transferred to differentiation medium, 4-morpholinepropanesulfonic acid; BFA, brefeldin A; GTP[S], guanosine 5′-O-(thio)triphosphate; DMEM, Dulbecco’s modified Eagle’s medium.

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medium (DMEM containing 10% fetal bovine serum, 0.5 mM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, and 1.7 mM insulin). After 48 h, the differentiation medium was replaced with maintenance medium (DMEM supplemented with 10% fetal bovine serum). The maintenance medium was changed every 48 h. The cells were used after 8 days of differentiation.

**Preparation of Cytosol from Rat Brain and 3T3-L1 Adipocytes**—
Brains were dissected from male Sprague-Dawley rats, thoroughly washed with ice-cold budding buffer (9) (38 mM potassium aspartate, 38 mM potassium glutamate, 38 mM potassium gluconate, 20 mM MOPS, 5 mM sodium carbonate, 2.5 mM magnesium sulfate, 5 mM reduced glutathione, 1 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin, 1 μM aprotinin, 1 μM leupeptin, pH 7.2), and homogenized by 12 strokes with a Potter-Elvehjem Teflon pestle. The homogenate was centrifuged at 16,000 × g for 20 min at 4 °C, and the supernatant was re-centrifuged at 200,000 × g for 1 h at 4 °C. The resulting supernatant (brain cytosol) was aliquoted and stored at −80 °C. Cultured 3T3-L1 adipocytes were washed twice with ice-cold budding buffer and homogenized by 10 strokes in a ball-bearing homogenizer (Isobiotec, Heidelberg, Germany) with a 12-μm clearance. The cytoxic fraction was isolated from the homogenate according to the procedure described above and used fresh.

**In Vitro Reconstitution of Glut4 Vesicles**—
3T3-L1 adipocytes were washed and homogenized as described in the previous section. The homogenate were centrifuged at 16,000 × g for 20 min at 4 °C. The pellet (heavy membrane fraction) was washed twice with ice-cold budding buffer and re-suspended in ice-cold budding buffer. In some experiments, the pellet was washed with urea-containing stripping buffer (2.5 mM urea, 250 mM sorbitol, 20 mM HEPES, 5 mM magnesium acetate, 150 mM potassium acetate) prior to washes with budding buffer (9). This procedure generally decreased the efficiency of Glut4 vesicle budding but did not qualitatively change the results of the assay. Washed heavy membrane fraction (250 μg per sample) was mixed with cytosol and supplied with an ATP regeneration system (1 mM ATP, 8 mM creatine phosphate, 1.5 unit/ml creatine phosphokinase) in a total volume of 250 μl. Samples were kept on ice for 5 min and transferred to 37 °C for the indicated periods of time. Then, samples were centrifuged for the second time at 16,000 × g for 20 min at 4 °C. The pellets of the second centrifugation (donor fraction) were removed, and supernatants were centrifuged at 200,000 × g for 60 min in a Beckman Type 42.2 Ti rotor to harvest de novo formed small vesicles. This vesicle fraction along with the donor fraction were analyzed by Western blotting.

**Sucrose Gradient Centrifugation**—
Samples (0.2 ml) were loaded onto a 4.6-ml continuous 10–30% sucrose gradient and centrifuged for 55 min in a Beckman SW-55 Ti rotor at 48,000 rpm. Each gradient was collected into 25 fractions starting from the bottom of the tube.

**Gel Electrophoresis, Immunoblotting, and Protein Content**—Proteins were separated in SDS-polyacrylamide gels according to Laemmli (10), and were transferred to Immobilon-P membranes (Millipore) in 25 mM Tris, 192 mM glycine. Following transblotting, the membranes were blocked with 10% non-fat dry milk in PBST but without reducing agents, and were transferred to an Immobilon-P membrane (Millipore) in 25 mM Tris, 192 mM glycine. Following transblotting, the membranes were blocked with 10% non-fat dry milk in PBST (phosphate-buffered saline with 0.5% Tween 20) for 1 h at 37 °C. Proteins were visualized with specific antibodies, horseradish peroxidase-conjugated secondary antibodies (Sigma), and an enhanced chemiluminescence substrate kit (PerkinElmer Life Sciences). Protein content was determined with the BCA kit (Pierce) according to manufacturer’s instructions.

**RESULTS AND DISCUSSION**

3T3-L1 adipocytes were homogenized in a ball-bearing homogenizer, and the homogenate were centrifuged at 16,000 × g for 20 min. Under these conditions, 27 ± 7% of the total Glut4 was found in the heavy membrane fraction recovered in the pellet, whereas 73 ± 5% of Glut4 was present in the supernatant.²

The heavy membrane fraction includes the plasma membrane (11), endoplasmic reticulum (3, 11), mitochondria (11), Golgi membranes (11, 12), lysosomes, and endosomes (3). Numerous studies (see, for example, Refs. 13 and 14) including our own (15) demonstrate that, under basal conditions, there is practically no Glut4 in the plasma membrane. In addition, it was shown by several independent approaches that, upon homogenization of rat adipocytes, the plasma membrane has the right-side-out orientation and, therefore, cannot possibly serve as a donor compartment for the formation of any type of vesicles (12, 16). Although these studies were performed in primary rat adipocytes and not in 3T3-L1 cells, the plasma membrane isolated from primary and cultured adipocytes may have the same topology in vitro. The amount of Glut4 in the endoplasmic reticulum is negligibly small in comparison with its total pool (17). That leaves Golgi apparatus and endosomes as a possible source of Glut4 in the heavy membrane fraction.

This fraction was washed as described under “Experimental Procedures” and incubated at 4 °C or at 37 °C with and without brain cytosol and ATP regeneration system in budding buffer for the indicated periods of time. Heavy membranes were then re-pelleted at 16,000 × g for 20 min (donor fraction), and de novo formed vesicles were collected from the supernatant by centrifugation at 200,000 × g for 60 min. Both donor and vesicle fractions were analyzed by Western blotting (Fig. 1).

We found that both cellugyrin and Glut4 are re-distributed from heavy membranes into the vesicle fraction in a cytosol-, ATP-, temperature-, and time-dependent fashion (Fig. 1). It is seen in Fig. 1A that the amount of Glut4 re-distributed to the

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² M. Malikova and K. V. Kandror, unpublished results.
³ K. V. Kandror, unpublished data.
vesicle fraction is directly proportional to the concentration of brain cytosol, whereas the amount of cellugyrin in this fraction reaches its maximum at 2 mg/ml cytosol and is not increased any further. Also, the yield of Glut4 in the vesicle fraction is decreased after 20 min of incubation, probably due to fusion of de novo formed vesicles with other membranes present in the reaction mixture. On the contrary, the yield of cellugyrin-containing vesicles steadily increases with time. This suggests that Glut4 and cellugyrin may be localized in different types of vesicle formation, which may be controlled by different molecular mechanisms.

In agreement with this hypothesis, the sedimentational analysis shows that two types of vesicles are formed in vitro. These vesicles exactly correspond to cellugyrin-positive and cellugyrin-negative vesicles present in cell extracts (Fig. 2). Together with our previous results showing that these vesicles do not represent an artifact of cell homogenization (3, 4), these data suggest that small Glut4 vesicles exist in the living adipose cell and represent the major Glut4-storing compartment in vivo. Such a conclusion is consistent with results of immuno-electron microscopy, demonstrating that, in adipocytes, Glut4 resides in small vesicles and short tubules with an average diameter of 50–70 nm (13, 18).

It is known that vesicle budding requires GTP binding to Arf (19), so that this reaction is often stimulated in vitro by GTPγS. Indeed, we have determined that GTPγS increases recruitment of Glut4 from the cytosol into the forming Glut4 vesicles (20). Such vesicles were analyzed by Western blot with 1F8 antibody and polyclonal antibody against cellugyrin.

**Fig. 4. Adipocyte cytosol supports the formation of small vesicles in vitro.** Reconstitution assay was performed in vitro for the indicated periods of time as described in the legend to Fig. 1 in the presence of adipocyte cytosol (AC, 2 mg/ml), brain cytosol (BC, 2 mg/ml), or budding buffer (BB) alone. In vitro formed vesicles were analyzed by Western blot with 1F8 antibody and with polyclonal antibody against cellugyrin.
of the adaptor complex AP1 onto donor membranes (results not shown). This, however, does not significantly change the yield of small vesicles (Fig. 3). We believe therefore that, under our experimental conditions, GTP-bound Arf does not limit the budding reaction. Also, addition of wortmannin does not significantly affect the formation of Glut4- and cellugyrin-containing vesicles in vitro (Fig. 3). BFA demonstrates some inhibitory activity at concentrations that dramatically exceed its ID_{50} and even LD_{50} in vitro (20). Since BFA does not block the formation of Glut4 vesicles in vivo (21), a small inhibitory effect of BFA in vitro may not be specific. Aminoglycoside antibiotics neomycin (Fig. 3) and Geneticin (not shown) significantly inhibit formation of vesicles, suggesting that phospholipase D may be involved in regulation of vesicle budding (see also Ref. 22). In our experiments, the inhibitory effect of aminoglycosides in vitro does not exceed 50%. This may be related to the fact that these compounds not only block vesicle budding, but also inhibit vesicle fusion with endosomes (23), thus maintaining an equilibrium between small vesicles and donor membranes in the reaction mixture.

We believe that our data are, in general, consistent with recent results of Lim et al. (9) who studied the formation of Glut4-containing vesicles in vitro using brain cytosol and donor membranes from Myc-Glut4-transfected Chinese hamster ovary cells. In both systems, formation of Glut4 vesicles is inhibited by neomycin, whereas BFA does not have any major effect. Moreover, Lim et al. (9) also found that the yield of Glut4 vesicles formed de novo was decreased after the first 15 min of incubation due to fusion with other membranes present in the reaction mixture. A notable inconsistency in our results is that in their experiments, GTPγS powerfully inhibits vesicle budding. At present, the reason for this discrepancy is not clear.

We have determined that adipocyte cytosol can support the formation of small vesicles in vitro to the same extent as brain cytosol that is routinely used in budding assays (Fig. 4). Thus, we decided to explore whether or not insulin administration has any effect on the formation of small vesicles in vitro. Since it is not known whether or not insulin changes the amount of Glut4 and cellugyrin in donor membranes, we figured that the direct comparison of vesicle formation in extracts of insulin-treated and not treated cells would be difficult to interpret. Therefore, we isolated the heavy membrane fraction and the cytosol from basal and insulin-treated adipocytes, and we used these fractions in the budding reaction in different combinations (Fig. 5). We found that insulin, indeed, stimulates the formation of Glut4 vesicles in vitro by −50%. A similar result was recently reported by Kristiansen and Richter (24) who studied the formation of Glut4 vesicles in the extracts of skeletal muscle cells. This increase, although rather modest in in vitro experiments, may still be indicative of a potentially larger effect in vivo, so that the stimulatory effect of insulin on vesicle budding in the living cell may be more pronounced. This hypothesis is consistent with recent results of Foster et al. (25) who showed, by immunofluorescence microscopy, that insulin accelerates inter-endosomal traffic of Glut4 in the L6 cell line. Thus, the results obtained in vitro with the help of the vesicle reconstitution assay suggest that fusion of small preformed Glut4 vesicles with the plasma membrane may account for the initial stimulation of glucose transport by insulin in adipose cells. In addition, insulin may stimulate the formation of Glut4 vesicles de novo and thus increase the rate of Glut4 recycling through its intracellular compartments to the plasma membrane.

REFERENCES
1. Bryant, N. J., Govers, R., and James, D. E. (2002) Nat. Rev. Mol. Cell. Biol. 3, 267–277
2. Kupriyanova, T. A., and Kandror, K. V. (2000) J. Biol. Chem. 275, 36263–36269
3. Kupriyanova, T. A., Kandror, V., and Kandror, K. V. (2002) J. Biol. Chem. 277, 9133–9138
4. Kandror, K. V., Codere, L., Pushkin, A. V., and Pilch, P. F. (1995) Biochem. J. 307, 383–390
5. Heller-Harrison, R. A., Morin, M., Guilherme, A., and Czech, M. P. (1996) J. Biol. Chem. 271, 10209–10204
6. Bogan, J. S., McKee, A. E., and Lodish, H. F. (2001) Mol. Cell. Biol. 21, 4785–4806
7. James, D. E., Brown, R., Navarro, J., and Pilch, P. F. (1988) Nature 333, 183–185
8. Stephens, J. M., Lee, J., and Pilch, P. F. (1997) J. Biol. Chem. 272, 971–976
9. Lim, S.-N., Bonzelius, F., Low, S. H., Wille, H., Weimbs, T., and Herman, G. A. (2001) Mol. Biol. Cell 12, 981–985
10. Laemmli, U. K. (1970) Nature 227, 680–685
11. Simpson, I. A., Yer, D. R., Hissin, P. J., Wardzala, L. J., Karniel, E., Salans, L. B., and Cushman, S. W. (1983) Biochim. Biophys. Acta 763, 393–407
12. Hedo, J. A., and Simpson, I. A. (1984) J. Biol. Chem. 259, 11083–11089
13. Slot, S. W., Geuze, H. J., Gigengack, S., Lienhard, G. E., and James, D. E. (1991) J. Cell Biol. 113, 125–135
14. Maide, D., Ramm, G., Cushman, S. W., and Slot, J. W. (2000) J. Cell Sci. 113, 4203–4210
15. Kandror, K. V. (1999) J. Biol. Chem. 274, 25210–25217
16. Jarrett, L., and Smith, R. M. (1974) J. Biol. Chem. 249, 7024–7031
17. Boh, C., Roduit, R., Thoren, B., Fried, B., and Kandror, K. V. (2001) J. Biol. Chem. 276, 35990–35994
18. Smith, R. M., Charron, M. J., Shah, N., Lodish, H., and Jarrett, L. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 6893–6897
19. Chavrier, P., and Goud, B. (1999) Curr. Opin. Cell Biol. 11, 466–475
20. Torii, S., Banno, T., Watanabe, T., Ikehara, Y., Murakami, K., and Nakayama, K. (1995) J. Biol. Chem. 270, 11574–11580
21. Martin, S., Ramm, G., Lyttle, C. T., Meerslo, T., Stoorvogel, W., and James, D. E. (2000) Traffic 1, 652–660
22. Emoto, M., Klarlund, J. K., Waters, S. B., Hu, V., Buxton, J. M., Chawla, A., and Czech, M. P. (2000) J. Biol. Chem. 275, 7144–7151
23. Jones, A. T., and Wessling-Resnick, M. (1998) J. Biol. Chem. 273, 25301–25309
24. Kristiansen, S., and Richter, E. A. (2002) Am. J. Physiol. 283, E374–E382
25. Foster, L. J., Li, D., Randhawa, V. K., and Klip, A. (2001) J. Biol. Chem. 276, 44212–44221