Identification and characterization of leptin-containing intracellular compartment in rat adipose cells

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Roh, Cecilia, Galini Thoidis, Stephen R. Farmer, and Konstantin V. Kandror. Identification and characterization of leptin-containing intracellular compartment in rat adipose cells. Am J Physiol Endocrinol Metab 279: E893–E899, 2000.—The major leptin-containing membrane compartment was identified and characterized in rat adipose cells by means of equilibrium density and velocity sucrose gradient centrifugation. This compartment appears to be different from peptide-containing secretory granules present in neuronal, endocrine, and exocrine cells, as well as from insulin-sensitive GLUT-4-containing vesicles abundant in adipocytes. Exocytosis of both leptin- and GLUT-4-containing vesicles can be induced by insulin; however, only leptin secretion is responsive to serum stimulation. This latter effect is resistant to cycloheximide, suggesting that serum triggers the release of a stored pool of presynthesized leptin molecules. We conclude that regulated secretion of leptin and insulin-dependent translocation of GLUT-4 represent different pathways of membrane trafficking in rat adipose cells. NIH 3T3 cells ectopically expressing CAAT box enhancer binding protein-α and Swiss 3T3 cells expressing peroxisome proliferator-activated receptor-γ undergo differentiation in vitro and acquire adipocyte morphology and insulin-responsive glucose uptake. Only the former cell line, however, is capable of leptin secretion. Thus different transcriptional mechanisms control the developmental onset of these two major and independent physiological functions in adipose cells.

regulated secretion; glucose transport; GLUT-4

Adipocytes produce and secrete a variety of physiologically important proteins (28), such as leptin (34), adipin (8), adipocyte complement-related protein (ACRP30) (29), tumor necrosis factor-α (14), and lipoprotein lipase (11). Studies both in vivo and in vitro have demonstrated that secretion of these proteins from adipose cells has two components, constitutive and regulated. In other words, adipocytes continuously release leptin (3, 5), adipin (18, 33), ACRP30 (4, 29), and lipoprotein lipase (11, 24) into the medium, but this process may be acutely and substantially stimulated by insulin without any marked changes in the constitutive secretory pathway (4). The acute effect of insulin on secretion precedes major changes in the biosynthesis of secreted proteins, and it is preserved, at least partially, in the presence of cycloheximide (5, 24, 29). This suggests that fat cells may possess regulatable pools of presynthesized secreted proteins that may be discharged by insulin. Secretory pathways in adipocytes, however, have not yet been characterized at the molecular level, and intracellular membrane structures that are responsible for accumulation and storage of secreted proteins have not been identified. It is also not known whether or not secretion from adipose cells may be regulated by any secretagogue other than insulin.

In addition to secretion, adipose cells possess another pathway of intracellular protein trafficking that has been studied to a much greater extent. These cells translocate glucose transporter isoform 4 (GLUT-4) and several other co-localized proteins from intracellular vesicles to the cell surface in a strictly insulin-dependent fashion (9, 16, 23, 25). The role of GLUT-4-containing vesicles in the regulated secretion of soluble proteins from adipocytes has not been elucidated until recently, when two research groups demonstrated by immunofluorescence that GLUT-4 does not co-localize with either leptin (3) or ACRP30 (4). These results suggest that the “GLUT-4 pathway” may, in fact, be different from the regulated secretory pathway(s) in adipose cells.

Here, we studied compartmentalization of intracellular leptin in rat adipocytes and found that this protein is localized in a novel type of a secretory compartment that is different from both GLUT-4-containing vesicles and “classical” peptide-containing secretory granules present in endocrine cells. We have also demonstrated that, although both leptin secretion and glucose uptake in adipocytes are stimulated by insulin, only the former process is responsive to serum. This suggests that different signaling mechanisms may control regulated secretion and GLUT-4 translocation in fat cells. Finally, we show that different genetic programs are required for the developmental onset of leptin secretion and insulin-stimulated glucose uptake in differentiating adipose cells. Thus regulated secretion of leptin and translocation of GLUT-4-containing vesicles represent different pathways of membrane trafficking in adipose cells.
EXPERIMENTAL PROCEDURES

Antibodies and cell lines. In this study, we used monoclonal anti-GLUT-4 antibody 1F8 (15), rabbit polyclonal antibodies against calnexin (StressGen) and secretogranin II (Biodesign International), goat polyclonal antibody against chromogranin B (Research Diagnostics), and mouse monoclonal antibodies against trans-Golgi network marker 38 (TGN38, Affinity Bioreagents) and synaptophysin (Chemicon International). NIH 3T3 cells ectopically expressing CAAT box enhancer binding protein-α (C/EBPα) and Swiss 3T3 cells expressing peroxisome proliferator-activated receptor-γ (PPARγ) were described recently (10).

Isolation and fractionation of rat adipocytes. Adipocytes were isolated from epididymal fat pads of male Sprague-Dawley rats (150–200 g) by collagenase digestion (26) and were transferred to DMEM or to Krebs-Ringer phosphate (KRP) buffer (12.5 mM HEPES, 120 mM NaCl, 6 mM KCl, 1.2 mM MgSO4, 1 mM CaCl2, 0.6 mM Na2HPO4, 0.4 mM Na2PO4, 2.5 mM d-glucose, and 2% BSA, pH 7.4) for 15–20 min. Then, the medium was changed, and the cells were placed in the incubator (37°C, 5% CO2). Insulin (Eli Lilly) or fetal bovine serum (FBS, GIBCO) was administered to the cells where indicated for 1–2 h. The medium was saved for 2-deoxy-[3H]glucose uptake.

Primary rat adipocytes were fractionated in sucrose gradients. For velocity fractionation, 1–2 mg of light and heavy microsomes (LM and HM, respectively) resuspended in 150–200 µl of HES or PBS were loaded on a 10–50% (wt/vol) sucrose gradient for 16 h at 48,000 rpm in a Beckman SW-50.1 rotor. For equilibrium density centrifugation, the same material was fractionated in a 10–50% (wt/vol) sucrose gradient for 16 h at 48,000 rpm. After each centrifugation, fractions were collected starting from the bottom of the tube and analyzed for the total protein content and for specific proteins by radioimmunoassay, Western blot, and dot blot assay.

2-Deoxy-[3H]glucose uptake. Primary rat adipocytes were incubated in KRP without glucose in the absence or in the presence of insulin or FBS for 1 h at 37°C. One confluent 10-cm plate was washed three times with buffer A (150 mM NaCl, 10 mM HEPES, pH 7.4, 1 mM EGTA, 0.1 mM MgCl2) at 4°C. Cells were scraped from the dish into 1 ml of buffer A with protease inhibitors (1 mM PMSF, 5 mM benzamidine, 10 ng/ml each of aprotinin, pepstatin, and leupeptin) and homogenized with 13 strokes in a Potter-Elvehjem Teflon pestle, and subcellular fractions were prepared by differential centrifugation (30).

Cell culture. NIH 3T3 and Swiss 3T3 cells expressing retroviral C/EBPα and PPARγ were grown in DMEM supplemented with 10% FBS. Differentiation was induced (day 0) by changing the medium to DMEM containing 10% FBS, 0.39 µg/ml dexamethasone, 115 µg/ml IBMX, and 100 µg/ml insulin. After 48 h, the cells were transferred to DMEM containing 10% FBS and 25 µg/ml insulin. The cell medium was changed every 48 h. In the case of Swiss-PPARγ cells, 10 µM troglitazone was added to the medium (days 0–5) to promote differentiation. Differentiation of C/EBPα-transfected NIH 3T3 cells does not require troglitazone (10).

The pheochromocytoma cell line PC12 was grown in DMEM supplemented with 5% FBS, 100 units/ml penicillin, and 100 µg/ml streptomycin in 5% CO2 at 37°C. One confluent 10-cm plate was washed three times with buffer A (150 mM NaCl, 10 mM HEPES, pH 7.4, 1 mM EGTA, 0.1 mM MgCl2) at 4°C. Cells were scraped from the dish into 1 ml of buffer A with protease inhibitors (1 mM PMSF, 5 mM benzamidine, 10 ng/ml each of aprotinin, pepstatin, and leupeptin) and homogenized with 13 strokes in a Potter-Elvehjem homogenizer with a 12-µm clearance, followed by 13 strokes in a Dounce homogenizer. The cell homogenate was centrifuged at 750 g for 5 min, and 200 µl of the resulting supernatant were fractionated in a 10–50% sucrose velocity gradient as described below.

2-Deoxy-[3H]glucose uptake. Primary rat adipocytes were incubated in KRP without glucose in the absence or in the presence of insulin or FBS for 1 h at 37°C in the incubator with 5% CO2. An aliquot of fat cells (80 µl) was mixed with 40 µl of 2-deoxy-[3H]glucose (NEN) diluted with 2.5 mM of cold d-glucose to specific activity of 15 Ci/mM for 30 s in a long microfuge tube. Then, silicon oil (~40 µl) was added to the mixture, and the tube was centrifuged for 10 s in an Eppendorf microcentrifuge. The tube was cleaved in the middle of the oil layer, and floating fat cells were transferred to a scintillation vial. The amount of 2-deoxy-[3H]glucose taken by adipocytes was counted in an LKB scintillation counter. Each determination was done four times in parallel.

Fractionation of intracellular microsomes from rat adipocytes. For velocity fractionation, 1–2 mg of light and heavy microsomes (LM and HM, respectively) resuspended in 150–200 µl of HES or PBS were loaded on a 10–30% (wt/vol) sucrose gradient in 10 mM HEPES, 150 mM NaCl, 0.1 mM MgCl2, 1 mM EGTA, pH 7.4) and centrifuged for 50 min at 48,000 rpm in a Beckman SW-50.1 rotor. For equilibrium density centrifugation, the same material was fractionated in a 10–50% (wt/vol) sucrose gradient for 16 h at 48,000 rpm. After each centrifugation, fractions were collected starting from the bottom of the tube and analyzed for the total protein content and for specific proteins by radioimmunoassay, Western blot, and dot blot assay.

Fig. 1. Equilibrium density fractionation of intracellular microsomes from rat adipocytes. Light (LM) and heavy (HM) microsomes (1 mg in 0.15–0.2 ml of PBS with protease inhibitors) were fractionated in a 10–50% equilibrium sucrose gradient for 16 h at 48,000 rpm in a Beckman SW-50.1 rotor. Fractions were collected from the bottom of the tube and analyzed for total protein content. Concentration of leptin in fractions was determined by RIA. Equal volume aliquots of even fractions were subjected to SDS-PAGE, and individual proteins were analyzed by Western blotting. Figure shows a representative result of 3 independent experiments.
Immunoadsorption of GLUT-4-containing vesicles. Protein A-purified 1F8 antibody and nonspecific mouse IgG (Sigma) were each coupled to acrylic beads (Reacti-gel GF 2000, Pierce) at a concentration of 0.50 and 0.56 mg of antibody/ml of resin, respectively, according to the manufacturer’s instructions. Before use, the beads were saturated with 2% BSA in PBS for ≥1 h and washed with PBS. LM and HM (200–600 mg) from rat adipocytes were incubated separately with 100–200 ml each of the specific and nonspecific antibody-coupled beads overnight at 4°C. The beads were washed five times with PBS and eluted with 1% Triton X-100 in PBS. This fraction was used for leptin determination. The beads were then washed again with PBS and with 10 mM Tris-HCl, pH 7.8, and eluted with Laemmli sample buffer (19) for the analysis of immunoadsorbed GLUT-4 by Western blotting.

Gel electrophoresis and immunoblotting. Proteins were separated by SDS-PAGE according to Laemmli (19) but without reducing agents and were transferred to Immobilon-P membranes in 25 mM Tris, 192 mM glycine, pH 8.3. After transfer, the membrane was blocked with 10% nonfat dry milk in PBS for 1 h at room temperature. Proteins were visualized with specific antibodies, horseradish peroxidase (HRP)-conjugated secondary antibodies (Sigma), and an enhanced chemiluminescent substrate kit (NEN). Autoradiograms were quantitated in a PhosphorImager (Molecular Dynamics).

Dot blot analysis. Nondenatured proteins were bound to Immobilon-P membrane with the help of a dot blot apparatus. The membrane was washed thoroughly and probed with anti-TGN38 antibody (1:500) in 5% BSA overnight at 4°C. Then, the membrane was washed and treated with HRP-conjugated secondary antibodies and chemiluminescent substrate similar to Western blot membranes. Resulting autoradiograms were quantitated in a computing densitometer (Molecular Dynamics).

Radioimmunoassay. Leptin content was determined with the help of a 125I-leptin radioimmunoassay kit (Linco) according to the manufacturer’s instructions. In some experiments, when the leptin content was analyzed in the cell medium, samples were lyophilized and solubilized in water. Each determination (except for the gradient fractions) was done three to five times in parallel.

Fig. 2. Sucrose velocity gradient fractionation of intracellular microsomes from rat adipocytes (A) and postnuclear extract from PC12 cells (B). Same amounts of LM and HM as well as postnuclear extract from PC12 cells were fractionated in 10–30% sucrose gradients for 50 min at 48,000 rpm in a Beckman SW-50.1 rotor. Gradient fractions were analyzed as described in Fig. 1 legend. Trans-Golgi network marker 38 (TGN38) was determined in even fractions by dot blot analysis (see EXPERIMENTAL PROCEDURES). Figure shows a representative result of 3 independent experiments.
RESULTS

Intracellular pools of leptin and GLUT-4 are localized in different vesicular carriers. Rat adipocytes were separated into five crude subcellular fractions by differential centrifugation according to Simpson et al. (30). These fractions include: 1) first low-speed pellet (mitochondria, nuclei, and lysosomes), 2) plasma membrane (PM), 3) HM (intermediate fraction enriched with endoplasmic reticulum, but also containing PM and LM markers), 4) LM, which contain Golgi apparatus, trans-Golgi network, and endosomes, and 5) cytosol. Noteworthy, this fractionation procedure allows the separation of the whole adipocyte into subcellular fractions without any “leftover” with the exception of the “lipid cake,” which is discarded after the first centrifugation. Of all these fractions, only HM and LM contained significant amounts of leptin; therefore, they were fractionated further in a 10–50% equilibrium density sucrose gradient. Under these conditions, leptin-containing membranes form a distinct peak with a buoyant density roughly corresponding to the density of other microsomal structures in the cell (compare the distribution of leptin and the total microsomal protein in Fig. 1).

Next, we fractionated LM and HM in a 10–30% continuous sucrose velocity gradient (Fig. 2A) and found that leptin-containing material has a rather narrow sedimentational distribution and is very well separated from the bulk of the total microsomal protein. In both types of gradients, however, (Figs. 1 and 2A), the distribution of leptin-containing membranes recovered in LM and HM is the same, which suggests that there is only one major leptin-containing compartment in the adipose cell. As is seen in Fig. 2A, this material sediments much faster than LM and somewhat slower than HM. This may explain the roughly equal distribution of leptin-containing membranes between HM and LM upon differential centrifugation of adipocyte homogenates.

Although the distribution of leptin-containing membranes in sucrose velocity gradient overlaps somewhat with that of the endoplasmic reticulum marker, calnexin, and TGN38, our results indicate that the major pool of intracellular leptin is separated from and, hence, is not localized in these compartments.

To compare the sedimentational properties of leptin-containing vesicles and classical secretory granules, we centrifuged the postnuclear extract prepared from the pheochromocytoma cell line PC12 in a parallel sucrose gradient (Fig. 2B). Under these conditions, two marker proteins for the secretory granules, secretogranin II and chromogranin B, were recovered in the pellet of the gradient. On the other hand, the position of small synaptic vesicles marked by synaptophysin is rather close to that of GLUT-4-containing vesicles and, also, to leptin-containing membranes, with the latter being significantly heavier (compare Fig. 2A with 2B). We thus conclude that the major leptin-containing compartment in adipocytes is different from classical peptide-containing secretory granules.

As is seen in Fig. 2A, leptin-containing membranes partially overlap with GLUT-4-containing vesicles, especially in the LM fraction. To determine whether or not leptin is present in GLUT-4 vesicles, we purified the latter by immunoadsorption with 1F8 beads (see EXPERIMENTAL PROCEDURES). This procedure allows us to isolate ~95% of GLUT-4 vesicles; however, only a small albeit statistically significant amount of leptin was recovered in the immunoadsorbed material [compare the results of specific (1F8) and nonspecific (Ig) immunoadsorptions in Fig. 3]. On the basis of these data, we believe that leptin is largely excluded from insulin-sensitive GLUT-4-containing vesicles, which is consistent with the previous results of Barr et al. (3) obtained by immunofluorescence.

The origin of leptin-containing vesicles is, at present, unclear (see DISCUSSION). Their highly specific and uniform distribution on sucrose gradient centrifugation suggests that these vesicles may exist in the cell as an individual secretory compartment. There is still a possibility, however, that leptin-containing vesicles may, at least partially, be a product of artificial fragmenting of larger membrane structures. We are now trying to address this question experimentally.

Leptin secretion but not glucose transport is stimulated by serum at the post translational level. As has been shown recently (3, 5), secretion of leptin from
adipocytes is acutely and substantially stimulated by insulin. Here, we confirm those results and demonstrate that serum also has an analogous stimulatory effect on leptin secretion (Fig. 4A). In control experiments, we have shown that leptin is stable in the adipocyte medium, even after overnight incubation at 37°C (results not shown). Therefore, our results should not be affected by leptin processing in the medium. Cycloheximide does not prevent serum-induced acute increase in leptin secretion (Fig. 4B, left); neither does it block a concomitant decrease in intracellular leptin content (Fig. 4B, right). Noteworthy, the amount of intracellular leptin in serum-treated adipocytes falls acutely below the detection threshold. On the basis of this result and the data shown in Figs. 1 and 3, we suggest that the major pool of intracellular leptin in adipocytes is localized in a regulatable population of small storage vesicles. In our preliminary experiments, we checked several downstream secondary messengers to “shortcut” signaling pathways that have the potential to trigger acute leptin secretion. However, in these experiments, the Ca\(^{2+}\) ionophore A-23187 and the phorbol ester PMA (phorbol 12-myristate 13-acetate) were without effect (data not shown).

Serum administration does not substantially increase glucose uptake into adipose cells (Fig. 4C). This result may be expected, because insulin concentration in FBS, as specified by the manufacturer, does not exceed 40 pM. Therefore, final concentration of insulin in our assay is <4 pM, which is much below its effective dose for stimulation of glucose uptake (17). We thus believe that the differential effect of serum on leptin

Fig. 4. The effect of insulin and serum on leptin secretion and glucose uptake. A: freshly isolated rat adipocytes (20% suspension) were incubated in the absence (open bars) and presence (closed bars) of 10% fetal bovine serum (FBS, left) or 40 nM insulin (right) for 1 h at 37°C in the incubator with 5% CO\(_2\). Aliquots of the cell medium were collected at the beginning and end of incubation and concentrated 10-fold by lyophilization, and leptin content was determined by RIA. Figure shows mean values ± SE of ≥3 independent measurements. Absence of error signs on some bars indicates that error is virtually undetectable. A representative result of 3 independent experiments is shown. B, left: freshly isolated rat adipocytes (20% suspension) were preincubated for 20 min with (+CHX) or without (-CHX) 100 μg/ml of cycloheximide. The medium was changed, and incubation was continued for 1 h in the absence (open bars) or presence (closed bars) of 10% FBS for 1 h at 37°C in the incubator with 5% CO\(_2\). Cycloheximide (100 μg/ml) was added in the incubation medium where indicated. Aliquots of the cell medium were collected at the beginning and end of incubation and were concentrated 10-fold by lyophilization, and leptin content was determined by RIA. Right: adipocytes were homogenized at the end of the incubation with serum and cycloheximide, and intracellular microsomes (HM + LM) were isolated. Leptin content in this material was determined by RIA (closed bar) and compared in the same experiment with leptin content in intracellular membranes of untreated adipocytes (open bar). Figure shows mean values ± SE of ≥3 independent measurements. Absence of error signs on some bars indicates that error is virtually undetectable. A representative result of 3 independent experiments is shown. C: freshly isolated rat adipocytes were incubated in KRP buffer without glucose in the absence and presence of 40 nM insulin (Ins) or 10% FBS (Serum) for 1 h at 37°C in the incubator with 5% CO\(_2\). Aliquots (80 μl) of packed cells were taken for analysis of 2-deoxy-[\(^3\)H]glucose (2-DOG) uptake as described in EXPERIMENTAL PROCEDURES. Figure shows mean values ± SE of 4 independent measurements. A representative result of 2 independent experiments is shown.
secretion and glucose uptake indicates that these processes may be controlled by different regulatory mechanisms.

Developmental onset of leptin secretion and GLUT-4 translocation. It has been demonstrated previously (13, 20, 22) that GLUT-4 and leptin represent important markers of adipocyte differentiation. Because regulated secretion of leptin and GLUT-4 translocation are likely to represent different and independent pathways of intracellular membrane traffic, it is interesting to determine whether or not the same transcriptional mechanism is responsible for the developmental onset of these important physiological functions.

Differentiation of adipocytes is controlled mainly by two families of transcription factors, the C/EBP family and the PPAR family (21). It has been shown that, upon ectopic expression of PPARγ in Swiss 3T3 cells and C/EBPα in NIH 3T3 cells, these normally nondifferentiating fibroblastic cell lines acquire an adipocyte-like morphology as well as insulin-stimulated glucose uptake (10). We show here that only in the latter case are these cells able to secrete leptin, despite the fact that the former cell line produces more GLUT-4 protein (Fig. 5). Thus insulin-regulated glucose transport can be uncoupled from leptin secretion in differentiating adipocytes. Previous results demonstrate that, in the process of differentiation in vitro, lipid accumulation can be uncoupled from insulin-activated glucose uptake (10). We think, therefore, that there may be no “common program” for adipocyte differentiation. Rather, the acquisition of the individual adipocyte features, such as lipid storage, insulin-activated glucose uptake, and secretion of physiologically important proteins, may require their own genetic programs.

DISCUSSION

We have compared the biochemical properties and regulation of leptin- and GLUT-4-containing vesicular carriers in adipose cells. All aspects of this study indicate that regulated secretion of leptin and insulin-dependent translocation of GLUT-4 represent different pathways of membrane trafficking. Also, the leptin-containing compartment from adipocytes is obviously different from the peptide-containing secretory granules present in neuronal, endocrine, and exocrine cells in buoyant density, sedimentational behavior, and kinetics of secretion. What, then, could be the nature of leptin-containing vesicles? A related question is whether this type of secretory vesicles is unique to adipocytes, or whether there are analogous compartments present in other cells.

Recent evidence suggests that cells that have previously been known as constitutive secretory cells may, in fact, possess regulated secretory pathways. Results obtained in Palade’s laboratory (27) demonstrate that, in hepatocytes, different vesicular carriers are responsible for secretion of soluble proteins (albumin, apolipoprotein B, prothrombin, C3 component of the complement, and caeruloplasmin) and membrane proteins (polymeric IgA receptor, transferrin receptor, asialoglycoprotein receptor, etc.). These two types of vesicular carriers have different sizes but a similar buoyant density, which is much less than the buoyant density of classical dense core secretory granules. Thus hepatocytes sort their soluble secretory products away from the constitutive pathway of membrane protein delivery (as is the case with regulated secretion), but they discharge these products continuously without the involvement of a secretagogue.

A “real” regulated secretory pathway has recently been elucidated in other constitutive cell lines, L and CHO (7). In these cells, a fraction of newly synthesized glucosaminoglycans is retained inside the cell in a population of rab3D-containing post-Golgi storage vesicles with a buoyant density much less than that of classical secretory granules.

In our laboratory, we have recently found a novel type of secretory vesicles in brain and PC12 cells that can be separated from both secretory granules and synaptic vesicles (31, 32). These vesicles contain a novel enzyme, aminopeptidase B (6). Similar to adipocyte products, aminopeptidase B is constitutively released from cells, but its secretion can be substantially stimulated by various secretagogues (2).
Sedimentation properties and the buoyant density of leptin-containing vesicles in fat cells (Figs. 1 and 2), aminopeptidase B-containing vesicles from neurons (31, 32), regulated vesicles in CHO cells (7), and soluble protein-carrying secretory vesicles in hepatocytes (27) are all rather similar. In addition, adipocytes contain large amounts of rab3D (1) whose expression mimics that of leptin in differentiation (1, 20) and is upregulated along with leptin in adipocytes from obese rats (12). Thus adipocytes may secrete their physiologically important products via a novel regulated secretory pathway, the molecular aspects of which are just beginning to emerge.

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REFERENCES


