Adipose cells produce and secrete several physiologically important proteins, such as lipoprotein lipase (LPL), leptin, adipsin, Acrp30, etc. However, secretory pathways in adipocytes have not been characterized, and vesicular carriers responsible for the accumulation and transport of secreted proteins have not been identified. We have compared the intracellular localization of two proteins secreted from adipose cells: leptin and LPL. Adipocytes accumulate large amounts of both proteins, suggesting that neither of them is targeted to the constitutive secretory pathway. By means of velocity centrifugation in sucrose gradients, equilibrium density centrifugation in iodixanol gradients, and immunofluorescence confocal microscopy, we determined that LPL and leptin were localized in different membrane structures. LPL was found mainly in the endoplasmic reticulum with a small pool being present in low density membrane vesicles that may represent a secretory compartment in adipose cells. Virtually all intracellular leptin was localized in these low density secretory vesicles. Insulin-sensitive Glut4 vesicles did not contain either LPL or leptin. Thus, secretion from adipose cells is controlled both at the exit from the endoplasmic reticulum as well as at the level of “downstream” secretory vesicles.

Fat is now emerging as an important endocrine tissue in mammalian organism. Adipose cells produce and secrete various physiologically important proteins, such as leptin (1), resistin (2), tumor necrosis factor-α (3), Acrp30 (4), adipsin (5), lipoprotein lipase (LPL) (6), etc., but the cellular biological nature of adipocyte secretion remains obscure. According to the current paradigm, secreted proteins are transported by specialized vesicular carriers through either the constitutive secretory pathway that exists in all cell types or through regulated secretory mechanisms that control the translocation of Glut4 and Glut4-containing membrane vesicles to the cell surface in response to insulin stimulation (19). This raises a possibility that glucose transporter vesicles may contain secreted proteins as soluble “cargo” and, therefore, may represent a specialized secretory organelle in insulin-sensitive cells (13).

Although it may still be true for Glut4-vesicles, it turned out that Glut4 and several secreted proteins, such as leptin, adipsin, Acrp30, and LPL, are localized in different vesicular carriers (see Refs. 9, 11, 12, and 14 and Fig. 3). Moreover, regulatory mechanisms that control the translocation of Glut4 and secretion of adipsin (14) and leptin (11) are different. These data strongly indicate that the “Glut4 pathway” is different from secretory pathway(s) in adipose cells, but these data give us no insight into the molecular nature of adipocyte secretion.

Here, we compared the intracellular localization of leptin and lipoprotein lipase in rat adipocytes by biochemical fractionation and immunofluorescence confocal microscopy. We found that the major intracellular pools of these proteins do not overlap, with LPL being localized largely in the endoplasmic reticulum and leptin in light vesicles that may represent a downstream secretory compartment. This suggests that adipocytes may have multiple “checkpoints” along their secretory pathway such that the release of different proteins may be regulated at several levels.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—Monoclonal anti-leptin antibody and polyclonal anti-calnexin antibody were from StressGen. Monoclonal anti-transferrin receptor antibody was from Zymed Laboratories Inc. Monoclonal anti-TGN38 antibody was from Transduction Laboratories. Affinity-purified polyclonal anti-calreticulin antibody was from Affinity BioReagents.

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The abbreviations used are: LPL, lipoprotein lipase; HM, heavy microsomes; LM, light microsomes; PM, plasma membranes; PBS, phosphate-buffered saline; ER, endoplasmic reticulum; FITC, fluorescein isothiocyanate; KRP, Krebs–Ringer phosphate.
Rabbit polyclonal anti-sortilin antibody was raised against the peptide acetyl-CFGQSKLYRSEDYGKNFKD-amide (amino acids 17–34) and affinity-purified by Quality Controlled Biochemicals, Inc., Hopkinton, MA. Rabbit polyclonal anti-leptin antibody was raised against the N-terminal 70 amino acids of leptin and affinity-purified on leptin-Sepharose columns. Monoclonal anti-Chip antibody 1F8 (Western Blotting) and polyclonal antibody against lipoprotein lipase (21) were described previously. Cy2-, Cy3-, and FITC-labeled secondary antibodies were from Jackson ImmunoResearch Laboratories, Inc., West Grove, PA.

**Isolation and Fractionation of Rat Adipocytes—**Adipocytes were isolated from epididymal fat pads of male Sprague-Dawley rats (150–200 g) by collagenase digestion (22). The fat pads were immersed in Krehbiel-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% bovine serum albumin, pH 7.4), minced, and subjected to collagenase ( Worthington) digestion for 35 min at 37 °C with constant shaking at 125 cycles/min. Adipocytes were filtered through 400-μm nylon mesh (Tetko) and washed three times with KRP. Cells were then equilibrated at 37 °C for 25 min with constant shaking at 25 cycles/min, washed twice with HES buffer (20 mM HEPES, 250 mM sucrose, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin, 1 μM aprotinin, and 1 μM leupeptin, pH 7.4) at room temperature, and homogenized with a Potter-Elvejem Teflon pestle. The cell homogenate was centrifuged for 20 min at 15,800 × g at 4 °C. After removal of the fat layer, the remaining supernatant was centrifuged for 20 min at 15,800 × g at 4 °C for 20 min to pellet high density microsomes (HM). Light microsomes were pelleted by centrifugation of the supernatant at 150,000 × g for 90 min at 4 °C. The first pellet was centrifuged through a sucrose cushion (1.12 M sucrose in 20 mM HEPES) at 71,000 × g for 90 min at 4 °C. The second pellet was centrifuged through a 32% (w/v) sucrose solution (750 ml of chloroform, 846 ml of methanol, 600 ml of heptane, 1 part concentrated substrate, 6 parts distilled water, 1 part 1M Tris, 4% phosphatidylcholine, and 392 ml of HES) at 100,000 × g for 90 min at 4 °C. The third pellet was resuspended in 6 ml of glycerol, and sonicated for 6 min. This material was collected and pelleted at 32,000 × g for 25 min at 4 °C. For the preparation of intracellular microsomes (HM + LM), the first supernatant was centrifuged at 150,000 × g at 4 °C for 90 min.

**LPL Activity—**The activity was measured according to Eckel et al. (18) with minor modifications. Aliquots (25 μl) of subcellular fractions from rat adipocytes were mixed with the same volume of extraction buffer (0.2 M Tris, 1% BSA, 10 units/ml heparin, 0.73% sucrose, 0.5% deoxycholate, and 0.02% Nonidet P-40, pH 8.3), incubated on ice for 30 min, and supplied with 0.8 ml of extraction buffer without deoxycholate and Nonidet P-40. Aliquots of these samples (150 μl) were mixed with the same volume of working substrate solution and incubated at 37 °C for 1 h with constant shaking at 50 cycles/min. Then, 3.25 ml of stop solution (750 ml of chloroform, 846 ml of methanol, 600 ml of heptane, and 0.2 ml of 100 mg/ml oleic acid) was added to the reaction mixture. Samples were briefly vortexed, mixed with 1.05 ml of borate-carbonate buffer (6.2 g of boric acid and 13.8 g of potassium carbonate in 1 liter, pH 10.5), vigorously vortexed, and centrifuged for 25 min at room temperature at 2000 rpm. The top phase was collected, and 1H radioactivity was counted using an LKB scintillation counter. Working substrate solution was prepared as described previously (23). Briefly, a 1% solution of triolein (PerkinElmer Life Sciences, 200 μl, 5 mCi/ml), 216 μl of phosphatidylcholine, and 392 μl of triolein were dried under nitrogen, resuspended in 6 ml of glycerol, and sonicated for 6 min. This material was called concentrated substrate. Working substrate solution included 1 part concentrated substrate, 6 parts distilled water, 1 part 1 M Tris, pH 8.6, 1 part 15% BSA, and 1 part serum.

**Fractionation of Intracellular Microsomes in Sucrose and Iodixanol Gradients—**For sucrose velocity fractionation, intracellular microsomes (2 mg of the total protein) were resuspended in 150–200 μl of HES and loaded on a 10–30% (w/v) sucrose gradient prepared in 10 mM HEPES, 150 mM NaCl, 0.1 mM MgCl2, and 1 mM EGTA, pH 7.4. Centrifugation lasted for 50 min at 150,000 × g in a Beckman SW-50.1 rotor at 4 °C. For equilibrium density fractionation in iodixanol gradients, the same amount of intracellular microsomes was layered on a 10–20% or on a 10–30% (w/v) iodixanol gradient in HES and centrifuged for 5 h at 29,000 rpm at 4 °C. After centrifugation, fractions were collected starting from the bottom of the tube and were analyzed for total protein content with the help of BCA kit (Pierce). Individual proteins along with LPL activity were analyzed in the gradient fractions by immunoblotting or radioimmunoassay.

**Immunoadsorption of Glut4-containing Vesicles—**Protein A-purified 1F8 antibody as well as nonspecific mouse IgG (Sigma) were each coupled to acrylic beads (Reacti-gel GF 2000, Pierce) according to the manufacturer’s instructions. Before use, the beads were saturated with KRP for at least 1 h and washed with PBS. Intracellular membranes (200 μg) from rat adipocytes were incubated separately with each of the specific and nonspecific antibody-coupled beads overnight at 4 °C. The beads were washed four times with PBS and once with 10 mM Tris-HCl, pH 7.8, and eluted with 1% Triton X-100 in PBS. Then, the beads were washed again and eluted with Laemmli sample buffer. The eluates along with nonadsorbed material were used for the analysis of individual proteins by Western Blotting. LPL was measured in Triton eluates with the help of radioimmunoassay (see below).

**Immunofluorescence Staining—**Isolated rat adipocytes were fixed at room temperature with 4% paraformaldehyde and 4% sucrose in PBS, pH 7.4, for 15 min with mild shaking. Cells were then washed twice with PBS by microcentrifugation for 3 s, incubated in 0.1% Triton X-100 for 5 min, and washed again three times with PBS. Permeabilized cells were blocked with 3% donkey serum and 1% BSA in PBS overnight on a rocking platform. Cells were then divided into 500-μl aliquots, placed into microcentrifuge tubes, and incubated with primary antibodies for 2 h on a rocking platform. Cells were washed twice with PBS, incubated with secondary antibodies (1:100 in blocking solution) for 1 h, and washed again twice with PBS. Cells were then diluted 1:20 with 10% glycerol in PBS, and an aliquot (5 μl) of this suspension was placed into a specialized deep-well slide, covered with a square-shaped coverslip, and sealed. Staining was examined with the help of confocal laser scanning microscopy (Zeiss LSM 510).

**Gene Electrophoresis, Immunoblotting, and Radioimmunoassay—**Proteins were separated by SDS-polyacrylamide gel electrophoresis according to Laemmli (24) and transferred a large amount of enzymatically active LPL, which was enriched in heavy microsomes and also in the plasma membrane fraction. This is consistent with earlier results showing that secreted LPL may associate with cell surface heparan sulfate proteoglycans (6) or may be attached to the plasma membrane via the glycosylphosphatidylinositol anchor (26). In addition, a fraction of LPL activity in the PM fraction may be derived from heavy microsomes that contaminate plasma membranes (25). Fig. 1 shows that the activity of LPL does not necessarily correlate with its amount in subcellular fractions as determined by Western blot analysis (Fig. 1B). This effect is likely to be explained by post-translational regulation of the enzyme by glycosylation, dimerization, and possibly, by other unknown factors (27–30). We found that the specific activity of LPL is the highest in the PM fraction, which is consistent with the idea that the plasma membrane represents the functional site of the enzyme. In any case, the results of both the Western blot analysis and LPL activity assay showed that the major pool of intracellular LPL was present in heavy microsomes (Fig. 1), a subcellular fraction that contains mostly endoplasmic reticulum (25). Fig. 1C demonstrates that intracellular leptin is recovered mainly in light microsomes, a fraction that is enriched in Golgi complex, trans-Golgi network, and endosomes. Thus, the pools of leptin and LPL can be partially separated simply by crude subcellular fractionation.

To confirm this result, we isolated total intracellular microsomes (HM + LM) and fractionated this material in a 10–30% continuous sucrose velocity gradient (Fig. 2). Under these conditions, leptin-containing vesicles were very well separated from the bulk of microsomal protein and sedimented as a homogenous peak in the first third of the gradient, whereas enzymatically active LPL was found in heavier structures in a broad sedimentational distribution. LPL-containing membranes...
sedimented faster than Golgi apparatus and trans-Golgi net-
work marked with TGN38 but overlapped significantly with
calnexin, a marker for the endoplasmic reticulum. This sug-
gests that in adipose cells, a large pool of enzymatically active
LPL may be stored in the endoplasmic reticulum. We obtained
similar results when we fractionated HM and LM separately
(results not shown).

Despite a clear difference in the sedimentational properties
of LPL- and leptin-containing carriers (Fig. 2), their positions
in the sucrose gradient partially overlap. To further study the
compartmentalization of these secreted proteins, we fraction-
ated intracellular microsomes in a 10–20% equilibrium density
gradient of iodixanol (Fig. 3A). It turned out that leptin-con-
taining vesicles (fractions 14–15) had very low buoyant den-
sity. Although some LPL was also present in low density ves-
icles, its major pool was found in membranes with much higher
buoyant density (fractions 3–11) that may represent rough
endoplasmic reticulum. Unfortunately, we were unable to use
ER proteins as markers for dense LPL-containing membranes
because under the conditions used, they were more or less
equally distributed throughout the gradient. This is to be ex-
pected, however, because ER resident proteins are present in
both rough and smooth endoplasmic reticulum, which have
different buoyant densities.

These results were confirmed by analogous experiments in
which we used more concentrated 10–30% equilibrium density
gradients of iodixanol (Fig. 3B). In a steeper gradient, the
analyzed material was concentrated in more narrow density
zones so that the peaks of LPL- and leptin-containing vesicular
carriers became more distinct. However, regardless of the ex-
perimental conditions used, our conclusion remained the same.
Namely, the major intracellular pool of leptin was present in
low density vesicles, whereas the majority of LPL was accumu-
lated in the material with the higher buoyant density, which is
likely to represent the endoplasmic reticulum.

Intracellular localization of LPL and leptin was further stud-
yed by immunofluorescence confocal microscopy. Primary adi-
pocytes represent a difficult cell type for microscopy because
almost the whole volume of the cell is occupied by the central
lipid droplet surrounded by a thin rim of cytoplasm. Nonethe-
less, Fig. 4A clearly shows the differential localization of LPL
and leptin in the rat adipose cell. At the same time, the distri-
bution of LPL significantly overlaps with that of calreticulin,
another ER marker (Fig. 4B). In special control experiments,
we determined that the LPL staining of heparin-washed and
untreated adipocytes is similar (not shown). Therefore, Fig. 4,
A and B, may show the localization of the intracellular LPL
The intracellular localization of leptin and calreticulin, as revealed by double immunofluorescence staining, is different (see both the low and high magnification panels of Fig. 4C). These data confirm the results of sucrose and iodixanol gradient centrifugations and strongly suggest that LPL and leptin reside in different subcellular structures. In the case of LPL, that structure is likely to be the endoplasmic reticulum, whereas leptin is localized in homogenous secretory vesicles.

Upon centrifugations in the velocity sucrose (Fig. 2) and equilibrium density iodixanol (Fig. 3) gradients, the positions of both LPL and leptin significantly overlap with that of Glut4. This raises a possibility that a fraction of these secreted proteins may reside in Glut4-containing vesicles. Such a hypothesis would be consistent with the results of the recent report showing that sortilin, one of the major component proteins of Glut4 vesicles, directly binds to LPL and may mediate endocytosis and degradation of LPL in transfected Chinese hamster ovary (CHO) cells (31). To determine whether or not sortilin and LPL co-localize in the same compartment in adipose cells, we immunoadsorbed Glut4-containing vesicles and analyzed this material for the presence of sortilin and LPL by Western blot (Fig. 5). We have also measured the amount of leptin in Glut4 vesicles by radioimmunoassay. We found that virtually all sortilin may be immunoadsorbed with monoclonal anti-

**Fig. 3.** Fractionation of intracellular microsomes from rat adipocytes in equilibrium density iodixanol gradients. Intracellular microsomes (2 mg in 0.2 ml of HES) were layered on top of a 10–20% iodixanol gradient (A) or a 10–30% iodixanol gradient (B) and centrifuged for 5 h at 29,000 rpm in a Beckman SW-50.1 at 4 °C. Fractions were collected from the bottom of the tube and analyzed for LPL and Glut4 by Western blotting (bottom panels) and for leptin content by radioimmunoassay (closed circles). LPL bands were quantitated in a computing densitometer (Molecular Dynamics) and plotted as open circles. Arrows indicate the direction of sedimentation. A representative result of three independent experiments is shown. a.u., arbitrary units.

**Fig. 4.** Double immunofluorescence staining of rat adipocytes. A, cells were stained with anti-LPL antibody (1:100) and Cy3-conjugated donkey anti-chicken IgG (red staining) and with polyclonal anti-leptin antibody (1:100) and FITC-conjugated donkey anti-rabbit IgG (green staining). B, cells were stained with anti-LPL antibody (1:100) and Cy3-conjugated donkey anti-chicken IgG (red staining) and with polyclonal anti-calreticulin antibody (1:100) and Cy2-conjugated donkey anti-rabbit IgG (green staining). C, cells were stained with monoclonal anti-leptin antibody (1:100) and Cy3-conjugated donkey anti-mouse IgG (red staining) and with polyclonal anti-calreticulin antibody (1:100) and FITC-conjugated donkey anti-rabbit IgG (green staining). The optical section of the cell shown in this panel goes through the nucleus that can be seen on the left. The bottom panel demonstrates high magnification of the bottom part of the cell.

**Fig. 5.** Immunopurified Glut4-containing vesicles do not contain either LPL or leptin. Intracellular membranes (0.2 mg) were immunoadsorbed with 100 µl of 1F8- or nonspecific IgG-bound beads. Beads were washed and eluted with 300 µl of 1% Triton X-100 in PBS and then with 300 µl of SDS-containing Laemmli sample buffer. The eluates were analyzed for Glut4, LPL, and sortilin by Western blotting. For leptin determination (bottom panel), intracellular membranes (0.5 mg) were immunoadsorbed with 200 µl of immunobeads. Leptin content was measured in the unbound material (sup) and in Triton eluates by radioimmunoassay. The bottom panel shows the mean values ± S.E. of three independent measurements. The absence of error bars indicates that error is virtually nondetectable. A representative result of at least three independent experiments is shown. n/d, not detectable.
Glut4 antibody 1F8. However, neither LPL nor leptin is present in Glut4 vesicles because both proteins stay in the supernatant (see also Ref. 11).

**DISCUSSION**

Despite their simple morphology, adipose cells possess a complicated network of membrane trafficking pathways that cannot be limited to ubiquitous endosomal recycling and constitutive secretion. In particular, these cells possess abundant moveable membrane compartments, such as Glut4-containing vesicles and regulated secretory vesicles. It has been shown by several independent approaches that in adipocytes, Glut4 vesicles do not contain any known secreted cargo proteins (see Refs. 9, 11, 12, and 14 and Fig. 5), the intracellular compartmentalization of which remains largely unknown. We report here that a large pool of enzymatically active LPL resides in the endoplasmic reticulum with a relatively small fraction of LPL being present in low density vesicles that may represent a distal compartment in the secretory pathway. This conclusion is consistent with earlier results of Doolittle et al. (29), who studied glycosylation of LPL in whole adipose tissue. Thus, LPL is different from typical constitutively secreted proteins that normally are not accumulated inside the cell in any significant quantities (8).

At the same time, the major fraction of intracellular leptin is localized not in the endoplasmic reticulum but in low density membrane vesicles that behave similarly to LPL-containing vesicles. Leptin-containing vesicles represent a regulated secretory compartment in adipocytes because incubation with 1 h with insulin completely depletes intracellular leptin stores (11). This compartment may also be analogous to Acrp30-containing vesicles found by Bogan and Lodish (12), who showed recently that a significant pool of Acrp30 resided in peripheral secretory vesicles that lacked ER marker proteins. Thus, secretion from adipose cells may be regulated at different levels: at the exit from the endoplasmic reticulum, as is the case for LPL, and at the level of downstream vesicular storage compartment (leptin, Acrp30). Constitutively secreted proteins should be able to pass freely through all checkpoints on the secretory pathway.

What could be the cellular biological nature of low density secretory vesicles described here? Is this compartment unique for adipose cells, or is it present in other cells as well? In fact, small low density secretory vesicles have been found in other cell types, such as hepatocytes (32) and PC12 cells (33). Moreover, it has also been shown that regulated secretory pathways may be present in “constitutive” L- and CHO cells (34) in which a fraction of newly synthesized glucosaminylcogalactosamine is retained inside the cell in a population of low density post-Golgi storage vesicles sensitive to Ca^{2+} and phorbol esters. We suggest, therefore, that leptin may represent a cell-specific cargo of a widely distributed regulated secretory compartment of yet unknown biochemical composition and regulation.

Interestingly, ablation of endosomes with HRP-conjugated transferrin, dianisobenzidine, and H_{2}O_{2} dramatically inhibits adipin secretion from 3T3-L1 adipocytes (14), indicating that the endosomal system may mediate secretion of this protein. Such a conclusion is indirectly supported by our data showing that leptin-containing vesicles partially overlap with transferrin receptor-containing endosomes in iodixanol and sucrose gradients (data not shown). However, the immunofluorescence data of Bogan and Lodish (12) demonstrated that the distribution of Acrp30 in 3T3-L1 cells is different from that of the transferrin receptor. Thus, it is not known whether or not the endosomal system is involved in secretion of leptin or Acrp30.

A part of the reason is that primary rat adipocytes are not an appropriate cell type for ablation experiments, whereas cultured 3T3-L1 adipocytes do not produce a sufficient amount of leptin for analysis. Thus, novel technical approaches are required to further investigate the molecular nature of the secretory pathways in adipose cells.

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