ADP-ribosylation factor 6 (ARF6) appears to play an essential role in the endocytic/recycling pathway in several cell types. To determine whether ARF6 is involved in insulin-regulated exocytosis, 3T3-L1 adipocytes were infected with recombinant adenovirus expressing wild-type ARF6 or an ARF6 dominant negative mutant (D125N) that encodes a protein with nucleotide specificity modified from guanine to xanthine. Overexpression of these ARF6 proteins affected neither basal nor insulin-regulated glucose uptake in 3T3-L1 adipocytes, nor did it affect the subcellular distribution of Glut1 or Glut4. In contrast, the secretion of adipin, a serine protease specifically expressed in adipocytes, was increased by the expression of wild-type ARF6 and was inhibited by the expression of D125N. These results indicate a requirement for ARF6 in basal and insulin-regulated adipin secretion but not in glucose transport.

Our results suggest the existence of at least two distinct pathways that undergo insulin-stimulated exocytosis in 3T3-L1 adipocytes, one for adipin release and one for glucose transporter translocation.

ADP-ribosylation factors are members of the Ras superfamily of low molecular weight GTP-binding proteins. Although ARFs1 were identified as cofactors required for the cholera toxin-catalyzed ADP-ribosylation of heterotrimeric G protein, Gs (1), they have subsequently been shown to play an important role in numerous membrane trafficking events. In addition, ARFs also stimulate the activity of phospholipase D (PLD) in vitro (2–5), suggesting that they may exert their effects at least in part by altering membrane phospholipid metabolism.

ARF6, one member of the ARF family, has been suggested to play a role in vesicle trafficking and in cytoskeletal organization (2, 6–13). The available data suggest that ARF6 plays a key role in membrane trafficking along the endocytic pathway (6, 10). However, overexpressed ARF6 in HeLa cells is localized to the plasma membrane and to a tubulovesicular compartment that is distinct from transferrin-positive endosomes (11, 16). Others have found that ARF6 is localized to chromaffin granules and is involved in exocytosis during regulated secretion (2, 9). Finally, in normal rat kidney (NRK) cells, endogenous and overexpressed ARF6 localizes to the plasma membrane and to the juxtanuclear region and may play a role in modeling the plasma membrane and in cortical actin organization (12).

In adipocytes, insulin affects several processes associated with intracellular membrane trafficking. Insulin enhances glucose transporter 4 (Glut4) translocation from intracellular compartments to the plasma membrane (17), stimulates accumulation of transferrin receptors and insulin-like growth factor II receptors on the cell surface (18), and increases the secretion of several proteins (19, 20). The molecular mechanism by which insulin induces intracellular redistribution of Glut4 is still unclear, but it probably stimulates exocytosis (21) and inhibits endocytosis of the transporter (22). Adipsin, a serine protease specifically expressed in adipocytes, is constitutively secreted from adipocytes, and its secretion is augmented 2–3-fold by insulin treatment (19, 20). In addition, insulin stimulates PLD activity in rat adipocytes (23), which may be important for activation of signaling and targeting processes in the plasma membrane.

Although it has been shown that ARF proteins are associated with the insulin receptor (24), it is unknown whether ARF6 is required for insulin-stimulated membrane trafficking. In this study, we examined the role of ARF6 in two insulin-regulated processes in 3T3-L1 adipocytes: glucose transport and adipin secretion. Our data strongly suggest the involvement of ARF6 in basal and insulin-regulated adipin secretion but not in insulin-stimulated glucose transport.

Materials and Methods

Reagents—Insulin, dexamethasone, isobutylmethylxanthine, [3H]-2-deoxyglucose, and Dulbecco’s modified Eagle’s medium (DMEM) were from Sigma. The production of mouse anti-ARF6 antibody was described previously (14). Goat anti-adipin antibody was kindly provided by Dr. Jess Miner (University of Nebraska).

Cell Culture—3T3-L1 fibroblasts were grown to confluence and 2 days later were differentiated as described previously (25). 3T3-L1 adipocytes were used for experiments between 10 and 14 days after differentiation. 293 cells were grown at 37 °C in DMEM with 10% fetal bovine serum in the presence of 50 units of penicillin per ml and 50 μg of streptomycin per ml and 5% CO2.

Plasmid Construction—The human ARF6 cDNA, provided by M. Vaughan and J. Moss (National Institutes of Health) (26), was amplified using the polymerase chain reaction and primers containing BglII restriction sites. Wild-type ARF6 and its mutant D125N were subcloned into the BamH I site of the adenovirus vector pACCMV (provided by C. Newgard, University of Texas Southwestern Medical Center, Dallas, TX) which was previously linearized by BamHI digestion and treated with calf alkaline phosphatase. The ARF6 mutant, D125N, was con-
structed by the method of Kunkel (27). The mutagenic oligonucleotide used was 5′-GCCAACAGAGGACGAGTGCAG-3′.

**Generation of Recombinant Adenovirus**—Recombinant viruses were generated as described previously (28). Briefly, the plasmid pACCMV-ARF6 (1 μg) was cotransfected with the plasmid pJM17 (4 μg, provided by C. Neward) into 293 cells by using the transfection kit from Stratagene (Transfection MBS, mammalian transfection kit). Cell lysis indicative of recombination occurred 1–2 weeks following cotransfection. Cell lysates were subjected to immunoblot analysis to assay for the expression of ARF6 protein. The recombinant viruses were amplified and purified as described (28). Purified viruses (1–5 × 10⁸ plaque-forming units/ml) were stored at −80 °C in Tris-buffered saline containing 1% bovine serum albumin and 10% glycerol. 

5 × 10⁶ plaque-forming units of virus were used to infect 35-mm dishes of 3T3-L1 adipocytes overnight. The infected cells were fed with fresh DMEM containing 10% fetal bovine serum for an additional 48 h before use in experiments.

**Glucose Transport Assays**—3T3-L1 adipocytes were serum-starved for at least 3 h, washed three times with Krebs-Ringer phosphate buffer and then treated or not with insulin (1 μM) for 20 min at 37 °C. [3H]-2-Deoxyglucose uptake was measured as described previously (25). Nonspecific background uptake in the presence of the inhibitor cytochalasin B (20 μM) was subtracted from all values. Glucose transport activity was normalized to protein concentration measured using the bicinchoninic acid assay (Pierce).

**Plasma Membrane Lawn Preparation**—3T3-L1 adipocytes grown in 35-mm dishes were serum-starved and treated as described above. Plasma membrane (PM) lawns were prepared as described (29) and solubilized in 150 μl of 1% SDS in PBS. The protein concentration was determined using the Pierce bicinchoninic acid assay kit. 1.6–2 μg of total protein was used for immunoblot analysis.

**Measurement of Adipsin Secretion in 3T3-L1 Adipocytes**—3T3-L1 adipocytes grown in 35-mm dishes were serum-starved for at least 3 h before the start of an experiment. Cells were treated or not with insulin (1 μM) for 30 min, and the cell culture media were collected and precipitated with 10% trichloroacetic acid. Precipitated proteins were used for immunoblot analysis with anti-adipsin antibody (30).

**GTP Overlay Blot**—Polyacrylamide gel electrophoresis and immunoblotting—Polyacrylamide gel electrophoresis and immunoblot analysis of ARF6 were performed as described (14). Immunoblot analysis for Glut1 and Glut4 were performed as described by Hresko et al. (31).

**GTP-Overlay Blot**—GTP overlay blot was performed as described. Proteins were resolved on SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose as described above (14). Briefly, nitrocellulose sheets were preincubated for 30 min at room temperature in 50 mM Tris-HCl, pH 7.4, containing 0.3% Tween 20, 5 mM MgCl₂, and 100 μM ATP. Incubation with 1 μCi [α-32P]-GTP/milliliter was carried out for 1 h in the same buffer. Nitrocellulose filters were then washed four times (10 min each) with the same buffer, dried, and autoradiographed at −80 °C.

**RESULTS**

**Expression of ARF6 in 3T3-L1 Adipocytes**—To determine whether ARF6 is involved in insulin-regulated membrane trafficking in 3T3-L1 adipocytes, we transiently expressed wild-type or GTP binding-deficient (D125N) mutant ARF6 proteins in 3T3-L1 adipocytes using an adenovirus expression system. Expression of ARF6 proteins was assessed by immunoblot analysis. As shown in Fig. 1A, much higher levels (10–20-fold) of ARF6 were detected in 3T3-L1 adipocytes infected with the recombinant adenoviruses compared with uninfected adipocytes. The use of immunocytochemistry revealed that more than 90% of the 3T3-L1 adipocytes were infected by the same quantity of virus expressing β-galactosidase (data not shown).

The ARF6-D125N mutation lies in a domain of the protein that, by analogy to Ras, is predicted to interact with the guanine nucleotide binding (32). This domain and the aspartate are highly conserved among all small GTP-binding proteins. We therefore examined the capacity of the wild-type and the mutant to bind GTP. Total cell lysates were prepared from 3T3-L1 adipocytes that were either not infected or infected with recombinant adenovirus expressing wild-type ARF6 and its mutant D125N, and the lysates were subjected to immunoprecipitation with anti-ARF6 antibody (14). Immunoprecipitates were then subjected to either immunoblot or GTP-overlay blot analysis. As shown in Fig. 1B, the ARF6-D125N mutant protein failed to show binding to [α-32P]-GTP, whereas the wild-type ARF6 bound GTP in the expected manner.

**ARF6 Does Not Affect Glucose Transport in 3T3-L1 Adipocytes**—Insulin stimulates translocation of the glucose transporters Glut1 and Glut4 from intracellular compartments to the cell surface and increases glucose uptake in differentiated 3T3-L1 adipocytes. To determine whether overexpression of ARF6 affects glucose uptake, 3T3-L1 adipocytes were either not infected (control) or infected with recombinant adenovirus expressing β-galactosidase, wild-type ARF6, or the ARF6 mutant, D125N. The uninfected or infected, serum-starved 3T3-L1 adipocytes were either treated or not with insulin for 30 min, and 2-deoxyglucose uptake was then measured. As shown in Fig. 2, insulin-stimulated glucose uptake was not affected by the overexpression of wild-type or mutant ARF6. Infection with all adenoviruses did result in a slight increase in basal glucose uptake.

To determine whether ARF6 affects Glut1 or Glut4 translocation, PM lawns were prepared from control and adenovirus-infected 3T3-L1 adipocytes. The collected PM lawns were used for quantitative immunoblot analysis using antibodies directed against Glut1 or Glut4. Fig. 3 shows that overexpression of wild-type ARF6 or its mutant D125N did not alter the amount of Glut1 or Glut4 in the plasma membrane under basal or insulin-stimulated conditions relative to uninfected control cells or cells that expressed β-galactosidase. The elevation in basal glucose transport induced by viral infection alone may thus be because of an increase in the intrinsic activity of Glut1 or Glut4 in the PM, or to a small increase in the basal PM content of either transporter that could not be detected by this method.

**ARF6 Stimulates Basal and Insulin-induced Adipsin Secretion in 3T3-L1 Adipocytes**—It has been shown that ARF6 is involved in regulated exocytosis in chromaffin cells (2, 9), therefore, we determined the effect of ARF6 on a regulated secretory pathway in 3T3-L1 adipocytes. Adipsin, a serine protease specifically expressed in fat cells, is constitutively secreted into the medium and its secretion is stimulated by insulin. 3T3-L1 adipocytes were either not infected or infected with adenovirus expressing β-galactosidase, wild-type ARF6, or the D125N mutant. As shown in Fig. 4, the expression of wild-type ARF6 increased the release of adipsin into the medium under both basal and insulin-stimulated conditions. In contrast, the expression of the ARF6/D125N mutant inhibited both pro-
cesses. Infection with β-galactosidase virus did not alter adipsin secretion.

DISCUSSION

In this study we present evidence that ARF6 is involved in basal and insulin-stimulated adipsin secretion, but that overexpression of ARF6 does not affect the amount of Glut1 or Glut4 in the plasma membrane under basal or insulin-stimulated conditions. Our results suggest the existence of at least two compartments or pathways that undergo insulin-stimulated exocytosis in 3T3-L1 adipocytes, one for adipsin secretion and one for the glucose transporters.

Under steady-state conditions Glut1 and Glut4 undergo constitutive endocytosis and recycling (for review, see Ref. 33). As a result, the amount of Glut1 and Glut4 on the cell surface reflects the relative rates of their internalization and recycling. Insulin causes the accumulation of Glut1 and Glut4 on the plasma membrane principally by increasing their exocytosis and/or by decreasing the rate of their internalization (for review, see Ref. 33). Significant advances have been made in our understanding of the compartmentalization of the insulin-sensitive pool of Glut4. Most of the emerging models suggest a specialized compartment for Glut4 storage in either adipocytes and muscle (for review, see Ref. 33). Given the hypothesis that ARF6 is involved in both endocytosis and exocytosis of Glut1 and Glut4 in 3T3-L1 adipocytes, any positive or negative effect on both processes by overexpressing ARF6 wild-type or its dominant negative mutant, D125N, might not affect the steady-state amount of Glut1 or Glut4 on the plasma membrane under basal or insulin-stimulated conditions. Alternatively, it is possible that ARF6 is involved in neither endocytosis nor exocytosis of Glut1 and Glut4 in 3T3-L1 adipocytes. To distinguish these hypotheses, measurement of the rates of endocytosis and exocytosis for photolabeled glucose transporters in 3T3-L1 adipocytes will be required.

The available data indicate that ARF6 is not only required for various membrane trafficking events, including endocytosis (6), membrane recycling (7, 16), and regulated exocytosis (9), but also plays an important role in cytoskeletal organization (8, 11). In addition, a recent study showed that, in chromaffin cells, ARF6 may participate in exocytosis by controlling PLD activity on the plasma membrane (2). Similar results have recently been reported for Rac1 and Rho. It has been shown that Rac1 and Rho regulate secretion in addition to influencing cytoskeletal organization (34–36). Furthermore, it has been reported that overexpression of the activated form of Rac1 decreases the efficiency of receptor-mediated endocytosis (37) and that the actin cytoskeleton is required for receptor-medi-
ARF6 in Exocytosis

ARF6 is distinguished from other members of the ARF family by its insensitivity to brefeldin A (BFA), a fungal macrolide antibiotic. It has been shown that BFA disrupts the organization of the Golgi complex (52), prevents vesicular budding from Golgi and trans-Golgi membranes (53), and leads to retrograde movement of Golgi proteins back to the endoplasmic reticulum. To determine the effect of BFA on the release of adipsin from 3T3-L1 adipocytes, adipisin secretion was measured in uninfected or adenovirus-infected 3T3-L1 adipocytes treated with or without 10 μg/ml BFA for 30 min. No effect of BFA on basal or ARF6-regulated adipisin secretion was observed in our experiments (data not shown), consistent with the role of ARF6 in this process. In addition, we did not observe any significant effect of BFA on basal or insulin-regulated glucose uptake (data not shown), which is consistent with previously published results (54, 55).

It will be of interest to determine whether ARF6 plays a role in other examples of regulated exocytosis in addition to adipisin secretion. We are currently determining the effect of ARF6 on glucose-regulated insulin secretion in β cells. We are also testing the effect of the mutant ARF6/D125N on GTPγS-stimulated glucose transport in 3T3-L1 adipocytes since it appears that insulin and GTPγS-stimulated glucose transport involves different GTPases.2

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