The role of ADP-ribosylation factor 1 (ARF-1) in the assembly of very low density lipoproteins (VLDL) was investigated by expressing dominant-negative mutants in McA-RH7777 cells. Transient expression of ARF-1(T31N), a GDP-restrictive mutant, significantly inhibited apolipoprotein B-100 (apoB-100) VLDL production without influencing the biosynthesis of apoB-100 low density lipoproteins or total apoB production (indicating that it inhibited the second step of VLDL assembly) and without altering total protein production or biosynthesis of transferrin, phosphatidylcholine, or triglycerides. These effects were confirmed in stable inducible transfectants. In contrast, expression of an ARF-1 mutant lacking the N-terminal 17 amino acids, which has no myristoylation site and cannot interact with the microsomal membrane, did not affect VLDL assembly. Thus, active ARF-1 is needed for the second step of the process. To further explore these observations, we developed a cell-free system based on the postnuclear supernatant isolated from McA-RH7777 cells. In this system, 10–15% of the apoB-100 pool was converted to VLDL in a time- and temperature-dependent way. The assembly process was highly dependent on a heat-stable factor in the d > 1.21 g/ml infranatant of fetal calf serum; this factor was not present in low density lipoproteins or VLDL. Brefeldin A inhibited VLDL assembly in this system, as did a synthetic peptide (corresponding to N-terminal amino acids 2–17 of ARF-1) that displaces ARF-1 from the membrane. Thus, active ARF-1 is also needed for cell-free assembly of VLDL. Guanosine 5′-3′-(thio)triphosphate also inhibited VLDL assembly in this system, indicating that the process requires ongoing hydrolysis of GTP. 1-Butanol, which inhibits the formation of phosphatidic acid (PA) and instead gives rise to phosphatidylbutanol, inhibited VLDL assembly, whereas 2-butanol, which does not inhibit PA formation, failed to do so. Thus, phospholipase D (PLD)-catalyzed formation of PA from phosphatidylcholine is essential for VLDL assembly. In support of this conclusion, exogenous PLD prevented brefeldin A from inhibiting the assembly process. Our results indicate that ARF-1 participates in the second step of VLDL assembly through a process that involves activation of PLD and production of PA.

Very low density lipoproteins (VLDL) are assembled in liver cells and contain a structural protein called apolipoprotein B-100. VLDL assembly occurs in at least two steps (Refs. 1 and 2; see also Ref. 3 for a review). The first step is completed shortly after translation of apoB-100 and translocation of the protein to the lumen of the endoplasmic reticulum (ER) (Refs. 1 and 2; see also Refs. 3 and 4). This step requires microsomal triglyceride transfer protein (for a review, see Ref. 5) and gives rise to a VLDL precursor that contains full-length apoB-100 (2–4, 6), which may be associated with the membrane of the ER (4, 6, 7). In the second step, the major amount of lipid is added to the core of the precursor particle to form VLDL (2). Immunoelectron microscopy studies indicate that the core lipids occur as apoB-free “droplets” in the lumen of the secretory pathway (1) and that the second step involves the fusion of these droplets with the VLDL precursor (for a review, see Ref. 3). How these lipid droplets are formed is unknown, but microsomal triglyceride transfer protein appears to be involved (8).

The second step of VLDL assembly can be inhibited by brefeldin A (BFA) (6). Best known for inhibiting intracellular transport, BFA blocks a guanine nucleotide exchange (GDP for GTP) on ADP-ribosylation factor 1 (ARF-1) (9–11), a member of the Ras superfamily of GTP-binding proteins. This exchange is important for activation of ARF-1 and for interactions between ARF-1 and other proteins. We hypothesized that ARF-1 has a central role in VLDL assembly.

To test this hypothesis, we expressed ARF-1 mutants in McA-RH7777 cells and assessed the effects on VLDL assembly. ARF-1(T31N), a GDP-restrictive mutant, inhibited VLDL assembly in McA-RH7777 cells. To elucidate the pathway between ARF-1 and the VLDL assembly, we developed a cell-free system that can assemble VLDL from full-length apoB-100. VLDL assembly in this system also required active ARF-1. Moreover, the activation of phospholipase D (PLD) and the subsequent production of phosphatidic acid (PA) appeared to be essential for the assembly process.

EXPERIMENTAL PROCEDURES

Materials—Engel’s minimum essential medium with and without methionine was obtained from Life Technologies (Paisley, Scotland). Nonessential amino acids, glutamine, penicillin, leupeptin, and streptomycin were purchased from ICN Biomedicals (Costa Mesa, CA). Fetal calf serum (FCS) was obtained from Biochrom KG (Berlin, Germany), and brefeldin A was from Epicentre Technologies (Madison, WI). Metionine, fatty acid-free bovine serum albumin, phenylmethylsulfonyl
fluoride, oleic acid, PLD (type V), ATP, GTP, creatine phosphate, and creatine kinase were obtained from Sigma. Rabbit immunoglobulin was purchased from Dako (Glostrup, Denmark). GTP·S was obtained from Roche Molecular Biochemicals (Mannheim, Germany). 1-Butanol and 2-propanol were from Merck (Darmstadt, Germany). Phosphatidyl-
butanol was from Biomol Research Laboratories (Plymouth, PA). Am-
plify, Redivue Pro-mix L[35S] in vitro cell labeling mix (t-[35S]methionine and t-[35S]cysteine), [3H]myristic acid, Rainbow molecular weight markers, PD-10 columns, and the enhanced chemiluminescence Western blotting system were from Amersham Pharmacia Biotech (Uppsala, Sweden). Ready 70% ethanol was from Beckman (Pomona, CA). Precast gels were purchased from Novex (San Diego, CA). Synthetic peptides were purchased from the Biomedical Unit at the University of Lund, Sweden. The T-Rex inducible expression system, blastocidin, and zeocin were from Invitrogen (Groningen, The Netherlands). Tfx-50 reagent was from Promega (Madison, WI).

**Cell Culture and Transfection**—Mca-RH7777 cells were cultured as described (2). At 80% confluency, the cells were transiently transfected with wild-type ARF-1 or ARF-1 mutants (T31N and ΔN wt) ligated to green fluorescent protein in the pEGFP-N1 vector (12) (kindly provided by Dr. G. Romero) with Tfx-50. The cells were allowed to recover for 48 h before the experiments.

To obtain stable inducible expression of ARF-1(T31N), we used the T-REx system (Invitrogen) as described by Dr. G. Romero (with Tfx-50). The cells were allowed to recover for 48 h with wild-type ARF-1 or ARF-1 mutants (T31N and ΔN wt) ligated to green fluorescent protein in the pEGFP-N1 vector (12) (kindly provided by Dr. G. Romero) with Tfx-50. The cells were allowed to recover for 48 h before the experiments. The same amount of ethanol was added to the culture medium of the uninduced control cells.

**Metabolic Labeling and Protein Isolation**—For metabolic studies, cells were preincubated with 360 μM oleic acid for 120 min, labeled with t-[35S]methionine and t-[35S]cysteine (300 μCi/ml) for 15 min, and chased for 45 min in the presence of 360 μM oleic acid. ApoB-100 and transferrin were isolated from whole cells as described (2). For radioactivity measurements, labeled cells were lysed, and the proteins were precipitated with perchloric acid. After a 30-min incubation at 98 °C, the precipitated proteins were collected on nitrocellulose filters, and the radioactivity was measured.

The microsomal fraction was isolated as described (2), with the exception that 10 mM Hepes-KOH was used instead of 3 mM imidazole, and the luminal content was extracted by the deoxycholate/carbonate method essentially as described (4) except that the 1.25 M KCl was omitted. The extracts were subjected to deoxycholate/carbonate extraction as described above. LDL were isolated from the extract by gradient ultracentrifugation.

**Cell-free Incubation**—Mca-RH7777 cells were grown to 80% confluency in 56-cm² dishes and labeled with [3H]glycerol for 120 min and measured with the BCA kit (Pierce). The PNS was subjected to deoxycholate/carbonate extraction as described above. LDL were isolated from the extract by gradient ultracentrifugation.

Expression of ARF-1(T31N) significantly decreased apoB-100 radioactivity in the LDL fraction compared with wild-type ARF-1, whereas expression of ARF-1(ΔN wt) had no effect (Fig. 1B). Neither mutant decreased apoB-100 radioactivity in the LDL fraction, and the total amount of radioactivity in apoB-100 and transferrin was the same in cells expressing mutant ARF-1 as in those expressing wild-type ARF-1 (Fig. 1B).

Expression of ARF-1(T31N) also inhibited protein secretion, as judged from the decrease in the accumulation of radioactive transferrin in the medium (58.3 ± 17.7% of that obtained with wild-type ARF-1; n = 9, while ARF-1(ΔN wt) had no effect (97.0 ± 17.6% of wild-type, n = 6). There were no significant differences in the incorporation of radioactivity into total lipids in cells expressing wild-type ARF-1, T31N, and ΔN wt ARF-1 (not shown). To determine if transfection affected lipid biosynthesis, we pulse-labeled the cells with [3H]glycerol for 120 min and measured the incorporation of radioactivity into triacylglycerols and phosphatidylcholine. The biosynthesis rates were the same in cells transfected with wild-type, T31N, and ΔN wt ARF-1 (not shown).
D1.1RH7777 cells at 80% confluence were transfected with wild-type ARF-1 apoB-100, and SDS-PAGE were used to isolate apoB from the fractions, total and the VLDL and LDL fractions were recovered. Immunoprecipitation and the culture medium were subjected to gradient ultracentrifugation, the microsomal fraction was recovered, and the luminal content was extracted. The deoxycholate/carbonate method. The luminal content and the culture medium were subjected to gradient ultracentrifugation, and the VLDL and LDL fractions were recovered. Immunoprecipitation and SDS-PAGE were used to isolate apoB from the fractions, total apoB-100 (ΔN wt apoB and T31N apoB) from the cell lysate and medium, and transferrin from the luminal content and the culture medium. In these experiments, the sum of cellular and secreted apoB-100 (VLDL, LDL, or total apoB-100) or transferrin are given as a percentage of corresponding observations (mean ± S.D.) in cells transfected with wild-type ARF-1-GFP (ΔN wt VLDL, T31N VLDL, ΔN wt LDL, T31N LDL), ΔN wt transferrin, and transferrin T31N, n = 6; ΔN wt apoB and T31N apoB, n = 3.

shown). Thus, ARF-1(T31N) inhibited the formation of bona fide VLDL without affecting the lipidation of apoB-100, the expression of apoB-100 or transferrin, or the total biosynthesis of proteins and lipids in the cell.

Effect of Stable Inducible Expression of ARF-1(T31N) on VLDL Assembly—In the next series of experiments, stable inducible ARF-1(T31N) expression was used, allowing uninduced cells to serve as controls and thereby avoiding cloning artifacts associated with the noninducible stable transfectants. Induction of ARF-1(T31N) expression was confirmed by Western blotting with antibodies to the Myc tag (Fig. 2A) and decreased by ~80% of the amount of radioactive apoB-100 VLDL recovered after a 15-min pulse and a 15-min chase (Fig. 2, B and C). Total apoB-100, apoB-100 LDL, and transferrin incorporated similar amounts of radioactivity (Fig. 2C). The difference in the effect of ARF-1(T31N) on the incorporation of apoB-100 into LDL and VLDL is also illustrated by the ratio of apoB radioactivity in the LDL density region to that in the VLDL density region (after a 15-min pulse and a 15-min chase) (1.09 ± 0.13 and 4.30 ± 0.83 in uninduced and induced cells, respectively; mean ± S.D., n = 4). These results confirm that expression of ARF-1(T31N) inhibits VLDL assembly without giving rise to a general inhibition of the lipidation of apoB-100.

The incorporation of radioactivity into total apoB-100 after induction was in the range of that seen for apoB-100 LDL (Fig. 2C) and transferrin. The recovery of similar amounts of transferrin and total apoB-100 suggests that the loss of total apoB-100 reflect a general influence of the ARF-1 mutant on the secretory pathway. However, part of the decrease in total apoB-100 can be explained by the loss of VLDL. We calculated that the decrease in VLDL apoB-100 seen after the induction of ARF-1(T31N) corresponded to 21.6 ± 12.3% (mean ± S.D., n = 4) of total apoB-100 radioactivity in the uninduced control.

The induction of wild type ARF-1 (Fig. 2D) did not decrease the expression of apoB-100 in the VLDL density fraction, nor did it affect the expression of total apoB-100, apoB-100 in the LDL density fraction, or transferrin.

VLDL Assembly in the Cell-free System—To further address the role of ARF-1 in VLDL assembly, we constructed a cell-free system that carries out the second step of the process. McA-RH7777 cells were incubated with 360 μM oleic acid for 105 min; BFA was added, and the incubation was continued for 15 min. The cells were then pulsed for 30 min with L-[35S]methionine and L-[35S]cysteine in the presence of BFA and oleic acid and chased for 30 min (in the presence of BFA and oleic acid). The PNS obtained after this treatment had a relatively small amount of labeled VLDL; most of the labeled apoB-100 was in denser, high density lipoprotein-like particles. These observations agree with our previous results (4).

The standard cell-free system consisted of this PNS supplemented with 100 mM potassium acetate, 5 mM magnesium acetate, 5 mM methionine, 250 mM sucrose, 1 mM GTP, 1 mM ATP, 20 mM creatine phosphate, 0.5 units/ml creatine kinase, 3 mg/ml FCS, and 360 μM oleic acid. As shown by the increase in the radioactivity of apoB-100 VLDL during the incubation, 10–15% of the total apoB-100 pool was converted into VLDL in a time-dependent (Fig. 3) and temperature-dependent fashion; during a 30-min incubation, four times more apoB-100 VLDL was formed at 37 °C than on ice (not shown).

To determine the density range in which the assembly-competent organelles banded, we subjected the PNS to gradient ultracentrifugation (18). Five fractions were recovered from the bottom of the tube. Each fraction was incubated (at 37 °C for 30 min) with 100 mM potassium acetate, 5 mM magnesium acetate, 5 mM methionine, 250 mM sucrose, 1 mM GTP, 1 mM ATP, 20 mM creatine phosphate, 0.5 unit/ml of creatine kinase, 3 mg/ml FCS, and 360 μM oleic acid. Since the subcellular fractions were depleted of cytosol during the centrifugation, 2 mg/ml cytosol was added during this experiment. The distribution of the VLDL assembly activity over the gradient (the total assembly activity in each fraction; mean of two experiments) was 16% in fraction 1, 47% in fraction 2, 28% in fraction 3, 3% in fraction 4, and 0% in fraction 5. Thus, VLDL assembly occurred predominantly in fractions 2 (d = 1.183 g/ml) and 3 (d = 1.149 g/ml).

We previously observed that FCS stimulates VLDL assembly in intact (2) and permeabilized cells. Therefore, we assessed the effects of FCS on VLDL assembly in the cell-free system. Virtually no VLDL were formed in the absence of FCS, indicating that FCS strongly stimulated VLDL production; heating the serum to 95 °C significantly increased VLDL production (Fig. 4). The active factor was present in the d > 1.21 g/ml infranatant; neither the d < 1.063 nor the d < 1.006 supernatant stimulated the incorporation of radioactive apoB-100 into VLDL. These observations exclude the possibility that radiolabeled apoB-100 in the microsomes associated with "premade" lipoproteins in the FCS and therefore might appear in the VLDL density range. Fatty acid-free bovine serum albumin (3

K. Lindberg, C. Claesson, L. Asp, J. Borén, and S.-O. Olofsson, unpublished data.
mg/ml) failed to stimulate VLDL assembly (data not shown), suggesting that the stimulation is not simply explained by the addition of proteins to the system. These findings suggest that FCS contains a factor that specifically stimulates VLDL assembly.

BFA and a Synthetic ARF-1 Peptide Inhibit Assembly of ApoB-100 VLDL in the Cell-free System—Next, we tested whether ARF-1 is important for VLDL assembly in the cell-free system. First, we investigated the effect of BFA. BFA inhibited

![Figure 2](image-url)  
**Fig. 2.** Effect of ARF-1(T31N) or wild type ARF-1 expression on VLDL assembly in stably transfected McA-RH7777 cells. cDNA for T31N-ARF-1 or wild type ARF-1 was amplified by polymerase chain reaction with Pfu polymerase, digested with EcoRI and ApoI, and subcloned in the pcDNA4/TO/Myc-His A vector. The resulting plasmids were transfected into McA-RH7777 cells expressing the pcDNA6/TR plasmid. ARF-1(T31N) or wild type ARF expression was induced by incubating the cells with tetracycline (1 μg/ml of culture medium) for 24 h. (Control experiments indicated that tetracycline did not influence VLDL assembly in untransfected cells.) The cells were also incubated with 360 μM oleic acid for 2 h before the experiment. A, the induction of ARF(T31N) or wild type ARF-1 was verified by immunoblotting with an antiserum to the Myc tag. B, a representative pulse-chase experiment. The cells were pulse labeled with L-[35S]methionine and L-[35S]cysteine for 15 min and chased for 15 min both in the presence of 360 μM oleic acid. The total luminal content of the microsomes was recovered as described in the legend to Fig. 1. The VLDL fraction was isolated from the luminal content and the medium, and apoB-100 in these fractions was isolated by immunoprecipitation and SDS-PAGE. The upper panel shows the autoradiograms obtained from induced and uninduced cells. The lower panel shows the corresponding radioactivity in apoB-100 VLDL and the sum of apoB-100 VLDL from the microsomal lumen and from the medium. Open bars indicate uninduced cells; filled bars indicate induced cells. C, effect of ARF-1(T31N) induction. Data are given as a percentage of the uninduced control (mean ± S.D.) on the incorporation of radioactivity into apoB-100 in the VLDL (apoB-100 VLDL) and the LDL (apoB-100 LDL) density regions (n = 4) and into total apoB-100 (n = 4) and transferrin (Tfr, n = 4). The cells were treated as described above, and the VLDL, LDL, and high density lipoprotein density regions were isolated by gradient ultracentrifugation. ApoB-100 was recovered from the VLDL and LDL density regions as described in the legend to Fig. 1B. Total apoB-100 and transferrin were recovered as described in the legend to Fig. 1B. D, effect of wild-type Arf-1 induction. Data are given as percentage of uninduced control (mean ± S.D.). Radiolabeled apoB-100 in the VLDL and LDL density fractions and total apoB-100 and transferrin were measured as described in the legend to Fig. 1C (apoB-100 VLDL, apoB-100 LDL, and transferrin, n = 2; total apoB-100, n = 3).
**Fig. 4. A factor in FCS is essential for the assembly of VLDL in the cell-free system.** McA-RH7777 cells were treated, pulse-labeled, and chased as described in the legend to Fig. 3. The PNS was recovered and incubated for 30 min at 37 °C in the basic cell-free system or in the same system in which FCS was omitted (No FCS) or replaced with FCS preincubated at 95 °C for 20 min (FCS 95 °C) or with the d > 1.21 g/ml infranatant, the d < 1.063 g/ml supernatant, or the d < 1.006 supernatant of FCS. To prepare the density fractions of FCS, the serum was adjusted to the given density and centrifuged at 44,000 rpm for 40 h at 12 °C in a Beckman Ti 50 rotor. The upper 4 ml (one-third of the tube) was collected as the supernatant, and the bottom 3 ml was used as the corresponding infranatant. In all cases, the final concentration of the FCS or the isolated serum fraction was 3 mg/ml. After the incubation, the incubation mixture was subjected to deoxycholate/carbonate extraction, and VLDL apoB-100 was recovered as described in the legend to Fig. 1. The amounts of VLDL apoB-100 formed under the different conditions are expressed as percentages of that formed in the basic cell-free system. Each value is the mean of two experiments run in parallel; the bar indicates the spread (maximum and minimum) of these two experiments.

The incorporation of radioactive apoB-100 into the VLDL fraction in a dose-dependent fashion (Fig. 5A). We also investigated the effects of a synthetic peptide corresponding to N-terminal amino acids 2–17 of ARF-1, which inactivates ARF-1 by displacing it from the membrane. Compared with a “scrambled” peptide containing the same amino acids, the synthetic ARF-1 peptide significantly, and in a dose-dependent manner, inhibited VLDL assembly (Fig. 5, B and C). These results show that ARF-1 is important for the assembly of VLDL in the cell-free system as it was in intact cells.

**Hydrolysis of GTP and Production of PA Are Essential for VLDL Assembly**—To investigate the role of GTP hydrolysis in the assembly process, we replaced GTP in the basic cell-free system with the nonhydrolyzable analogue GTPγS. At low levels, GTPγS had no inhibiting effect. At concentrations of 100 μM and higher, however, it significantly decreased VLDL assembly (Fig. 6). Adding GTP overcame this inhibition (data not shown). These results indicate that the assembly of VLDL is dependent on ongoing hydrolysis of GTP. Failure to detect inhibition at low levels of GTPγS can be explained by the presence of endogenous GTP in the PNS.

ARF-1 is important for the activation of PLD (23–25). To determine if PLD activity is important in VLDL assembly, we investigated whether the production of PA is essential for the assembly process. To do this, we took advantage of the fact that the primary alcohol 1-butanol serves as a nucleophil acceptor during PLD-catalyzed degradation of phosphatidylcholine, yielding phosphatidylbutanol instead of PA. As a control, we used the secondary alcohol 2-butanol, which does not serve as a nucleophil acceptor in the reaction. As shown in Fig. 7, 1-butanol inhibited VLDL assembly in a dose-dependent manner, whereas 2-butanol had no effect.

To exclude the possibility that 1- or 2-butanol nonspecifically interferes with the assembled VLDL or decreases the recovery of apoB-100 VLDL in other ways, we added the alcohols to the incubation mixture after the 30-min incubation (i.e. before apoB-100 VLDL were isolated and quantified), and the incubation mixture after the 30-min incubation (Fig. 6). Adding GTP overcame this inhibition (data not shown). These results indicate that the assembly of VLDL is dependent on ongoing hydrolysis of GTP. Failure to detect inhibition at low levels of GTPγS can be explained by the presence of endogenous GTP in the PNS.

**Fig. 5. BFA and a synthetic polypeptide corresponding to amino acids 2–17 of ARF-1 inhibit VLDL assembly in the cell-free system.** McA-RH7777 cells were treated, pulse-labeled, and chased as described in the legend to Fig. 3. The PNS was incubated for 30 min at 37 °C in the basic cell-free system with the indicated amount of BFA (A) or one of two synthetic peptides (B and C), one corresponding to the N terminus of ARF-1 (amino acids 2–17) and the other containing the same amino acids in scrambled order. (In these experiments, the PNS was preincubated for 15 min with BFA or the synthetic peptides.) After the incubation, VLDL apoB-100 was recovered, and the radioactivity was determined as described in the legend to Fig. 3. A, results of a representative experiment with BFA. B, VLDL apoB-100 formed in the presence of the ARF-1 peptide (squares) or the scrambled peptide (diamonds), expressed as radioactivity recovered in apoB-100 of the VLDL fraction (mean ± S.D., n = 3). C, VLDL apoB-100 formed in the presence of the ARF-1 polypeptide, expressed as a percentage of that formed in the presence of the scrambled peptide (see Fig. 5B).
tion continued for 5 min. The system could tolerate up to 3% of either 1- or 2-butanol without any detectable decrease in the recovery of apoB-100 VLDL (data not shown). The formation of phosphatidylbutanol in the presence of 1-butanol, but not 2-butanol, was confirmed by thin layer chromatography (data not shown).

In the next experiment, we inhibited VLDL assembly with BFA and added increasing amounts of exogenous PLD to the cell-free system. The exogenous PLD overcame the BFA-induced inhibition of VLDL assembly (Fig. 8). Together, these results indicate that PLD is essential for the assembly of VLDL.

**DISCUSSION**

Our previous results (6) indicate that BFA inhibits the conversion of full-length apoB-100 to VLDL, the second step of VLDL assembly. BFA inhibits a guanine nucleotide exchange protein involved in nucleotide exchange on ARF-1 (9–11), a small GTP-binding protein belonging to the Ras superfamily (for a review, see Ref. 23). To determine if ARF-1 is involved in the assembly of VLDL, we began by transiently expressing dominant negative ARF-1 mutants in McA-RH7777 cells and assessing the effects on VLDL assembly. One mutant, ARF-1(T31N), is GDP-restrictive (19) (i.e., blocks removal of GDP from the active site of ARF-1 catalyzed by guanine nucleotide exchange factor) and competes with wild-type ARF-1 for the guanine nucleotide exchange factor, inactivating ARF-1. Expression of this mutant decreased VLDL assembly by 40% (in the range expected from the estimated transfection efficiency of ~50%) without inhibiting protein or lipid biosynthesis.

These results were confirmed in experiments with stable transfectants. To avoid cloning artifacts, we used an inducible expression system, which allows uninduced clones to serve as controls. Induction of ARF(T31N) expression caused a striking decrease in the amount of assembled apoB-100 VLDL. Total apoB-100 decreased as well; however, this decrease is probably explained by a general effect on protein secretion, as shown by the decrease in intracellular transferrin. Such an effect is not unexpected, since expression of the GDP-restrictive mutant of ARF-1 (like BFA) inhibits transport through the secretory pathway and influences the structure of this pathway (19).

Moreover, the decrease in VLDL assembly was more than 2-fold larger that the decreases in transferrin and total apoB-100. The specificity of the inhibition of the VLDL assembly was underlined by the observation that induction of ARF(T31N) did not influence apoB-100 LDL. Thus, inhibition of ARF-1 selectively inhibits VLDL assembly without interfering with the general lipiddation of apoB-100 in the cell. The inhibition of VLDL assembly was not due to the transfection of the cells, since expression of wild type ARF-1 did not decrease the amount of assembled VLDL and did not influence the expression of LDL, total apoB, or transferrin. Taken together, our observations demonstrate that ARF-1 has an important role in the assembly of VLDL.

As expected, both stable and transient expression of ARF-1(T31N) inhibited secretion from the cell, as shown by the accumulation of radioactive transferrin during a 15-min pulse and 15-min chase. The brief duration of the experiment allowed us to measure the leading edge of the intracellular transfer and to avoid problems with degradation and reuptake from the medium. The extent of inhibition of intracellular transport agreed well with the estimated transfection rate (in the transient transfection experiments) and with extent of inhibition of VLDL assembly. These results confirm our previous finding that BFA affects intracellular transport as well as VLDL assembly (6).

Expression of ARF-1(ΔN wt) did not inhibit VLDL assembly.
ARF-1 and Its Activation of PLD in VLDL Assembly

produced in the cell-free system corresponded relatively well to the proportion of apoB-100 that exists as VLDL in the secretory pathway.

To establish that VLDL assembly in the cell-free system depends on ARF-1, as it did in intact cells, we used two approaches. First, we demonstrated that BFA inhibited cell-free assembly of apoB-100 VLDL. Previously, we found that BFA completely inhibited the assembly of apoB-48 VLDL at concentrations that did not completely inhibit intracellular transport (6). This difference was less pronounced for apoB-100 VLDL; however, the assembly of apoB-100 VLDL was completely inhibited at BFA concentrations of 0.5–1 μg/ml. The concentration needed to inhibit assembly of apoB-100 VLDL by 50% was at least 5 times higher in the cell-free system than in intact cells. The reason for this discrepancy is not known; however, to obtain effects on ARF-1 activity, other authors have also been forced to use higher concentration of BFA in cell-free in vitro systems than in intact cells (see, for example, Ref. 29).

Second, we demonstrated that a synthetic peptide derived from the N terminus of ARF-1 also inhibited VLDL assembly. This polypeptide interferes with the interaction between ARF-1 and the membrane (20) (an interaction that is important for the activity of ARF-1) and has been used extensively to investigate the role of ARF-1 in other cellular processes (20, 30–32). To further support our hypothesis that the ARF-1–PLD pathway is involved in VLDL assembly, we employed two approaches. First, we demonstrated that BFA inhibited cell-free assembly of apoB-100 VLDL. Previously, we found that BFA completely inhibited the assembly of apoB-48 VLDL at concentrations that did not completely inhibit intracellular transport (6). This difference was less pronounced for apoB-100 VLDL; however, the assembly of apoB-100 VLDL was completely inhibited at BFA concentrations of 0.5–1 μg/ml. The concentration needed to inhibit assembly of apoB-100 VLDL by 50% was at least 5 times higher in the cell-free system than in intact cells. The reason for this discrepancy is not known; however, to obtain effects on ARF-1 activity, other authors have also been forced to use higher concentration of BFA in cell-free in vitro systems than in intact cells (see, for example, Ref. 29).

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Since ARF-1 is a GTPase, we investigated the role of GTP hydrolysis in VLDL assembly by assessing the effects of replacing GTP in the basic cell-free system with a nonhydrolyzable nucleotide analogue, GTPγS. The results indicated that the assembly process requires the hydrolysis of GTP. ARF-1 also activates PLD (23–25). In a series of experiments with 1-butanol and 2-butanol, we found that PLD-mediated production of PA was a prerequisite for the assembly of VLDL. Moreover, adding active PLD to the system overcame the inhibition of ARF-1 and of VLDL assembly by BFA. These results suggest that ARF-1 influences VLDL assembly by activating PLD, leading to the formation of PA.

A similar pathway (ARF-1 to PLD and the formation of PA) has been suggested to be functional (33–36) in the ARF-1-mediated recruitment of the heptameric coat protein 1 (COP-1) complex (for reviews, see Refs. 27 and 37) during the formation of transport vesicles. Thus, the second step in the assembly of VLDL may be dependent on the formation of COP-1 vesicles, which are involved in transport within the Golgi apparatus and in retrograde transport from the cis-Golgi network to the ER (38). Budding of COP-1 vesicles from the ER has also been shown (31, 39). One possibility is that an ARF-1-mediated budding of COP-1 vesicles gives rise to a compartment that is needed for the second step of VLDL assembly. This possibility is consistent with the results of immunoelectron microscopy studies demonstrating the presence of the apoB-free VLDL precursor in a compartment outside rough ER (1) and by our recent finding that the second step is carried out in a compartment other than the rough ER (40).

The importance of PA formation in the budding of transport vesicles, however, has been questioned. A direct interaction between ARF-1 and β-COP has been demonstrated (41, 42), and it has been proposed that PA formation and ARF-1-mediated recruitment of COP-1 are parallel events (43). In a study of chemically defined liposomes, the budding of COP-1-coated vesicles required only ARF, the coatomers, and the cytoplasmic domain of the coat/cargo receptors (the p24 family). The budding was completely independent of the lipid composition (Ref. 26; for an overview, see also Ref. 27). Thus, PA may participate in VLDL assembly by a mechanism that does not involve vesicle formation. For example, diacylglycerol can be formed from PA. Diacylglycerol is a non-bilayer-forming lipid that can perturb a membrane (44, 45). Membrane perturbation has been suggested to be important for the budding process (44, 45), and it could, of course, be important for the lipidation of apoB-100. Diacylglycerol is also the substrate for triglycerol formation, and a substantial proportion of the triglycerol in VLDL is derived from phosphatidylcholine (46). Thus, formation of the lipid core of VLDL may require conversion of critical phosphatidylcholine molecules in the microsomal membrane to triglycerols.

In summary, we have demonstrated that ARF-1 is involved in VLDL assembly. We have reconstituted the second step of VLDL assembly in a novel cell-free system and confirmed that this step is dependent on ARF-1. Finally, we have shown that ARF-1 appears to participate in VLDL assembly through the activation of PLD and the production of PA.

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Phospholipase D (Units/ml)

FIG. 8. Exogenous PLD can overcome the BFA-induced inhibition of VLDL assembly in the cell-free system. McA-RH7777 cells were treated, pulse-labeled, and chased as described in the legend to Fig. 3. The PNS was preincubated with 20 μg/ml BFA for 15 min and then incubated for 30 min at 37 °C in the basic cell-free system with the indicated amount of PLD. VLDL apoB-100 was recovered, and radioactivity was determined as described in the legend to Fig. 3. VLDL apoB-100 formed in the presence of BFA and PLD is expressed as a percentage of that formed in the basic cell-free system (mean ± S.D., n = 3).
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