Subcellular Distribution and Differential Expression of Endogenous ADP-ribosylation Factor 6 in Mammalian Cells

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ADP-ribosylation factor (ARF) 6 has been shown to play a role in vesicular transport; however, the expression and subcellular localization of the endogenous protein have not been clearly delineated. In this study, an ARF6-specific monoclonal antibody was raised and used to examine the subcellular distribution and expression of ARF6 in various tissues and during the differentiation of several well characterized cell types. We found that ARF6 localizes in both the cytosol and membranes of all tissues and cells tested. Moreover, ARF6 in 3T3-L1 adipocytes is principally localized on the plasma membrane, but substantial amounts are detected in the cytosolic and intracellular membrane fractions. We observed an increased expression of ARF6 during the differentiation of B lymphocytes to plasmacytes. However, the expression of ARF6 decreased during adipogenesis and monococyte differentiation. In contrast, the expression of other ARFs, detected by the monoclonal antibody 1D9, did not significantly change during differentiation of the aforementioned cell types. Taken together, our results indicate that ARF6 is a broadly expressed, differentially regulated GTPase that is present in cytoplasm and on both cell-surface and intracellular membranes and whose functions may include tissue-specific effects on vesicular trafficking during cellular differentiation.

ADP-ribosylation factors are members of the Ras superfamily of low molecular weight GTP-binding proteins. Although ARFs have been identified as cofactors required for the cholera toxin-catalyzed ADP-ribosylation of the heterotrimeric G protein Gs (1), they have been shown to play an important role in membrane trafficking. Six mammalian ARF genes have been identified, of which five corresponding proteins have been found in humans. The most extensively studied is ARF1, which has been used, almost exclusively, to study the biology of ARFs. This protein appears to cycle between the cytosol and its membrane targets in the Golgi apparatus (2–4). ARF regulates membrane trafficking along the secretory pathway by facilitating coatamer binding to Golgi membranes and by maintaining the integrity of the Golgi complex (5, 6). ARFs also stimulate the activity of phospholipase D in vitro (7–9), suggesting that they may exert their effects at least in part by altering membrane phospholipid metabolism.

ARF6 has been suggested to play a role in vesicle trafficking and cytoskeletal organization (10–13). Overexpressed ARF6 is localized on the plasma membrane and endosomes, suggesting a role for this protein in membrane trafficking along the endocytic pathway (10, 11). However, a recent report suggested that endogenous ARF6 is exclusively localized on the plasma membrane in CHO cells (14). Others have found that ARF6 is localized on chromaffin granules, suggesting that it mediates exocytosis during regulated secretion (15).

Based on the above results, it appears that ARF6 has a cell type-dependent subcellular distribution. To investigate this question further, we developed an ARF6 monoclonal antibody that allowed us to determine the subcellular localization of endogenous ARF6 in various cells and tissues and its expression during cellular differentiation. We demonstrate that ARF6 is recovered in cytosolic and membrane fractions in all cells and tissues tested. In addition, we found that the expression of ARF6 is ubiquitous and differentially regulated during cellular differentiation. We postulate that ARF6 might play a role in membrane trafficking by a mechanism in which it transiently shuttles between the cytosol and various membrane compartments.

EXPERIMENTAL PROCEDURES

Monoclonal Antibody Production—Recombinant human ARF6 protein was expressed in Pichia pastoris and purified by two successive columns: anion exchange on DEAE-Sepharose and gel filtration on Sepharose 100. Purified recombinant ARF6 was used for immunization of BALB/c mice (Charles Rivers Laboratories) following standard procedures. Cell fusions and cultures were performed at the Hybridoma Center at Washington University. Culture supernatants from hybridomas positive by enzyme-linked immunosorbent assay were used for immunohotting to test their specificity for ARF6. Eight clones specific for ARF6 were obtained after three rounds of cloning by limited dilution. Only one of these, termed 3F8.1, was used in this study. The IgG fraction was purified from ascites of mice injected with hybridoma 3F8.1 by affinity chromatography on a protein G-agarose column. The purified antibody was dialyzed against phosphate-buffered saline, aliquoted, and stored at −80 °C.

Preparation of Mouse Spleen B Lymphocytes—Mice (female or male C57Black 6 (6–8 weeks old); Charles River Laboratories) were killed by cervical dislocation. Spleens were mechanically dissociated, and cell clumps and debris were removed from the cell suspension by gravity sedimentation for 5 min. Spleen cells were collected by centrifugation at
Localization and Expression of ARF6

Differentiation of HL-60 Cells to Monocyte-like Cells—HL-60 cells were grown in suspension in RPMI 1640 medium supplemented with 10% calf serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. Maturation of HL-60 cells was induced as described by Maridonneau-Parini et al. (16). Briefly, HL-60 cells were cultured at a density of 2 × 10⁶ cells/ml and exposed to 20 ng/ml phorbol 12-myristate 13-acetate for 4 days. The differentiating cells were collected with a rubber policeman and washed twice with phosphate-buffered saline. Cell viability, as determined by trypan blue exclusion, was >90%.

Differentiation of 3T3-L1 Fibroblasts to Adipocytes—3T3-L1 fibroblasts were grown in Dulbecco’s modified Eagle’s medium containing 20% calf serum and differentiated to adipocytes by an established protocol (17). Briefly, confluent monolayers of 3T3-L1 fibroblasts were cultured for 2 days in Dulbecco’s modified Eagle’s medium containing 20% calf serum. Cells were incubated in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 670 nM insulin, 25 nM dexamethasone, and 500 µM isobutylmethylxanthine for 2 days to induce differentiation. Fresh Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum was added every 2 days. Cells were used between 8 and 12 days after the removal of the differentiation medium.

Preparation of Crude Cytosolic and Membrane Fractions—The postnuclear supernatant (PNS), crude cytosol, and membranes were prepared as described previously (18). Briefly, cells were homogenized in HES buffer (20 mM HEPES, pH 7.4, 1 mM EDTA, and 250 mM sucrose) plus 1 mM phenylmethylsulfonyl fluoride and a mixture of protease inhibitors (leupeptin, aprotinin, chymostatin, antipain, and pepstatin at 1 µg/ml final concentrations) at 4 °C by 10 strokes in a ball-bearing homogenizer. The cell lysates were centrifuged at 400 g for 5 min to pellet nuclei and unbroken cells. The supernatant was centrifuged at 150,000 g for 1 h at 4 °C to fractionate cytosolic and membrane fractions. The supernatant was then homogenized with a Polytron homogenizer in the same buffer.

Subcellular Fractionation of 3T3-L1 Adipocytes—Fully differentiated 3T3-L1 adipocytes were washed twice with HES buffer at 4 °C and gently scrapped with a policeman in HES buffer containing a mixture of protease inhibitors. Subcellular fractionation of 3T3-L1 adipocytes was performed as described previously (19). Briefly, homogenization was performed by 10 strokes in a Potter homogenizer with a Teflon pestle. After removal of the unbroken cells by a 5-min centrifugation at 400 g, the homogenates were centrifuged at 16,000 × g for 15 min. The 16,000 × g supernatant was centrifuged at 30 min at 44,000 × g. This pellet was resuspended in the same buffer, loaded onto a sucrose cushion (32% sucrose in HES buffer), and centrifuged at 210,000 × g for 75 min to obtain the LDL fraction. The protein concentration of the fractions was measured by the BCA assay (Pierce) using bovine serum albumin as a standard.

Electrophoresis and Immunoblotting—Two-dimensional gel electrophoresis was carried out essentially as described by O’Farrell (20) with the following minor modifications. For isoelectric focusing, a mixture of 1% ampholines (pH 5–8 and pH 3–10 (Sigma), was used; for electrophoresis, 14% SDS-PAGE was used. Bovine serum albumin was added to all samples as an internal standard. For SDS-PAGE, samples were diluted in sample buffer and subjected to electrophoresis on 14% gels. Proteins were transferred to nitrocellulose. The sheets were incubated for 2 h at room temperature or overnight at 4 °C with blocking buffer (5% skim milk in phosphate-buffered saline) and incubated for 2 h at room temperature or overnight at 4 °C with primary antibodies (diluted with 1:4 blocking buffer/phosphate-buffered saline). After four washes (15 min each) with buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 0.1% Tween 20), the nitrocellulose sheets were incubated for 1 h with a secondary antibody (horseradish peroxidase-conjugated goat anti-mouse IgG, washed as described above, and detected by chemiluminescence (ECL kit, Amersham Corp.). 125I-Labeled secondary antibody (goat anti-mouse IgG) was used to detect ARF6 in CHO cells (as described for Fig. 3A).

GTP Overlay Blot—Proteins were resolved by SDS-PAGE and transferred to nitrocellulose as described above. 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 mM ATP. Incubation with 1 µCi/ml [α-32P]GTP 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**

Characterization of an ARF6 Monoclonal Antibody—Although several previous studies have reported on the subcellular localization and cellular effects of overexpressed ARF6 (10–13), the localization and function of endogenous ARF6 remain incompletely understood (14, 15). To address this issue, we generated a monoclonal antibody against purified recombinant human ARF6 expressed in P. pastoris. As shown in Fig. 1A, clone 3F8.1 specifically reacted with ARF6, and no cross-reaction was found with other known members of the ARF family that were expressed in bacteria.

To confirm that 3F8.1 does not cross-react with unidentified ARFs, we tested its specificity by two-dimensional PAGE. As shown in Fig. 1B, only one spot of 19 kDa with an isoelectric point of 8.6 could be detected in a total lysate of rat lung, indicating that 3F8.1 is specific for ARF6 and that the antibody is not directed against the highly conserved domains of the ARF proteins.

Cytosol- and Membrane-associated Distribution of ARF6 in Different Cells and Tissues—To date, two groups have reported on the subcellular localization of endogenous ARF6 in CHO cells (14) and bovine adrenal chromaffin cells (15). In CHO...
cells, endogenous ARF6 was localized exclusively on the plasma membrane. However, the second study reported that ARF6 is associated with secretory granules in bovine adrenal chromaffin cells. These results suggest a cell type-dependent subcellular localization of endogenous ARF6. To further address this question, we analyzed the subcellular distribution of ARF6 in six rat tissues using the 3F8.1 antibody (Fig. 2). Although the level of expression of ARF6 varied from one tissue to another, the protein was detectable in all tissues analyzed. This result is in agreement with the finding that mRNA encoding ARF6 is detectable in all mouse tissues analyzed (21). In addition, when equivalent quantities of cytosolic and membrane proteins obtained from 50 μg of post-nuclear supernatant were used for ARF6 blot analysis, a significant percentage of total cellular ARF6 was found in the cytosol in all tissues tested, although this percentage varied from 12% (spleen) to 50% (liver and lung).

To determine whether the cytosolic distribution of ARF6 was specific only for rat tissues, the subcellular distribution of ARF6 was analyzed in other cell types. First, ARF6 distribution in CHO cells was examined. As shown in Fig. 3A (left panel), a short exposure time (12 h) of the immunoblot showed an exclusive membrane-associated distribution of ARF6 in these cells. However, after a longer exposure time (3 days), we indeed found that ~5% of the total ARF6 was in the cytosol (Fig. 3A, right panel). We further analyzed ARF6 distribution in another cell line, J774 macrophages. Surprisingly, ARF6 was equally distributed between the cytosol and membranes (Fig. 3B). Total membrane and cytosolic fractions were also prepared from nondifferentiated and LPS-differentiated mouse B lymphocytes (described below). Equivalent quantities of cytosol and membranes obtained from 50 μg of PNS were analyzed by Western blotting. We found that ARF6 was distributed equally between the cytosolic and membrane fractions both in nondifferentiated B lymphocytes and after 2 days of exposure to LPS. However, the fraction of membrane-associated ARF6 was greatly increased in B lymphocytes after 5 days of exposure to LPS (Fig. 3C). Taken together, our results showed that the quantity of ARF6 localized in the cytosol varies substantially from one cell type to another.

**Subcellular Localization of ARF6 in 3T3-L1 Adipocytes**—To further explore the subcellular distribution of ARF6, we analyzed the localization of ARF6 in fully differentiated 3T3-L1 adipocytes, a cell type amenable to subcellular fractionation and the preparation of relatively enriched fractions. The plasma membrane, LDMs (mixture of endosomes, secretory vesicles, and Golgi apparatus), cytosol, and HDMs (mixture of endoplasmic reticulum, trans-Golgi network, and small fraction endosomes) were prepared following established procedures (19). When the same amounts of proteins of the plasma membrane, LDM, cytosolic, and HDM fractions were analyzed by immunoblotting, ARF6 was found predominantly in the plasma membrane, LDM, and HDM fractions (data not shown).

Increased Expression of ARF6 during Plasmocyte Differentiation—The varied expression and differential subcellular localization of ARF6 in the tissues and cells analyzed above prompted us to examine its level of expression during cell differentiation. For these studies, mouse B lymphocytes were differentiated to Ig-secreting plasmocytes by stimulation with...
LPS. Differentiated B cells have a well developed synthetic/secretory pathway (endoplasmic reticulum and Golgi apparatus). Plasmocyte differentiation was verified by the production and secretion of IgM. After 5 days of exposure to LPS, >80% of B cells from mouse spleen were positive for IgM (data not shown), and [35S]methionine-pulse-labeled Ig was completely secreted into the medium after a 1-h chase (data not shown), indicating that the B cells were differentiated to plasmocytes that produced and secreted high quantities of IgM.

Compared with undifferentiated resting B lymphocytes, differentiated B cells were found to express much higher levels of ARF6. The level of ARF6 in mouse spleen B cells increased with increased exposure to LPS (2.1 ± 0.5- and 3.5 ± 0.4-fold increases over undifferentiated B cells after 2 and 5 days of exposure to LPS, respectively) (Fig. 5A, upper panel). In contrast, the expression of other ARF proteins was not significantly changed (Fig. 5A, middle panel), as detected by Western blotting using monoclonal antibody 1D9 (14), which recognizes all known ARF isoforms except ARF4 (Fig. 1A). These results suggest that in B cells the quantity of ARF6 could be much lower than that of other ARF members. Consequently, a 2–3-fold increase in ARF6 may not significantly change the total amount of ARF. Alternatively, one of the ARFs (ARF1, ARF3, or ARF5) could be decreased during B cell differentiation. Also, the expression of other small GTP-binding proteins, as detected by GTP overlay blotting (Fig. 5A, lower panel), or Rab proteins, in particular, as detected by immunoblotting,2 did not increase during B cell differentiation.

To rule out the possibility of contamination of B lymphocytes by other cell types from mouse spleen, the B lymphoma cell line A-20 was also differentiated by exposure to LPS. As shown in Fig. 5B, the expression of ARF6 also greatly increased after a 2-day exposure to LPS (2.3 ± 0.4-fold increase over undifferentiated cells), and we observed a very slight increase from 2 to 5 days of treatment (2.3 ± 0.4 to 2.7 ± 0.6-fold increase over undifferentiated cells). Thus, the level of ARF6 expression was very similar during the differentiation of mouse spleen B cells and A-20 B lymphoma cells, which suggests that our B lymphocyte preparation from mouse spleen was highly enriched and not heavily contaminated by other cell types. The expression of ARFs (detected by 1D9) and small GTP-binding proteins (detected by GTP overlay blotting) was not altered (data not shown).

Decreased Expression of ARF6 during Adipogenesis and Monocyte Differentiation—To delineate that increased expression of ARF6 is commonly associated with cell differentiation, we further examined the level of ARF6 expression in other differentiating cell types. Two other examples of cell-type differentiation were chosen to study the expression of ARF6: 3T3-L1 fibroblasts and HL-60 cells. Murine 3T3-L1 fibroblasts were differentiated to adipocytes as described under “Experimental Procedures.” 3T3-L1 adipocytes have induced expression of the insulin-sensitive glucose transporter isoform 4 (Glut4) and respond to insulin by enhanced Glut4 translocation from intracellular compartments to the plasma membrane (22).

Insulin has also been shown to stimulate the accumulation of transferrin receptors and insulin-like growth factor II receptors on the cell surface (23). In addition, adipocytes also secrete several proteins potentially important in homeostatic control of glucose and lipid metabolism. Complement factors C3 and B are secreted by adipocytes. Adipsin, equivalent to factor D of the alternative complement pathway, is synthesized exclusively in adipocytes, and its secretion is enhanced severalfold by insulin (24). Therefore, 3T3-L1 adipocytes have an enhanced recycling and secretory activity compared with 3T3-L1 fibroblasts. As illustrated in Fig. 6A (upper panel), ARF6 expression could be detected in fully differentiated 3T3-L1 adipocytes, but its expression was much lower than in 3T3-L1 fibroblasts. This is consistent with a previous report that the level of ARF6 mRNA was reduced during 3T3-L1 differentiation, suggesting that the decrease of ARF6 protein in differentiated 3T3-L1 adipocytes may result from changes at the level of transcription (25). Immunoblots with 1D9 showed that the overall expression of ARF GTPases was not significantly altered during adipogenesis (Fig. 6A, middle panel). Although it has been reported that the levels of Rab3A and Rab3D increase in fully differentiated 3T3-L1 adipocytes compared with 3T3-L1 fibroblasts (26, 27), the total amount of small GTP-binding proteins, as detected by GTP overlay blotting (Fig. 6A, lower panel), was not significantly altered in these cells.

HL-60 human leukemic cells can be induced to differentiate to monocyte-like cells, which have differentiated functions such as phagolysosome formation and directed locomotion that presumably require enhanced organized intracellular traffic (28). HL-60 cells were induced to differentiate to monocytes by exposure to phorbol 12-myristate 13-acetate (20 ng/ml) for 4 days. The differentiation was determined by their adhesion to substratum and a substantial increase in the expression of total small GTP-binding proteins (Fig. 6B, lower panel), as previously reported (16). When equal amounts of total proteins prepared from undifferentiated and differentiated HL-60 cells were used for immunoblotting by the anti-ARF6 monoclonal antibody (3F8.1), the levels of ARF6 unexpectedly decreased in differentiated HL-60 cells (Fig. 6B, upper panel), whereas those

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**Fig. 4.** Subcellular localization of ARF6 in 3T3-L1 adipocytes.

The cytosol (CYT), HDMs, plasma membrane (PM), and LDMs (20 μg of protein of each fraction) were used for ARF6 immunoblot analysis as described for Fig. 1A.

**Fig. 5.** Increased expression of ARF6 during plasmocyte differentiation. B cells from mouse spleen (A) or the A-20 cell line (B) were differentiated by treatment with LPS (25 μg/ml) for 0, 2, or 5 days as indicated. Total cell lysates were either analyzed by immunoblotting with either 3 μg/ml 3F8.1 IgG (upper panel) or 1 μg/ml 1D9 IgG (middle panel) and developed by ECL or analyzed by GTP overlay blotting (1 μCi/ml [γ-32P]GTP) (lower panel), followed by autoradiography at −80 °C.
Localization and Expression of ARF6

Two previous reports (14, 15) allowed us to speculate that endogenous ARF6 has a cell type-dependent subcellular localization. To further investigate this question, we generated an ARF6-specific monoclonal antibody (3F8.1) that can easily detect endogenous ARF6. Using the 3F8.1 monoclonal antibody, we showed that ARF6 localizes in the cytosol in all cell types examined, but the quantity present in the cytosol varied from 5 to 50% of the total ARF6. The question that therefore arises is why previous studies in CHO cells and bovine adrenal chromaffin cells did not report its cytosolic localization. One possibility is that the majority of ARF6 is associated with membranes in these two types of cells. For example, only 5% of ARF6 was found in the cytosol of CHO cells (Fig. 3A). In addition, it is likely that the polyclonal antibody R1471 (14) could not detect the relatively small amount of ARF6 present in the cytosol. Furthermore, in the study by Galas et al. (15), equal amounts of cytosolic and membrane proteins were used for immunoblot analysis instead of equivalent quantities of cytosol and membranes as in our study. This is significant because 70–80% of proteins of the post-nuclear supernatant are cytosolic and 20–30% are membrane-associated. Thus, three to four times more cytosolic protein was analyzed in our experiments than in the study by Galas et al. (15). An important future undertaking will be to confirm the cytosolic localization by immunofluorescence, confocal microscopy, and electron microscopy since we cannot rule out some artifactual dissociation of ARF6 from membranes to the cytosol during the procedures of cytosol and membrane preparation.

We also showed in this study that ARF6 is mainly localized on the plasma membrane in 3T3-L1 adipocytes, which is similar to its localization in CHO cells (14). In addition to its plasma membrane localization, we did observe some ARF6 in the cytosol and low density microsomes, which are relatively enriched in endosomes, secretory vesicles, and Golgi apparatus. In addition, Hosaka et al. (21) have also shown intracellular and plasma membrane staining in monkey kidney Vero cells transiently transfected with an ARF6-hemagglutinin construct. However, it has been shown by subcellular fractionation that ARF6 is exclusively localized on granules in bovine adrenal chromaffin cells and that it plays a general role in the exocytotic pathway in endocrine and neuroendocrine cells (15). Our results combined with previously published reports suggest that ARF6 localization is dependent on cell types in which ARF6 may control different steps of vesicular transport. It will be interesting to compare the subcellular localization of ARF6 in 3T3-L1 fibroblasts and 3T3-L1 adipocytes. If ARF6 displays function-dependent subcellular localization, we would expect to see a varying localization in the same cells in the two differentiated states. We are also in the process of determining whether ARF6 is involved in insulin-sensitive glucose transporter translocation from intracellular compartments to the plasma membrane. We speculate that the localization of ARF6 on the plasma membrane in 3T3-L1 adipocytes is important for the exocytic fusion between Glut4-containing vesicles and the plasma membrane, where ARF6 might regulate fusogenic lipid metabolism.

We have also shown that the expression of ARF6 during cell differentiation varies depending on the cell type examined. Increased expression was observed during plasmocyte differentiation, whereas decreased expression was recorded during the differentiation of HL-60 cells to monocyte-like cells and during adipogenesis. Differentiated plasmocytes have a well developed secretory apparatus including the endoplasmic reticulum and Golgi apparatus and higher secretory activity compared with undifferentiated lymphocytes. The increased quantity of ARF6 in plasmocytes and the higher percentage of ARF6 associated with membranes could reflect its involvement in membrane trafficking in these cells, i.e. exocytosis or membrane recycling. Interestingly, the total amount of ARF proteins, as detected by monoclonal antibody 1D9, was not significantly changed, despite the fact that they have been shown to be regulators of the late steps of secretion. One possibility is that the activity of these proteins in plasmocytes is increased independently of the level of their expression. Rab1, Rab2, and Rab6 are proteins that are involved in the synthetic/secretory pathway, but their expression does not increase during plasmocyte differentiation. It will be interesting to determine the activity of appropriate regulatory molecules such as GTPase-activating proteins, guanine nucleotide exchange factors, or GDP dissociation inhibitor for ARF and Rab proteins in plasmocytes compared with resting B lymphocytes.

The expression of ARF6 decreased during differentiation of 3T3-L1 fibroblasts to adipocytes and of HL-60 human leukemic cells to monocyte-like cells. These results suggested that ARF6 expression is not directly linked to the acquisition of the differentiated phenotype found in these cells.

DISCUSSION

Two previous reports (14, 15) allowed us to speculate that endogenous ARF6 has a cell type-dependent subcellular localization. To further investigate this question, we generated an ARF6-specific monoclonal antibody (3F8.1) that can easily detect endogenous ARF6. Using the 3F8.1 monoclonal antibody, we showed that ARF6 localizes in the cytosol in all cell types examined, but the quantity present in the cytosol varied from 5 to 50% of the total ARF6. The question that therefore arises is why previous studies in CHO cells and bovine adrenal chromaffin cells did not report its cytosolic localization. One possibility is that the majority of ARF6 is associated with membranes in these two types of cells. For example, only 5% of ARF6 was found in the cytosol of CHO cells (Fig. 3A). In addition, it is likely that the polyclonal antibody R1471 (14) could not detect the relatively small amount of ARF6 present in the cytosol. Furthermore, in the study by Galas et al. (15), equal amounts of cytosolic and membrane proteins were used for immunoblot analysis instead of equivalent quantities of cytosol and membranes as in our study. This is significant because 70–80% of proteins of the post-nuclear supernatant are cytosolic and 20–30% are membrane-associated. Thus, three to four times more cytosolic protein was analyzed in our experiments than in the study by Galas et al. (15). An important future undertaking will be to confirm the cytosolic localization by immunofluorescence, confocal microscopy, and electron microscopy since we cannot rule out some artifactual dissociation of ARF6 from membranes to the cytosol during the procedures of cytosol and membrane preparation.

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The expression of ARF6 decreased during differentiation of 3T3-L1 fibroblasts to adipocytes and of HL-60 human leukemic cells to monocyte-like cells. These results suggested that ARF6 expression is not directly linked to the acquisition of the differentiated phenotype found in these cells. In contrast, the expression of other members of the ARF family did not vary significantly, but certain Rab proteins greatly increased during the differentiation of HL-60 leukemic cells to monocyte-like cells. These findings highlight a potentially attractive feature of small GTP-binding proteins where the regulation of membrane trafficking may involve increased protein levels, changes in subcellular localization, or increased activity via modulation of interacting molecules.

In conclusion, our findings provide the first evidence that endogenous ARF6 is localized in both the cytosol and membranes. These results suggest that endogenous ARF6, similar to other ARFs, undergoes repeated transitions between membrane-bound and soluble states during its activity cycle. In addition, the observation that ARF6 is differentially expressed...
suggests that ARF6 plays a tissue- or cell-specific role in vesicular trafficking.

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