Adipocytes support cAMP-dependent translocation of aquaporin-2 from intracellular sites distinct from the insulin-responsive GLUT4 storage compartment

Giuseppe Procino,1,2* Donne Bennett Caces,1* Giovanna Valenti,2 and Jeffrey E. Pessin1

1Department of Pharmacological Sciences, Stony Brook University, Stony Brook, New York; and 2Dipartimento di Fisiologia Generale ed Ambientale, University of Bari, Bari, Italy

Submitted 8 September 2005; accepted in final form 15 November 2005

Procino, Giuseppe, Donne Bennett Caces, Giovanna Valenti, and Jeffrey E. Pessin. Adipocytes support cAMP-dependent translocation of aquaporin-2 from intracellular sites distinct from the insulin-responsive GLUT4 storage compartment. Am J Physiol Renal Physiol 290: F985–F994, 2006. First published November 22, 2005; doi:10.1152/ajprenal.00369.2005.—Aquaporin-2 (AQP2), when expressed in fully differentiated 3T3-L1 adipocytes, displays cAMP-dependent plasma membrane translocation in a manner similar to its behavior in renal epithelial cells. The translocation of AQP2 required phosphorylation at serine 256, as the expression of AQP2/S256D was constitutively plasma membrane localized, whereas AQP2/S256A was refractory to forskolin stimulation. Unlike GLUT4, this property is not inhibited by depolymerization of cortical actin. In addition, coexpression with the dominant negative form of TC10 (TC10/T31N) or inhibition of phosphatidylinositol 3-kinase did not abrogate the cAMP-mediated response. Under basal conditions, AQP2 is localized in both the perinuclear region and in punctate vesicles scattered within the periphery of the cell. Two- and three-dimensional confocal immunofluorescence microscopy demonstrated that the adipocyte AQP2 cAMP-responsive compartment was distinct from the GLUT4 insulin-responsive compartment. Consistent with this conclusion, insulin was an effective stimulator of GLUT4 translocation but had no effect on AQP2. Conversely, forskolin induced AQP2 translocation but not GLUT4. Colocalization studies with the early endosomal marker EEA1 and transferrin receptor suggested that the AQP2 compartment is mostly distinct from endosomal vesicles. Interestingly, however, the peripheral AQP2 vesicles significantly overlapped vesicle-associated membrane protein-2, underscoring the role of the latter in hormone-regulated exocytosis. To acquire insulin responsiveness following biosynthesis, GLUT4 undergoes a slow sorting step that requires 6–9 h. In contrast, AQP2 rapidly acquires forskolin responsiveness (3 h following biosynthesis) and directly enters the cAMP-regulated compartment without transiting the plasma membrane. Together, these data demonstrate that adipocytes display two different intracellular sorting mechanisms that direct distinct hormone-sensitive partitioning of GLUT4 and AQP2.

hormone-regulated exocytosis; trans-Golgi network; renal epithelial cells

IN GENERAL, HORMONE-REGULATED EXOCYTOSIS is mechanistically dependent on the formation of a distinct pool of hormone-responsive proteins that are readily accessible and can easily be mobilized in response to hormone stimulation. Such a compartmentalization scheme has been observed in several cell types that support hormone-inducible translocation. Although there are numerous examples of regulated secretion of soluble factors, there are only a few types of integral membrane proteins that undergo hormone-stimulated trafficking. For example, in gastric parietal cells the H+-K+-ATPase subunits are contained in distinct tubulovesicular compartments until mobilized to the cell surface following histamine stimulation (7, 9). In striated muscle cells and adipocytes, the rate of plasma membrane exocytosis of the facilitative glucose transporter GLUT4 undergoes a dramatic increase after insulin stimulation, resulting in the redistribution of the protein from intracellular storage sites to the periphery (41). Similarly, the water channel aquaporin-2 (AQP2) expressed in renal collecting duct principal cells is retained in vesicles distinct from lysosomes, the Golgi apparatus, and the endoplasmic reticulum (32). In the presence of vasopressin, PKA-dependent phosphorylation of AQP2 at Ser256 leads to apical membrane translocation (5, 6, 12, 17, 26, 39).

Analysis of hormone-regulated membrane protein trafficking has primarily relied on steady-state expression. However, in the case of regulated secretion, the initial sorting and formation of the secretory compartment are intimately dependent on trans-Golgi network (TGN) function to direct newly synthesized proteins into the appropriate subcellular destination (23, 36). In this regard, we have recently observed that following initial biosynthesis and before entry into the insulin-responsive compartment in adipocytes, the insulin-responsive aminopeptidase and GLUT4 undergo a slow Golgi-sorting step that requires 6–9 h (21, 42). This sorting process is dependent on the TGN-associated coat adaptor protein GGA, which we have observed can also regulate the sorting of adiponectin, the antidiabetic adipokine (Xie L, Boyle D, Sanford D, Scherer PE, Pessin JE, and Mora S, unpublished observations).

To examine the complex hormone-regulated sorting pathways, subcellular compartmentalization and trafficking mechanisms present in adipocytes, we directly compared the properties of insulin-stimulated GLUT4 and PKA-stimulated AQP2 translocation in this cell line. Similar to renal collecting duct cells, phosphorylation at Ser256 is necessary and sufficient to induce translocation of AQP2 in fully differentiated adipocytes. This process is facilitated independently of known downstream effectors of insulin signaling (e.g., TC10, phosphatidylinositol 3-kinase). We demonstrate that unlike the perinuclear localization of GLUT4 in the basal condition, the intracellular distribution of AQP2 is marked by the presence of
peripheral cytoplasmic vesicles. These AQP2-bearing compartments are mostly distinct from endosomal vesicles but significantly colocalize with vesicle-associated membrane protein-2 (VAMP-2). In addition, we show that newly synthesized AQP2 rapidly acquires hormone responsiveness and, like GLUT4, does not transit the plasma membrane before entering a specialized storage compartment. Together, these data demonstrate the presence of a separate and unique sorting mechanism that can facilitate the spatial and functional segregation of AQP2 from GLUT4 in adipocytes.

MATERIALS AND METHODS

Materials. A FastPlasmid Mini-prep DNA kit was purchased from Eppendorf (Westbury, NY) and DNA gel extraction kits from Qiagen (Valencia, CA). Pfu Turbo Polymerase was obtained from Stratagene (La Jolla, CA) and Vectashield from Vector Laboratories (Burlingame, CA). All other chemicals used were purchased from Sigma unless otherwise stated. Plasmids. The TC10/T31N and CAPDH3 constructs were provided by Dr. Alan Saltiel (University of Michigan). The human wild-type AQP2 (AQP2/WT) cDNA was a gift from Dr. Irene Konings (Radboud University of Nijmegen Medical Center, The Netherlands). The AQP2 cDNA was cloned in-frame into the EcoRI-NorI sites of the mammalian expression vector pcDNA3 obtained from Invitrogen. The previous construct was used as a template in overlapping PCR procedures to generate the AQP2-S256A and AQP2-S256D mutants. The bold underlines indicate the codon mutations generated. The forward sequences of the mutagenesis primers used for the PCR reactions are as follows: 5'-ggccggttgggagcctggtcagtcggag-3' for acquiring the AQP2-S256A mutant and 5'-ggccggttgggagcctggtcagtcggag-3' for acquiring the AQP2-S256D mutant. The bold underlines indicate the codon mutations generated. The forward primer 5'-ggccggttgggagcctggtcagtcggag-3' and reverse primer 5'-ggccggttgggagcctggtcagtcggag-3' were used to introduce an EcoRI and NorI site to facilitate sub cloning into pcDNA3. The PCR products were cloned into Topo vector (Invitrogen), digested, and subcloned into the pcDNA3 vector using the same restriction sites. The final constructs were then sequenced in their entirety to ascertain that the appropriate mutations have been introduced. The human VAMP-2 and EE1 cDNA were inserted into the pEGFP-C1 vector (Clontech) using standard cloning techniques, where EGFP is enhanced green fluorescence protein. The human transferrin receptor (TIR), COOH-terminal EGFP-tagged GLUT4 (GLUT4-EGFP) and myc epitope-tagged GLUT4 (myc-GLUT4) constructs were prepared as previously described (10, 13, 37).

Culture and transfection of 3T3-L1 adipocytes. The murine 3T3-L1 preadipocyte cell line was purchased from the American Type Tissue Culture repository. Cells were maintained in DMEM supplemented with 25 mM glucose, 10% bovine calf serum, and 1% penicillin/streptomycin at 37°C and 8% CO2. Two days postconfluence, differentiation into adipocytes was induced by shifting media into DMEM supplemented with 25 mM glucose, 10% fetal bovine serum, 1% penicillin/streptomycin, 1 μg/ml insulin, 0.25 μM dexamethasone, and 0.5 mM IBMX. After 4 days, the same media less the dexamethasone and IBMX was used to bathe the cells for 4 additional days. Fully differentiated adipocytes were used for transfection by electroporation using 100–150 μg of plasmid DNA under low-voltage conditions (160 V, 950 μF). After electroporation, the cells were plated on collagen-coated glass coverslips and maintained in complete media to allow full recovery.

Indirect immunofluorescence, colocalization, and time course experiments. Differentiated 3T3-L1 adipocytes transfected with the appropriate cDNA and expressing GLUT4-EGFP, AQP2/WT, AQP2/S256A, or AQP2/S256D were grown on coverslips and serum-starved in DMEM for 2 h before each experiment. For the colocalization studies, the adipocytes were cotransfected with 50 μg each of myc-GLUT4 and AQP2/WT cDNA, or 50 μg of AQP2/WT and 50 μg of EE1-AFP, VAMP-2-GFP, or hTfR construct. For the signaling studies, fully differentiated adipocytes were cotransfected with 50 μg of GLUT4-GFP or AQP2/WT and 200 μg of TC10/T31N construct. The cells were then incubated with either insulin (100 nM) or forskolin (FK; 50 μM) for 30 min, and the coverslips were processed for confocal microscopy. For the time course experiments, stimulations were done at exactly 3, 6, 9, or 12 h after transfection. For the phosphatidylinositol 3-kinase study, the cells were preincubated with 1 nM wortmannin for 10 min before the appropriate stimulation was done. Shortly after each stimulation, the cells were fixed using 4% paraformaldehyde supplemented with 0.18% Triton X-100 for 20 min at room temperature and blocked using 1% bovine serum albumin solution with 5% donkey serum for 1 h at room temperature. After blocking, the coverslips were incubated in primary antibody solution for 1 h at 37°C. The primary antibodies used were as follows: c-myc monoclonal antibody (Sigma), TGN38 sheep polyclonal antibody (Serotec), GM130 mouse monoclonal antibody (BD Transduction Lab), hTfR mouse monoclonal antibody (Molecular Probes), and AQP2-COOH rabbit polyclonal antibody (38). Each antibody solution was a 1:100 dilution of the stock (1–20 μg/ml) prepared in blocking solution. After incubation with the primary antibody, the coverslips were washed three times with PBS and incubated in secondary antibody for another hour at 37°C. The secondary antibodies used were as follows: anti-rabbit IgG Alexa Fluor 594 and anti-mouse IgG Alexa Fluor 488 (Molecular Probes). After incubation with the secondary antibody, the coverslips were again washed with PBS and mounted on Vectashield Medium. Plasma membrane translocation was determined by visualization on a Zeiss LSM510 confocal fluorescence microscope and counting of 50 representative cells/condition. The number of expressing cells displaying a continuous plasma membrane ring was calculated (means ± SE) from two to five independent determinations.

RESULTS

GLUT4 and AQP2 are localized to distinct intracellular compartments in adipocytes. GLUT4 primarily expressed in muscle and adipose tissue, whereas AQP2 is expressed in the principal cells of the kidney collecting duct. Because no cell naturally expresses both hormone-responsive translocating proteins, we hypothesized that by virtue of their secretory nature adipocytes could provide an appropriate context for as comparison of the sorting and trafficking properties of AQP2 with GLUT4. We first examined the relative compartmentalization of these proteins by cotransfecting 3T3-L1 adipocytes with myc-GLUT4 and WT/AQP2 (Fig. 1). Under basal conditions, both proteins maintained their characteristic distribution pattern, with GLUT4 mostly in the perinuclear region and AQP2 partly perinuclear and partly peripheral (Fig. 1A, a–c). Insulin stimulation resulted in robust translocation of GLUT4 but not AQP2 (Fig. 1A, d–f), whereas FK stimulated AQP2 translocation but not GLUT4 (Fig. 1A, g–i). Quantification of the relative extent of insulin- and FK-stimulated GLUT4 and AQP2 translocation is shown in Fig. 1B.

To ensure that the apparent partial colocalization observed in the two-dimensional images in the XY plane were not a result of the protein localized in separate compartments that were on top of each other (stacked), the XZ and YZ reconstruction of representative cells was also determined (Fig. 2A). These observations confirm that GLUT4 and AQP2 lie in distinct compartments in the cell periphery but are colocalized within the perinuclear region. Comparison with the localization of the
cis-Golgi marker GM130 (Fig. 2B, a–d) and the trans-Golgi marker TGN38 (Fig. 2B, e–h) suggests that this overlapped region is more likely a shared compartment after the cis-Golgi network.

Because AQP2 appears to localize to intracellular membrane compartments distinct from GLUT4, we next compared the distribution of AQP2 with several endosome markers (Fig. 3).

The early endosome marker (EEA1) displayed a punctate pattern that was poorly colocalized with AQP2 except at a few locations underneath the plasma membrane (Fig. 3, A–D). TfR was also peripherally located in dispersed regions throughout the cell but was poorly colocalized with AQP2 (Fig. 3, E–H). Although the vesicle trafficking protein VAMP-2 only partially overlapped with AQP2, there was a much greater degree of

---

**Fig. 1.** Coexpression of myc-GLUT4 and wild-type aquaporin-2 (AQP2/WT) in 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes were electroporated with 50 μg each of GLUT4-enhanced green fluorescent protein (EGFP) and AQP2/WT cDNAs. A: cells were allowed to recover for 24 h and either left untreated (a–c) or stimulated with either 100 nM insulin (d–f) or 50 μM forskolin (FK; g–i) for 20 min at 37°C. The cells were then fixed, processed for indirect whole cell immunofluorescence, and subjected to confocal microscopy. Representative composite images are shown. B: quantification of the number of cells displaying plasma membrane (PM) localization was determined by counting the fraction of cells showing continuous labeling of the PM rim. The extent of translocation is expressed as the percentage (means ± SE) of cells showing a continuous PM ring by counting 50 random cells/experiment from 3–4 independent determinations.
colocalization in peripheral regions adjacent to the plasma membrane (Fig. 3, I–L).

_Intracellular signals regulating AQP2 translocation in 3T3-L1 adipocytes._ To determine whether the FK-dependent AQP2 translocation was due to increases in intracellular cAMP levels, we compared the sensitivity and responsiveness of AQP2 translocation to FK, isoproterenol, 8-bromoadenosine 3′,5′-cyclic monophosphate (8-Br-cAMP; a cell-permeable form of cAMP), and the phosphodiesterase inhibitor IBMX (Fig. 4). These data demonstrate that AQP2 responds to these agonists at concentrations that induce or mimic physiological levels of intracellular cAMP: isoproterenol (10 nM-100 μM), IBMX (1 μM-1 mM), FK (100 nM-100 μM), and 8-Br-cAMP (1 μM-1 mM).

Several studies have also demonstrated that hormone-regulated plasma membrane translocation is dependent on cortical actin polymerization. In particular, depolymerization of cortical actin in adipocytes inhibits insulin-stimulated GLUT4 translocation (2, 15) but can potentiate cAMP-stimulated AQP2 translocation in renal epithelial cells (20, 33–35). As expected, depolymerization of adipocyte cortical actin with the actin monomer-sequestering agent latrunculin B resulted in an inhibition of insulin-stimulated GLUT4 translocation (Fig. 5A). In contrast, latrunculin B treatment in the adipocytes has no significant
Fig. 3. Peripheral AQP2-bearing compartments are mostly distinct from endosomal vesicles. 3T3-L1 adipocytes were cotransfected with 50 μg of AQP2/WT and 50 μg of early endosomal marker (EEA1)-EGFP, human transferrin receptor (TfR), or vesicle-associated membrane protein-2 (VAMP-2)-EGFP constructs. Twenty-four hours after transfection, cells were fixed and processed for fluorescent microscopy. Representative composite images showing AQP2/WT colocalization with EEA1 (A–D), TfR (E–H), and VAMP-2 (I–L) are shown. Arrowheads (D, H, and L), overlapping red and green signals, signifying colocalization.

Fig. 4. Effect of isoproterenol and FK (A) and IBMX and 8-bromoadenosine 3′,5′-cyclic monophosphate (8-Br-cAMP; B) on AQP2 translocation to the PM in 3T3-L1 adipocytes. Fully differentiated 3T3-L1 adipocytes were electroporated with 50 μg of AQP2/WT cDNA. Twenty-four hours after transfection, cells were stimulated with isoproterenol (10 nM-100 μM), FK (100 nM-100 μM), IBMX (1 μM-1 mM), and 8-Br-cAMP (1 μM-1 mM) for 20 min at 37°C. The cells were then fixed, stained with anti-AQP2 antibody, and subjected to confocal fluorescence microscopy.

A

B

AJP-Renal Physiol • VOL 290 • MAY 2006 • www.ajprenal.org

Downloaded from http://ajprenal.physiology.org/ by 10.220.33.3 on June 11, 2017
effect on FK-stimulated AQP2 translocation. The lack of latrunculin B potentiation probably reflects that at the dose of FK used, as AQP2 translocation was already maximally stimulated. In any case, these data demonstrate that the expressed AQP2 protein in adipocytes displays the same characteristics and cAMP-dependent responsiveness as in renal epithelial cells.

It was previously shown that overexpression of the mutant form of TC10, a Rho family member of GTP binding proteins, potently inhibits insulin-stimulated GLUT4 translocation (4). For controls, we coexpressed the dominant-interfering TC10/T31N mutant with GLUT4 and examined insulin-stimulated GLUT4 translocation (Fig. 5B). Consistent with these previous findings, expression of TC10/T31N markedly inhibited insulin-stimulated GLUT4 translocation. In contrast, expression of TC10/T31N had no significant effect on FK-stimulated AQP2 translocation. Similarly, the dominant-interfering CAP mutant (CAPΔSH3) also inhibited insulin-stimulated GLUT4 translocation without affecting FK-stimulated AQP2 translocation (data not shown). It has also been established that the phosphatidylinositol 3-kinase inhibitor wortmannin can block insulin-stimulated GLUT4 translocation (27). As expected, GLUT4 translocation in the presence of insulin was completely inhibited by pretreatment with wortmannin, whereas FK-stimulated AQP2 translocation remained unaffected (Fig. 5C). Together, these data demonstrate that adipocytes can fully support the regulated trafficking of AQP2 and GLUT4 through distinct intracellular localization and signaling pathways.

To further characterize the cAMP dependence of AQP2 translocation, we compared the cellular distribution of AQP2/WT with the S256A (AQP2/S256A) and S256D (AQP2/S256D) mutants (Fig. 6). In renal cells, the mutant with the alanine substitution at Ser256 mimics a constitutively nonphosphorylated state and is retained intracellularly. The aspartic acid substitution, on the other hand, mimics a constitutively phosphorylated state and creates a mutant that is expressed on the plasma membrane (40). Adipocytes expressing AQP2/S256A displayed a persistent intracellular localization of AQP2 that was unresponsive to FK (Fig. 6A, e and f). In contrast, the expressed AQP2/S256D protein was persistently localized to the plasma membrane in both the absence and presence of FK (Fig. 6A, c and d). These data demonstrate that similar to renal principal cells, cAMP-dependent Ser256 phosphorylation is sufficient to induce AQP2 plasma membrane translocation.

Time-dependent acquisition of FK-stimulated AQP2 translocation in 3T3-L1 adipocytes. Having established that adipocytes can function as an appropriate cell line for a comparison of the trafficking properties of GLUT4 and AQP2, we next examined the time-dependent acquisition of FK responsiveness of newly synthesized AQP2 protein. Recently, we have re-

Fig. 5. Comparison of signaling events that regulate the translocation of GLUT4 and AQP2 in adipocytes. A: fully differentiated 3T3-L1 adipocytes were electroporated with 50 μg of AQP2/WT or GLUT4-EGFP cDNAs and allowed to recover for 24 h. The cells were then left untreated or incubated with 20 μM latrunculin B. GLUT4-EGFP-transfected cells were subsequently incubated in the presence or absence of 100 nM insulin for 20 min, whereas AQP2/WT transfected cells were incubated in the presence or absence of 50 μM FK for 20 min. B: 3T3-L1 adipocytes were cotransfected with 50 μg of AQP2/WT or GLUT4-EGFP and 200 μg of TC10/T31N cDNAs. Twenty-four hours after transfection, cells were stimulated with insulin or FK as previously described. C: 3T3-L1 adipocytes expressing GLUT4-EGFP or AQP2/WT were pretreated with 1 nM wortmannin for 10 min, then stimulated with insulin or FK. All data represent the counting of 100 representative cells/condition and determination of the fraction of cells displaying a continuous GLUT4-EGFP or AQP2/WT plasma membrane rim. Shown are average values from 3 independent experiments.
ported that newly synthesized GLUT4 protein undergoes a slow (6–9 h) biosynthetic sorting step before entry into the insulin-responsive storage compartment (18, 42). Consistent with these previous studies, there was no significant plasma membrane localization of GLUT4 in the basal state (Fig. 7A). However, the time-dependent acquisition of insulin-stimulated GLUT4 translocation occurred 6–9 h following biosynthesis, with maximal insulin stimulation by 12 h (Fig. 7A). In contrast, FK-stimulated plasma membrane translocation of AQP2 was near maximal 3 h following biosynthesis, with no
significant cell surface accumulation in the basal state (Fig. 7B). These data demonstrate that the biosynthetic processing of AQP2 to the cAMP-responsive compartment is markedly faster than the trafficking of GLUT4 to the insulin-responsive compartment.

Entry of newly synthesized AQP2 into the FK-responsive compartment is not affected by inhibition of endocytosis. In general, there are two pathways by which newly synthesized AQP2 could traffic to its intracellular storage compartment. Following biosynthesis and exit from the Golgi, AQP2 could default to the plasma membrane and undergo subsequent endocytosis and recycling to the cAMP-responsive storage compartment. Alternatively, AQP2 could directly traffic to its storage compartment without transiting the plasma membrane. Because AQP2 at the plasma membrane is localized to coated pits and is recycled by clathrin and dynamin-dependent endocytosis (31), we determined whether newly synthesized AQP2 transits the plasma membrane before entering its storage compartment by inhibiting endocytosis with a dominant-interfering dynamin mutant (Dyn/K44A). To ensure that endocytosis is inhibited before the secretory trafficking of AQP2 despite the coexpression of the two proteins, we took advantage of the fungal metabolite brefeldin A (BFA), which reversibly blocks the endoplasmic reticulum to Golgi anterograde trafficking (3, 19, 28, 30) and reversibly accumulates AQP2 in the endoplasmic reticulum (29). However, because dynamin is a soluble protein, BFA treatment does not affect dynamin’s ability to associate with the plasma membrane (42). Thus cells were transfected with wild-type (Dyn/WT) or a dominant-interfering dynamin mutant (Dyn/K44A) plus AQP2 and were immediately treated with BFA for 3 h. Because the BFA block on endoplasmic reticulum exit is rapidly reversible, the cells were extensively washed to remove BFA, and the time-dependent acquisition of FK-stimulated translocation for the newly synthesized AQP2 was determined (Fig. 8). Expression of Dyn/WT did not affect the intracellular localization of AQP2 under basal conditions at any of the time points examined or the acquisition of FK responsiveness that occurred by 3 h post-BFA washout (Fig. 8, ○). In contrast, the expression of Dyn/K44A increased, in a time-dependent manner, the localization of AQP2 at the plasma membrane even in unstimulated cells (Fig. 8, □). These findings are consistent with previous reports demonstrating that AQP2 continually recycles even in the basal state and that inhibition of endocytosis results in plasma membrane accumulation (22). Despite the relatively slower plasma membrane accumulation, the expression of Dyn/K44A did not prevent the acquisition of FK responsiveness of the newly synthesized AQP2 protein at 3 h (Fig. 8, △). These data demonstrate that the newly synthesized AQP2 protein directly acquires cAMP responsiveness without transiting the plasma membrane or undergoing endocytosis.

DISCUSSION

Regulated exocytosis is a highly complex process that occurs in cells to modify their surface or their immediate surroundings in response to changes in stimuli in the extracellular environment. Although there are numerous examples of regulated secretion in numerous cell types, only a few cell-specific systems display regulated exocytosis of membrane proteins. To date, the best-characterized hormone-regulated trafficking systems are the insulin regulation of GLUT4 exocytosis in adipocytes (14) and antidiuretic hormone-stimulated AQP2 exocytosis in renal collecting duct cells (1). In general, proteins that are destined to function on the cell surface enter the secretory pathway shortly after synthesis from the endoplasmic reticulum and transport into the Golgi apparatus. In the Golgi complex, further processing and maturation are facilitated by glycolipid- and glycoprotein-trimming enzymes. The proteins are then sorted and packaged in the TGN into transport intermediates that will deliver them directly to the plasma membrane or bring them to specialized storage compartments where they are retained until needed. From the plasma membrane, these proteins may enter the endocytic pathway via another sorting station, the early endosomes. The retrieved proteins may be returned to the same domain from which they originated (recycling) or to a different domain of the plasma membrane (transcytosis). In certain cell types, specialized storage pools may also originate from early endosomes and these compartments provide the ready supply of proteins that can be delivered to the cell surface in response to external stimuli.

In the case of GLUT4 and AQP2 in the basal state, there is a slow but continuous recycling to and retrieval from the plasma membrane such that the steady-state equilibrium favors intracellular sequestration (8, 11, 16). Stimulation primarily results in an increase in the rate of exocytosis, resulting in a new steady-state redistribution favoring the cell surface membrane (11, 24). Following hormone withdrawal, exocytosis returns to a low rate and restoration of the basal steady-state distribution ensues. Because the endocytic transporters are fully capable of undergoing subsequent rounds of stimulated exocytosis, it has been generally assumed that entry into these specialized storage compartments results exclusively from plasma membrane endocytosis. Although the plasma membrane recycling of GLUT4 into the insulin-responsive storage compartment certainly occurs, we have also demonstrated that...
the newly synthesized GLUT4 protein is directly sorted into this regulated compartment without first transiting the plasma membrane (42).

For a variety of reasons, we speculated that adipocytes might also express the appropriate compartments and regulatory machinery to support cAMP-dependent AQP2 trafficking and thereby serve as a model system to directly compare the trafficking and sorting properties of AQP2 with GLUT4. To test this hypothesis, we initially observed that when expressed in adipocytes, AQP2 is localized to the perinuclear region that overlaps with GLUT4. However, a significant population of AQP2 is distinct from GLUT4 and is scattered in small punctate vesicles in the periphery and in close proximity to the plasma membrane. These AQP2-bearing compartments are mostly separate from endosomal vesicles, as evidenced by their minimal colocalization with the EEA1 marker and TIR, but partially colocalize with VAMP-2. These data agree with independent studies done using the Madin-Darby canine kidney epithelial cell line showing that AQP2 is retained in a distinct subapical compartment and utilizes the EEA1-positive early endosomal system shortly after retrieval from the plasma membrane (32). Moreover, the association with VAMP-2-containing compartments is also consistent with the established role of VAMP-2 in AQP2 fusion with the plasma membrane in epithelial cells (25). In a broader sense, these data demonstrate that each protein is equipped with a unique set of targeting motifs that establishes the transporter’s trafficking itinerary and allows for functional segregation. Consistent with a distinct intracellular compartmentalization, elevation of cAMP levels by FK treatment was an effective inducer of AQP2 trafficking without any significant effect on GLUT4. Similarly, insulin stimulation mobilized the facilitative glucose transporter without perturbing the sequestration of AQP2. Moreover, the translocation of AQP2 in adipocytes requires cAMP-mediated phosphorylation of Ser256, analogous to that in renal epithelial cells (6, 17). Overall, these data demonstrate that not only do adipocytes provide an appropriate cellular environment for regulated AQP2 trafficking but that this process is distinct from the retention mechanism that facilitates regulated exocytosis of GLUT4. In future studies, it will be interesting to determine the endogenous adipocyte membrane and intraluminal cargo proteins that are responsive to increased cAMP levels.

In any case, the ability of adipocytes to differentially translocate GLUT4 and AQP2 prompted us to investigate further the machinery involved in sorting both proteins. To address this issue, we examined the initial sorting of both GLUT4 and AQP2 immediately following their biosynthesis by transient expression of a dominantly-interfering dynamin mutant did not affect the ability of newly synthesized AQP2 to rapidly display cAMP responsiveness. These data are more consistent with a rapid TGN sorting of AQP2 directly to the cAMP-responsive compartment without having to initially transit the plasma membrane.

The ability to now functionally distinguish between the initial trafficking of GLUT4 and AQP2 in the same cell system will allow for more detailed analysis of the functional domains and mechanisms responsible for these distinct sorting decisions. In particular, this will facilitate efforts to identify the protein and lipid components of each transporter’s sorting machinery and will expand our molecular understanding of the role of the TGN in directing distinct trafficking decisions leading to appropriate intracellular compartmentalization.

ACKNOWLEDGMENTS
We thank Bintou Diouf and Jeffery Smith for the care and maintenance of 3T3-L1 adipocytes.

GRANTS
This study was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-55811 and DK-33823.

REFERENCES