cAMP-dependent exocytosis and vesicle traffic regulate CFTR and fluid transport in rat jejunum in vivo

NADIA A. AMEEN,1 CHRISTOPHER MARINO,2 AND PEDRO J. I. SALAS3

Departments of 1Pediatrics and 3Cell Biology, University of Miami School of Medicine, Miami, Florida 33101; and 2Department of Medicine (Gastroenterology), University of Tennessee, Memphis, Tennessee 38104

Submitted 11 June 2002; accepted in final form 27 August 2002

The cystic fibrosis transmembrane conductance regulator (CFTR) channel is regulated by cAMP-dependent vesicle exocytosis and insertion to the apical membrane in some cell types, but this has not been demonstrated in the intestinal crypt. The distribution of CFTR, lactase (control), and fluid secretion were determined in rat jejunum after cAMP activation in the presence of nocodazole and primaquine to disrupt vesicle traffic. CFTR and lactase were localized by immunofluorescence, and surface proteins were detected by biotinylation of enterocytes. Immunoprecipitates from biotinylated and nonbiotinylated cells were analyzed by streptavidin detection and immunoblots. Immunolocalization confirmed a cAMP-dependent shift of CFTR but not lactase from a subapical compartment to the apical surface associated with fluid secretion that was reduced in the presence of primaquine and nocodazole. Analysis of immunoblots from immunoprecipitates addressing this issue except for our previous analysis of endogenous CFTR (8, 39). In the intestine, CFTR channel gating is regulated by cAMP- and cGMP-dependent phosphorylation of protein kinases A and G (12). Whether vesicle traffic regulates CFTR and fluid secretion in the intestine is unclear. Published studies using a variety of techniques to examine this question with T84 cells (a widely used chloride-secreting colonic cell line expressing CFTR) resulted in conflicting conclusions (9, 28, 39), and there are no in vivo studies addressing this issue except for our previous analysis (4) of CFTR high expresser (CHE) cells in rat small intestine.

In a recent study of isolated rat colonic crypts, membrane capacitance was used to determine cAMP-stimulated exocytosis of CFTR; however, the authors could not demonstrate an increase in membrane surface area on cAMP activation and therefore concluded that activation of CFTR by cAMP does not involve detectable exocytosis (13). However, cAMP-dependent chloride secretion in the rat colon has been shown to be dependent on intact microtubules, the molecular motors of vesicle transport (14). This latter evidence suggests a role for cAMP-dependent vesicle traffic in regulating chloride secretion in the rat intestine.

Although CHE cells are a predominantly villous cell population with undefined ion transport properties, the number of CFTR channels expressed on the surface of these cells was demonstrated to be regulated by cAMP-dependent exocytosis. The observation that

Address for reprint requests and other correspondence: P. Salas, Dept. of Cell Biology and Anatomy, R-124, Univ. of Miami School of Medicine, PO Box 016960, Miami, FL 33101 (E-mail: psalas@miami.edu).
CHE cells are also present in the human small intestine suggests that cAMP-dependent exocytosis of CFTR is also a potentially important regulatory mechanism in the human intestine (3, 34).

The extent to which vesicle traffic regulates fluid secretion and the number of CFTR channels expressed on the surface of the small intestinal lumen (the predominant site of CFTR-mediated fluid secretion) is unknown. In previous studies (2), we used immunoelectron microscopy to examine and quantify the subcellular distribution of CFTR. These observations confirmed that CFTR was associated with subapical vesicles and the plasma membrane of both crypt and CHE cells. Furthermore, quantification of the subcellular distribution in these cells supported a role for CFTR regulation by vesicle traffic in the rat small intestine (2). On the basis of these observations, we used independent morphological and biochemical methodologies in conjunction with fluid secretion measurements in the current study to determine whether cAMP and vesicle traffic regulate the exocytosis of CFTR to the apical membrane of the crypts and the whole mucosa of rat proximal small intestine.

MATERIALS AND METHODS

Animal preparation and fluid secretion measurements. The study was approved by the Animal Research Committee of the University of Miami School of Medicine. Male Sprague-Dawley rats (Charles River Laboratories) weighing 250–300 g were fed standard chow. Rats were fasted overnight and cannulated proximally and distally, and the lumen was opened and intestinal loops were excised, animal was observed for 2 h. At the end of the study period, the loops were removed, embedded in OCT embedding medium, frozen in liquid nitrogen-cooled isopentane, and prepared for immunocytochemistry as described previously (3, 4).

Independent studies were performed to confirm our observations of CFTR distribution in the intestine after cAMP stimulation with the receptor agonist vasoactive intestinal peptide (VIP) as described previously (4). Thirty minutes after VIP administration tissues from rat jejunum were removed, embedded in OCT embedding medium, frozen, and prepared for indirect immunofluorescence labeling, and enterocytes were isolated and used in immunoprecipitation and surface biotinylation studies.

Reagents. All chemicals were obtained from Sigma (St. Louis, MO) except where stated. R3194 and R3195 are affinity-purified polyclonal antibodies raised against rodent CFTR and were provided by C. R. Marino. The specificity of both antibodies has been documented in rats (1, 2, 4, 44). The previously characterized antibody to lactase, YBB2/61, was a gift from Dr. A. Quaroni (Cornell University, Ithaca, NY; Ref. 29). The antibody to AKAP149 was purchased from Alomone Laboratories (Jerusalem, Israel).

Isolation of intestinal enterocytes. Segments of rat jejunum were removed 30 min after VIP administration, and villus and crypt enterocytes were isolated as described previously (24). Suspensions of freshly isolated enterocytes were subject to Trypan blue exclusion, immunofluorescence immunocytochemistry, and surface biotinylation studies.

Surface biotinylation of isolated enterocytes. Suspensions of freshly isolated cells from rat small intestine after VIP or diltuent infusion were biotinylated by rotating in the cold for 30 min in freshly prepared 1.0 mM sulpho-NHS biotin [bicinchoninic acid (BCA) protein assay kit; Pierce Laboratories, Rockford, IL] in PBS-CM (PBS supplemented with 1.3 mM CaCl2 and 1.0 mM MgCl2). Control cells were incubated with PBS-CM. After biotinylation, cells were washed in the cold with 50 mM NH4Cl in PBS-CM to quench unreacted free biotin and immunoprecipitation was performed. Surface biotinylation of CFTR from the lumen of the intact intestine was also attempted; however, complete diffusion into the deep crypts (a major site of CFTR expression) could not be ensured. Surface biotinylation was therefore performed in isolated cells because the yield of isolated crypt and villus enterocytes was high in our hands and polarity and membrane preservation could be ensured in the majority of cells. Indeed, it has been shown that isolated enterocytes remain well polarized for hours (45), a fact that we tested further in our experiments (see Fig. 3E).

Immunoprecipitation of CFTR. Enterocytes were lysed in immunoprecipitation buffer (IP) containing 0.5% Triton X-100, 0.1% SDS, and 0.5% sodium deoxycholate in PBS pH 7.4 supplemented with a protease inhibitor cocktail (Sigma). Samples were homogenized and sonicated and then centrifuged for 15 min in the cold at 14,000 rpm. Protein content was measured by UV absorption (Pierce Laboratories), and immunoprecipitation was performed with a minimum of 1 mg/ml protein in supernatants. Supernatants were pre-cleared with protein A-Sepharose beads (Amersham Pharmacia, Piscataway, NJ) for 1 h in the cold. Immunoprecipitations were performed with the anti-CFTR antibody R3194, antibody to lactase YBB2/61, or nonspecific rabbit IgG. Antibody or serum was added to supernatants and incubated for 2 h at 4°C. Protein A agarose (5 mg/sample) was added to samples and resuspended in IP buffer containing 1% (wt/vol) globulin-free bovine serum albumin (BSA), and then samples were rotated overnight in the cold. Samples were then centrifuged (14,000 rpm), supernatants were discarded, and the beads were washed in IP buffer supplemented with 0.5 M KCl. The immunoprecipitates were eluted in 1% SDS, 4 M...
urea, and 1 mM Tris·HCl, pH 6.8, precipitated on ice in trichloroacetic acid, acetone extracted, and air dried. The dried pellets were resuspended in sample buffer before analysis by Western blot.

**Western blotting.** Immunoprecipitates were analyzed by SDS-PAGE using a 7.5% gel and proteins transferred to polyvinylidene difluoride (PVDF) membranes by a semi-dry transfer method. After transfer of proteins, membranes were washed in deionized water, nonspecific proteins were blocked in PBS-0.05% Tween 20 containing 5% nonfat dry milk for 2 h, and biotinylated proteins were detected by streptavidin peroxidase binding (Sigma). Western blots of nonbiotinylated immunoprecipitates were analyzed with antibodies to CFTR (R3194) and lactase (YBB2/61). Western blots of CFTR or IgG immunoprecipitates were also analyzed with a commercial antibody to AKAP 149 (Alomone Labs). Detection of primary immunoprecipitates were also accomplished with using goat anti-rabbit (1:10,000) or anti-mouse (1:8,000) peroxidase secondary antibodies (Sigma). After immunodetection, membranes were exposed to chemiluminescence (previously (4)). On the basis of these observations, we examined the distribution of CFTR in the crypt after VIP (Fig. 1E). We compared the distribution with that of lactase, an integral apical membrane protein that is not regulated by cAMP-dependent vesicle traffic (Fig. 1, A and B; Refs. 23, 26). Although lactase is absent in proliferative undifferentiated crypt cells and the highest levels of CFTR are found in this compartment, both lactase and CFTR are present on the apical pole of newly differentiated crypt cells that are more superficially located (Fig. 1, A and D; Refs. 3, 29, 34). In fact, lactase is mostly found in a subapical compartment in the crypts (Fig. 1, A and B), whereas it is expressed in the brush border in the villus (29). Examination of crypt sections from rat jejunum revealed that lactase did not redistribute to the cell surface after VIP administration (Fig. 1, B compared with A). CFTR, on the other hand, was distributed in a broad subapical region under control conditions (Fig. 1D) and redistributed to the apical surface in a narrow band (correlating with the region of phalloidin label) after VIP (Fig. 1E). To further confirm that the effect of VIP was mediated by cAMP, similar experiments were conducted after luminal 8-BrcAMP stimulation. Examination of labeled sections by confocal microscopy after administration of the membrane-permeant agonist 8-BrcAMP similarly confirmed a redistribution of CFTR from a predominant subapical compartment (Fig. 2A) to the region corresponding to the apical microvilli of crypt epithelial cells (Fig. 2B). The 8-BrcAMP-dependent shift of CFTR from the subapical to apical domain corresponded with an almost threefold increase in the ratio of apical to subapical CFTR fluorescence (6.15 ± 3.08) compared with unstimulated PBS controls (2.14 ± 1.03; P < 0.001) and was associated with net fluid movement into the lumen of the jejunum (Table 1).

To determine whether the cAMP-dependent shift of CFTR from the subapical compartment to the apical domain of crypt cells was dependent on vesicle traffic, we examined whether the same shift of CFTR signal occurred when vesicle traffic was interrupted. The antimalarial drug primaquine is a lysosomotropic amine that inhibits vesicle trafficking and prevents the fusion of vesicles with the plasma membrane (16). It was recently shown to be a potent inhibitor of CFTR vesicle traffic in oocytes (39). Examination of crypt sections from jejunum labeled for CFTR after pretreatment with 8-BrcAMP and primaquine (Fig. 2C) revealed a prominent rim of CFTR fluorescence label (extending ∼1.5 μm) beneath the apical microvilli, similar to the subapical distribution of CFTR after control conditions (Fig. 1A). The CFTR apical fluorescence was reduced in the presence of primaquine both in terms of pixel values and in the apical-to-subapical ratio (Table 1). Accordingly, the reduction in the shift of CFTR from

**RESULTS**

**Morphological distribution of CFTR in rat jejunum after cAMP stimulation.** Our previous light microscopic localization of CFTR in rat proximal small intestinal crypts revealed a subapical distribution for CFTR, suggesting the presence of CFTR in a vesicular compartment. Immuno electron microscopic examination revealed that although CFTR was detected on the apical membrane, the majority of CFTR was associated with subapical vesicles, supporting a role for vesicle insertion in regulating CFTR and anion secretion in the crypt (2). VIP, a cAMP agonist, also induced a redistribution of CFTR from the subapical compartment to the apical domain in villus CHE cells as observed previously (4). VIP, a cAMP agonist, also induced a redistribution of CFTR from the subapical compartment to the apical domain in villus CHE cells as observed previously (4). On the basis of these observations, we examined the distribution of CFTR in the crypt after VIP (Fig. 1E). We compared the distribution with that of lactase, an integral apical membrane protein that is not regulated by cAMP-dependent vesicle traffic (Fig. 1, A and B; Refs. 23, 26). Although lactase is absent in proliferative undifferentiated crypt cells and the highest levels of CFTR are found in this compartment, both lactase and CFTR are present on the apical pole of newly differentiated crypt cells that are more superficially located (Fig. 1, A and D; Refs. 3, 29, 34). In fact, lactase is mostly found in a subapical compartment in the crypts (Fig. 1, A and B), whereas it is expressed in the brush border in the villus (29). Examination of crypt sections from rat jejunum revealed that lactase did not redistribute to the cell surface after VIP administration (Fig. 1, B compared with A). CFTR, on the other hand, was distributed in a broad subapical region under control conditions (Fig. 1D) and redistributed to the apical surface in a narrow band (correlating with the region of phalloidin label) after VIP (Fig. 1E). To further confirm that the effect of VIP was mediated by cAMP, similar experiments were conducted after luminal 8-BrcAMP stimulation. Examination of labeled sections by confocal microscopy after administration of the membrane-permeant agonist 8-BrcAMP similarly confirmed a redistribution of CFTR from a predominant subapical compartment (Fig. 2A) to the region corresponding to the apical microvilli of crypt epithelial cells (Fig. 2B). The 8-BrcAMP-dependent shift of CFTR from the subapical to apical domain corresponded with an almost threefold increase in the ratio of apical to subapical CFTR fluorescence (6.15 ± 3.08) compared with unstimulated PBS controls (2.14 ± 1.03; P < 0.001) and was associated with net fluid movement into the lumen of the jejunum (Table 1).

To determine whether the cAMP-dependent shift of CFTR from the subapical compartment to the apical domain of crypt cells was dependent on vesicle traffic, we examined whether the same shift of CFTR signal occurred when vesicle traffic was interrupted. The antimalarial drug primaquine is a lysosomotropic amine that inhibits vesicle trafficking and prevents the fusion of vesicles with the plasma membrane (16). It was recently shown to be a potent inhibitor of CFTR vesicle traffic in oocytes (39). Examination of crypt sections from jejunum labeled for CFTR after pretreatment with 8-BrcAMP and primaquine (Fig. 2C) revealed a prominent rim of CFTR fluorescence label (extending ∼1.5 μm) beneath the apical microvilli, similar to the subapical distribution of CFTR after control conditions (Fig. 1A). The CFTR apical fluorescence was reduced in the presence of primaquine both in terms of pixel values and in the apical-to-subapical ratio (Table 1). Accordingly, the reduction in the shift of CFTR from
the subapical to the apical domain after pretreatment with primaquine and 8-BrcAMP was associated with an increase in CFTR signal detected in the subapical compartment in the presence of primaquine. The shift in the subcellular distribution of CFTR in the crypt paralleled a functional effect of primaquine pretreatment in blocking the fluid secretory response of 8-BrcAMP by 35% (Table 1). These observations are consistent with an effect of primaquine in inhibiting the 8-BrcAMP-dependent insertion of CFTR-containing vesicles from the subapical compartment into the apical membrane and suggest that cAMP and vesicle insertion of CFTR to the apical membrane regulates fluid secretion in the jejunum.

Microtubules serve as molecular motors in the transport of vesicles from the Golgi complex to the apical domain of polarized cells and have been shown to play a role in cAMP-dependent exocytosis of CFTR in T84 cells and in cAMP-dependent chloride secretion in rat colon (14, 10, 21, 38). However, studies of polarized Madin-Darby canine kidney (MDCK) cells and airway epithelial cells could not confirm a role for microtubules in regulating the exocytosis of CFTR (22, 25). We examined the distribution of CFTR after 8-BrcAMP

**Fig. 1.** Vasoactive intestinal peptide (VIP) induces a redistribution of the cystic fibrosis transmembrane conductance regulator (CFTR), but not lactase, to the apical membrane of crypt cells. Lactase fluorescence labeling in crypt cells under control conditions (A) reveals a distribution under the apical domain that does not change after VIP stimulation (B). Control section labeled with nonimmune serum reveals lack of specific staining (C). Under unstimulated conditions, CFTR fluorescence (D) is distributed in a broad band in the subapical region (arrows) and redistributes to the apical surface after VIP stimulation (E). Control section labeled with CFTR antibody (R3195) preincubated with peptide (F). Bars, 10 μm.
stimulation in the presence of nocodazole, an agent that blocks microtubule polymerization and thereby prevents vesicle transport in cells. Nocodazole also blocked the subapical-to-apical shift in CFTR signal, although to a lesser extent than primaquine, and reduced the 8-BrcAMP-induced fluid secretory response by 33% (Table 1). Because the mechanisms of action of primaquine and nocodazole are different and they have in common their effect on exocytic membrane traffic, these results strongly suggest a role of cAMP-induced exocytosis of CFTR as a mechanism to regulate CFTR-mediated anion transport and fluid secretion.

Detection of CFTR exocytosis by surface biotinylation in vivo. Although immunofluorescence examination of the distribution of CFTR in intestinal sections after cAMP agonist treatment suggested a shift of CFTR from a subapical compartment to the apical domain, we wished to independently confirm that cAMP indeed stimulated exocytosis of CFTR to the surface of intestinal cells. Immunolocalization in toto could not confirm this because our antibodies were raised against the cytoplasmic COOH terminus of CFTR. We therefore used the technique of surface biotinylation, a sensitive method that is widely used to quantify surface proteins in cells and to study membrane traffic of proteins and has been used in studies examining CFTR membrane traffic (28, 30).

Morphological examination of fixed intestinal segments after isolation confirmed that we could successfully retrieve most cells for biotinylation and immunoprecipitation, including those from the crypts, within 30 min. Lack of damage to isolated enterocytes was confirmed by Trypan blue exclusion in cell suspensions. Furthermore, immunofluorescence labeling of freshly isolated cells confirmed preservation of polarity by the presence of apical markers (Fig. 3, E and F) in isolated cells. Immunoprecipitations were performed on freshly isolated cells with the CFTR antibody R3194 and nonspecific rabbit IgG as negative controls, and immunoprecipitates were analyzed by CFTR by Western blots using the same CFTR antibody. Western blot analysis of immunoprecipitates from VIP-stimulated or control cells with R3194 detected a broad band of mature CFTR (band C) of molecular mass of 170–185 kDa (Fig. 3A, lane 2) and a smaller band of immature CFTR of ~148 kDa in native rat tissues but not in IgG

Table 1. CFTR fluorescence values

<table>
<thead>
<tr>
<th></th>
<th>Apical Fl</th>
<th>Subapical Fl</th>
<th>Ratio A/SA Fl</th>
<th>Fluid, μg·min⁻¹·cm⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>190.19 ± 59.41</td>
<td>98.93 ± 36.56</td>
<td>2.14 ± 1.03</td>
<td>−0.29 ± 0.04</td>
</tr>
<tr>
<td>8-BrcAMP</td>
<td>210.72 ± 38.93</td>
<td>43.33 ± 23.33</td>
<td>6.15 ± 3.08</td>
<td>+0.105 ± 0.03</td>
</tr>
<tr>
<td>Nocodazole + 8-BrcAMP</td>
<td>166.05 ± 70.03</td>
<td>131.53 ± 69.78</td>
<td>1.24 ± 0.65</td>
<td>P&lt;0.001 †</td>
</tr>
<tr>
<td>P&lt;0.001 †</td>
<td>166.05 ± 70.03</td>
<td>131.53 ± 69.78</td>
<td>1.24 ± 0.65</td>
<td>P&lt;0.001 †</td>
</tr>
<tr>
<td>8-BrcAMP + primaquine</td>
<td>125.58 ± 89.42</td>
<td>159.54 ± 71.95</td>
<td>0.97 ± 0.92</td>
<td>P&lt;0.001 †</td>
</tr>
<tr>
<td>P&lt;0.001 †</td>
<td>125.58 ± 89.42</td>
<td>159.54 ± 71.95</td>
<td>0.97 ± 0.92</td>
<td>P&lt;0.001 †</td>
</tr>
</tbody>
</table>

Values for fluorescence (Fl) are expressed in pixels as means ± SD determined from a minimum of 30 crypts and 10 random sections. n = 4 animals per condition. A, apical; S, subapical; 8-BrcAMP, 8-bromo-CAMP; CFTR, cystic fibrosis transmembrane conductance regulator. P values: *vs. PBS, †vs. 8-BrcAMP (t-test).
controls (Fig. 3A, lane 1) as shown previously (1, 9). These results confirmed the success of the immunoprecipitation.

Before surface biotinylation experiments, we confirmed that NHS-biotin was effective in surface biotinylation of freshly isolated crypt and villus enterocytes by immunofluorescence labeling with Texas red streptavidin (not shown). Having confirmed that we could detect CFTR in immunoprecipitates from isolated enterocytes, we then proceeded with surface biotinylation/immunoprecipitation studies on VIP-stimulated and control cells. Streptavidin detection of R3194 or IgG immunoprecipitates (Fig. 3B) after biotinylation of VIP-stimulated (Fig. 3B, lane 4) and control (Fig. 3B, lane 5) cells revealed a band consistent with mature CFTR of ~175–183 kDa (Fig. 3B) that was more intense in VIP-treated samples than control. The total material from stimulated and control samples was carefully normalized for total protein, and the amounts of total immunoprecipitated CFTR were almost identical as determined in parallel immunoblots. Densitometric analysis of the CFTR band in independent experiments revealed a 3.8 ± 1.7-fold (P < 0.005) increase in surface biotinylated CFTR in immunoprecipitates after VIP treatment (Fig. 3B). In addition to CFTR, we identified at least two other CFTR antibodiespecific biotinylated bands in both VIP and controls (Fig. 3B), apparent molecular mass of ~162 and 110 kDa, that appeared more prominent in VIP-stimulated immunoprecipitates. Although one of these bands appears at a level that may be confused with immature CFTR, analysis of that band revealed it to be a polypeptide of ~162 kDa, ~14,000 kDa larger than the size of...
immature band B of CFTR in the same preparation (Fig. 3A).

We explored the possibility that the results described above are due to leaking of sulfo-NHS-biotin into the cells and therefore labeling intracellular proteins including CFTR. We tested this hypothesis by performing biotinylation/immunoprecipitation of CFTR in the presence and absence of saponin. Streptavidin detection of R3194 immunoprecipitates after saponin treatment and biotinylation revealed at least one additional band that could not be identified in non-saponintreated immunoprecipitates. These experiments suggest that in our system of isolated enterocytes, the plasma membrane remains intact both in the presence and absence of cAMP stimulation, preventing sulfo-NHS-biotin from entering the cytoplasm unless the cells are permeabilized with saponin.

We also entertained the possibility that cAMP may be inducing a generalized exocytosis of membrane proteins. To test the specificity of CFTR exocytosis, we used the same biotinylation/immunoprecipitation procedure to analyze the apical membrane protein lactase in VIP-stimulated and control immunoprecipitates with the antibody YBB2/61 as shown in immunofluorescence localization (Fig. 1, A and B). Streptavidin detection of biotinylated immunoprecipitates of lactase with the antibody YBB2/61 or IgG is shown in Fig. 3C. Under the same conditions that we used to detect CFTR, blot analysis of biotinylated lactase immunoprecipitates revealed a single antibody-specific protein band of ~150 kDa consistent with lactase in VIP and unstimulated controls (Fig. 3, lanes 8 and 9) but not in IgG immunoprecipitates of VIP-stimulated or control (Fig. 3, lanes 6 and 7; Ref. 29). Densitometric analysis revealed no difference in the intensities of the biotinylated lactase band identified in VIP or control immunoprecipitates. These observations are consistent with our immunofluorescence data indicating no change in the distribution of lactase in intestinal cells on VIP stimulation, and they suggest that cAMP stimulates exocytosis of a specific population of apical membrane proteins that comprises CFTR but not lactase. In addition, this result further supports the notion that the two additional bands in CFTR immunoprecipitates were true antibody-specific immunoprecipitating peptides and not just contaminants.

In fact, we were puzzled by these two additional biotinylated bands that coimmunoprecipitated with CFTR and not with lactase under the same conditions. One possible explanation for this observation is that CFTR exists in a multiprotein complex that includes other membrane proteins, as suggested by others (33, 35). CFTR was recently shown to be physically linked to regulatory complexes containing PKA and PKA anchoring proteins (AKAPs), sodium-hydrogen exchanger regulatory factor (NHERF), and ezrin in a complex insoluble scaffold (35). To test whether our immunoprecipitation conditions preserve some of the protein-protein interactions in that scaffold, we analyzed whether AKAP coimmunoprecipitates with CFTR in our system. Western blot analysis was performed using an antibody to AKAP 149, a PKA type II anchoring protein that is highly expressed in the small intestine. The antibody recognized a specific protein band of 149 kDa in CFTR immunoprecipitates (Fig. 3D, lane 11) but not in IgG controls (Fig. 3D, lane 10), indicating that we were indeed immunoprecipitating a multiprotein complex.

**DISCUSSION**

In the current study, two independent techniques were used to confirm that physiological cAMP stimulation and vesicle traffic regulate the number of CFTR channels on the surface of the rat small intestinal epithelium. This observation resolves the current controversy regarding the role of membrane traffic in regulating CFTR in the intestine. Although cAMP-dependent exocytosis of CFTR to the apical membrane has been demonstrated in villus CHE cells (4), the physiological relevance of that observation remains unknown. Our observation in this work that CFTR is regulated in vivo by cAMP-dependent vesicle traffic and channel insertion in both crypt and villus cells in association with fluid secretion, however, provides strong support for physiological membrane traffic regulation of CFTR and intestinal anion secretion.

Both receptor (VIP)- and non-receptor (8-BrcAMP)-mediated cAMP agonists induced a redistribution of CFTR from the subapical to the apical domain in the jejunum. The lack of change in the distribution of lactase in crypt cells after cAMP agonist stimulation confirmed that the cAMP-dependent redistribution of CFTR was specific, because lactase is not regulated by cAMP-dependent vesicle insertion (23, 26). To determine whether the cAMP-induced redistribution of CFTR from the subapical to the apical domain is regulated by vesicle traffic, we disrupted vesicle traffic in vivo with primaquine and nocodazole. In rat jejunum, primaquine (0.1 mM) was a potent inhibitor of the cAMP-dependent shift of CFTR from the subapical to the apical domain in the crypt and reduced the fluid secretory response of 8-BrcAMP (Table 1). The accumulation of CFTR in the subapical compartment in the crypt in the presence of primaquine is consistent with the observations by others of its effect in inhibiting receptor recycling and in producing an intracellular accumulation of endocytosed receptors, blocking the exit of receptors from the early endosomes and recycling to the plasma membrane (41). The reduction in the fluid secretory response to 8-BrcAMP in vivo in the presence of primaquine (~35%) supports the notion that insertion of new CFTR channels into the membrane contributes importantly to augmenting fluid secretion.

These experiments, however, may allow an alternative albeit nonexclusive interpretation. If CFTR is continuously recycling between the apical domain and the subapical compartment, primaquine may interrupt the cycle and accumulate CFTR in the early endosomal compartment. The fluorescence measurements in confocal images actually point to this scenario. In that
case, we could conclude that the balance between exocy-
tosis and endocytosis of CFTR is almost as important
as channel gating as a regulatory factor. Our observa-
tions in the intestine support the results of recent
studies in oocytes demonstrating that primaquine
drastically reduced cAMP-dependent CFTR chloride
currents and effectively blocked vesicle and protein
traffic (40). Further studies will be necessary to assess
the relative contributions of exocytosis and endocytosis
to the steady-state levels of surface CFTR on cAMP
stimulation.

Although the effects of microtubule disruption on
CFTR distribution and fluid movement were less strik-
ing than those of primaquine, they also suggest a role
of membrane traffic in regulating the number of CFTR
channels on the apical surface and provide support for
the previous observation that cAMP-dependent chlor-
ide secretion in rat intestine is regulated by microtu-
bules (14).

Surface biotinylation, a well-established technique
used to assess the delivery of proteins to the plasma
membrane (30, 31), confirmed cAMP-stimulated CFTR
exocytosis. However, the finding that other unidentified
polypeptides were communoprecipitating with CFTR
and were also upregulated by cAMP, as shown in
Fig. 3, was unexpected. Our first interpretation was
that other proteins possessing at least one ectoplasmic
domain capable of biotinylation may be nonspecific
contaminants of the immunoprecipitation. This, how-
ever, was unlikely for the following reasons: 1) these
other biotinylated proteins were CFTR antibody spe-
cific in the immunoprecipitation and did not appear in
controls immunoprecipitated with nonimmune IgG
(Fig. 3B, lane 3); 2) the conditions for immunoprecipi-
tation were rather stringent, detergents were present
in all washes, and one of the washes was performed in
high salt (0.6 M KCl) conditions; and 3) the sucrase-
isomaltase immunoprecipitation experiments (Fig. 3C)
supported the notion that our immunoprecipitations
were “clean” (in those cases no additional bands were
observed).

Another potential artifact that could explain biotiny-
lation of multiple bands is damage to the plasma mem-
brane during the isolation of enterocytes before bioti-
nylation. This possibility was ruled out by verifying
Trypan blue exclusion in parallel cell suspensions and
by actually permeabilizing some cell suspensions with
saponin. The latter resulted in an increase in the
number of biotinylated bands, indicating that in the
absence of saponin the plasma membrane was tight.

The observation that AKAP communoprecipitates
with CFTR (Fig. 3D) in the intestine supports the
notion that the physiological regulation of CFTR by
PKA involves a physical and functional association
with AKAP as demonstrated recently (19). The com-
munoprecipitation of AKAP with CFTR indicated that
under the conditions of homogenization, detergent sol-
ubilization, and immunoprecipitation used here, the
NHERF-ezrin insoluble scaffold that normally holds
CFTR (35, 36) is at least partially preserved.

At least one other transmembrane protein, Na+/H+
exchanger (NHE), is known to be attached to this
scaffold in addition to CFTR (42). Although the appar-
ent molecular masses of the biotinylated bands that we
found do not correspond to that of NHE-3 (97 kDa; Ref.
5), it is conceivable that other membrane proteins are
also attached to the same scaffold. Furthermore, be-
cause some membrane proteins do not biotinylate and
because we cannot assert that the scaffold is totally
intact, the actual number of membrane proteins at-
tached to the same scaffold of CFTR may be actually
greater than three (CFTR and the 2 unknown proteins
found in this work). On the other hand, the data pre-
sented here do not rule out the possibility that the
additional unidentified proteins may be directly bound
to CFTR and not to a NHERF-type scaffold. Identification
of these proteins in future investigations will be
important before any mechanistic model can be postu-
lated.

In previous work from our laboratory (7) and others
(27), it was found that cAMP stimulates exocytosis of
apical membrane proteins at a post-Golgi step. The
lack of effect of cAMP stimulation on lactase would
suggest, however, that cAMP-dependent exocytosis is
restricted to a subpopulation of apical membrane
proteins. It has been generally accepted that cAMP oper-
ates by increasing membrane traffic (7, 27). If that is
the case, the results in this work would suggest that at
least two subpopulations of subapical vesicles must
exist, one that carries CFTR and other proteins regu-
lated by cAMP-dependent delivery and another cAMP-
independent pathway that facilitates the transport of
proteins such as lactase. Such a scenario raises inter-
esting questions regarding potentially different path-
ways that may regulate the formation and sorting of
these two different subpopulations of vesicles.

Another alternative explanation that by no means
excludes differences in vesicle traffic pathways is that
cAMP may actually increase the number of binding
sites available in the scaffold itself. Because the scaf-
fold contains PKA and AKAP, it is conceivable that its
binding capacity may be modulated by cAMP. In that
scenario, cAMP stimulation would increase the num-
ber of surface molecules for all the membrane proteins
that bind to the same scaffold, disregarding the ves-
icles that transport them to the cell surface. In other
words, retention in the apical domain would be respon-
sible for the increase of surface CFTR and some other
proteins. In both cases, the results of this study suggest
that the increase in the number of CFTR channels on
the surface of intestinal epithelial cells on cAMP stim-
ulation contributes substantially to regulating fluid
secretion and is regulated by vesicle traffic. The obser-
vations in this study should provide the basis for a
critical examination of membrane traffic in the patho-
genesis of CFTR-mediated diseases in the intestine.

We thank Dr. A. Quaroni for the generous gift of antibodies and
Dr. G. McLaughlin and M. Hernandez for technical assistance.

Present address of N. A. Ameen: Pediatric Gastroenterology and
Cell Biology, University of Pittsburgh School of Medicine, Pitts-
burgh, PA 15213.
REFERENCES


37. Takahashi A, Watkins SC, Howard M, Frizzell RA. CFTR-dependent membrane insertion is linked to stimulation of the


