The role of regulated CFTR trafficking in epithelial secretion

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Bertrand, Carol A., and Raymond A. Frizzell. The role of regulated CFTR trafficking in epithelial secretion. Am J Physiol Cell Physiol 285: C1–C18, 2003; 10.1152/ajpcell.00554.2002.—The focus of this review is the regulated trafficking of the cystic fibrosis transmembrane conductance regulator (CFTR) in distal compartments of the protein secretory pathway and the question of how changes in CFTR cellular distribution may impact on the functions of polarized epithelial cells. We summarize data concerning the cellular localization and activity of CFTR and attempt to synthesize often conflicting results from functional studies of regulated endocytosis and exocytosis in CFTR-expressing cells. In some instances, findings that are inconsistent with regulated CFTR trafficking may result from the use of overexpression systems or non-physiological experimental conditions. Nevertheless, judging from data on other transporters, an appropriate cellular context is necessary to support regulated CFTR trafficking, even in epithelial cells. The discovery that disease mutations can influence CFTR trafficking in distal secretory and recycling compartments provides support for the concept that regulated CFTR recycling contributes to normal epithelial function, including the control of apical CFTR channel density and epithelial protein secretion. Finally, we propose molecular mechanisms for regulated CFTR endocytosis and exocytosis that are based on CFTR interactions with other proteins, particularly those whose primary function is membrane trafficking. These models provide testable hypotheses that may lead to elucidation of CFTR trafficking mechanisms and permit their experimental manipulation in polarized epithelial cells.

cystic fibrosis transmembrane conductance regulator; membrane traffic; chloride channel; protein secretion

THE CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR (CFTR) is responsible for the cAMP-activated anion conductance of epithelial cell apical membranes. As a principal apical conductance pathway, the CFTR anion channel is involved in cAMP-dependent Cl and bicarbonate secretion in airway and intestinal epithelia and exocrine glands. It also influences the activity of other conductive pathways, through either linked regulatory interactions or electrochemical coupling that influences the driving forces for ion transport (49, 93). Subsequent to the cloning of the CFTR gene, methods for in situ hybridization and the development of antibodies to CFTR permitted the characterization of its distribution in a variety of tissues (52, 104, 107). The results generally correlated with the presence of cAMP/PKA-stimulated anion secretory processes in various epithelial tissues, identified in earlier studies using physiological methods. Mutations in the gene encoding CFTR produce cystic fibrosis (CF), whereas hyperactivity of this transport pathway underlies secretory diarrheas. CF disease arises from a reduced density and/or activity of CFTR in the apical membranes of secretory epithelial cells, such as those in the airways, exocrine pancreas, salivary glands, reproductive tissues, and intestines. A regulated CFTR conductance pathway is also important for the normal functions of several absorptive cells, including epithelia lining the airway surface and sweat gland ducts (for a general review, see Ref. 82).

The subject of this review is CFTR trafficking in the distal portion of the protein secretory pathway, which lies between the trans-Golgi network and the plasma membrane. Historically, studies of this pathway were motivated by the question of whether it would be possible to explain all of the manifestations of CF disease on the basis of a missing anion conductance at the apical membranes of epithelial cells. Accordingly, functional roles for CFTR other than its primary role as an apical membrane anion conductance pathway have
been proposed to contribute to CF pathology. These include disease-associated changes in regulated protein secretion (65), the chemistry of macromolecular secretions (22, 33, 90), and the properties of the airway microenvironment that lead to bacterial colonization of CF airways (89).

Although the attention of numerous investigators has focused on CFTR trafficking through the distal protein secretory pathway as a possible contributor to the complexity of CF disease, a consensus regarding the importance of intracellular CFTR function and the process of regulated CFTR trafficking within this subcellular domain has yet to emerge. As an integral membrane protein, CFTR will reside at least transiently in all compartments of the protein secretory pathway as it migrates toward the cell surface. Thus one can expect to find CFTR in membranes of the endoplasmic reticulum (ER), Golgi, and endosomal and lysosomal compartments of epithelial cells (Fig. 1).

Although there is no question that CFTR channels function as a regulated conductance pathway when they are resident in the apical membrane, numerous studies (see CFTR LOCALIZATION IN CELLS) have also indicated that a significant quantity of mature (i.e., post-Golgi) CFTR is contained within intracellular compartments, that CFTR within these compartments is functional (9, 78), and that cAMP stimulation can modulate the distribution of CFTR between these compartments and the plasma membrane (see REGULATED CFTR TRAFFIC). Therefore, the issue is not whether apical CF conductance is due to regulation of the gating of membrane-resident CFTR channels or whether it results from the insertion of CFTR channels into the membrane, because both processes occur. We want to know to what extent these forms of CFTR regulation are linked mechanistically and how trafficking of CFTR contributes to the overall conductance response.

This area of CFTR cell biology has been among the most contentious and unresolved subjects in CF research, and it continues to be so. In a relatively recent review of this subject, Bradbury (10) argued that disparate conclusions regarding the regulation of the distribution of CFTR between plasma membrane and intracellular compartments might be reconciled if regulated CFTR trafficking was a property specific to epithelial cells, i.e., studies showing a lack of CFTR trafficking were generally performed in fibroblasts. Nevertheless, four years after the Bradbury review, the literature has not been clarified. Accordingly, the task of bringing some synthesis to this conflicting data set is not an easy one. Yet, we feel that it is possible to do so and that the attempt should add to our understanding of the control of plasma membrane CFTR density, a critical issue in CF disease.

CFTR LOCALIZATION IN CELLS

This review is primarily concerned with the steady-state distribution of CFTR between plasma membrane and intracellular compartments and with the effect of cAMP/PKA-mediated stimulation on its distribution. The methods used to determine cellular CFTR localization have relied primarily on its detection by antibodies in cultured cells or tissue sections. Other studies have employed functional assays subsequent to cell fractionation, and others have relied on the expression of epitope-tagged or green fluorescent protein (GFP)-CFTR fusion proteins as a method of locating exogenously expressed CFTR. Cellular targets for these studies have included both polarized and non-polar cells. Detection of a reliable CFTR signal has often required overexpression of this normally low-abundance protein in heterologous systems.

Location of mature CFTR (band C). From a biochemical standpoint, the prevailing view is that core-glycosylated, immature (band B) CFTR is localized in the ER, whereas complex glycosylated, mature (band C) CFTR is present primarily in compartments distal to the Golgi, including the apical membrane. In contrast with many heterologous expression systems, relatively
little band B is observed by immunoblot in most epithelial cells expressing CFTR endogenously at steady state. For example, the ratio of band C to B in Calu-3 airway cells is ~10 (100). This indicates that most of the CFTR expressed endogenously in epithelial cells is located in distal compartments of the protein secretory pathway. The first reported studies of CFTR localization, performed in COS-7 cells transiently expressing CFTR, showed a weak, diffuse plasma membrane staining pattern. Most of the signal was intracellular, probably due to protein overexpression (23). In polarized epithelial cells that express CFTR endogenously (T84, HT29, Caco-2, and airway epithelia), this signal was located unambiguously at the apical membrane domain (27, 28). No staining was detected in the basolateral membrane, and little perinuclear staining of CFTR was observed in epithelial monolayers, implying that little CFTR is present in the ER at steady state (consistent with the low levels of band B). The studies of Denning et al. (27) highlighted the importance of localizing CFTR within epithelial cells that express the protein endogenously. In the same study, a mostly intracellular location of exogenously expressed CFTR was found in non-polar HeLa and 3T3 cells.

Biochemical methods. The central question concerning the issue of regulated CFTR trafficking is whether the protein that contributes to the plasma membrane anion conductance is present strictly in the apical plasma membranes of epithelial cells or whether it is present also in a subapical, post-Golgi compartment from which it is mobilized to the cell surface during stimulation. A related issue is whether CFTR is stable in the plasma membrane or undergoes recycling to intracellular compartments. In this regard, a rapid rate of CFTR endocytosis from the cell surface has been observed in both epithelial and nonepithelial systems (13, 61, 85). These studies used biotinylation procedures to monitor the rate of CFTR internalization from the cell surface. In T84 cells, it was estimated that ~50% of cell surface CFTR is retrieved within a few minutes (85); in Chinese hamster ovary (CHO) cells the internalization rate was ~5% per minute (61). These rates of endocytosis are not different from those of constitutively recycling receptors, such as those for transferrin or LDL. These findings, together with the estimated 24- to 48-h half-life of mature, plasma membrane CFTR (7, 41), indicate that virtually all of the CFTR that is internalized by endocytosis must be returned to the plasma membrane by a recycling/exocytic pathway. The presence of the rapid CFTR recycling pathway is a minimal requirement for a regulated insertion-retrieval model that controls cellular CFTR distribution. Thus the question should not be whether CFTR undergoes trafficking at the plasma membrane but whether this process contributes to a change in the distribution of CFTR between plasma membrane and intracellular compartments in response to cAMP/PKA regulation.

In addition to these kinetic considerations, the presence of CFTR in endosomes under steady-state conditions has been established with the use of a combination of biochemical techniques and functional assays that rely on CFTR’s anion channel activity. Bradbury et al. (12) resolved CFTR in the early stages of endosomal retrieval within clathrin-coated vesicles from epithelial cells. CFTR was demonstrated to be present in this compartment by immunoblot. After removal of the clathrin lattice by uncoatase and fusion of the resulting membranes with planar lipid bilayers, currents with biophysical and regulatory properties characteristic of CFTR were observed. Because clathrin removal was a necessary prerequisite for reconstitution, CFTR was definitely a component of this endocytic compartment. Additional studies have demonstrated that the rapid endocytosis of CFTR from the cell surface depends on clathrin-mediated pathways and that CFTR is not significantly internalized into caveolae (11, 85). Several transport and/or channel proteins have been found to internalize via clathrin-dependent endocytosis, including aquaporin-2 (AQP-2) (101), Na/H exchanger 3 (NHE3) (24), renal outer medulla K channels (ROMK) (114), and epithelial Na channels (ENaC) (96). Recent studies of the mechanism of CFTR endocytosis (see Functional studies of endocytosis) indicate a direct physical interaction of CFTR with the plasma membrane adapter complex, making CFTR a specific target of this endocytic machinery.

Other methods for demonstrating the presence of CFTR in endosomes have relied on the ability of CFTR to provide a pathway for anion transport that parallels the vacuolar H-ATPase. Lukacs et al. (60) allowed CHO cells expressing CFTR to take up the pH-sensitive dye FITC-dextran into endosomes, where the rate of dissipation of a pH gradient across endosomal membranes was stimulated by addition of a proton ionophore. In these intact cells, pH gradient dissipation was limited by the counterion. Activation of cAMP production elicited a twofold increase in the rate of pH change in cells expressing CFTR but not in parental or mock-transfected cells. The results were duplicated in microsomes isolated from these cells. Biwersi and Verkman (9) used a similar method for monitoring endosomal pH in CFTR-transfected 3T3 cells and in T84 cells endogenously expressing CFTR. Treatment of the cells with forskolin before microsome isolation produced an approximately twofold increase in the rate of subsequently measured pH gradient dissipation but had no effect on microsomes isolated from nontransfected or ΔF508 CFTR-expressing 3T3 cells. These data are also consistent with the presence of functional CFTR in endosomes that, in principle, could contribute to CFTR recycling.

Morphological methods. Using immunofluorescence and confocal microscopy for detection of cellular CFTR, investigators have generated data that arrive at somewhat divergent conclusions with respect to the importance of regulated CFTR trafficking. On one side of this issue is the study of Lehrich et al. (55) in shark rectal gland, a tissue that expresses CFTR at high levels endogenously. These investigators used quantitative confocal microscopy to show that CFTR immunofluo-
Fluorescence extended from the apical membrane into subapical, supernuclear regions of the cell and that during stimulation with secretagogues, the overall depth of this CFTR signal decreased by ~50%. Similar conclusions were reached when the histogram of CFTR fluorescence intensity as a function of distance from the apical membrane was quantitated. These cAMP agonist effects on CFTR distribution, imposed in perfused, intact rectal glands before fixation, were reversible. Whether this shift in fluorescence signal represents insertion of vesicles containing CFTR into the apical membrane remains unknown; however, the findings are consistent with the acute hormonal regulation of CFTR trafficking in an intact epithelial tissue.

Similarly, Ameen et al. (1, 2) used immunofluorescence microscopy to quantify the cellular location of CFTR in rat small intestine and the influence of cAMP-dependent agonists on its distribution. Vasoactive intestinal peptide (VIP) elicited a bicarbonate-rich fluid secretory response in isolated perfused intestinal loops and elicited, within the same time frame, a redistribution of CFTR to the apical membrane domain. CFTR association with the apical membrane was quantified by colocalization of F-actin as a brush border marker. In duodenal villus cells, VIP elicited a reversible, threefold increase in brush border associated CFTR, and it redistributed CFTR within the cell apex to the brush border within 30 min. These studies were confirmed by immunoelectron microscopic methods (see Immunoelectron microscopy). The results provide support for a physiological role for cAMP-induced CFTR-containing membrane traffic in the regulation of the apical anion conductance in a native, endogenously expressing epithelium.

Other investigators have reached different conclusions with respect to regulated CFTR trafficking. Moyer et al. (70) expressed a GFP-CFTR fusion protein in MDCK type I epithelial cells and used quantitative confocal fluorescence microscopy and cell surface biotinylation to determine the effect of cAMP stimulation on apical membrane CFTR localization. Their findings showed that cAMP did not stimulate detectable GFP-CFTR translocation from intracellular compartments to the apical membrane, along an apical-to-basal GFP fluorescence gradient that extended throughout the cell. In these cells, cAMP did not regulate GFP-CFTR endocytosis, determined by apical biotinylation and subsequent immunoprecipitation. Likewise, disruption of microtubules with colchicine did not affect cAMP-stimulation of Cl secretion or the expression of GFP-CFTR in the apical membrane. Thus the authors concluded that cAMP stimulates CFTR-mediated Cl secretion in MDCK type I cells by activating only the channels that are resident in the apical membrane. A regulated CFTR trafficking event may be lacking in this cell type; however, it is also reasonable to ask whether the exogenous expression of CFTR driven by the CMV promoter leads to saturation of regulated trafficking pathways (see Functional studies of exocytosis).

A related study (58) involving measurements of cellular CFTR immunolocalization in endogenously expressing Calu-3 epithelia by fluorescence confocal microscopy detected no change in apical CFTR staining upon cAMP stimulation. Surface biotinylation experiments reported in the same study (58) also showed no stimulation-dependent increase in apical membrane CFTR. Two biotinylated proteins were detected by CFTR antibody precipitation following biotinylation; they were of equal intensity and 200–220 kDa in molecular size. Yet, others have demonstrated by immunoblot that CFTR in Calu-3 cells shows the typical pattern of a predominant band C of 150–180 kDa and relatively little band B; the latter is presumably intracellular (c.f., Refs. 18 and 100). Thus it is not clear that the biotin-labeled proteins identified in these experiments were CFTR.

It is possible that differences in the conclusions drawn from fluorescence measurements in native and cultured epithelial cells stem from variations in the distribution of CFTR in subapical compartments under nonstimulated conditions. In the studies of native secretory tissues (shark rectal gland and rat intestine), the apical fluorescence signal extended into the cell sufficiently to detect a clear change in its distribution with stimulation. In MDCK cells expressing GFP-CFTR (70), there was an even deeper distribution of expressed protein, but this may reflect its deposition in nonphysiological compartments, as described earlier in this article. Given quantitative limitations of GFP visualization, ~7 molecules/pixel (79), it may be necessary to express CFTR at nonphysiological levels to obtain a CFTR signal. In Calu-3 cells (58), the CFTR signal was much more densely localized at the apical membrane domain. Nevertheless, it is reasonable to ask whether a band of apical fluorescence detected in epithelial cross sections can resolve apical membrane CFTR from that present in a nearby subapical recycling pool. Consideration of the point-spread function of fluorescence intensity variation with distance suggests that structures separated by >0.2 μm will not be resolved using light microscopy. Thus immunofluorescence, even coupled with confocal microscopy, may lack the ability to resolve fluorescence signals from membrane vesicles having a diameter of 0.1 μm or less, which are components of the CFTR recycling pathway. This vesicle size expectation is based on neurosecretory and other regulated trafficking vesicles (see APPENDIX). For example, intracellular GLUT-4 staining by thin-section immunoelectron microscopy was localized to vesiculotubular structures 50–70 nm in diameter (62). Thus it is possible that different conclusions at the light microscopic level may emerge from different cellular distributions of intracellular CFTR trafficking compartments and their constituents (see Consequences of regulated CFTR trafficking).

Immunoelectron microscopy. The results of several studies using immunoelectron microscopic methods are consistent with a subapical population of post-Golgi CFTR (c.f. Refs. 26, 42, 86, and 107). Puschelle et al. (86) used immunogold labeling to localize CFTR in...
vesicles present beneath the plasma membrane of human airway epithelial cells. They also observed vesicles in the process of fusion or retrieval from the plasma membrane. Immunogold labeling of CFTR-expressing L cells and SF9 cells also demonstrated the presence of CFTR beneath the plasma membrane as well as in the rough ER (26). Studies of the striated duct of rat submandibular gland cells by Webster et al. (107) showed immunogold labeling of CFTR along the apical membrane as well as in numerous subapical membrane vesicles. Their estimates of labeling density suggested that more CFTR is present in subapical intracellular compartments than in the plasma membrane under “resting” conditions. Some of the CFTR-labeled vesicles also stained with antibodies against the transferrin receptor and rab4, two endosomal markers, indicating that these vesicles are likely part of an endosomal/recycling pathway for CFTR.

The fluorescence measurements of Ameen et al. (1), performed in rat small intestine, were confirmed by immunoelectron microscopy. CFTR was identified in subapical vesicles in several intestinal cell types, including cells from the crypt, Brunner glands, and a subpopulation of villar cells where CFTR expression predominated (so-called CFTR high expressors, or CHE cells). cAMP stimulation elicited a two- to threefold increase in CFTR labeling of the apical microvilli of CHE cells in response to cholera toxin, providing evidence for regulated insertion of CFTR into the apical membranes of a native epithelium.

The study of Howard et al. (42) examined apical membrane domain CFTR localization as a function of CFTR expression level. In an earlier study (43), these investigators showed that CFTR located in the plasma membrane could be discriminated from intracellular CFTR by using nonpermeabilized MDCK cells expressing an extracellular epitope-tagged CFTR (where the flag, M2 epitope, was added to the 4th extracellular loop of CFTR, after amino acid 901). They found that cAMP stimulation increased the cell surface signal of CFTR two- to threefold in the steady state. In the more recent studies, they induced CFTR expression by using a recombinant adenovirus that encoded M2-901/CFTR. Virally expressed, FLAG-tagged CFTR was functional and could be detected on the apical surface of forskolin-stimulated, polarized MDCK (type II) cells by immunofluorescence performed on nonpermeabilized epithelial monolayers. At a low multiplicity of infection (MOI) (i.e., lower CFTR expression level), forskolin stimulated the insertion of M2-901/CFTR into the apical membrane, but at higher MOI and M2-901/CFTR expression levels, no agonist-dependent increase in surface expression could be detected. Immunoelectron microscopy confirmed the redistribution of CFTR to the apical membrane upon forskolin stimulation at the lower CFTR expression levels and demonstrated that the apically inserted CFTR originated from a population of subapical vesicles. Results similar to these have been obtained with the use of immunoelectron microscopy in Calu-3 cells endogenously expressing CFTR, where a two- to threefold increase in apical CFTR was observed in response to acute forskolin stimulation (Hug MJ and Frizzell RA, unpublished observation).

The observations of Howard et al. (42) may reconcile at least some of the prior conflicting reports regarding the effect of cAMP stimulation on CFTR trafficking. They show that a high level of CFTR expression, obtained by using strong promoters, for example (70), can saturate pathways for regulated CFTR trafficking, even in epithelial systems. Also in oocytes, the CFTR trafficking signal (cAMP-induced increase in membrane capacitance) saturated as the CFTR expression level was elevated by injection of more RNA (102). Inappropriate trafficking of AQP-2, in relation to the actions of dominant-negative AQP-2 mutants, has also been observed at high protein expression levels (51). Later in this article, we discuss candidate protein interactions that may mediate regulated CFTR trafficking at the apical membrane. If cells express different levels of the relevant traffic regulatory proteins, then it is likely that the contribution of regulated CFTR trafficking to the total anion conductance (as opposed to regulated gating of membrane resident CFTR) will vary in different epithelial or nonepithelial systems.

**REGULATED CFTR TRAFFIC**

**Functional studies of endocytosis.** The concept that the CF gene product may play a role in regulated membrane trafficking is not new. The implication, from defective mucin and glycoprotein secretion found in CF airways and other organs (64, 67), was that the CF gene product, once identified, would be involved somehow in the control of protein secretory processes. Because the secretion of mucin in several CFTR-expressing tissues is stimulated by cAMP (65), and because cAMP/PKA regulatory pathways also control CFTR gating, it was reasonable to anticipate that protein secretion by exocytosis may lead also to an increase in the numbers of CFTR channels in the plasma membrane (Fig. 1).

The first studies that implicated CFTR function in regulated endocytosis were performed in colonic and pancreatic epithelial cell lines, with the latter transduced to express CFTR using retrovirus vectors. Bradbury et al. (13, 14) measured detergent-sensitive horseradish peroxidase and FITC-dextran uptake to monitor fluid phase endocytosis in T84 and CFPAC-1 cells. They found that cAMP significantly inhibited fluid-phase endocytosis, and in pancreatic cells, this depended on the expression of wild-type (wt) CFTR. No cAMP inhibition of endocytic activity was observed in parental CFPAC-1 cells or those transduced by empty vector. A similar ~60% inhibition of endocytosis was observed during cAMP stimulation of endogenous CFTR-expressing T84 (13) and 9HTEo- cells (94). In T84 cells, PKC stimulation did not affect either basal endocytosis or the inhibitory effect elicited by cAMP stimulation.

Methods employing biotinylation of the cell surface have also demonstrated that CFTR internalization is inhibited by cAMP/PKA. Prince et al. (85) biotinylated
Recent findings have been able to establish certain structural features of CFTR that govern its rapid removal from the cell surface. Studies by Prince et al. (84) and Weixel and Bradbury (109) have demonstrated that the internalization of CFTR via clathrin-dependent mechanisms depends on the presence of a YXXΨ motif (where Ψ is a bulky hydrophobic residue) at the CFTR COOH terminus. Similar tyrosine-based endocytic motifs are present on other proteins that are rapidly retrieved from the cell surface (see Ref. 80 for review). In vivo cross-linking and in vitro pull-down assays showed that CFTR binds to the plasma membrane endocytic adaptor complex AP-2. The CFTR COOH terminus was able to bind AP-2 but did not bind the Golgi-specific adaptor complex AP-1. Mutation of the tyrosine residue at position 1424 of CFTR significantly reduced the ability of an isolated CFTR COOH terminus to bind AP-2. The YD3I sequence of CFTR interacts specifically with the μ2 subunit of AP-2 (108), a site implicated in adapter binding to similar endocytic motifs (74). Protein binding studies showed that the COOH terminus binds selectively to this adapter subunit. Cells expressing either a dominant negative μ2 or a CFTR lacking the tyrosine-based internalization motif at the COOH terminus (Y1424A) fail to endocytose CFTR efficiently. These studies indicate that the interaction of CFTR’s COOH terminus with AP-2 guides it into the clathrin-mediated endocytic pathway, as observed for many cell surface receptors. Although these studies provide compelling evidence for specific protein interactions that mediate CFTR retrieval at the cell surface, they do not yet provide an indication of how this process may be regulated by cAMP/PKA. We have speculated on possible mechanisms for regulated CFTR endocytosis (see MECHANISMS OF REGULATED CFTR TRAFFICKING: PERPETRATOR OR BYSTANDER? and Fig. 2).

 Functional studies of exocytosis. The studies of Bradbury et al. (13) on CFTR-dependent endocytosis also examined CFTR-dependent exocytic recycling to the cell surface. To monitor exocytosis, the plasma membranes were pulse-labeled with a biontylated lectin (WGA), and after its removal from the bath, the cells were allowed to take up lectin into intracellular compartments. Remaining cell surface WGA was blocked with unlabeled avidin, and at various times the recycling of internalized WGA to the cell surface was assayed using Texas Red-labeled avidin. In cells expressing wt CFTR, recycling and exocytosis of internalized marker was increased approximately threefold by forskolin, whereas cAMP stimulation had no effect on WGA recycling in either the parental CFPAC-1 cell line or in empty vector controls. Importantly, these studies showed that cAMP-responsive recycling/exocytosis required CFTR expression.

 Similar findings were obtained in human airway cell lines (94) where recycling/exocytosis was monitored as the release of previously internalized FITC-dextran (a fluid-phase marker of endocytosis) from human airway cells. Treatment of cells expressing wt CFTR with a cAMP analog elicited increased release of FITC-dex-
tran, and this was accompanied by increases in membrane capacitance monitored during whole cell patch-clamp measurements. Again, trafficking was CFTR dependent; cAMP had no effect on membrane capacitance or recycling/exocytosis in airway cells derived from a CF patient. The investigators concluded that cAMP stimulates exocytosis and the CFTR Cl conductance of normal but not CF cells and that it does so by stimulating the delivery of CFTR channels from an intracellular pool to the plasma membrane.

Much of the effort to determine whether cAMP stimulates CFTR-dependent membrane trafficking has employed measurements of membrane capacitance in cells that express CFTR endogenously or cells in which CFTR expression was induced. These studies are summarized in Table 1, which tabulates the conditions, methods, and experimental results of these studies. As discussed below, the diversity of methods and conditions used may contribute to their variable outcome.

Previous work in nonepithelial cell lines (e.g., CHO cells) failed to show CFTR- and cAMP-dependent membrane trafficking (29, 47), again suggesting that the machinery for regulated CFTR recycling may be exercised only in epithelial cells. However, studies of Xenopus oocytes generally have shown a good correlation between cAMP stimulation of CFTR currents and increases in membrane capacitance (81, 102, 105). In the studies of Takahashi et al. (102), the apparent changes in cell surface area with stimulation, determined from membrane capacitance measurements, were confirmed by using blinded measurements of membrane morphometry in stimulated and nonstimulated cells. In addition, recruitment of CFTR to the cell surface was demonstrated by using epitope-tagged M2-901/CFTR labeling of nonpermeabilized cells (81). Together, these findings are consistent with the exocytic insertion of CFTR into the oocyte plasma membrane during cAMP stimulation. The cell surface immunofluorescence studies of Peters et al. (81) suggested a low level of CFTR expression in the plasma membrane of nonstimulated oocytes (i.e., fluorescence levels were near background in the absence of stimulation). These data suggested that recruitment of CFTR to the cell surface is a significant component of CFTR current stimulation in this system. However, this conclusion assumes that the flag-CFTR labeling approach can detect low levels of cell surface CFTR. Although technically difficult, it would be optimal to determine a dose-response relation between cAMP-dependent cell surface CFTR labeling and some independent parameter such as the corresponding change in membrane capacitance. It is possible, for example, that some threshold level of CFTR residing in the plasma membrane cannot be detected by this method, underestimating levels of CFTR at the cell surface under basal conditions. However, this concern does not compromise the data, obtained from three different methods, consistent with stimulation-dependent recruitment of CFTR to the surface of these cells.

The electrophysiological aspects of these studies were confirmed by the work of Weber et al. (105), who used impedance analysis to assess membrane conductance and capacitance and correlated the changes in these parameters induced by cAMP. In agreement with

Table 1. Measurement of stimulated capacitance in CFTR-expressing tissue

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Method</th>
<th>ΔC_{m,stim}/C_{m,basal}</th>
<th>ΔC_{m}</th>
<th>ΔG_{m}</th>
<th>Stimulation Agonists</th>
<th>Temperature, °C</th>
<th>[Ca]_{i} adjustments, mM</th>
<th>Transfected</th>
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<tr>
<td>16HBE14ae; HT29-19A</td>
<td>1</td>
<td>0; 0*</td>
<td>0.8 nS; 0.6 nS</td>
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<td>RT</td>
<td>5 EGTA</td>
<td>1 MgATP</td>
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<tr>
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<td>0.023/10 pF</td>
<td>375 pA</td>
<td>400 μM 8-CPT-cAMP</td>
<td>1 mM IBMX</td>
<td>0.5 CaCl_{2}</td>
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<td></td>
</tr>
<tr>
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<td>3</td>
<td>14/190 nF</td>
<td>557 nA</td>
<td>10 μM FSK</td>
<td>23</td>
<td>10 EGTA</td>
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<tr>
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<td>1 mM IBMX</td>
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<td>(50 ng)</td>
<td>N</td>
<td>46</td>
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<tr>
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<td>1330 nA</td>
<td>100 μM 8-Br-cAMP</td>
<td>23</td>
<td>0.5 EGTA</td>
<td>1 NaATP</td>
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<td>105</td>
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<td>5000 nA</td>
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<td>37</td>
<td>0.1 GTP</td>
<td>N</td>
<td>(50 ng)</td>
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<td>0.5 mM db-cAMP</td>
<td>1 mM IBMX</td>
<td>None</td>
<td>(1 ng)</td>
<td>N</td>
<td>81</td>
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<tr>
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<td>0.5 mM adenosine</td>
<td>37</td>
<td>0.73 Ca-glucogon</td>
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<td>0.1 μM FSK</td>
<td>37</td>
<td>1 EGTA</td>
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<td>1 EGTA</td>
<td>0.1 Ca^{2+}</td>
<td>N</td>
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</tbody>
</table>

The following capacitance measurement methods were used: I) whole cell patch clamp (WCPC), sine + square; 2) WCPC, 4-frequency sine wave; 3) 2-electrode voltage clamp (TEVC), single sine wave; 4) TEVC, step; 5) TEVC, 5 sine wave; 6) WCPC, 2-frequency sine wave; and 7) WCPC. *Basal membrane capacitance (ΔC_{m,basal}) values were not given. ΔC_{m} change in membrane capacitance; ΔG_{m} change in membrane current; ΔΔC_{m} change in membrane capacitance; FSK, forskolin; 8-CPT-cAMP, 8-[4-chlorophenylthio]-cAMP; IBMX, isobutylmethylxanthine; db-cAMP, dibutyryl-cAMP; RT, room temperature.
prior reports (81, 102), cAMP stimulation of CFTR-expressing oocytes evoked significant increases in both current and capacitance that were not observed in noninjected or ΔF508-expressing cells. Injection of Rp-cAMP (a specific cAMP antagonist) abolished the effects of the cAMP stimulation on both current and capacitance. Interestingly, the less specific PKA inhibitors, KT5720 and H8, after prolonged (overnight) exposure, primarily blocked the capacitance increases evoked by cAMP while having less effect on the corresponding current activation. However, these reagents have been shown to alter cytoskeletal properties. For example, KT5720 induces microtubule rearrangement by inhibition of mitogen-activated protein kinase (76), and H8 has been shown to alter cytoskeletal protein phosphorylation through inhibition of PKC (63). It would be interesting to explore these actions further, because both of these less selective kinase inhibitors produce cytoskeletal disruption, which may account for their inhibitory effects on membrane trafficking. Indeed, Weber et al. (106) showed that microtubule disruption markedly inhibits current and capacitance increases evoked by cAMP in CFTR-expressing oocytes, and similar conclusions have been reached in CFTR-expressing epithelial cells (103).

Although two groups have independently demonstrated cAMP-dependent changes in membrane capacitance and other parameters indicative of regulated CFTR trafficking in Xenopus oocytes, using different analytic methods Liu et al. (57) arrived at a different conclusion. They employed a CFTR construct containing a cysteine substitution in the postulated CFTR conduction pathway to arrive at the conclusion that essentially all conductance activation by cAMP in oocytes was due to CFTR channels that are already present in the plasma membrane. This approach relied on changes in conductance induced by treatment of this CFTR mutant with cysteine-reactive methanethiosulfonate (MTS) reagents, whose effects have been attributed to altered charge shielding properties in the CFTR conduction pathway. When oocytes were treated briefly with 2-(trimethylammonium)ethyl methanethiosulfonate (MTSET; a positively charged modifier) for as little as 20 s before cAMP activation, the activated channels behaved as if their properties were already modified. Because MTSET has been demonstrated in other systems to be cell impermeant, the data suggest that the modified channels must have already been present at the cell surface. These findings are consistent with a lack of regulated CFTR redistribution in oocytes, but they need not negate the results of prior studies for two reasons. First, this study used CFTR expression levels (based on conductance) that are 5–10 times higher than the prior work in which capacitance measurements were made. As discussed earlier in this article, overexpression of CFTR can saturate pathways available for regulated trafficking. Consistent with this idea, Takahashi et al. (102) observed that increasing CFTR expression levels increased CFTR currents, but the corresponding capacitance changes plateaued, as if regulated trafficking pathways had saturated. Constitutive delivery of CFTR to the plasma membrane at high expression levels would limit one’s ability to detect a population of trafficking channels. Second, Liu et al. (57) did not measure membrane capacitance, cell surface CFTR, or any other measure of CFTR trafficking. Nevertheless, there is not a facile explanation for differences between these carefully performed studies and those cited above. It should be determined, using MTS CFTR modification together with capacitance measurements or surface CFTR labeling, whether high CFTR expression levels would account for these results.

Two recent studies, performed in mammalian epithelial cells that endogenously express CFTR, have reported negative findings with respect to changes in membrane capacitance during cAMP stimulation. As an independent assay, both studies also employed FM 1-43 dye labeling in an attempt to monitor membrane addition to the plasma membrane that would result from exocytosis stimulated by cAMP. In the studies of Chen et al. (21), membrane capacitance was measured in Calu-3 airway cells by imposing alternating sinusoidal and square voltage waveforms and monitoring the resulting currents to assess membrane capacitance and conductance. The calculated capacitance changes induced by cAMP were insignificant. In addition, they found no increase in steady-state plasma membrane labeling by FM 1-43 when the cells were stimulated by cAMP during fluorescence measurements. Enhanced dye labeling (fluorescence intensity) would be expected if additional membrane were exposed at the cell surface as a result of exocytosis. Thus the authors concluded that cAMP does not stimulate CFTR currents by increasing CFTR trafficking to the plasma membrane but stimulates only membrane-resident CFTR channels in Calu-3 cells.

In a similar study, Chang et al. (20) used sinusoidal voltage-current phase-lag analysis to estimate cell capacitance and fluorescence measurements of FM 1-43 membrane dye labeling in colonic HT29-C1.19A and airway 14HBEo- cells, both of which express CFTR endogenously. The outcome in the airway cell line was similar to that in study by Chen et al. (21), i.e., no detectable cAMP-induced changes in membrane capacitance or FM dye labeling. In HT29 cells, increases in membrane capacitance with cAMP stimulation occurred only when endocytosis was blocked with the use of a dynamin antibody. As the authors indicated, the absence of a change without dynamin inhibition may result from parallel increases in both exocytosis and endocytosis during stimulation, i.e., no net increase in membrane area.

The studies of Chen et al. (21) and Chang et al. (20), despite the sophisticated methodologies employed, have two significant shortcomings that preclude definitive conclusions regarding regulated CFTR trafficking in these systems. First, both studies were performed at room temperature, a condition that is known to impair regulated membrane traffic in a variety of systems. For example, the release of amylase from pancreatic exocrine cells is decreased 70% at 25°C compared with
normal temperature. The release of insulin from rat pancreatic islets is markedly inhibited at 25°C, with a 90% reduction in the membrane capacitance response to stimulation (the Q10 was 11.6) (75, 87). In addition, cAMP-regulated AQP-2 recycling in renal cells is blocked at 20°C (38). Indeed, recent data collected in this laboratory (45) indicate that studies performed at 37°C in Calu-3 cells using either sinusoidal waveform analysis or current transient analysis show a ~1 pF increase in membrane capacitance (basal membrane capacitance averages 20 pF) in response to cAMP stimulation that parallels the increase in membrane conductance. When identical recordings were made at room temperature with 5 or 10 mM EGTA in the pipette solution (conditions of the Chen and Chang studies), cAMP elicited no significant increase in membrane capacitance. It is interesting that early recordings of capacitance changes in airway cells (94) showed cAMP-dependent increases at 23°C (Table 1); however, these experiments employed low EGTA concentrations (Ca buffering at normal resting levels) in the recording pipette. Thus the recording conditions have not been consistent.

The assays of FM dye labeling (also performed at room temperature) were conducted by using a protocol that is insensitive to small increases in membrane area. The cells were labeled to steady-state intensity with FM dye, and their fluorescence intensity was monitored during cAMP stimulation. However, if the increase in plasma membrane area induced by cAMP is only ~3% over the basal membrane area (suggested from capacitance measurements at 37°C; Ref. 46), this signal would not be detected during steady-state dye labeling. Rather, subsequent to stimulation, it is necessary to wash out dye and agonist from the membrane to minimize plasma membrane (background) staining. This permits visualization of the dye retrieved by subsequent endocytosis of labeled plasma membrane, assuming that the cell recovers to its original area following reversal of stimulation. As recent studies in this laboratory performed at 37°C have demonstrated, measurements performed in this manner indicate that FM 1–43 labeling is increased by cAMP stimulation in Calu-3 (45). The protocol used in the studies of Chen et al. (21) and Chang et al. (20) is similar to methods employed to monitor secretory granule release in systems that incorporate large amounts of membrane into the cell surface during stimulation (e.g., pancreatic acinar cells), generally in response to stimulation by a cellular calcium rise (36). In these systems, the addition of membrane (and increase in FM dye signal) at steady state is easily demonstrated, as it is in HT29–16E cells during stimulation of mucin secretion by Ca-dependent agonists (Bertrand CA, Laboisse C, Hopfer U, Bridges RJ, and Frizzell RA, unpublished observations).

Summary of functional data. Estimates of the numbers of CFTR channels in intracellular vesicles obtained by using data derived from Calu-3 cell and oocyte measurements are provided in the APPENDIX. Important assumptions in these calculations include the single-channel parameters and assumed vesicle radius (100 nm). The calculations are based on reported values of membrane current and capacitance changes from patch-clamp studies of cAMP-stimulated cells (46, 102). These calculations and assumptions yield estimates of 1–10 channels per vesicle in these systems; they are not likely to provide more than one order of magnitude estimate of channel density. The mean oocyte current and capacitance changes reported by Weber et al. (105) were both approximately fourfold smaller than those in the Takahashi study (102) so that the calculated outcome would be unaffected. For comparison, Wright et al. (110) estimated the number of Na-dependent glucose transporters per oocyte vesicle at 10–20, based on freeze-fracture scanning electron microscopy. The size of the imaged oocyte vesicles was 100–120 nm. The value of 1–10 CFTRs per vesicle is instructive when considering whether one should be able to visualize vesicular GFP-CFTR by light microscopic methods. As discussed earlier in this article, the sensitivity of GFP detection (79), together with these considerations, suggests that CFTR density in a single transport vesicle is near the limit of detection.

Relation to other systems. It is worth emphasizing that the source of CFTR current generation, whether membrane-resident or inserted channels, need not be a black-and-white issue. In most systems, plasma membrane CFTR currents are likely to arise from both stimulation of membrane-resident channels and acutely trafficked CFTR channels, and the proportions of these may differ among cell types or experimental conditions (see CONSEQUENCES OF REGULATED CFTR TRAFFICKING). Data from studies of the regulated trafficking of GLUT-4 or AQP-2 indicate that they undergo recycling even under basal conditions (15, 17). During stimulation, the rate coefficients of the steps involved in recycling these proteins are altered, changing their distribution to increase the number of transporters and/or channels in the plasma membrane. Presumably, regulated CFTR recycling behaves similarly. As appears to be true of CFTR, the cellular background that can support regulated GLUT-4 or AQP-2 trafficking is very important. Thus the expression of GLUT-4 in nonmuscle or nonadipose derived cells eliminates the insulin-regulated redistribution of GLUT-4 (40, 44). Similarly, for AQP-2, its endogenous expression in vas deferens results in a constitutive apical membrane localization; the channel is not found in a cAMP/PKA-regulated internal compartment as it is in renal epithelia, and there is no effect of stimulation on apical channel density (99). A requirement for the proper cellular context is likely to also characterize the regulated trafficking of CFTR. The AQP-2 data suggest that such differences can exist not only between epithelial and nonepithelial cells but also between epithelial systems. This conclusion underscores the need to determine which cellular components provide the proper context.
MECHANISMS OF REGULATED CFTR TRAFFICKING: PERPETRATOR OR BYSTANDER?

The studies discussed above do not provide a mechanistic basis for the CFTR-dependent proteins and regulatory events involved in the control of CFTR distribution at the apical membrane. Positive reports of regulated CFTR trafficking (increased exocytosis, decreased endocytosis, and increased endosome fusion) have indicated that these events require both the expression of CFTR and its stimulation by cAMP/PKA. In addition, with the exception of Xenopus oocytes, these processes appear to be epithelial cell specific. In view of the relatively recent discovery of the extensive interactions of CFTR with other proteins, speculation regarding possible molecular mechanisms governing at least some of these trafficking reactions seems warranted. Although firm conclusions cannot be drawn at this stage, the principal value of the models we propose is to suggest testable hypotheses that may lead to an understanding of mechanism. Moreover, it is our contention that the importance of regulated CFTR trafficking for anion secretion will continue to generate controversy until its mechanistic basis is understood to the extent that it can be manipulated experimentally (e.g., in the same manner that identification of adapter protein interactions with CFTR has provided a mechanistic basis for CFTR endocytosis).

Endocytosis. The physical interaction between the CFTR COOH terminus and the μ2 subunit of the AP-2 adapter complex, indicated by the results of recent studies (108), suggests a model like that shown in Fig. 2 for PKA-mediated regulation of endocytosis. This scheme is based on observations concerning the inherently rapid rate of CFTR-dependent endocytosis and the inhibitory effect of cAMP on this process (13, 14, 61, 85). It assumes that CFTR is normally endocytosed at a rapid rate because of its interaction with the AP-2 adapter complex at the YDSI motif of the COOH terminus. Recent data indicate that this interaction requires also phosphorylation of the μ2 subunit, which facilitates AP-2 interactions with membrane lipids (25). The model of Fig. 2 implies that the PKA-mediated inhibition of CFTR endocytosis may result from occlusion or obstruction of the adapter protein binding site at the COOH terminus by a CFTR phosphorylation event. For example, this could result from an as yet unidentified physical interaction of the phosphorylated R domain with the COOH-terminal tail where adapters bind, or it could be mediated by another interacting protein whose association with the COOH terminus is proportional to CFTR phosphorylation. The key feature of this model relies on phosphorylation-dependent accessibility of the COOH terminus site for adapter protein binding.

A second possibility is that the YXXΨ motif itself is altered by phosphorylation. In species where CFTR’s sequence has been described, the YDSI motif is highly conserved (108). Although it does not conform to a canonical PKA consensus sequence, it is possible that the Y+2 serine is phosphorylated by PKA or another kinase, which acts to disrupt the adapter binding motif. The possibility that AMP kinase, implicated in binding to this region of CFTR (39), is involved in phosphorylation of the YDSI internalization motif requires evaluation. However, the function of this kinase is presumably to downregulate CFTR during metabolic stress, which would be expected to promote, not inhibit, CFTR retrieval. Nevertheless, a phosphorylation event at or near this site could reduce the endocytic rate of CFTR in parallel with the activation of channel gating. It remains to be determined whether such a mechanism can account for the finding, made in both epithelial and nonepithelial systems (13, 14, 85, 94), that PKA phosphorylation inhibits CFTR endocytosis, leading to retention of the channel on the cell surface.

Fig. 2. Model for molecular interactions regulating endocytosis of CFTR. A: the COOH terminus of phosphorylated (asterisks) CFTR is not free to bind the adapter protein complex AP-2, limiting its internalization. B: phosphorylated AP-2 adapter (see text) associates with the plasma membrane and with the COOH terminus of dephosphorylated CFTR. Small blue “bubbles” indicate the CFTR site of AP-2 binding. Binding of AP-2 to the plasma membrane initiates assembly of the clathrin lattice (structure simplified for this 2-dimensional representation). C: AP-2-bound CFTR is sequestered in the forming clathrin-coated pit. D: after the clathrin-coated vesicle has been internalized, coat and adaptor proteins are shed.
**Exocytosis.** What determines the ability of CFTR to enter a regulated (as opposed to constitutive; Fig. 1) secretory pathway during its progression to the cell surface? In *Xenopus* oocytes, a redistribution of CFTR between intracellular and plasma membrane compartments is demonstrable by immunofluorescence measurements, as discussed above. These data can be interpreted to suggest that CFTR is stabilized, kinetically, in an intracellular compartment under nonstimulated conditions. Therefore, it is reasonable to ask what structural feature(s) of CFTR provides for its entry into a regulated secretory pathway under basal conditions. CFTR carries its own regulatory domain, and the phosphorylation of this domain stimulates both channel gating and trafficking. Therefore, it is logical to ask whether the R domain is responsible for the entry of CFTR into a regulated pathway. For this purpose, CFTR lacking the R domain has been expressed from two injected RNAs, each corresponding to a CFTR half molecule: N-TM1-NBD1 and TM2-NBD2-C (56). Oocytes expressing these constructs showed spontaneous currents quantitatively similar to the stimulated currents of cells expressing wt CFTR, and they were not further augmented by cAMP stimulation. In addition, there was no change in membrane capacitance during stimulation, indicating that regulated CFTR trafficking required the R domain. The direct targeting of AR-CFTR to the plasma membrane in these cells is consistent with the concept that the nonphosphorylated R domain may allow CFTR to access a regulated intracellular compartment and that CFTR within this compartment can be redistributed to the plasma membrane when the R domain is phosphorylated. Therefore, it is reasonable to ask the following: What are the mediators of this process? Do protein interactions involving the R domain govern the movement of CFTR through this regulated trafficking pathway?

Although these questions cannot be answered with certainty at present, the relatively recent discovery by Kirk and colleagues (72, 73) that CFTR interacts physically and functionally with syntaxin 1A (S1A) suggests soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins as candidates for participation in this regulated trafficking process. The interaction of S1A with the NH2 terminus of CFTR (73, 81) and the inhibition of CFTR currents during S1A overexpression (72) have been interpreted as the consequence of a direct interaction of S1A with CFTR that reduces its open probability (20). However, in addition, Peters et al. (81) showed that S1A overexpression decreased the density of CFTR channels in the plasma membrane. They concluded that disruption of SNARE complex formation by overexpressed S1A interfered with CFTR trafficking (presumably with exocytosis). As regards the functional involvement of syntaxin with CFTR, there is no reason to discount one mechanism in favor of the other; that is, syntaxin may regulate CFTR gating and also function as a component of the machinery that brings CFTR into the plasma membrane, or it may act as a regulator of that process.

How might SNARE proteins be involved in regulated CFTR exocytosis? To pose an answer to this question, we should recount several observations. First, the Kirk laboratory has shown that the NH2 terminus of CFTR, to which S1A binds, also interacts with a proximal portion of the R domain in protein binding studies (71). A series of basic amino acid residues at the NH2 terminus is thought to mediate this association. Mutation of these residues altered the magnitude and kinetics of CFTR current stimulation in oocytes and decreased the open probability of single CFTR channels (34), perhaps by interfering with NH2 terminus-R domain interactions that influence CFTR gating. The NH2 terminus is also the site of S1A binding (73). Thus it is possible that NH2 terminus-R domain interactions influence the association of CFTR with S1A or that it affects the ability of S1A to interact with other proteins necessary for CFTR trafficking.

In relation to another possible scenario, we recently reported physical and functional interactions of another synaptic terminal protein, the cysteine string protein (Csp), with CFTR (115). Csp is a resident of synaptic vesicles, and its knockout interferes with regulated neurotransmitter release (116), as does a S1A knockout (111). In protein binding studies, Csp interacted with both the CFTR NH2 terminus and R domain. It seems likely that the interactions of S1A and Csp with the NH2 terminus and the additional interactions of the NH2 terminus and Csp with the R domain may provide a means for linking proteins involved in regulated membrane trafficking to the regulatory (phosphorylation) status of the R domain (similar in general concept to the proposal above for regulated CFTR endocytosis). According to the model shown in Fig. 3, phosphorylation of the R domain would alter these protein interactions in a manner that would lead to SNARE protein associations appropriate for fusion of CFTR-containing vesicles with the plasma membrane. It is also of interest that Csp can bind to several SNARE proteins, including S1A, syntaxin 4, and vesicle-associated membrane protein 2 (VAMP-2) and that Csp has been proposed as a modulator of SNARE protein interactions (19). According to this concept, NH2 terminus-R domain interactions within CFTR can alter single-channel properties, but they may also couple the phosphorylation-dependent activation of CFTR channel gating to proteins that regulate CFTR trafficking. It is possible that a protein such as Csp, through its ability to interact with SNARE proteins on one hand and with both the NH2 terminus and R-domain of CFTR on the other, provides a transduction mechanism for coupling CFTR channel activation to channel trafficking mediated by the SNARE machinery.

**CONSEQUENCES OF REGULATED CFTR TRAFFICKING**

**CFTR channel density.** The most obvious consequence of regulated CFTR insertion/retrieval processes...
is that the distribution of CFTR between intracellular compartments and the apical plasma membrane will vary with agonist stimulation. The analogy of this process to other examples of transporters whose overall plasma membrane activity is modulated by trafficking, such as the vasopressin-stimulated AQP-2 water channel or the insulin-dependent GLUT-4 transporter, have been cited throughout this article. For CFTR, there is no doubt that the gating of this channel is regulated by phosphorylation when the channel is already present in the plasma membrane. Why, then, should the trafficking of CFTR also be regulated? Control of insertion/retrieval is a common mechanism for regulation of transporters having slow activation kinetics. Control of channel number would provide for amplification of the secretory response to agonist while preserving an option to sequester CFTR within a compartment where it cannot affect the plasma membrane Cl conductance. High intracellular salt levels can be deleterious to cells, whose intracellular ionic composition and volume must be closely regulated (30).

In addition, the contribution of trafficking events to anion secretion may vary with the differentiation status of the epithelium (6, 37). It has been found, for example, that CFTR in some epithelial cells does not find its way to the plasma membrane until the tight junctions have formed and the cells polarize (68). As the epithelium is developing, cells may utilize regulated trafficking as a means to fine tune the apical Cl conductance until the expression levels of other transporters, metabolic or regulatory components, on which overall secretory or absorptive ion movements depend are expressed at appropriate levels. At that stage, the mature cell may find it reasonable to better anchor CFTR in the plasma membrane by virtue of PDZ domain (69, 97) and other interactions, to shift the balance of channel distribution in favor of the plasma membrane. Yet, even in well-differentiated native epithelia, significant CFTR trafficking signals can be demonstrated (see REGULATED CFTR TRAFFIC). Not until we know, from a mechanistic viewpoint, the protein interactions that mediate CFTR trafficking events will we be able to determine how such a balance is achieved or how it may be perturbed to counter the effects of disease.

Relation to CF disease. Disease-related mutations that influence CFTR trafficking in distal compartments of the protein secretory pathway have been identified. It is probably true that mutations in proteins that control general trafficking pathways would have a negative selective advantage and may be lethal; however, CFTR mutations that influence its trafficking have now been described. Several reports have indicated that the half-life of F508 CFTR at the plasma membrane, and perhaps in other post-Golgi compartments, is markedly reduced relative to that of wt CFTR. Using functional measurements of CFTR activity in CHO cells, Lukacs et al. (59) found that the plasma membrane currents associated with F508 CFTR (recruited to the cell surface at low temperature) decayed with a half-time of <4 h, whereas the functional half-life of wt CFTR currents was >24 h.

Heda et al. (41) measured the half-life of F508 vs. wt CFTR in the plasma membrane of LLC-PK1 cells using cell surface biotinylation, streptavidin-mediated isolation, and immunoblot analysis. Mutant CFTR was
brought to the cell surface with the use of a combination of low temperature and butyrate preincubation. They found that the half-life of plasma membrane ΔF508 CFTR was ~4 h, whereas the corresponding half-life of wt CFTR exceeded 48 h in these cells. The cAMP-dependent iodide efflux correlated with CFTR expression at the cell surface. These authors concluded that ΔF508 CFTR is more rapidly internalized and perhaps targeted to a degradation pathway. The parallel study of Sharma et al. (95) reached a similar conclusion. Mutant CFTR was encouraged to escape the ER with the use of a combination of reduced temperature and chemical chaperone (glycerol) treatment. They observed also a rapid degradation rate of ΔF508 CFTR, which they attribute to decreased conformational stability: the protein was more susceptible to aggregation and protease digestion. These authors favor the concept that folding defects of this CFTR mutant persist in the mature, post-Golgi protein (most studies agree that it lacks normal channel gating kinetics and open probability; c.f. Ref. 92) and that this leads to its more rapid degradation, perhaps by ubiquitin-proteasome pathways. It is also possible, in view of the model for regulated CFTR endocytosis illustrated in Fig. 2, that the rapid degradation of ΔF508 CFTR reflects its more rapid endocytic rate (41, 95), increasing its exposure to degradation mechanisms. It is evident from these findings that the altered stability of mutant CFTR in distal trafficking pathways will require attention should rescue of ΔF508 CFTR from ER degradation pathways be an achievable therapeutic target for CF disease.

Another interesting disease mutation that influences distal pathway CFTR trafficking has been identified by Silvis et al. (98). These investigators searched the mutation database for disease mutations that may lead to increased CFTR endocytosis through creation of an endocytic motif. The N287Y mutant resides in the second intracellular loop of CFTR and results in mild disease when expressed in combination with ΔF508 CFTR. This mutant did not exhibit a folding defect, because the N287Y and wt CFTRs showed similar maturation kinetics. However, there was roughly 50% of the mutant at the plasma membrane at steady state relative to wt CFTR. An increased colocalization of the mutant with the endocytic marker EEA1 suggested that this mutation alters the distribution of CFTR between plasma membrane and intracellular compartments. CI transport was reduced in proportion to altered cell surface CFTR, but the single-channel properties of N287Y were similar to those of wt CFTR. Biotinylation experiments showed that N287Y CFTR was internalized approximately twice as fast as wt CFTR, which is expected to alter its distribution between plasma membrane and intracellular compartments. These findings provide evidence of disease mutations where the primary defect lies in altered kinetics of CFTR recycling at the plasma membrane, resulting in subnormal apical membrane CFTR levels. Intracellular CFTR channel function. A second consequence of regulated CFTR trafficking is that intracellular CFTR distribution may vary with the secretory status of the epithelium. Despite indications that intracellular CFTR channels can be activated by cAMP/PKA (9, 77), the model of Barasch et al. (5), suggesting that vesicular CFTR activity alters internal compartment pH, cellular glycoprotein processing, and bacterial colonization, has not been supported by subsequent studies (32, 35, 60, 83). Nevertheless, there is a fundamental need for resolving the causes for increased bacterial adherence to CF cells and their glycoprotein secretory products and for the preferential binding of P. aeruginosa to undersialylated CF mucins (16, 31, 48, 53, 91, 113). These findings argue that the deletion of CFTR from the distal secretory pathway influences epithelial cell surface chemistry. These properties of CF cells and their secretions could be affected by CFTR-dependent membrane trafficking events in intracellular compartments as well as at the plasma membrane domain. For example, Biwersi et al. (8) demonstrated that CFTR promotes endosome-endo-}


important role in cAMP-mediated glycoconjugate secretion. Although not explicitly implicating CFTR in mucin release, the studies of Kuver et al. (54) have demonstrated cAMP-dependent regulation of protein or mucin secretion in CFTR-expressing canine gallbladder epithelial cells. CFTR was detected by immunostaining on mucin granule membranes.

In studies that may have implications for epithelial protein secretion, Yilla et al. (112) examined the influence of vacuolar H-ATPase inhibition on the constitutive protein secretory pathway in HepG2 cells. Although treatment with concanamycin B did not influence ER-to-Golgi transit, the kinetics of protein traffic between the Golgi and plasma membrane were significantly impaired. The secretion of albumin, α1-antitrypsin, and transferrin was delayed, and processing of N-linked glycans by sialyltransferases was inhibited, resulting in the secretion of less extensively modified glycoproteins. This study implicates intracellular compartment acidification in the sialylation and N-linked glycan modification of secreted glycoproteins and with the rate of protein secretion. In a related study, Jilling and Kirk (50) demonstrated that cAMP increased the secretion of several proteins, including α1-antitrypsin, into the apical but not the basolateral compartment of T84 cells. This effect was Cl dependent and resulted also in an increase in protein sialylation. An involvement of CFTR in regulated exocytosis could underlie the differences in protein secretion observed in studies of cells and/or tissues derived from normal and CF patients or CF knockout mice.

Common threads. Once we understand the reasons for the ability of CFTR to alter membrane traffic, it may become apparent that the underlying mechanisms involved in the progression of CFTR between different compartments have certain features in common. A critical question in this regard concerns the exit of CFTR protein from the ER. Reasons for thinking along these lines include the common features controlling vesicle transit along compartments of the secretory pathway, e.g., a combination of SNARE and coat protein associations, which often involve interactions with the cargo being conveyed (3).

Our recent findings regarding Csp interactions with CFTR (115) also may provide an example of how common trafficking events may occur in the early and later components of the protein secretory pathway. Csp regulates exocytosis in neurosecretory cells, and implications for a similar role in CFTR exocytosis were discussed above. In addition, Csp plays a role in CFTR maturation in the ER. Csp antibodies co-precipitate a large proportion of band B CFTR and localize Csp protein to the ER, in addition to its presence at the apical domain. The major influence of Csp overexpression was a disruption of the biogenesis of mature CFTR (band C). Because Csp is an Hsc70 binding protein, which also binds to CFTR, it is likely that Csp serves as a CFTR co-chaperone. Csp overexpression may decrease CFTR biogenesis by prolonging its association with Hsc70, which can lead to CFTR degradation (88).

Much work needs to be done to determine the role of Csp in CFTR maturation, but it is interesting to speculate that the steps involved in the egress of nascent CFTR from the ER and from a regulated compartment of the distal secretory pathway to the plasma membrane may involve similar protein interactions. Therefore, identifying the mechanisms underlying stage-specific CFTR trafficking events may also shed light on traffic occurring at other compartments of the cell.

SUMMARY

As with any active field of study, the above discussion leaves several issues unresolved. On balance, there is good evidence that epithelial cells exhibit regulated CFTR trafficking as a mechanism to increase the density of CFTR channels at the cell surface. This process may contribute to the cAMP-dependent secretion of proteins contained within this trafficking compartment. Some results that show a lack of regulated CFTR trafficking may be attributable to the use of overexpression systems or nonphysiological experimental conditions. Yet, there are other findings that cannot be similarly rationalized, and this may reflect the need for expression of the right set of interacting proteins, perhaps some of them as yet unidentified. The finding that disease mutations influence CFTR trafficking events in the distal protein secretory pathway and thereby contribute to the CF phenotype further supports the concept that regulated CFTR recycling is important for normal epithelial function. The era of CFTR interactions with other proteins, particularly with recognized traffic regulatory components, is beginning to shed light on the potential mechanism of regulated CFTR trafficking. We suggest testable hypotheses (Figs. 2 and 3) regarding the manner in which CFTR phosphorylation may alter protein interactions and thereby affect the distribution of CFTR between apical membrane and intracellular compartments. Yet, it is likely that controversy regarding the significance of this process will continue until we can identify the specific components of the CFTR-dependent trafficking machinery, permitting their expression or activity to be modulated experimentally in polarized epithelial cells and, ultimately, in the organism.

APPENDIX

The calculated CFTR channel density in transport vesicles from reported (46, 102) changes in whole cell current and capacitance during CFTR stimulation by cAMP is shown. Common values for CFTR single-channel parameters are used to estimate channel number.

\[
N_C = \frac{I_m}{P_C \gamma \Delta V}
\]

where \(I_m\) is the measured membrane current (Calu-3 cell, 500 pA; *Xenopus* oocyte, 4 μA), \(P_C\) is the open probability of a CFTR channel (0.4), \(\gamma\) is the conductance of a CFTR channel

(8 pS), and \( \Delta V = V_m - V_R \), or membrane potential minus reversal potential (20 mV);¹ and
\[
N_V = \Delta C_m / (C_r \cdot 4\pi r_G^2)
\]
where \( \Delta C_m \) is the measured increase in membrane capacitance (Calu-3 cell, 0.9 pF; Xenopus oocyte, 80 nF), \( C_r \) is the specific capacitance of a biological membrane (1 \( \mu \)F/cm²), and \( r_G \) is the radius of a spherical vesicle (100 nm).

For a Calu-3 cell
\[
N_C = (500 \text{ pA}/(0.4 \times 8 \text{ pS} \times 20 \text{ mV}) = 7,800 \text{ channels}
\]
and
\[
N_V = (0.9 \text{ pF}) / (1 \text{ \mu F/cm}^2 \times (100 \text{ cm/m})^2 \times 4 \times \pi \times (100 \text{ nm})^2)
\]
= 716 vesicles

The channel density in a Calu-3 vesicle is 7,800/716, or 11 channels/vesicle.

For a Xenopus oocyte
\[
N_C = (4.0 \text{ \mu A}/(0.4 \times 8 \text{ pS} \times 20 \text{ mV}) = 63,125,000 \text{ channels}
\]
and
\[
N_V = (80 \text{ nF}) / (1 \text{ \mu F/cm}^2 \times (100 \text{ cm/m})^2 \times 4 \times \pi \times (100 \text{ nm})^2)
\]
= 63,662,000

The channel density in an oocyte vesicle is 1 channel/vesicle.

References


C16 INVITED REVIEW


