Regulated trafficking of the CFTR chloride channel

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The cystic fibrosis transmembrane conductance regulator (CFTR), the ABC transporter encoded by the cystic fibrosis gene, is localized in the apical membrane of epithelial cells where it functions as a cyclic AMP-regulated chloride channel and as a regulator of other ion channels and transporters. Whereas a key role of cAMP-dependent phosphorylation in CFTR-channel gating has been firmly established, more recent studies have provided clear evidence for the existence of a second level of cAMP regulation, i.e. the exocytotic recruitment of CFTR to the plasma membrane and its endocytotic retrieval. Regulated trafficking of the CFTR Cl⁻ channel has sofar been demonstrated only in a subset of CFTR-expressing cell types. However, with the introduction of more sensitive methods to measure CFTR cycling and submembrane localization, it might turn out to be a more general phenomenon that could contribute importantly to both the regulation of CFTR-mediated chloride transport itself and to the regulation of other transporters and CFTR-modulated cellular functions. This review aims to summarize the present state of knowledge regarding polarized and regulated CFTR trafficking and endosomal recycling in epithelial cells, to discuss present gaps in our understanding of these processes at the cellular and molecular level, and to consider its possible implications for cystic fibrosis.

Introduction

Cystic fibrosis (CF), the most common lethal genetic disease among Caucasians, is the consequence of mutations in the CF gene encoding a cAMP-regulated chloride channel designated cystic fibrosis transmembrane conductance regulator (CFTR) (Riordan et al., 1989). The CFTR protein is expressed at high levels in the apical membrane of epithelial cells where it fulfills a major role in the vectorial transport of electrolytes and water. CFTR is also present at much lower levels in the plasma membrane of several non-epithelial cells including cardiomyocytes (Gadsby et al., 1998), T-lymphocytes (Krauss et al., 1992), hypothalamic neurons (Weyler et al., 1999) and endothelial cells (Tousson et al., 1998), but its function in those cell types is still ill-understood. Mislocalization or dysfunctioning of the CFTR-Cl⁻ channel results in the characteristic symptoms of CF including elevated sweat Cl⁻ concentration, pulmonary disease, pancreatic insufficiency, intestinal obstruction and male infertility (Kant et al., 1995). Unlike any other known ion channel, with the exception of the recently discovered cholesterol-export protein ABC1 that was characterized first as an anion transporter (Becq et al., 1997), CFTR belongs to the ATP-binding cassette superfamily of transporters (class III, sub-family C, member 7; cf. Fig. 1). The channel is a dimer of two CFTR polypeptides, each composed of two cytoplasmic nucleotide-binding domains (NBD1 and 2) involved in ATP binding and hydrolysis, two transmembrane domains (TMD1 and 2), each consisting of six membrane-spanning α-helices that contribute to the channel pore, and one unique cytoplasmic regulatory (R) domain containing multiple sites for phosphorylation by cAMP- and cGMP-dependent protein kinases (PKA and the type II isoform of PKG) (Lohmann et al., 1997) and protein kinase C (PKC) (Gadsby and Nairn, 1999a). Multi-site phosphorylation of the R domain triggers cycles of channel opening and closing through ATP binding and hydrolysis at the two NBDs (Gadsby and Nairn, 1999a).

CFTR not only comprises a metabolically gated Cl⁻ pore but additionally modifies the activity of a multitude of other ion channels and transporters including epithelial Na⁺ channels (ENaC), outwardly rectifying Cl⁻ channels (ORCC), renal K⁺ channels (ROMK) (Schwiebert et al., 1999), Ca²⁺-activated Cl⁻ channels (CaCC) (Wei et al., 1999), Cl⁻/HCO₃⁻ exchangers (AE2) (Lee et al., 1999), intestinal volume-regulated K⁺ channels (Valverde et al., 1995), ATP-release channels (Schwiebert, 1999), gap junctional channels (connexin 45) (Chanson et al., 1999) and Na⁺/H⁺ exchangers (NHE3) (Clarke and Harline, 1996). Remarkably, a reciprocal regula-
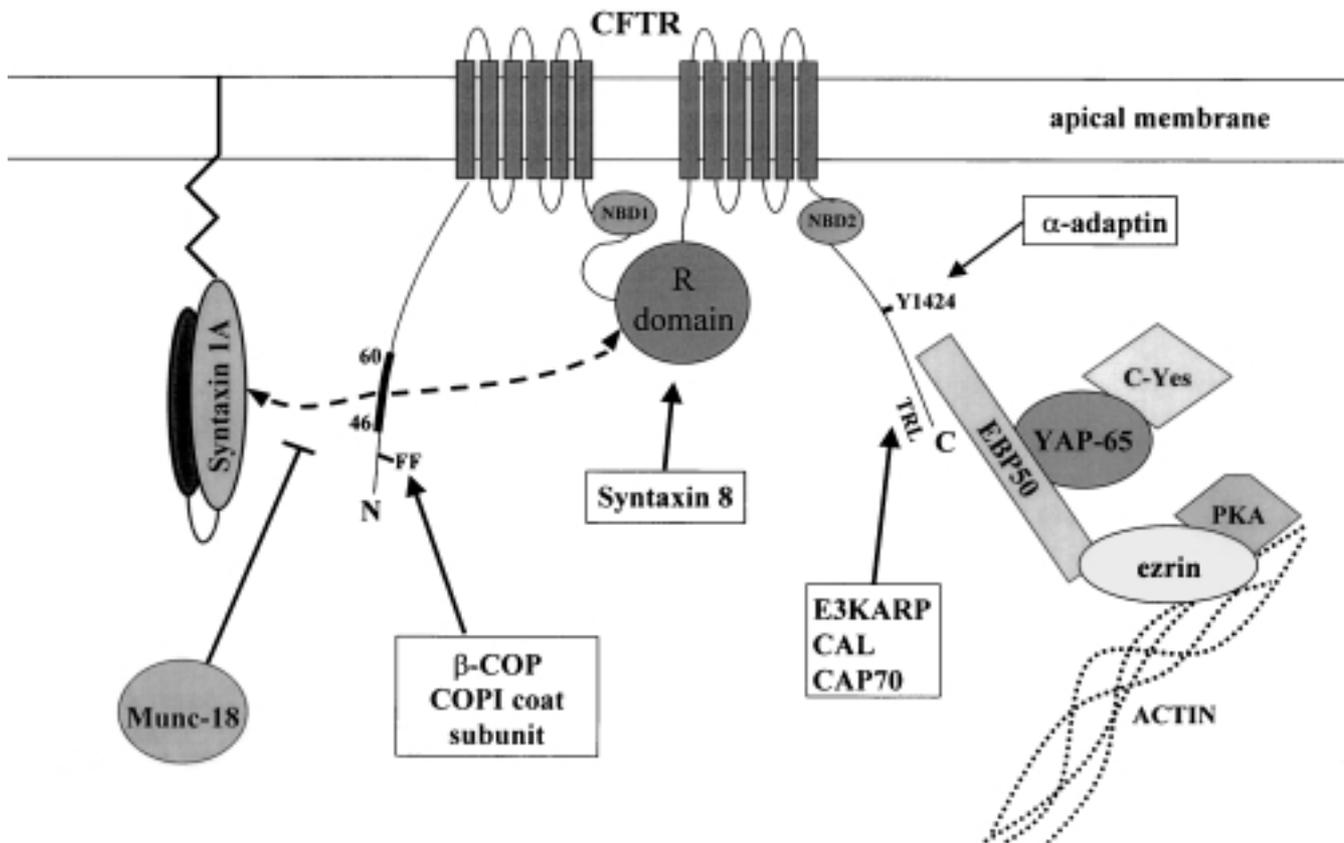
Fig. 1. Map of binding sites for CFTR-associated proteins within the CFTR structure. For explanation, see text.

tion of CFTR expression and CFTR channel open probability by co-expression of ENaC has also been observed (Ji et al., 1999). However it should be pointed out that some of these studies have been carried out solely in heterologous cell expression systems and that the results still need to be confirmed in native epithelia under physiologically relevant conditions. Whether the CFTR crosstalk with other transporters involves direct intramembrane or cytoplasmic protein-protein interaction; a chain of protein interactions involving additional regulatory proteins or cytoskeletal elements; or indirect ways of communication through changes in membrane potential, pH, autocrinic puringeric signaling or other CFTR-affected cellular functions has not been established firmly for any of the CFTR-regulated transporters but is presently a field of active investigation. Importantly, phosphorylation of CFTR through the cAMP/PKA pathway turned out to be a pre-requisite for most (Schwiebert et al., 1999) but not all (Lee et al., 1999) CFTR interactions with the other transporters. The cAMP dependency suggests that cAMP-stimulated exocytotic insertion of CFTR from subplasma membrane vesicles could potentially serve as an additional mechanism to up-regulate other transporters such as the ATP release channel (Schwiebert, 1999) by facilitating their recruitment to the plasma membrane.

Localization of CFTR to the plasma membrane is essential for CFTR function; hence mutations that alter the intracellular CFTR distribution result in CF. The most common CFTR mutation, deletion of phenylalanine at position 508 (ΔF508) occurring in nearly 70% of CF patients and accounting for 90% of all mutant CFTR alleles (Kant et al., 1995) does not

severely impair channel function but causes misfolding and trapping of CFTR in the endoplasmic reticulum (ER) resulting in the virtual absence of CFTR at the plasma membrane (Seibert et al., 1997). Therefore one important therapeutic strategy in CF is to improve anterograde trafficking of CFTR-ΔF508 through the secretory pathway to the apical plasma membrane (Riordan, 1999).

Trafficking of CFTR from the ER to the plasma membrane

Wild-type CFTR is synthesized in the ER and co-translationally undergoes core glycosylation as an initial step in CFTR processing. Upon completion of the chaperone-assisted folding process, CFTR is exported from the ER in COPII (coatomer protein)-coated vesicles that bud off from specialized sites called the transitional elements (Schekman and Orci, 1996). COPII vesicles are composed of coatomer proteins Sec23p/Sec24p, Sec31p/Sec13p and the Sar1 GTPase (Kuehn and Schekman, 1997). As the cargo-loaded COPII vesicles uncoat they can fuse to a structure called the ER/Golgi intermediate compartment (ERGIC) or the vesiculo-tubular clusters (VTCs) (Aridor et al., 1995). Within the ERGIC, cargo proteins are incorporated into anterograde COPI vesicles for transport to the Golgi and ultimately into the plasma membrane, or alternatively into retrograde COPI vesicles for recycling back to the ER (Orci et al., 1997). COPI vesicles contain seven COPI protein subunits (α-, β-, β'-, δ-, ϵ-, and ξ-COP) and a GTPase (ARF1) that are distinct from COPII proteins (Chavrier and Goud, 1999).

As exemplified by type I integral membrane proteins from the p24 family that function as cargo receptors and cycle between the secretory pathway compartments (Fiedler et al., 1996), the segregation between anterograde and retrograde traffic could occur in the COPI coated VTCs by selective binding of COPI subunits to a sorting motif in the cytosolic domain of a protein. A p24 protein dilysine (KK) retrieval signal binds to a subcomplex of α-, β'-, and ε-COP subunits, known as COPI "B" and directs p24 into retrograde COPI vesicles (Cosson and Letourneur, 1994), whereas a p24 protein diphenylalanine (FF) sorting motif interacts with a β-, γ-, and ξ-COPI subunit complex, known as the COPI "F" and functions in directing the protein into anterograde vesicles (Fiedler et al., 1996). A similar diphenylalanine trafficking signal has been identified recently in the N-terminal cytoplasmic tail of CFTR (F16/F17) and was shown to greatly improve the efficiency of CFTR transport through the secretory pathway, apparently through binding to a β-COP complex in the anterograde COPI-coated vesicles (Olenych and Teem, 1999). Currently none of the 890 CF-causing mutations reported have been mapped at the F16/F17 position, suggesting that a ∼80% reduction in the amount of processed CFTR as observed with the F16A/F17A double mutant is still insufficient to cause CF disease.

Anterograde transport of CFTR from trans-Golgi network to the plasma membrane

During CFTR traffic through the Golgi complex, its two N-linked oligosaccharide chains are converted from high mannose to complex structures prior to its trafficking to the plasma membrane. The trans-Golgi network is the main sorting station where proteins are concentrated in coated vesicles and trafficked to specific organelle membranes (endocytic pathway) or to the plasma membrane (biosynthetic pathway). Since CFTR is mainly expressed in polarized cells like the enterocyte and airway epithelial cell, we will focus on the polarized biosynthetic pathway of CFTR. Selective targeting of proteins to apical or basolateral domains is achieved by distinct sorting or retention signals.

Basolateral sorting
Basolateral membrane targeting determinants reside in the cytoplasmic domains of transmembrane proteins and often rely on critical tyrosine-dependent or dileucine-dependent motifs, frequently followed by a cluster of acidic residues (Matter, 2000). Similar motifs function as clathrin-coated pit determinants in endosomal or lysosomal targeting by virtue of their interaction with specific adaptor proteins (Aps), hetero-tetramers of adaptins that are part of the clathrin vesicle coat (Bonifacino and Dell'Angelica, 1999). Adaptor proteins mediating endocytosis at the plasma membrane (AP-2, consisting of adaptins α, β2, μ2, σ2) are distinct from adaptins involved in lysosomal targeting at the trans-Golgi network and endosomes (AP-1 γ, β1, μ1A/B, σ1 and AP-3 δ, β3, μ3, σ3). Generally, μ subunits interact with tyrosine motifs and β subunits with dileucine signals (Matter, 2000). Very recently μ1B, a novel isoform of the μ1 subunit of AP-1, was found to rescue basolateral expression of membrane proteins with tyrosine-based sorting signals in μ1B-deficient LLC-PK1 kidney cells and was therefore identified as part of a specific adaptor complex acting as a sorter for basolateral membrane

proteins in both endosomes and the trans-Golgi network (Folsch et al., 1999).

Does basolateral sorting have any relevance for the CFTR protein? Sofar immunological studies of CFTR localization in polarized epithelial cells have identified the protein exclusively in the apical membrane and the subapical compartment, but not in the basolateral membrane (Kartner et al., 1992). Moreover a recent study of the subcellular distribution of green fluorescent protein (GFP)-tagged wild-type (WT) CFTR in MDCK cells and human airway epithelial cells confirmed that WT-CFTR is expressed exclusively in the apical membrane (Moyer et al., 1998). However, expressing the C-terminally truncated green fluorescent protein (GFP)-CFTR-S1455X construct shifted the CFTR localization to the basolateral membrane, suggesting that the deleted 26 amino acids somehow mask interaction of basolateral targeting signals in the CFTR deletion mutant and thereby prevent basolateral localization (Moyer et al., 1999). Potential basolateral targeting motifs in CFTR include a dileucine motif (residues 1430–1431) and a tyrosine-based motif (residues 1424–1427); the latter motif has been recently identified as a clathrin-coated pit determinant that is required for the formation of the AP-2 complex and therefore for CFTR internalization ((Weixel and Bradbury, 2000); discussed in more detail later). In light of these studies it would be of interest to assess whether CFTR-S1455X (but not WT-CFTR) has acquired the capacity to interact with the AP-1 complex in cells expressing the novel μ1B adaptor subunit (Folsch et al., 1999).

Apical sorting
Until recently, less was known about the signals that sort or retain proteins in the apical membrane. Apical targeting has been attributed to at least three classes of sorting signals (Rodriguez-Boulan and Gonzalez, 1999): N-linked or O-linked carbohydrates; specific transmembrane domains or glycosylphosphatidylinositol (GPI) anchors interacting with sphingolipid- and cholesterol-rich microdomains of the lipid bilayer known as rafts (Benting et al., 1999); and cytoplasmic domains interacting with a protein machinery associated with the cytosolic surface of the trans-Golgi network. In the case of CFTR the first two signals are most plausibly only of minor importance. First, inhibitors of N-linked glycosylation did not impair CFTR-mediated transepithelial Cl⁻ currents in T84 and HT-29 human colonocytes (Morris et al., 1993), and none of the CF disease-causing mutations have been mapped nearby or at the fourth-loop asparagines anchoring the N-glycans. In contrast in the *Xenopus* oocyte expression model the mutation of both sites for carbohydrate addition, but not the mutation of a single site resulted in a marked reduction (∼80%) of the CFTR current (Howard et al., 1995). Whether this defect is caused by impaired intracellular trafficking or other changes in CFTR properties, e.g. a reduced stability in the plasma membrane, remains to be determined.

Secondly, the signal for GPI attachment to proteins consists of a short hydrophobic sequence (13–17 residues) and an appropriate upstream cleavage or attachment site, sequences that are lacking in CFTR. Moreover endogenous CFTR in polarized epithelial cells could not be detected in raft-enriched, specialized domains of the plasma membrane named caveolae (Bradbury et al., 1999), arguing against the concept that CFTR is a raft-associated protein.

By contrast there is overwhelming evidence from numerous recent studies, mainly on the basis of yeast two-hybrid screens, in vitro pulldown assays and GFP- or immuno-fluorescence localization, that PDZ-binding motifs at the cytoplasmic tail of many receptors, transporters and ion channels, including CFTR, play an essential role in their cellular localization and polarization (Fanning and Anderson, 1999). PDZ domains are modular 70–90 amino acid domains in proteins that bind to short peptide sequences (called PDZ-interacting domains) generally located at the C-termini of target proteins. They were first identified as conserved sequence elements in the post-synaptic density protein PDS95/SAP90, the *Drosophila* tumor suppressor dlg-A, and the tight junction protein ZO-1, which resulted in the acronym PDZ (PSD95/Dlg/ZO-1). These 80–90 amino acids sequences have now been identified in well over 75 proteins and are typically expressed in multiple copies within a single protein. Because different PDZ domains often possess distinct peptide-binding specificity, a tandem arrangement of several PDZ domains in one protein allows for multivalent interactions to organize a supramolecular complex. However in addition to their role as central organizers of proteins in a functional complex at the plasma membrane, PDZ domains have also been implicated in the polarized localization of proteins to the apical or basolateral plasma membrane of epithelial cells (Muth et al., 1998).

Importance of PDZ-containing proteins in apical sorting and regulation of CFTR

In most proteins the PDZ-interacting domain is located at the C-terminus and characteristically contains the amino acid sequence (T/S)XV that is part of an antiparallel β strand which binds to an elongated surface groove in the PDZ domain (Fanning and Anderson, 1999). The C-terminal TRL sequence in CFTR is highly conserved and comprizes a PDZ-interacting motif that binds to the first PDZ domain of the ezrin-radixin-moesin (ERM)-binding phosphoprotein EBP50 (Short et al., 1998). This protein is a human homologue of the NHE-regulating factor (NHERF) that is bound through its second PDZ domain to an internal (T/S)XV motif in the rabbit kidney proximal tubule Na^+/H^+ exchanger NHE3 (Yun et al., 1997). NHERF and EBP50 are coupled through their C-terminal ERM-binding domain to the actin-binding protein ezrin, which possesses a dual function: anchoring the NHE3-NHERF/EBP50 complex to the actin cytoskeleton, and conferring cAMP/PKA sensitivity to NHE3 through its ability to anchor the regulatory subunit RII of PKA in the vicinity of the transporter (Lamprecht et al., 1998). A very similar model is now emerging for the cAMP/PKA regulation of CFTR (Short et al., 1998) (Fig. 1). Both EBP50 and ezrin segregated with CFTR in the same subcellular fraction, and GFP-WT-CFTR and EBP50 co-localized to, and could be co-immunoprecipitated from the apical membrane of MDCK cells (Moyer et al., 1998). Furthermore, intracellular dialysis of an AKAP (A kinase anchoring protein)-binding domain peptide (Ht31) or a peptide resembling the PKA-binding site of ezrin into Calu-3 serous airway epithelial cells (Hug et al., 1999a; Sun et al., 2000), or the addition of HT-31 to isolated membrane patches (Huang et al., 2000) blocked the cAMP activation of CFTR and its cAMP-dependent exocytotic recruitment in intact cells, as well as the cAMP/PKA-II stimulation of CFTR in the excised membrane patches, respectively.

The importance of the PDZ-interacting domain of CFTR as an apical targeting signal was demonstrated recently in MDCK type I cells and in human airway epithelial cells by showing that CFTR with a truncation in the C-terminus (CFTR-ΔTRL) had lost its apical localization and was distributed nearly equally between the apical and basolateral membrane (Moyer et al., 1999). Such alterations in the polarized distribution of CFTR are likely to result in a partially defective vectorial Cl^- transport and may therefore explain why deletions of the C-terminal region of CFTR including the PDZ-interacting domain (approximately 10% of all mutations in CFTR) cause CF disease without altering the cAMP-stimulated Cl^- permeability of the CFTR channel.

Though EBP50 is a good candidate for acting as an apical CFTR sorter, at least three other PDZ-domain proteins have been identified that might fulfill a similar function: The NHE3 kinase A regulatory protein E3KARP, another human homologue of NHERF capable of interacting with both the C-terminus of NHE3 (Yun et al., 1997) and the PDZ-interacting domain of CFTR (Sun et al., 1999); CAP70, a CFTR-associated protein expressed in the apical region of airway and intestinal epithelial cells that contains four PDZ domains and, through specific binding to the C-terminus, is able to potentiate CFTR Cl^- currents (Wang et al., 1999); and a CFTR-associated ligand protein named CAL that has been identified and cloned using the C-terminus of CFTR as a bait in yeast two-hybrid screens (Cheng et al., 1999) (Fig. 1). This 50-kDa protein contains a single PDZ domain and two coiled-coil domains. CAL has been found in several cell types including T84 colonocytes, 16HBE14o airway cells, and MDCK type I cells and could be specifically co-precipitated with CFTR in T84 intestinal epithelial cells. Unlike EBP50, CAL has been localized beneath the apical membrane and is probably associated with the post-Golgi vesicles or TGN. Consequently brefeldin A treatment leading to a loss of Golgi structure resulted in dissociation of CAL from the Golgi and its redistribution into the cytosol. Therefore CAL is a promising candidate to be involved in sorting and targeting of CFTR at/from the trans-Golgi network. It might be postulated, however, that the apically sorted proteins EBP50 or E3KARP could exchange with CAL upon arrival of CFTR at the apical membrane. In this view CAL functions as an apical sorter whereas EBP50 or E3KARP determine the surface location of CFTR by retention rather than targeting, i.e. by linking CFTR through ezrin to the cortical actin cytoskeleton. A similar retention mechanism has recently been postulated to explain the PDZ-dependent localization of the BGT-1 GABA transporter at the basolateral membrane of MDCK cells (Perego et al., 1999). EBP50 and E3KARP, in contrast to CAL, may use their second PDZ domain to recruit other PDZ-interacting domain proteins like YAP65 (Yes-associated protein 65), resulting in a highly ordered signaling complex termed the transducisome (Mohler et al., 1999). YAP65 may function as a scaffolding protein to target c-Yes nonreceptor tyrosine kinase to the apical membrane. The function of c-Yes is not yet known in any detail but it could be involved in CFTR phosphorylation and fine-regulation of CFTR gating (Jia et al., 1999), or modulate other transporters in the vicinity of CFTR, e.g. ENaC (Mohler et al., 1999).

Role of SNARE proteins in CFTR trafficking and regulation

Vesicular transport of integral membrane proteins from the TGN to the apical or basolateral surface may also require the formation of a SNARE complex composed of multiple

proteins including the N-ethylmaleimide-sensitive ATPase and fusion protein (NSF), the soluble NSF attachment protein α-SNAP, small isoprenylated G-proteins of the rab family, and a vesicle SNAP receptor (t-SNARE) (Hanson et al., 1997). SNARE proteins are relatively small (15–40 kDa) and compartment-specific carboxy-terminally anchored integral membrane proteins with their amino-terminal and central regions present in the cytoplasm. SNAREs participate in protein trafficking mediated by coiled-coil domain interactions. Analogous to the established role of the SNARE complex in regulated exocytosis of synaptic vesicles in nerve terminals, v-SNAREs may interact with target membrane SNAP receptors (t-SNAREs) in the apical or basolateral plasma membrane of epithelial cells and promote vesicle docking and fusion (Low et al., 1998a). The t-SNARE consists of two subunits, which are members of the syntaxin and SNAP-25 families, respectively (Low et al., 1998b). Syntaxin 3 has been identified in the apical membrane of airway, intestinal and kidney epithelial cells (Naren et al., 2000) and, together with α-SNAP and SNAP-23, has been functionally implicated in trans-Golgi to apical surface trafficking in MDCK cells (Low et al., 1998a). Remarkably, functional inhibition of other components of the SNARE machinery, e.g. the botulinum neurotoxin F-sensitive v-SNARE synaptobrevin/VAMP-2, NSF, or Rab, appeared to block basolateral but not apical trafficking (Ikonen et al., 1995), suggesting that toxin-insensitive epithelial homologues of VAMP and NSF may exist which, in concert with syntaxin 3 and SNAP, mediate apical membrane fusion. Whereas the biosynthetic pathway of CFTR is likely to utilize a similar docking and fusion mechanism to insert CFTR in the apical membrane, direct evidence for a role of syntaxin 3 in CFTR trafficking is presently lacking.

However, a novel and exciting role of another t-SNARE protein, syntaxin 1A, in CFTR regulation has emerged from a series of recent studies (Naren et al., 1997). Syntaxin 1A is abundantly expressed in synaptic membranes but has also been identified in very low amounts (but still in a 10-fold molar excess over CFTR) near the apical membrane surface of human and mouse airway and intestinal epithelial cells and in physical association with the N-terminal cytoplasmic tail of the CFTR Cl⁻ channel (Naren et al., 2000) and possibly with the γ subunit of ENaC (Saxena et al., 1999) (cf. Fig. 1). As shown by mutational analysis and co-injection of N-tail peptides, a cluster of four acidic residues in a well-conserved region of the N-tail of CFTR (residues 46 to 60) partitions onto one surface of a putative α-helical domain and acts as a positive regulator of CFTR activity by virtue of its interaction with the N-terminal portion of the R domain (residues 595 to 740) (Naren et al., 1999). Binding of the N-terminal tail of CFTR to a membrane-anchored H3 helical domain of syntaxin 1A (but not of syntaxin 3) or to wild-type syntaxin 1A (but not to a cytosolic C-terminal deletion mutant of syntaxin 1A) was shown to disrupt its interaction with the R domain and to strongly reduce cAMP-activated CFTR Cl⁻ currents in *Xenopus* oocytes (Naren et al., 1998). Moreover a natural mutation within the acidic domain (Asp 58) of CFTR is associated with mild CF disease (Naren et al., 1999). Conversely, disruption of the syntaxin 1A-CFTR interaction by infusion of the soluble syntaxin-binding protein Munc-18 or the syntaxin 1A cytosolic domain, or by treatment with the botulinum neurotoxin C1 (i.e. a protease that cleaves syntaxin from the membrane) rescued the CFTR from syntaxin

inhibition in *Xenopus* oocytes (Naren et al., 1998) and augmented cAMP-activated CFTR Cl⁻ currents by 2- to 4-fold in airway epithelial cells (Naren et al., 2000). Syntaxin binding is observed also with CFTR mutant proteins (e.g. the processing mutant ΔF508; the regulation mutant G551D; the conduction mutant R117H) and, unlike typical t-SNARE/v-SNARE interactions, is specific for the syntaxin 1A isoform: CFTR is not physically complexed with other epithelial syntaxins (i.e. syntaxin 2–4; with the possible exception of syntaxin 8; see later (Thoreau et al., 1999)), and the cytosolic syntaxin 1A peptide did not augment CFTR Cl⁻ currents in syntaxin 1A-deficient but syntaxin 3-containing mouse fibroblasts (L cells) (Naren et al., 2000). The latter observation also demonstrates that syntaxin 1A, though being an important CFTR regulator, is not essential for the trafficking of CFTR along the biosynthetic pathway. It should be pointed out, however that these studies could not resolve the issue whether syntaxin regulates CFTR activity primarily at the level of channel gating, by affecting its cAMP-dependent plasma membrane insertion, or by both mechanisms acting in concert. Sofar, none of these experiments have been carried out in cell types in which cAMP-regulated trafficking of CFTR is apparently lacking (e.g. MDCK type I cells (Moyer et al., 1998)), or under conditions of cAMP-prestimulation and inhibition of endocytosis to eliminate the CFTR trafficking component. However the dual localization of syntaxin 1A in the apical pole and in multiple intracellular compartments including endosomes of various epithelial cell types (Naren et al., 2000) strongly supports the concept that syntaxin 1A plays an additional role in the cAMP-modulated internalization and recycling of CFTR to the apical membrane in polarized epithelial cells. This process will be discussed more extensively in the next section.

Endocytosis and recycling of CFTR at the apical membrane

Endocytic recycling of membrane proteins is an important and highly regulated process that contributes to the fine tuning of membrane protein activities due to environmental responses. Regulated recycling between the plasma membrane and intracellular compartments has been extensively studied for a number of membrane proteins including the insulin-sensitive GLUT4 glucose transporter, the water channel aquaporin AQP2, renal ion transporters and the EGF receptor and LDL receptor (see other articles in this issue (Deen et al., 2000; Dunbar and Caplan, 2000; Markovich, 2000) and (Levkowitz et al., 1998)). However there is growing evidence from biochemical, immunocytochemical and functional studies in cultured cells and native epithelia that CFTR, in a fashion analogous to the GLUT4 transporter (Pessin et al., 1999), is likewise able to adjust its density in the cell surface and therefore the Cl⁻ permeability of the plasma membrane by regulated exocytosis and retrieval (see (Bradbury, 1999) for a comprehensive review). The essential elements in this process are a functional cytoskeleton, protein sorting (clustering) at the apical membrane, clathrin coat formation, fusion of clathrin-coated vesicles to endosomes, and sorting/targeting of the recycling membrane proteins back to the apical membrane. We will now consider each step in the CFTR recycling process in more detail and highlight the underlying molecular mechanisms.

CFTR endocytosis
Early immunolocalization and surface biotinylation studies in
T84 colonocytes and chinese hamster ovary (CHO) cells stably
transfected with CFTR showed that approximately 50% of the
total pool of mature CFTR in the cell localized at the plasma
membrane and 50% localized intracellularly, suggesting the
existence of a large subapical CFTR pool (Lukacs et al., 1992).
Cell surface CFTR was constitutively internalized within
minutes (5–16% per min) (Lukacs et al., 1997) (Fig. 2). This
internalization rate is very similar to that of the low-density
lipoprotein (LDL) and transferrin receptor, both of which are
internalized through clathrin-coated vesicles. Indeed, func-
tionally active CFTR was identified in purified clathrin-coated
vesicles from colonocytes (Bradbury et al., 1994), and pertur-
bation of clathrin-coated vesicle formation was shown to
inhibit CFTR internalization from the plasma membrane
(Lukacs et al., 1997). Furthermore, stimulation of protein
phosphorylation by PKA or PKC caused a substantial
diminuation of the endosomal CFTR pool by partial inhibition
of CFTR internalization (Lukacs et al., 1997).

Internalization is initiated by formation of an AP-2 clathrin
coat around the budding membrane vesicle, which eventually
pinches off the plasma membrane (Fig. 2). As mentioned
earlier, the tyrosine-based, and dileucine-based targeting
signals in the cytoplasmic tails of integral membrane proteins
initiate internalization by binding to subunits of the endocytic

adaptor complex, AP-2 (Clague, 1998). Functionally active
CFTR was found in association with α-adaptin (but not with γ-
adaptin), indicating that CFTR is endocytosed via AP-2
clathrin coats (Bradbury et al., 1994). Construction of chi-
meras consisting of the C-terminal or N-terminal CFTR tail
regions fused to the transferrin receptor (TR) followed by
mutational analysis in chicken embryo fibroblasts revealed
that one specific mutation within the C-terminal CFTR-TR,
Y1424A, reduced the internalization rate by 40% (Prince
et al., 1999). As shown in a very recent study, fusion proteins
containing the C-terminus of CFTR (residues 1404–1480)
bound to AP-2 but not to the Golgi-specific adaptor complex
AP-1. As expected, the C-terminal CFTR mutation Y1424A
significantly reduced the AP-2 association with CFTR, con-
firming that the tyrosine-based signal is required for the
formation of the AP-2 complex (Weixel and Bradbury, 2000)
(Fig. 1).

Preliminary evidence indicates that CFTR co-localizes with
the microtubule-associated Rab protein Rab5 and that over-
expression of WT-CFTR in a CF cell is associated with a
redistribution of Rab5 from the plasma membrane into
discrete areas in the submembrane region (Cloutier and
Guernsey, 1999), in line with the established function of
Rab5 in regulating the homotypic SNARE docking and fusion
of clathrin-coated vesicles to eventually form the early
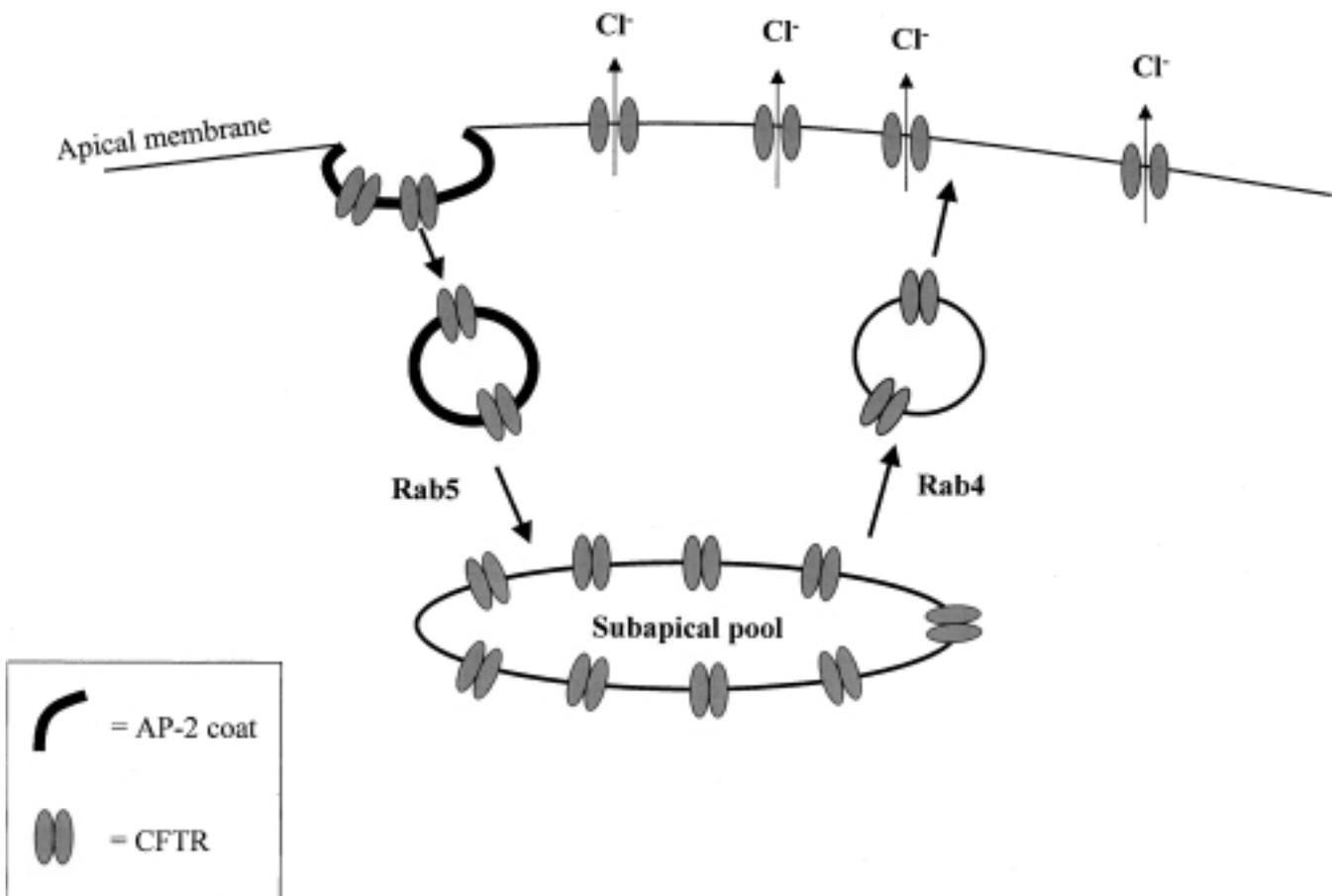endosomes (Fig. 2). Recent studies also identified Rab5 as

Fig. 2.   Role of AP-2 and Rab proteins in the constitutive endosomal recycling of CFTR. For explanation, see text.

initiator of recruiting a protein complex to the early endosomal membrane that is necessary for trafficking of early endosomes along microtubules (Pfeffer, 1999).

Endosomal recycling of CFTR
The early endosomes are thought to be an important sorting station of vesicle distribution throughout the cell. From the early endosomes proteins are targeted to the late endosomes or recycled back to the plasma membrane. Preventing the budding of clathrin-coated vesicles from the plasma membrane for 15 min resulted in a depletion of the CFTR content of early endosomes, implying that CFTR departs rapidly from this compartment (Lukacs et al., 1997). Considering its long metabolic half-life ($t_{1/2} = 12 - 16$ h) and rapid internalization rate, it is conceivable that CFTR is routed constitutively to the plasma membrane by bulk membrane flow, and avoids targeting to the late endosomes and premature lysosomal degradation. Accordingly, CFTR has been co-localized by immunogold electron microscopy with transferrin receptors and with Rab4, a regulator of membrane traffic from early endosomes back to the apical membrane, in ductal epithelium of submandibular glands (Webster et al., 1994). A role for other components of the endosomal recycling machinery has not yet been firmly established. However, considering the presence of a diphenylalanine β-COP-binding site at the N-terminus of CFTR, and the recently recognized role of COPI proteins in the assembly of endosomal COPI-coated pits (Matter, 2000) it is conceivable that COPI vesicles are involved in the recycling of CFTR from endosomes to the plasma membrane.

Exocytotic insertion of CFTR in the apical membrane

The early demonstration that CFTR expression confers cAMP regulation of endocytic and exocytic membrane traffick in epithelia suggested already that CFTR may itself undergo cAMP-regulated trafficking (Bradbury et al., 1992). Because standard immunofluorescence techniques do not have sufficient resolution to distinguish proteins in 100-nm submembrane vesicles from that in the plasma membrane, and highly specific extracellular loop CFTR antibodies are presently lacking, new approaches have been developed to monitor exocytotic insertion of CFTR into the cell surface: the use of CFTR with epitope sequences in the fourth external loop as a trafficking reporter (Schultz et al., 1997), and the monitoring of membrane capacitance (C_m) changes in conjunction with CFTR-dependent membrane ion conductance (G_m) and current measurements (I_{Cl}) (Takahashi et al., 1996). A parallel increase in surface area (as reflected by changes in C_m) and CFTR current was found upon cAMP stimulation in three different model systems expressing high levels of CFTR: primary cultures of shark rectal gland (Lehrich et al., 1998), *Xenopus* oocytes (Schultz et al., 1997), and Calu-3 cells (Hug et al., 1999b), a model for CFTR-rich airway submucosal gland cells. In the latter cell type, the increase in C_m was paralleled by a simultaneous increase in surface fluorescence of epitope-tagged CFTR, consistent with a model of cAMP-induced exocytotic CFTR insertion. Cyclic AMP-provoked increases in surface expression of CFTR have also been reported in HeLa cells (Howard et al., 1996), T84 colonocytes (Tousson et al., 1996), amphibian kidney A6 cells (Morris et al., 1998), and in the MDCK type II cell line transfected with epitope-tagged

CFTR (Howard et al., 1996). The first indication that regulated insertion of CFTR may also be of physiological relevance was provided in a recent quantitative confocal microscopical study of immunostained CFTR in rat intestinal high expressor (CHE) cells (Ameen et al., 1999). This cell type constitutes only a minor subpopulation (2.5%) of primarily villus enterocytes in rat and human small intestine and possesses very high levels of CFTR in association with a prominent subapical vesicular pool of CFTR (Ameen et al., 1995). In vivo infusion of the cAMP-linked intestinal secretagogue vasoactive intestinal peptide (VIP) in cycloheximide-pretreated rats resulted in an acute but reversible redistribution of CFTR from the cytoplasm to the apical surface, indicating that cAMP-activated exocytosis and/or cAMP-inhibited endocytosis of CFTR is not merely an artefact of cultured cells or heterologous expression systems (cf. *Xenopus* oocytes) (Ameen et al., 1999).

Others, however, were unable to document cAMP-provoked changes in C_m or endosomal recycling in CFTR-transfected CHO cells (Hug et al., 1997) (despite firm evidence for the occurrence of cAMP-inhibition of CFTR endocytosis in this cell type (Lukacs et al., 1997)) and gall bladder epithelium (Torres et al., 1996), or failed to demonstrate cAMP-stimulated GFP-CFTR translocation from an intracellular pool to the apical membrane (and regulated GFP-CFTR endocytosis) in polarized MDCK type I cells (Moyer et al., 1998). Moreover current evidence for cAMP-dependent CFTR recruitment in T84 colonocytes (Tousson et al., 1996) and Calu-3 cells (Hug et al., 1999b) is conflicting. There are at least two plausible explanations for these different results: regulated trafficking of CFTR, analogous to that of GLUT4 or aquaporin-2, may require the co-expression of specific components of the CFTR recycling machinery (e.g. SNAREs, rab proteins, possibly CAL), i.e. it is a cell type-specific function; alternatively the techniques employed for monitoring regulated CFTR trafficking are not sensitive enough to detect cAMP-induced CFTR recruitment in cells expressing a low level of endogenous CFTR or in cells in which only a small fraction of exogenously expressed CFTR is recruited to the plasma membrane in a cAMP-dependent manner.

Mechanism by which cAMP regulates CFTR traffic

Surface expression of CFTR in the *Xenopus* oocyte is almost completely cAMP-dependent, a rather unique feature that is not shared with any other known ion channel or transporter expressed in this cell model (Peters et al., 1999a). Although the molecular target of PKA-dependent protein phosphorylation responsible for cAMP-dependent recruitment of CFTR is not known with certainty, the CFTR specificity of this process and the known role of PKA in CFTR channel gating strongly suggests that phosphorylation of CFTR itself, rather than of endogenous components of the trafficking machinery, triggers its trafficking to the cell surface and/or inhibits its endocytosis. CFTR phosphorylation studies and mutational analysis of phosphorylation sites have established that PKA-dependent CFTR channel gating requires the phosphorylation of multiple serine residues (up to 10) contained in the R domain (Gadsby and Nairn, 1999b), and that mutant CFTR or CFTR half-molecules missing the R domain (CFTRΔR) are constitutively active (Csanady et al., 1999). Preliminary studies in *Xenopus* oocytes expressing R-domainless CFTR indicate that this CFTR mutant is present in the plasma membrane and yielded

a large CFTR Cl⁻ current under basal conditions (i.e. in the absence of cAMP), in line with the concept that the R-domain is essential for regulated trafficking of CFTR (Peters et al., 1999b). Clearly additional studies exploiting CFTR phosphorylation mutants (e.g. the 11SA mutant that virtually lacks PKA-mediated phosphorylation and channel gating; or constitutively active CFTR channels created by substitution of negatively charged aspartates for serines in the R domain (Sheppard and Welsh, 1999)) need to be performed to establish this model.

Currently we can only speculate about the mechanism by which R-domain phosphorylation may trigger CFTR insertion. First, one has to consider the possibility that activation of the CFTR Cl⁻ current by itself triggers exocytosis, perhaps by changing vesicular membrane potential or pH (Lukacs et al., 1993). However pharmacological modulation of CFTR channel activity by compounds acting distal of cAMP/PKA phosphorylation (e.g. genistein (French et al., 1997); the Cl⁻ channel inhibitor DPC) did not affect membrane traffic in *Xenopus* oocytes (Hug et al., 1999b). An opposite conclusion was reached in earlier studies of the G551D-CFTR regulation mutant showing that the strongly reduced Cl⁻ channel activity is accompanied by the loss of cAMP inhibition of CFTR internalization in T84 colonocytes (Bradbury, 1999). However it remains possible that the G551D mutation in NBD1 may have compromised CFTR trafficking independently of the loss of channel function, implying a need for additional studies using more specific and effective CFTR channel inhibitors like a CFTR-neutralizing antibody (Naren et al., 2000). Alternatively, considering the specific binding of the N-terminus of CFTR to the R domain (Naren et al., 1999), it is plausible that the phosphorylation of R, by changing its charge and conformation, may affect its interaction with the N-tail (i.e. increase the affinity as suggested by in vitro peptide binding data (Naren et al., 1999)) and therefore weaken the interaction of CFTR with the H3 helix of syntaxin 1A. According to this concept binding of syntaxin 1A in the plasma membrane to unphosphorylated CFTR-loaded vesicles in the subplasma membrane region might prevent the formation of a SNARE complex and membrane fusion; PKA-mediated phosphorylation of CFTR would allow the H3 helix of syntaxin 1A to dissociate from CFTR and to interact with an (unidentified) v-SNARE in the vesicles resulting in recruitment of CFTR to the cell surface (Fig. 3). Following the disassembly of the SNARE complex, syntaxin 1A might reassociate with CFTR in the apical membrane and thereby exert a dual action: inhibition of the CFTR activity, and inhibition of CFTR endocytosis (Fig. 3). This model is in line with two other recent observations: first, the ability of munc-18 and the cytosolic syntaxin 1A peptide to augment cAMP-activated CFTR Cl⁻ currents in both *Xenopus* oocytes and human airway epithelial cells, presumably through disruption of the syntaxin 1A-CFTR interaction in the plasma membrane (see above) (Naren et al., 2000); second the preliminary observation that the internalization rate of a CFTR chimera consisting of the N-tail of CFTR (residues 2–78) fused to the transmembrane and extracellular domains of the transferrin receptor and expressed in COS cells was reduced by more than 30% by the coexpression of syntaxin 1A (but not syntaxin 3) (Peter et al., 1999). On the other hand this model is difficult to reconcile with the inhibition of cAMP-induced increases in CFTR-Cl⁻ currents and C_m observed upon overexpression of syntaxin 1A but not of syntaxin 3 in the oocyte model (Peters et al., 1999a).

However, as discussed by the authors, it is possible that overexpression of a specific syntaxin isoform disrupts the pathway in which that isoform normally plays a role in membrane trafficking events. Examples of such actions include the inhibition of apical targeting of the immunoglobulin receptor by exogenous overexpression of syntaxin 3 (Low et al., 1998a), and the blockade of GLUT-4 trafficking by syntaxin 1A in pancreatic β cells (Nagamatsu et al., 1996).

Finally, the recent cloning of syntaxin 8 from a two-hybrid screen using the R domain of CFTR as a bait suggests that more direct interactions between the R domain and syntaxin isoforms may occur that may render the formation of the SNARE complex phosphorylation-dependent (Thoreau et al., 1999) (Fig. 3). Clearly additional studies are needed to elucidate the precise role of syntaxin 1A and syntaxin 8 in regulated CFTR trafficking.

Potential role of PDZ domain proteins in regulated CFTR trafficking

The role of another class of CFTR-binding partners, the PDZ domain proteins EBP50, CAP70 and CAL, in cAMP-regulated CFTR trafficking is ill-defined. Available evidence does not suggest any direct interactions between the R domain and the C-tail of CFTR, and there are no consensus sequences for PKA phosphorylation in the C-tail which potentially could affect the association of CFTR with EBP50 or CAL. However EBP50/NHERF (but not E3KARP) is a bona fide PKA substrate (Zizak et al., 1999), raising the possibility that the phosphorylation of EBP50 plays a role in CFTR trafficking, either by preventing endocytosis, or by releasing CFTR from an intracellular retention compartment (Fig. 3). The tight association of EBP50 with the AKAP ezrin is not known to be phosphorylation-dependent and might therefore ensure a close apposition of PKA II and CFTR in both the plasma membrane and the retention compartment. In this concept EBP50 may play a dual role in CFTR trafficking: providing an apical localization signal in polarized epithelial cells, and, by switching its phosphorylation status, allowing cAMP and PKA regulation of CFTR trafficking in a subset of epithelial cells equipped with the appropriate recycling machinery. Predictions of this hypothetical model, i.e. the loss of cAMP-regulated CFTR exo-and endocytosis in EBP50-deficient cells or in cells transfected with EBP50 phosphorylation mutants, and a possible overexpression of EBP50 in intestinal CHE cells or in serous airway epithelial cells, need to be verified experimentally.

Pathophysiological implications of defects in regulated CFTR trafficking

The cAMP-regulated membrane insertion of CFTR has been documented only in a limited number of tissues and cell types, expressing CFTR endogenously at relatively high levels. In some of these cell types, e.g. intestinal CHE cells; shark rectal gland epithelial cells; MDCK type II-like cells in the proximal tubule and thick ascending limb of the kidney, CFTR exocytosis may serve primarily as an amplification mechanism (in addition to cAMP-stimulated CFTR channel gating) to enhance the Cl⁻ and HCO₃⁻ secretory capacity of the epithelial cells. In other cell types, e.g. Calu-3-like serous epithelial cells in the submucosal glands of the airways (Shen et al., 1994); acinar cells in the submandibular gland (McPherson et al., 1999), this mechanism may additionally assist in the release of
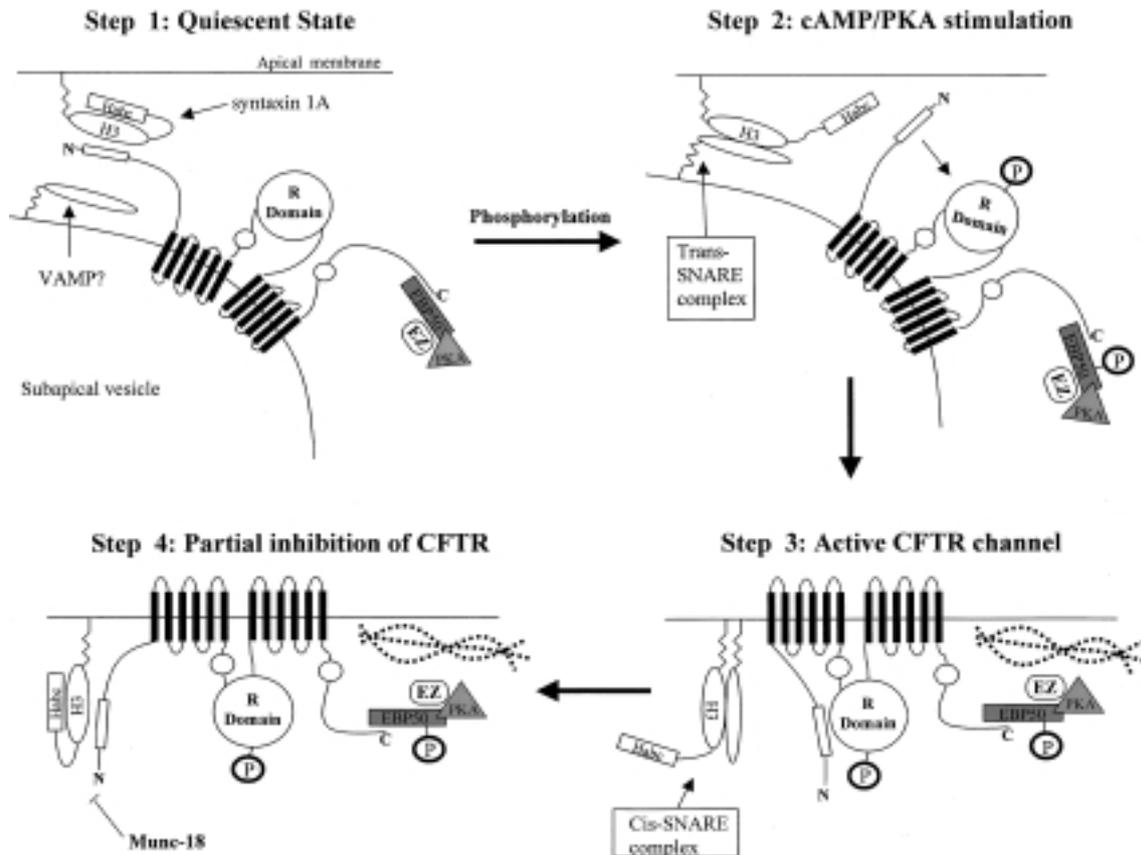
Fig. 3. Hypothetical model depicting cAMP-regulation of CFTR exo- and endocytosis at the apical membrane of epithelial cells. Cyclic AMP activates a subfraction of PKA type II associated with EBP50 and CFTR through the AKAP ezrin, resulting in phosphorylation of EBP50 and of the R domain of CFTR. Phosphorylation of R reinforces its binding to the N-tail and liberates the H3 helix of syntaxin 1A for binding to a v-SNARE and formation of a trans-SNARE complex. Following vesicle fusion and recruitment of CFTR to the cell surface, syntaxin 1A dissociates from the cis-SNARE complex and re-associates with CFTR in the apical membrane resulting in partial inhibition of CFTR Cl⁻ channel activity and inhibition of CFTR endocytosis. Dephosphorylation of R and EBP50 triggers endocytotic retrieval and recycling of CFTR to a subapical compartment.

gland secretory products, e.g. antibiotics or mucins. The submandibular gland is the clearest example of disturbed mucin secretion in response to β-adrenergic/cAMP signaling in CF cells (McPherson et al., 1986); in other mucin-secreting cell types, e.g. goblet cells in the intestine, secretion is triggered predominantly by a cAMP-independent purinergic signaling pathway rendering a direct role of CFTR in this process rather questionable (Guo et al., 1997).

As suggested almost a decade earlier (Bradbury et al., 1992), the coordinate regulation of membrane turnover as a result of cAMP-dependent CFTR exo-and endocytosis may also have implications for the recruitment and retrieval of other membrane proteins and in this way modulate additional cellular functions such as Cl⁻ transport through non-CFTR channels (e.g. ORCC (Schwiebert et al., 1999)) or ATP secretion (Schwiebert, 1999). However, unambiguous proof of such a relationship has not yet been attained in any of the model systems studied.

Because the cAMP-dependent recycling of CFTR requires the appropriate trafficking of CFTR to the apical membrane, most classes of CFTR mutations, i.e. those that affect CFTR protein synthesis, folding, export from the ER to the plasma membrane, apical sorting or protein stability, are predicted to result in disturbances of the cellular functions summarized above. Therefore, this dysfunctioning may contribute significantly to the CF phenotype such as impaired mucociliary clearance and microbial defense in the lungs or defective intestinal Cl⁻ and HCO₃⁻ secretion. In addition it is plausible that at least a portion of the class III CFTR regulation mutants suffering from defective cAMP-dependent channel gating (e.g. G551D (Bradbury, 1999)) may show additional defects in cAMP-dependent CFTR recruitment and retrieval. Provided that a role for syntaxin 1A and EBP50 in this process could be firmly established, mutations in the R domain or at the C-terminus could possibly exist that solely affect CFTR association with these proteins and result in defective cAMP-dependent trafficking of an otherwise normal CFTR channel. However, because such mutations may only lead to CF defects in a subset of epithelial cell types and tissues, CF diagnosis in such patients might be hampered by a lack of symptoms in tissues probed routinely for diagnosis, e.g. the sweat gland. Identification of these patients could be of great help in delineating the physiological importance of cAMP-dependent CFTR trafficking. Furthermore, in view of future strategies to increase anterograde trafficking of class II folding CFTR mutants (e.g. ΔF508) as a possible therapeutic approach in CF,

it would be of interest to learn whether these mutants, beside their virtually normal cAMP-dependent gating characteristics, also display a normal behaviour with regard to cAMP-regulated membrane insertion and retrieval.

Future directions

At this point in time detailed knowledge about the molecular mechanism underlying polarized and regulated trafficking of CFTR in epithelial cells and about its (patho)physiological importance is only beginning to emerge, and many questions remain to be answered. For example, the operation of cAMP-regulated trafficking of CFTR in cells showing a relatively low CFTR protein expression, such as (non-CHE) intestinal crypt and villus cells, endothelial cells (Tousson et al., 1998), and neuroendocrine cells (Weyler et al., 1999), has not yet been shown unambiguously but may become an important area of future research. The development of more sensitive and specific, optical and CFTR tagging methodologies to improve the visualization of CFTR trafficking and recycling (e.g. the use of GFP-CFTR in combination with multi-photon fluorescence microscopy), and the use of transgenic knock-in mice expressing tagged versions of CFTR may greatly facilitate such studies. Clearly, much has still to be learned about the identity and function of v- and t-SNAREs in CFTR-expressing epithelial cells and about the role of CFTR-associated proteins in regulated trafficking (e.g. EBP50; CAL; CAP70; syntaxin 1A and 8; possibly other candidate proteins that may emerge from yeast two-hybrid screens or co-immunoprecipitations). Again, transgenic mouse models such as epithelial-specific EBP50-, syntaxin 1A-, or CAL-knock-out mice or knock-in mice for CFTR bearing a specific regulated trafficking mutation may prove to be very useful in future analysis of the physiological role of these CFTR partners in native CFTR-expressing tissues. Currently there is also a paucity of information about the possible role of other, non-cAMP/PKA-mediated signal transduction mechanisms in regulating the density of CFTR in the cell surface. Possible candidates include *1)* the cGMP/PKG-II pathway that operates as a major CFTR phosphorylating and activating mechanism in the intestine (Lohmann et al., 1997); *2)* the protein kinase C pathway that not only phosphorylates and fine-regulates the CFTR channel itself but may additionally affect the channel density through the phosphorylation of cytoskeletal components (Gadsby and Nairn, 1999a); and, *3)*, the phosphoinositide 3-kinase (PI3kinase) pathway that, through generating specific inositol lipids recognized by pleckstrin-homology (PH) domains in other proteins including the protein kinases PDK1 and PKB (Vanhaesebroeck and Alessi, 2000), plays an important role in the endocytosis of ion transporters in the apical membrane of epithelial cells (e.g. the EBP50-associated Na^+/H^+ exchanger NHE3 (Janecki et al., 2000)). Finally, a more extensive knowledge of the endogenously expressed regulatory proteins involved in cAMP/PKA-dependent CFTR trafficking in *Xenopus* oocytes, i.e. homologues of syntaxin 1A or EBP50, would facilitate the interpretation of results obtained from studies in this important model system. Considering the potential importance of CFTR exo- and endocytosis in the CFTR regulation of multiple cellular functions discussed in this review it is anticipated that such studies may lead to a better understanding of CF pathophysiology and may assist in improving CF diagnosis and future CF therapy.

References

Ameen, N. A., Ardito, T., Kashgarian, M., Marino, C. R. (1995): A unique subset of rat and human intestinal villus cells express the cystic fibrosis transmembrane conductance regulator. Gastroenterology **108**, 1016–1023.

Ameen, N. A., Martensson, B., Bourguinon, L., Marino, C., Isenberg, J., McLaughlin, G. E. (1999): CFTR channel insertion to the apical surface in rat duodenal villus epithelial cells is upregulated by VIP in vivo. J. Cell Sci. **112**, 887–894.

Aridor, M., Bannykh, S. I., Rowe, T., Balch, W. E. (1995): Sequential coupling between COPII and COPI vesicle coats in endoplasmic reticulum to Golgi transport. J. Cell Biol. **131**, 875–893.

Becq, F., Hamon, Y., Bajetto, A., Gola, M., Verrier, B., Chimini, G. (1997): ABC1, an ATP binding cassette transporter required for phagocytosis of apoptotic cells, generates a regulated anion flux after expression in *Xenopus laevis* oocytes. J. Biol. Chem. **272**, 2695–2699.

Benting, J. H., Rietveld, A. G., Simons, K. (1999): N-Glycans mediate the apical sorting of a GPI-anchored, raft-associated protein in Madin-Darby canine kidney cells. J. Cell Biol. **146**, 313–320.

Bonifacino, J. S., Dell'Angelica, E. C. (1999): Molecular bases for the recognition of tyrosine-based sorting signals. J. Cell Biol. **145**, 923–926.

Bradbury, N. A. (1999): Intracellular CFTR: localization and function. Physiol. Rev. **79**, S175–191.

Bradbury, N. A., Clark, J. A., Watkins, S. C., Widnell, C. C., Smith, H. S. T., Bridges, R. J. (1999): Characterization of the internalization pathways for the cystic fibrosis transmembrane conductance regulator. Am. J. Physiol. **276**, L659–668.

Bradbury, N. A., Cohn, J. A., Venglarik, C. J., Bridges, R. J. (1994): Biochemical and biophysical identification of cystic fibrosis transmembrane conductance regulator chloride channels as components of endocytic clathrin-coated vesicles. J. Biol. Chem. **269**, 8296–8302.

Bradbury, N. A., Jilling, T., Berta, G., Sorscher, E. J., Bridges, R. J., Kirk, K. L. (1992): Regulation of plasma membrane recycling by CFTR. Science **256**, 530–532.

Chanson, M., Scerri, I., Suter, S. (1999): Defective regulation of gap junctional coupling in cystic fibrosis pancreatic duct cells. J. Clin. Invest. **103**, 1677–1684.

Chavrier, P., Goud, B. (1999): The role of ARF and Rab GTPases in membrane transport. Curr. Opin. Cell Biol. **11**, 466–475.

Cheng, J., Moyer, B. D., Milewski, M., Hazama, A., Mickle, J. E., Cutting, G. R., Stanton, B. A., Guggino, W. B. (1999): CFTR associates with the PDZ domain protein CAL at the trans-Golgi network. Pediatr. Pulmonol. Suppl. **19**, 168 (abstr.).

Clague, M. J. (1998): Molecular aspects of the endocytic pathway. Biochem. J. **336**, 271–282.

Clarke, L. L., Harline, M. C. (1996): CFTR is required for cAMP inhibition of intestinal Na^+ absorption in a cystic fibrosis mouse model. Am. J. Physiol. **270**, G259–267.

Cloutier, M. M., Guernsey, L. (1999): Rab5 and tubulin distribution in CF and normal airway epithelial cells. Pediatr. Pulmonol. Suppl. **19**, 194 (abstr.).

Cosson, P., Letourneur, F. (1994): Coatomer interaction with di-lysine endoplasmic reticulum retention motifs. Science **263**, 1629–1631.

Csanady, L., Chan, K. W., Seto-Young, D., Nairn, A. C., Gadsby, D. C. (1999): Coexpression of two halves of CFTR lacking the R domain results in near fully functional channels. Pediatr. Pulmonol. Suppl. **19**, 174 (abstr.).

Deen, P. M. T., van Balkom, B., Kamsteeg, E.-J. (2000): Routing of the aquaporin-2 water channel in health and disease. Eur. J. Cell Biol. **79**, 523–530.

Dunbar, L. A., Caplan, M. J. (2000): The cell biology of ion pumps: sorting and regulation. Eur. J. Cell Biol. **79**, 557–563.

Fanning, A. S., Anderson, J. M. (1999): PDZ domains: fundamental building blocks in the organization of protein complexes at the plasma membrane. J. Clin. Invest. **103**, 767–772.

Fiedler, K., Veit, M., Stamnes, M. A., Rothman, J. E. (1996): Bimodal interaction of coatomer with the p24 family of putative cargo receptors. Science **273**, 1396–1399.

Folsch, H., Ohno, H., Bonifacino, J. S., Mellman, I. (1999): A novel clathrin adaptor complex mediates basolateral targeting in polarized epithelial cells. Cell **99**, 189–198.

French, P. J., Bijman, J., Bot, A. G., Boomaars, W. E., Scholte, B. J., de Jonge, H. R. (1997): Genistein activates CFTR Cl⁻ channels via a tyrosine kinase- and protein phosphatase-independent mechanism. Am. J. Physiol. **273**, C747–753.

Gadsby, D. C., Dousmanis, A. G., Nairn, A. C. (1998): ATP hydrolysis cycles and the gating of CFTR Cl⁻ channels. Acta Physiol. Scand. Suppl. **643**, 247–256.

Gadsby, D. C., Nairn, A. C. (1999a): Control of CFTR channel gating by phosphorylation and nucleotide hydrolysis. Physiol. Rev. **79**, S77–S107.

Gadsby, D. C., Nairn, A. C. (1999b): Regulation of CFTR Cl⁻ ion channels by phosphorylation and dephosphorylation. Adv. Second Messenger Phosphoprotein Res. **33**, 79–106.

Guo, X. W., Merlin, D., Laboisse, C., Hopfer, U. (1997): Purinergic agonists, but not cAMP, stimulate coupled granule fusion and Cl⁻ conductance in HT29-Cl.16E. Am. J. Physiol. **273**, C804–809.

Hanson, P. I., Heuser, J. E., Jahn, R. (1997): Neurotransmitter release – four years of SNARE complexes. Curr. Opin. Neurobiol. **7**, 310–315.

Howard, M., DuVall, M. D., Devor, D. C., Dong, J. Y., Henze, K., Frizzell, R. A. (1995): Epitope tagging permits cell surface detection of functional CFTR. Am. J. Physiol. **269**, C1565–1576.

Howard, M., Jilling, T., DuVall, M., Frizzell, R. A. (1996): cAMP-regulated trafficking of epitope-tagged CFTR. Kidney Int. **49**, 1642–1648.

Huang, P., Trotter, K., Boucher, R. C., Milgram, S. L., Stutts, M. J. (2000): PKA holoenzyme is functionally coupled to CFTR by AKAPs. Am. J. Physiol. Cell Physiol. **278**, C417–422.

Hug, M. J., Sun, F., Bradbury, N. A., Watkins, S. C., Frizzell, R. A. (1999a): AKAPS are required for the activation of ion transport and membrane traffick in epithelial cells. Pediatr. Pulmonol. Suppl. **19**, 183 (abstr.).

Hug, M. J., Sun, F., Frizzell, R. A. (1999b): cAMP increases membrane conductance and membrane capacitance in Calu-3 cells. Pediatr. Pulmonol. Suppl. **19**, 183 (abstr.).

Hug, M. J., Thiele, I. E., Greger, R. (1997): The role of exocytosis in the activation of the chloride conductance in Chinese hamster ovary cells (CHO) stably expressing CFTR. Pflügers Arch. **434**, 779–784.

Ikonen, E., Tagaya, M., Ullrich, O., Montecucco, C., Simons, K. (1995): Different requirements for NSF, SNAP, and Rab proteins in apical and basolateral transport in MDCK cells. Cell **81**, 571–580.

Janecki, A. J., Janecki, M., Akhter, S., Donowitz, M. (2000): Basic fibroblast growth factor stimulates surface expression and activity of Na⁽⁺⁾/H⁽⁺⁾ exchanger NHE3 via mechanism involving phosphatidyl-inositol 3-kinase. J. Biol. Chem. **275**, 8133–8142.

Ji, H., Chalfant, M. L., Fuller, C. M., Stanton, B. A., Benos, D. J. (1999): Up-regulation by rENAC of CFTR current expressed in *Xenopus* oocytes. Pediatr. Pulmonol. Suppl. **19**, 201 (abstr.).

Jia, Y., Loo, M. A., Jensen, T. J., Chang, X. B., Riordan, J. R., Hanrahan, J. W. (1999): Tyrosines 625 and 627 are not essential for Src activation of CFTR chloride channel. Pediatr. Pulmonol. Suppl. **19**, 176 (abstr.).

Kant, J. A., Mifflin, T. E., McGlennen, R., Rice, E., Naylor, E., Cooper, D. L. (1995): Molecular diagnosis of cystic fibrosis. Clin. Lab. Med. **15**, 877–898.

Kartner, N., Augustinas, O., Jensen, T. J., Naismith, A. L., Riordan, J. R. (1992): Mislocalization of delta F508 CFTR in cystic fibrosis sweat gland. Nature Genet. **1**, 321–327.

Krauss, R. D., Bubien, J. K., Drumm, M. L., Zheng, T., Peiper, S. C., Collins, F. S., Kirk, K. L., Frizzell, R. A., Rado, T. A. (1992): Transfection of wild-type CFTR into cystic fibrosis lymphocytes restores chloride conductance at G1 of the cell cycle. EMBO J. **11**, 875–883.

Kuehn, M. J., Schekman, R. (1997): COPII and secretory cargo capture into transport vesicles. Curr. Opin. Cell Biol. **9**, 477–483.

Lamprecht, G., Weinman, E. J., Yun, C. H. (1998): The role of NHERF and E3KARP in the cAMP-mediated inhibition of NHE3. J. Biol. Chem. **273**, 29972–29978.

Lee, M. G., Wigley, W. C., Zeng, W., Noel, L. E., Marino, C. R., Thomas, P. J., Muallem, S. (1999): Regulation of Cl⁻/HCO₃⁻ exchange by cystic fibrosis transmembrane conductance regulator expressed in NIH 3T3 and HEK 293 cells. J. Biol. Chem. **274**, 3414–3421.

Lehrich, R. W., Aller, S. G., Webster, P., Marino, C. R., Forrest, J. N., Jr. (1998): Vasoactive intestinal peptide, forskolin, and genistein increase apical CFTR trafficking in the rectal gland of the spiny dogfish, *Squalus acanthias*. Acute regulation of CFTR trafficking in an intact epithelium. J. Clin. Invest. **101**, 737–745.

Levkowitz, G., Waterman, H., Zamir, E., Kam, Z., Oved, S., Langdon, W. Y., Beguinot, L., Geiger, B., Yarden, Y. (1998): c-Cbl/Sli-1 regulates endocytic sorting and ubiquitination of the epidermal growth factor receptor. Genes Dev. **12**, 3663–3674.

Lohmann, S. M., Vaandrager, A. B., Smolenski, A., Walter, U., De Jonge, H. R. (1997): Distinct and specific functions of cGMP-dependent protein kinases. Trends Biochem. Sci. **22**, 307–312.

Low, S. H., Chapin, S. J., Wimmer, C., Whiteheart, S. W., Komuves, L. G., Mostov, K. E., Weimbs, T. (1998a): The SNARE machinery is involved in apical plasma membrane trafficking in MDCK cells. J. Cell Biol. **141**, 1503–1513.

Low, S. H., Roche, P. A., Anderson, H. A., van Ijzendoorn, S. C., Zhang, M., Mostov, K. E., Weimbs, T. (1998b): Targeting of SNAP-23 and SNAP-25 in polarized epithelial cells. J. Biol. Chem. **273**, 3422–3430.

Lukacs, G. L., Chang, X. B., Kartner, N., Rotstein, O. D., Riordan, J. R., Grinstein, S. (1992): The cystic fibrosis transmembrane regulator is present and functional in endosomes. Role as a determinant of endosomal pH. J. Biol. Chem. **267**, 14568–14572.

Lukacs, G. L., Kapus, A., Nanda, A., Romanek, R., Grinstein, S. (1993): Proton conductance of the plasma membrane: properties, regulation, and functional role. Am. J. Physiol. **265**, C3–14.

Lukacs, G. L., Segal, G., Kartner, N., Grinstein, S., Zhang, F. (1997): Constitutive internalization of cystic fibrosis transmembrane conductance regulator occurs via clathrin-dependent endocytosis and is regulated by protein phosphorylation. Biochem. J. **328**, 353–361.

Markovich, D. (2000): Molecular regulation and membrane trafficking of mammalian renal phosphate and sulphate transporters. Eur. J. Cell Biol. **79**, 531–538.

Matter, K. (2000): Epithelial polarity: sorting out the sorters. Curr. Biol. **10**, R39–42.

McPherson, M. A., Dormer, R. L., Bradbury, N. A., Dodge, J. A., Goodchild, M. C. (1986): Defective beta-adrenergic secretory responses in submandibular acinar cells from cystic fibrosis patients. Lancet **2**, 1007–1008.

McPherson, M. A., Pereira, M. M., Lloyd Mills, C., Murray, K. J., Dormer, R. L. (1999): A cyclic nucleotide PDE5 inhibitor corrects defective mucin secretion in submandibular cells containing antibody directed against the cystic fibrosis transmembrane conductance regulator protein. FEBS Lett. **464**, 48–52.

Mohler, P. J., Kreda, S. M., Boucher, R. C., Sudol, M., Stutts, M. J., Milgram, S. L. (1999): Yes-associated protein 65 localizes p62(c-Yes) to the apical compartment of airway epithelia by association with EBP50. J. Cell Biol. **147**, 879–890.

Morris, A. P., Cunningham, S. A., Benos, D. J., Frizzell, R. A. (1993): Glycosylation status of endogenous CFTR does not affect cAMP-stimulated Cl⁻ secretion in epithelial cells. Am. J. Physiol. **265**, C688–694.

Morris, R. G., Tousson, A., Benos, D. J., Schafer, J. A. (1998): Micro-tubule disruption inhibits AVT-stimulated Cl⁻ secretion but not Na⁺ reabsorption in A6 cells. Am. J. Physiol. **274**, F300–314.

Moyer, B. D., Denton, J., Karlson, K. H., Reynolds, D., Wang, S., Mickle, J. E., Milewski, M., Cutting, G. R., Guggino, W. B., Li, M., Stanton, B. A. (1999): A PDZ-interacting domain in CFTR is an apical membrane polarization signal. J. Clin. Invest. **104**, 1353–1361.

Moyer, B. D., Loffing, J., Schwiebert, E. M., Loffing-Cueni, D., Halpin, P. A., Karlson, K. H., Ismailov, II, Guggino, W. B., Langford, G. M., Stanton, B. A. (1998): Membrane trafficking of the cystic fibrosis gene product, cystic fibrosis transmembrane conductance regulator,

tagged with green fluorescent protein in Madin-Darby canine kidney cells. J. Biol. Chem. **273**, 21759–21768.

Muth, T. R., Ahn, J., Caplan, M. J. (1998): Identification of sorting determinants in the C-terminal cytoplasmic tails of the gamma-aminobutyric acid transporters GAT-2 and GAT-3. J. Biol. Chem. **273**, 25616–25627.

Nagamatsu, S., Fujiwara, T., Nakamichi, Y., Watanabe, T., Katahira, H., Sawa, H., Akagawa, K. (1996): Expression and functional role of syntaxin 1/HPC-1 in pancreatic beta cells. Syntaxin 1A, but not 1B, plays a negative role in regulatory insulin release pathway. J. Biol. Chem. **271**, 1160–1165.

Naren, A. P., Cormet-Boyaka, E., Fu, J., Villain, M., Blalock, J. E., Quick, M. W., Kirk, K. L. (1999): CFTR chloride channel regulation by an interdomain interaction. Science **286**, 544–548.

Naren, A. P., Di, A., Cormet-Boyaka, E., Boyaka, P. N., McGhee, J. R., Zhou, W., Akagawa, K., Fujiwara, T., Thome, U., Engelhardt, J. F., Nelson, D. J., Kirk, K. L. (2000): Syntaxin 1A is expressed in airway epithelial cells, where it modulates CFTR Cl$^{(-)}$ currents. J. Clin. Invest. **105**, 377–386.

Naren, A. P., Nelson, D. J., Xie, W., Jovov, B., Pevsner, J., Bennett, M. K., Benos, D. J., Quick, M. W., Kirk, K. L. (1997): Regulation of CFTR chloride channels by syntaxin and Munc18 isoforms. Nature **390**, 302–305.

Naren, A. P., Quick, M. W., Collawn, J. F., Nelson, D. J., Kirk, K. L. (1998): Syntaxin 1A inhibits CFTR chloride channels by means of domain-specific protein-protein interactions. Proc. Natl. Acad. Sci. USA **95**, 10972–10977.

Olenych, S. G., Teem, J. L. (1999): A diphenylalanine motif in the amino terminus of CFTR binds beta-COP, a component of COPI secretory vesicles. Pediatr. Pulmonol. Suppl **19**, 185 (abstr.).

Orci, L., Stamnes, M., Ravazzola, M., Amherdt, M., Perrelet, A., Sollner, T. H., Rothman, J. E. (1997): Bidirectional transport by distinct populations of COPI-coated vesicles. Cell **90**, 335–349.

Perego, C., Vanoni, C., Villa, A., Longhi, R., Kaech, S. M., Frohli, E., Hajnal, A., Kim, S. K., Pietrini, G. (1999): PDZ-mediated interactions retain the epithelial GABA transporter on the basolateral surface of polarized epithelial cells. EMBO J. **18**, 2384–2393.

Pessin, J. E., Thurmond, D. C., Elmendorf, J. S., Coker, K. J., Okada, S. (1999): Molecular basis of insulin-stimulated GLUT4 vesicle trafficking. Location! Location! Location! J. Biol. Chem. **274**, 2593–2596.

Peter, K., Naren, A., Kirk, K. L., Schwiebert, M., Sorscher, E. J., Collawn, J. F. (1999): Regulation of CFTR endocytosis by syntaxin 1A. Pediatr. Pulmonol. Suppl. **19**, 174 (abstr.).

Peters, K. W., Qi, J., Watkins, S. C., Frizzell, R. A. (1999a): Syntaxin 1A inhibits regulated CFTR trafficking in *Xenopus* oocytes. Am. J. Physiol. **277**, C174–180.

Peters, K. W., Qi, J. J., Watkins, S. C., Frizzell, R. A. (1999b): SNARE proteins mediate cAMP-stimulated CFTR trafficking in *Xenopus* oocytes. Pediatr. Pulmonol. Suppl. **19**, 191 (abstr.).

Pfeffer, S. R. (1999): Transport-vesicle targeting: tethers before SNAREs. Nature Cell Biol. **1**, E17–22.

Prince, L. S., Peter, K., Hatton, S. R., Zaliauskiene, L., Cotlin, L. F., Clancy, J. P., Marchase, R. B., Collawn, J. F. (1999): Efficient endocytosis of the cystic fibrosis transmembrane conductance regulator requires a tyrosine-based signal. J. Biol. Chem. **274**, 3602–3609.

Riordan, J. R. (1999): Therapeutic strategies for treatment of CF based on knowledge of CFTR. Pediatr. Pulmonol. Suppl. **18**, 83–87 (abstr.).

Riordan, J. R., Rommens, J. M., Kerem, B., Alon, N., Rozmahel, R., Grzelczak, Z., Zielenski, J., Lok, S., Plavsic, N., Chou, J. L., et al. (1989): Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. Science **245**, 1066–1073.

Rodriguez-Boulan, E., Gonzalez, A. (1999): Glycans in post-Golgi apical targeting: sorting signals or structural props? Trends Cell Biol. **9**, 291–294.

Saxena, S., Quick, M. W., Tousson, A., Oh, Y., Warnock, D. G. (1999): Interaction of syntaxins with the amiloride-sensitive epithelial sodium channel. J. Biol. Chem. **274**, 20812–20817.

Schekman, R., Orci, L. (1996): Coat proteins and vesicle budding. Science **271**, 1526–1533.

Schultz, B. D., Takahashi, A., Liu, C., Frizzell, R. A., Howard, M. (1997): FLAG epitope positioned in an external loop preserves normal biophysical properties of CFTR. Am. J. Physiol. **273**, C2080–2089.

Schwiebert, E. M. (1999): ABC transporter-facilitated ATP conductive transport. Am. J. Physiol. **276**, C1–8.

Schwiebert, E. M., Benos, D. J., Egan, M. E., Stutts, M. J., Guggino, W. B. (1999): CFTR is a conductance regulator as well as a chloride channel. Physiol. Rev. **79**, S145–166.

Seibert, F. S., Loo, T. W., Clarke, D. M., Riordan, J. R. (1997): Cystic fibrosis: channel, catalytic, and folding properties of the CFTR protein. J. Bioenerg. Biomembr. **29**, 429–442.

Shen, B. Q., Finkbeiner, W. E., Wine, J. J., Mrsny, R. J., Widdicombe, J. H. (1994): Calu-3: a human airway epithelial cell line that shows cAMP-dependent Cl$^-$ secretion. Am. J. Physiol. **266**, L493–501.

Sheppard, D. N., Welsh, M. J. (1999): Structure and function of the CFTR chloride channel. Physiol. Rev. **79**, S23–45.

Short, D. B., Trotter, K. W., Reczek, D., Kreda, S. M., Bretscher, A., Boucher, R. C., Stutts, M. J., Milgram, S. L. (1998): An apical PDZ protein anchors the cystic fibrosis transmembrane conductance regulator to the cytoskeleton. J. Biol. Chem. **273**, 19797–19801.

Sun, F., Hug, M. J., Yun, C. H., Bradbury, N. A., Frizzell, R. A. (1999): The PDZ domain-containing protein, E3KARP couples ezrin to CFTR. Pediatr. Pulmonol. Suppl. **19**, 165 (abstr.).

Sun, F., Hug, M. J., Bradbury, N. A., Frizzell, R. A. (2000): Protein kinase A associates with CFTR via an interaction with ezrin. J. Biol. Chem. **275**, 14360–14366.

Takahashi, A., Watkins, S. C., Howard, M., Frizzell, R. A. (1996): CFTR-dependent membrane insertion is linked to stimulation of the CFTR chloride conductance. Am. J. Physiol. **271**, C1887–1894.

Thoreau, V., Berges, T., Callebaut, I., Guillier-Gencik, Z., Gressin, L., Bernheim, A., Karst, F., Mornon, J. P., Kitzis, A., Chomel, J. C. (1999): Molecular cloning, expression analysis, and chromosomal localization of human syntaxin 8 (STX8). Biochem. Biophys. Res. Commun. **257**, 577–583.

Torres, R. J., Altenberg, G. A., Cohn, J. A., Reuss, L. (1996): Polarized expression of cAMP-activated chloride channels in isolated epithelial cells. Am. J. Physiol. **271**, C1574–1582.

Tousson, A., Fuller, C. M., Benos, D. J. (1996): Apical recruitment of CFTR in T-84 cells is dependent on cAMP and microtubules but not Ca^{2+} or microfilaments. J. Cell Sci. **109**, 1325–1334.

Tousson, A., Van Tine, B. A., Naren, A. P., Shaw, G. M., Schwiebert, L. M. (1998): Characterization of CFTR expression and chloride channel activity in human endothelia. Am. J. Physiol. **275**, C1555–1564.

Valverde, M. A., O'Brien, J. A., Sepulveda, F. V., Ratcliff, R. A., Evans, M. J., Colledge, W. H. (1995): Impaired cell volume regulation in intestinal crypt epithelia of cystic fibrosis mice. Proc. Natl. Acad. Sci. USA **92**, 9038–9041.

Vanhaesebroeck, B., Alessi, D. R. (2000): The PI3K-PDK1 connection: more than just a road to PKB. Biochem. J. **346**, 561–576.

Wang, S., Yu, H., Derin, R. B., Guggino, W. B., Li, M. (1999): CAP70, a positive regulator for the chloride channel activity of CFTR. Pediatr. Pulmonol. Suppl. **19**, 246 (abstr.).

Webster, P., Vanacore, L., Nairn, A. C., Marino, C. R. (1994): Subcellular localization of CFTR to endosomes in a ductal epithelium. Am. J. Physiol. **267**, C340–348.

Wei, L., Vankeerberghen, A., Cuppens, H., Eggermont, J., Cassiman, J. J., Droogmans, G., Nilius, B. (1999): Interaction between calcium-activated chloride channels and the cystic fibrosis transmembrane conductance regulator. Pflügers Arch. **438**, 635–641.

Weixel, K. M., Bradbury, N. A. (2000): The carboxyl terminus of the cystic fibrosis transmembrane conductance regulator binds to AP-2 clathrin adaptors. J. Biol. Chem. **275**, 3655–3660.

Weyler, R. T., Yurko-Mauro, K. A., Rubenstein, R., Kollen, W. J., Reenstra, W., Altschuler, S. M., Egan, M., Mulberg, A. E. (1999): CFTR is functionally active in GnRH-expressing GT1–7 hypothalamic neurons. Am. J. Physiol. **277**, C563–571.

Yun, C. H., Oh, S., Zizak, M., Steplock, D., Tsao, S., Tse, C. M., Weinman, E. J., Donowitz, M. (1997): cAMP-mediated inhibition of the epithelial brush border Na$^+$/H$^+$ exchanger, NHE3, requires an associated regulatory protein. Proc. Natl. Acad. Sci. USA **94**, 3010 – 3015.

Zizak, M., Lamprecht, G., Steplock, D., Tariq, N., Shenolikar, S., Donowitz, M., Yun, C. H., Weinman, E. J. (1999): cAMP-induced phosphorylation and inhibition of Na$^{(+)}$/H$^{(+)}$ exchanger 3 (NHE3) are dependent on the presence but not the phosphorylation of NHE regulatory factor. J. Biol. Chem. **274**, 24753 – 24758.