Vesicular targeting and the control of ion secretion in epithelial cells: implications for cystic fibrosis

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Non-polarized HT-29 colonic epithelial cells fail to respond to cyclic AMP-generating agonists with increases in plasma membrane anion conduction. Radio-isotopic efflux and patch-clamp experiments revealed that both undifferentiated and differentiated HT-29 colonocytes possess volume- and Ca^{2+}-activated Cl^- channels. However, only within the apical plasma membranes of the latter were cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channels found. CFTR was expressed equally well in both non-polarized and polarized colonocytes. Lack of CFTR-dependent anion conduction was shown to be the result of CFTR retention within a peripheral intracellular compartment. We demonstrate that upon polarization, CFTR moves to the apical plasma membrane via a Brefeldin A (BFA)-sensitive intracellular trafficking pathway.

Over the last decade modern electrophysiological approaches have characterized many of the conductive pathways involved in epithelial cell fluid secretion. This exploration of ion channels has revealed that regulatory influences reside not only at the level of ion channel function but also at the level of ion channel expression within the epithelial cell plasma membrane. Modern cell biological approaches are now being used to complement studies concerned with the biophysical basis of ion secretion to address how channels arrive at their correct membrane location. The field of cystic fibrosis stands as a prime example of this paradigm. Following the discovery of the cystic fibrosis transmembrane conductance regulator gene (CFTR, Riordan *et al.* 1989), electrophysiological evidence was amassed describing the single channel basis of this cyclic AMP-stimulated Cl^- conductance (Anderson *et al.* 1991; Cheng *et al.* 1991; Kartner *et al.* 1991; Bear *et al.* 1992). Originally, it was hypothesized that mutations within the gene resulted in defective anion channel function. However, some recombinant expression systems have demonstrated that the majority of mutant CFTRs are capable of conducting anions if the protein is inserted into the plasma membrane (Drumm *et al.* 1991). Nevertheless it is clear that defective CFTR protein moves inefficiently through the early cellular biosynthetic pathway when heterologously expressed (Cheng *et al.* 1990) (Fig. 1). Thus it is believed that intracellular quality control processes lead to the retention of mutant CFTR channels within the biosynthetic pathway and account for the loss of cyclic AMP-stimulated fluid secretion observed in CF epithelia.

Our interest in understanding the cell biology behind intracellular transport of normal CFTR protein resulted from the observation that changes in cyclic AMP-dependent anion conduction occurred during epithelial cell polarization. To study this phenomenon we have employed the human colonocyte HT-29 cell line model in which many of the individual ion transporting processes involved in transepithelial solute movement have been characterized (Morris & Frizzell, 1994). We found that while non-polarized HT-29 cells responded to changes in both volume and intracellular Ca^{2+} with the activation of separate plasma membrane anion channel populations, hormones and cellular agonists which raised intracellular cyclic AMP levels failed to provoke a similar plasma membrane Cl^- conductive response. In contrast, the polarized counterparts of this cell line were capable of maintaining sustained transepithelial Na^+ and Cl^- secretion across electrically tight monolayers. In addition, patch clamp studies revealed that CFTR Cl^- channels were present in the apical plasma membranes of polarized cells challenged with cyclic AMP agonists (Morris *et al.* 1992).

Cellular polarity and anion secretion

The attainment of cyclic AMP-dependent anion conduction was correlated with the differentiation status of HT-29 cells by using a radioisotopic iodide efflux assay (Fig. 2). This method measures unidirectional radiotracer movement across all plasma membranes. Thus the assay is independent of cellular polarization (Fig. 2). Non-polarized HT-29 colonocytes monitored at 4 days post-seeding gave negligible changes in net efflux rate when challenged with the cyclic AMP-elevating agonist forskolin. However, the polarized

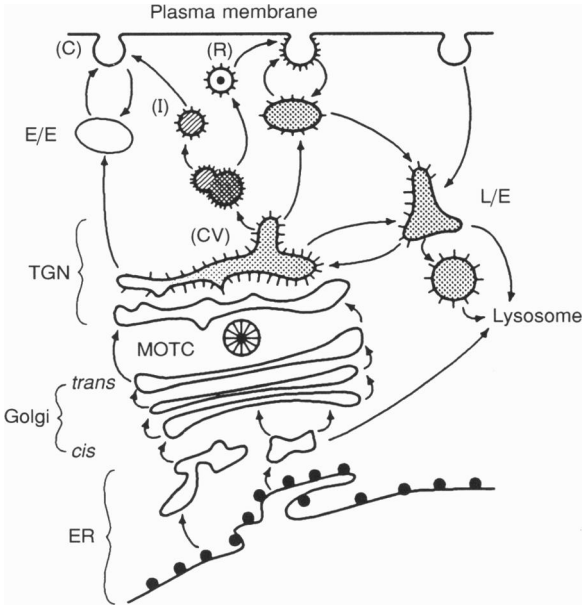

Fig. 1. ER = endoplasmic reticulum, E/E = early endosome, L/E = late endosome, TGN = *trans*-Golgi network, CV = condensing vacuole of the TGN, (R) = regulated secretion, (C) = constitutive secretion, (I) = immature secretory granule, MTOC = microtubule organizing centre, || = clathrin coat.

sub-clone of this cell line (Cl.19A), assayed at similar times post-seeding, gave a nearly fourfold increase in net efflux rate. High resolution electron microscopy performed on the same monolayers clearly demonstrated that this phenomenon could be linked to the lack of polarity observed within the non-polarized cell line. Corroborating these findings, growth of the parental cell line under conditions that induced differentiation induced cyclic AMP-sensitive ^{125}I efflux (Morris *et al.* 1992). These experiments were controlled by measuring intracellular cyclic AMP levels directly following forskolin addition. It was determined that differences in anion conducting responsiveness were not related to an inability of non-polarized cells to increase their levels of this second messenger.

The role of CFTR in the control of cyclic AMP-dependent anion secretion

The discovery that CFTR was responsible for cAMP-dependent exit from epithelial cells presented the opportunity to investigate the cellular basis of this differentiation-dependent Cl^- secretory phenomenon. CFTR is an N-linked glycoprotein predicted to consist of 1480 amino acids arranged into 12 membrane-spanning α-helices, two nucleotide-binding domains and a central cytoplasmic loop. This latter domain of approximately 240 amino acids has been postulated to regulate the anion-conducting properties of CFTR.

To demonstrate that CFTR-dependent effects on cyclic AMP-dependent Cl^- secretion were present in our colonocyte model, the levels of both CFTR mRNA and protein were quantified. Northern blot hybridization was performed on polyA^+-mRNA isolated from Cl.19A cells at days 1–21 post-seeding. A $\alpha^{32}P$-labelled PCR probe made against bases 1773–2654 of CFTR that corresponded to the cytoplasmic R-domain was employed. The results revealed that the levels of CFTR message remained constant during this period, as assessed by the ratio of CFTR to γ-actin message. This was surprising since Cl.19A colonocytes do not attain cyclic AMP-dependent Cl^- secretory capacity until monolayers have formed at 4–6 days post-seeding. Maximum secretory capacity is reached around 9–12 days and thereafter remains constant (up to 78 days post-seeding). This lack of correlation between CFTR message and cyclic AMP-dependant secretion was further emphasized when CFTR message levels were probed in the non-polarized parental colonocyte cell line. They appeared

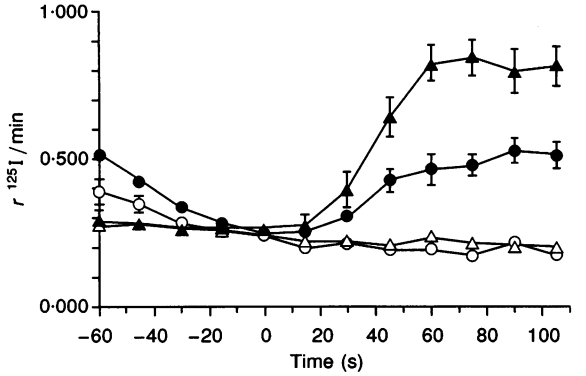

Fig. 2. The effect of forskolin on ^{125}I efflux rate coefficient (r) for 4-day-old non-polarized and polarized HT-29 cells. Agonist was added at time 0 s. ○ and △ represent control ^{125}I efflux rates for 4-day-old non-polarized and polarized HT-29 cell lines, respectively. ● and ▲ represent the same cell lines in the presence of 10 μM forskolin. Values are expressed as means ± S.E.M. of different experiments.

to express CFTR mRNA at similar levels to their polarized counterparts (Morris *et al.* 1992). Therefore the transcriptional control of CFTR did not correlate with the fluid secretory capacity of these cells.

In order to probe the possibility that CFTR protein levels were regulated at the translational level, CFTR protein was immunoprecipitated from the HT-29 cells. In both polarized and non-polarized (parental) cell lines CFTR was found to run as a single 170 ± 3 kDa band when electrophoresed on SDS gels (Morris *et al.* 1992). Furthermore, CFTR isolated from both sources was expressed as the fully glycosylated, endoglucosidase H-resistant form of the protein. This demonstrated that processing through the ER and Golgi biosynthetic compartments of the cell was complete. In other studies, we have shown that the degree of glycosylation for endogenously expressed CFTR in polarized Cl.19A colonocytes does not affect its ability to function within the apical plasma membranes (Morris *et al.* 1993). These latter findings are corroborated by recombinant expression studies where non-glycosylated CFTR was shown to function normally as an anion channel (Gregory *et al.* 1991). Our results indicated that during cytoplasmic polarization, the level of expression and post-translational processing of CFTR remained constant. Therefore, this suggests that mature CFTR protein is not inserted into the plasma membrane of undifferentiated cells.

Brefeldin A effects on cyclic AMP-dependent Cl^- secretion and the intracellular trafficking of CFTR

Our electrophysiological and biochemical studies concerning the appearance of cyclic AMP-dependent Cl^- current in polarizing epithelial cells led us to speculate that the native CFTR anion channel protein was retained intracellularly in non-polarized colonocytes. This hypothesis was investigated by immunolocalizing CFTR protein in both non-polarized and polarized HT-29 cells. Indeed we found that CFTR resided peri-nuclearly in cells that lacked a significant cAMP-activated Cl^- conductance. In contrast, CFTR was found within the apical domain of polarized colonocytes that were capable of actively secreting Cl^- in response to elevated cellular cyclic AMP levels (Morris *et al.* 1993). Thus differentiation dependent changes in the cellular location of CFTR accounted for the functional differences in CFTR-mediated anion exit between non-polarized and polarized cells.

The ER and Golgi provide a common route for the synthesis and delivery of proteins to the basolateral and apical membranes of the epithelial cell. Movement of proteins between compartments occurs by cycling through carrier vesicles which fuse between each subcellular organelle (Fig. 1). These vesicles are endowed with coat proteins, the particular constitution of which depends on their location within the trafficking pathway. The TGN plays a critical role in directing secretory vesicle movement to either membrane domain and is believed to act as a control point for the supply of most, if not all, proteins to their correct cellular location (Mellman & Simons, 1992). Since we demonstrated that mature (Golgi processed) CFTR was synthesized in non-polarized colonocytes, the regulatory mechanism for CFTR trafficking must lie beyond the Golgi cisternae compartments at the level of the TGN or peripheral vesicular pools. To investigate the role of the TGN in this controlled movement of CFTR to the apical plasma membrane, and hence the expression of cyclic AMP-stimulated Cl^- current, we relied on the vesicular coat dissociating properties of the fungal isoprenoid metabolite Brefeldin A (BFA).

Brefeldin A interferes with the outward migration of two classes of vesicular coat protein found within the early and late stages of the biosynthetic pathway: β-COP from the ER and cis-Golgi cisternae (Donaldson *et al.* 1990; Oprins *et al.* 1993) and γ-adaptin from the TGN and late endosomal pool (Rosa *et al.* 1992; Wong *et al.* 1992). Treatment of polarized Cl.19A colonocytes with BFA was found to inhibit the cyclic AMP-stimulated Cl^- current and simultaneously re-localize CFTR from the cellular apices into large coalesced intracellular vesicles located within the same plane as the cellular microtubule organization centre (MTOC, Morris *et al.* 1994). Because BFA does not affect the binding of plasma membrane α-adaptin and clathrin associated proteins, endocytotic membrane retrieval remains fully operative (Miller *et al.* 1992). This explains the accumulation of CFTR and apical plasma membrane at this structure (Fig. 3). BFA at the $1-5\ \mu g\ ml^{-1}$ doses used for these studies did not affect the anion-conducting properties of the basolateral membrane. The chloride current evoked by Ca^{2+} mobilizing agonists remained fully functional even after 24 h incubations with BFA. Thus while likely to affect ER to Golgi vesicular movement, under these conditions, BFA also appears to selectively interfere with the TGN to apical plasma membrane targeting of CFTR. To functionally localize the effects of this drug within the biosynthetic pathway, normal and BFA-treated Cl.19A monolayers were mounted in Ussing chambers and their basolateral membranes selectively permeabilized with nystatin to univalent ions. By employing this protocol we were able to effectively eliminate any contribution of the basolateral membrane to transepithelial Cl^- secretion. The results clearly showed that the inhibitory effects of BFA on cyclic AMP-dependent Cl^- secretion were localized to the apical plasma membrane and correlated inversely with the amount of CFTR found within this domain (Morris *et al.* 1994). A similar selective effect of BFA on TGN apical glycoprotein trafficking in polarized epithelial cells has since been recorded for the intestinal hydrolase dipeptidyl-peptidase IV (DPPIV, Low *et al.* 1992). Thus factors associated with the movement of CFTR out of the TGN during cellular polarization appear to control the onset of CFTR-mediated Cl^- secretion.

Whilst BFA may affect γ-adaptin binding, this coat associated protein is normally found bound to immature secretory granules of the regulated macromolecular

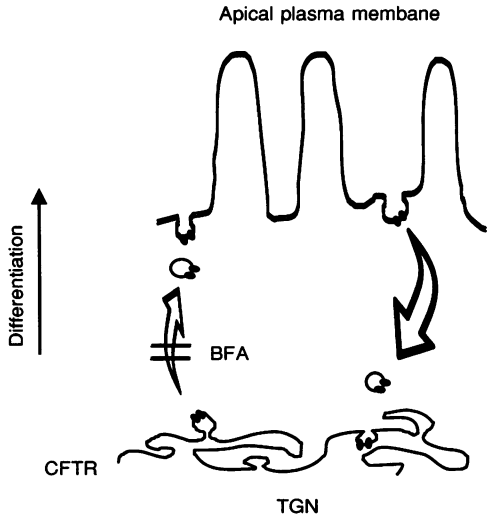

Fig. 3. Summary of the effects of Brefeldin A (BFA) on CFTR intracellular location in polarized epithelial cells. BFA inhibits the outward movement of glycoproteins in the apical membrane biosynthetic pathway but does not inhibit their recycling from the apical plasma membrane. Hence CFTR is cleared from this membrane with a half-life of approximately 8–12 h.

secretory pathway, or recycling between the TGN and late endosomal pools. Since there is currently no evidence predicting cross-talk between constitutive and regulatory traffic, the role of γ-adaptin in movement of CFTR-containing vesicles from the TGN to the apical plasma membrane remains elusive. To address this concern we are currently monitoring the expression of both β-COP and γ-adaptin mRNA and protein levels in differentiating colonocytes. We intend to determine whether changes in the production of these proteins relate to the appearance of the apical membrane CFTR targeting pathway and the control of mucosal fluid secretion. It is likely that other factors confer directionality to vesicle trafficking from the TGN structure. The most promising new candidates are monomeric ARF GTPases which have also been reported to bind to post-Golgi trafficking vesicles in a BFA-sensitive manner. ARFs were originally isolated as co-factors necessary for cholera toxin-induced secretory diarrhoea in the small intestine. This deleterious effect on mucosal fluid transport results from ADP ribosylation of heterotrimeric G_s, leading to the activation of adenylate cyclase, increases in cyclic AMP levels and the phosphorylation of target substrates including CFTR. We are presently investigating whether these monomeric G proteins serve as a link between the classical signal transduction role of G_s and its apparent effect on constitutive secretory vesicle trafficking in polarized epithelial cells. G_s stimulation has been shown to promote apical but not basolateral plasma membrane vesicular movement (Pimplikar *et al.* 1993). These studies may ultimately lead to the discovery of the sub-cellular mechanisms that control the apical plasma membrane expression of the CFTR anion channel in polarizing epithelial cells. In addition, this knowledge will be important in uncovering the problems encountered with targeting of mutant CFTR in epithelial cells.

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