CFTR Targeting in Epithelial Cells

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We used polarized and nonpolarized colonic cell lines (HT-29) to correlate CFTR function and expression with epithelial cell morphogenesis. Unpolarized cells express levels of CFTR mRNA and protein that are equivalent to those observed in polarized cells, and the extent of CFTR glycosylation is also similar. Despite these similarities in CFTR expression, the polarized cells secreted Cl in response to cAMP, but there was no cAMP-stimulated Cl conductance response in the unpolarized cells. In the polarized cells, CFTR is localized in the apical membrane domain, but in unpolarized cells the protein is retained at a perinuclear location. These findings indicate that a peripheral targeting mechanism, distal to the Golgi cisternae, controls the progression of N-linked glycoproteins like CFTR to the apical membrane. This targeting process does not become active until epithelial cells polarize. It may determine whether mutant forms of CFTR are targeted to the apical membrane.

INTRODUCTION

The gene responsible for cystic fibrosis encodes an integral membrane N-linked glycoprotein, the cystic fibrosis transmembrane conductance regulator, or CFTR (Riordan *et al.*, 1989). The predicted protein product consists of 1,480 amino acids, which are arranged in 12 membrane-spanning α-helices, two nucleotide-binding domains, and a central regulatory domain that contains phosphorylation sites for protein kinases. Mutations in CFTR's primary sequence produce the CF phenotype. The most common mutation, causing severe CF, involves the deletion of three base pairs in the first nucleotide-binding domain of CFTR, which omits a phenylalanine residue at position 508. Exogenous expression of wild-type CFTR cDNA in CF epithelial cells corrects the impaired activation of apical membrane Cl conductance by cAMP (Drumm *et al.*, 1990; Rich *et al.*, 1990; Jilling *et al.*, 1990), which is today widely recognized as a

primary, disease-causing defect. Stimulation of this Cl conductance pathway is a vital step in salt and water secretion by cAMP-dependent agonists in a variety of epithelial tissues. Moreover, heterologous expression of CFTR in a variety of cell types produces the same cAMP-activated Cl conductance pathway where none was present before. CFTR mutagenesis studies (Anderson *et al.*, 1991) and, most recently, the reconstitution of purified CFTR protein into planar lipid bilayers (Bear *et al.*, 1992), suggest that this glycoprotein functions as a Cl channel whose activity is regulated by cAMP-dependent phosphorylation. Accordingly, this CFTR Cl channel plays a central role in epithelial salt secretion, and impairment of this process leads to pathology in the secretory processes of the lungs, pancreas, liver, intestines, and other tissues where CFTR is expressed.

In a generic sense, there are now thought to be two ways in which CFTR mutations lead to disease (Collins, 1991). First, the mutant protein itself may be dysfunctional due to an alteration in a critical component of its primary structure. Second, the mutant protein may not be processed correctly after its synthesis, and thus fail to reach its appropriate functional destination. Since these protein-processing reactions

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occur within intracellular compartments where CFTR may serve an important function, these two mechanistic possibilities for the etiology of CF could be related. That is, a defect in intracellular CFTR function might impair the progression of CFTR itself to the plasma membrane.

Epithelial cell lines have contributed enormously to both functional and molecular studies of CFTR. Cell lines derived from human colonic tumors have enhanced our understanding of epithelial cell differentiation and the factors that govern the functional expression of a variety of polarized membrane glycoproteins (Zweibaum et al., 1991). Cell lines arrested at different stages of development provide models for intestinal morphogenesis and the cellular expression of transport and enzymatic processes. The Cl secretory functions of the intestine reside within the intestinal crypts (Welsh et al., 1982), which is also the site of CFTR expression, as detected by in situ hybridization (Trezise and Buchwald, 1991). Several colonic cell lines, including T84 and HT-29, have served as models for Cl secretion. Thus, it is possible to utilize these cells to correlate CFTR expression and Cl secretory functions with epithelial morphogenesis and the generation of epithelial cell polarity (Morris et al., 1992, 1993).

MODELS OF EPITHELIAL DIFFERENTIATION

The parental HT-29 cell line, when grown under standard culture conditions, remains unpolarized, i.e., these cells do not form separate apical and basolateral membrane domains. In this respect, they resemble fetal intestinal cells in that they express markers and transport processes that are normally associated with the basolateral, not apical, membranes of intestinal cells. However, they are pluripotent: chemical or metabolic induction of differentiation is accompanied by cellular polarization, the development of brush border microvilli, and the expression of apical membrane markers, including the intestinal hydrolases (Laboisse, 1990).

The transport properties of the undifferentiated, nonpolarized HT-29 cells are similar to those of the basolateral membranes of the irreversibly differentiated subclone, Cl.19A, which polarizes and forms discrete apical and basolateral membrane domains.
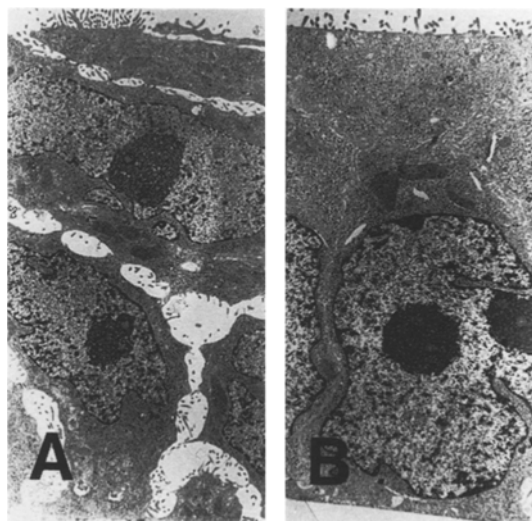

Fig. 1. Morphology of unpolarized (parental) and polarized (Cl.19A) HT-29 cell lines 4 days after seeding onto tissue culture dishes. Sections were cut perpendicular to the confluent cell layer surface and examined by low-power electron microscopy. Magnifications: (A) 2,500 ×, (B) 4,500 ×. For experimental details, see Morris et al. (1992).

The Cl.19A subclone has been used as a model Cl-secreting epithelium (Augeron et al., 1986). The morphology of the unpolarized and polarized HT-29 cell lines is shown in Fig. 1. At 4 days in culture, cells of the parental line (Fig. 1A) form a multicellular layer with no evidence of tight junctions. At the same time, cells of the Cl.19A clone (Fig. 1B) have formed an epithelial monolayer with an apical brush border, and clear evidence of cellular polarization is present. The Cl.19A cells do not immediately polarize in culture; depending on seeding density, approximately 4–6 days are required for an intact, electrically resistive monolayer to be established (Morris et al., 1993).

The differences in morphology shown in Fig. 1 are correlated with functional differences in the Cl conductance response of these cells to cAMP-generating agonists. Since the parental cells are unpolarized, the Cl transport assay used to compare the properties of these cells must be independent of cellular polarization. An isotopic anion efflux assay has been used for this purpose (Morris et al., 1992). Figure 2 shows a comparison of ^{125}I effluxes from the polarized and unpolarized cells and their response to the cAMP-generating agonist, forskolin. In the polarized cells, addition of forskolin leads to a 3.5-fold increase in the rate of ^{125}I efflux. In contrast, the unpolarized cells show a negligible change in efflux rate in response to
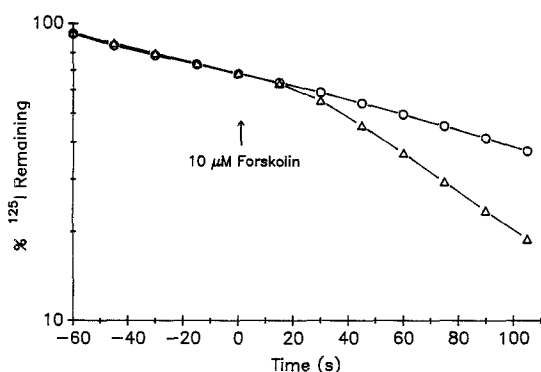
Fig. 2. Effect of forskolin on ^{125}I efflux from unpolarized (circles) and polarized (triangles) HT-29 cells 4 days post-seeding. Forskolin added at zero time, as indicated. The percent of loaded ^{125}I remaining within the cell layer is plotted as a function of time. Experiments performed as described by Venglarik et al. (1990).

Fig. 3. CFTR mRNA and protein expression in polarized and unpolarized HT-29 cells. (A) poly(A$^+$) mRNA was probed with an R domain fragment at high stringency and normalized to γ-actin expression (not shown). (a) unpolarized cells, (b) polarized cells. (B) Immunoprecipitates obtained using pre-immune (a,b) or monoclonal CFTR antibody (c,d) were phosphorylated in vitro with protein kinase A prior to SDS-PAGE. (a,c) unpolarized cells, (b,d) polarized cells. For details, see Morris et al. (1992).

forskolin. Similar results are obtained with cAMP analogues or primary cAMP-mediated agonists such as vasoactive intestinal peptide (VIP).

These findings indicate that the development of cell polarity is paralleled by development of the Cl conductance reponse to cAMP. This functional manifestation of cell polarity is not explained by differences in the degree to which forskolin stimulates cAMP generation in these cell lines (Morris et al., 1992). In the polarized Cl.19A cells, the development of a resistive monolayer permits transepithelial measurements in Ussing chambers. Forskolin or other cAMP-dependent agonists increase the short-circuit current (a measure of transepithelial Cl secretion) and increase the transepithelial conductance, indicative of the activation of Cl channels in the apical membranes of these Cl-secreting epithelial cells.

EXPRESSION AND POST-TRANSLATIONAL MODIFICATION OF CFTR IN POLARIZED AND UNPOLARIZED CELLS

Transfection of CFTR cDNA into a variety of cell types has been shown to produce a cAMP-responsive plasma membrane Cl conductance. The absence of this response in nonpolarized HT-29 cells is not explained by the level of CFTR expression (Fig. 3). mRNA blots performed on polarized and unpolarized HT-29 cells at similar cell densities and times in culture indicate that CFTR message levels are identical (Fig. 3A). Thus, differences in the level of CFTR mRNA do not account for differences in the ability of
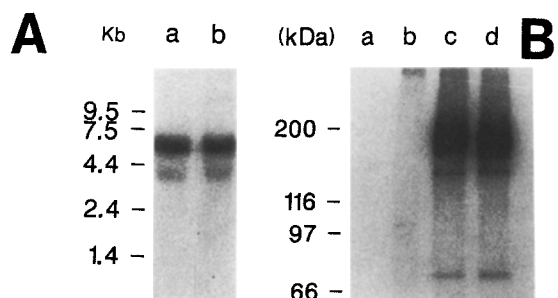
these cells to activate a plasma membrane Cl conductance in response to cAMP.

To determine whether differences in RNA translation or post-translational modification of CFTR are responsible for the observed differences in Cl transport phenotype, CFTR was immunoprecipitated from both polarized and unpolarized cells, again at similar culture densities and times after plating. The immunoprecipitates, after phosphorylation by protein kinase A (Fig. 3B), show that the level of CFTR protein expressed by these cell lines is indistinguishable. A similar intensity of the 170-kDa CFTR glycoprotein band was present in both the polarized and unpolarized cells. Thus, the post-translational glycosylation of CFTR in these cells is also similar. Treatment of the immunoprecipitates from both polarized and unpolarized cells with N-glycanase reduced the apparent molecular weight of CFTR to 140-kDa, and the glycosylated protein from both cell lines was resistant to endoglycosidase H (Morris et al., 1993). Thus, the level of CFTR protein expression and its post-translational glycoprocessing do not provide an explanation for the failure of unpolarized HT-29 cells to respond to cAMP with an increase in plasma membrane Cl conductance.

In other studies, we showed that the level of glycosylation of CFTR endogenously expressed by the polarized Cl.19A cells did not affect its ability to function as a cAMP-activated Cl conductance (Morris et al., 1993). Using inhibitors of various glycoprocessing reactions, we could reduce the apparent molecular weight of CFTR immunoprecipitates from these cells; however, the ability of CFTR to function in
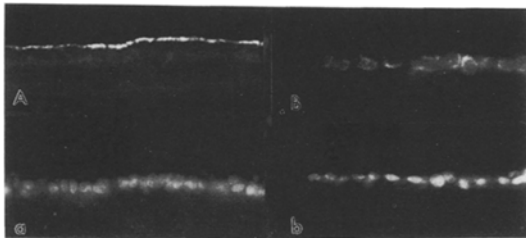
Fig. 4. Cellular CFTR location in polarized and unpolarized HT-29 cells. (A) pre-confluent Cl.19A cells at 3 days post-seeding; (B) post-confluent Cl.19A cells 12 days post-seeding. Cells were grown on filters, folded, and examined at the folded edge to provide an apex-to-basal view. Cells were fixed and stained with monoclonal CFTR antibody (A,B) or Hoechst dye (a,b). See Morris *et al.* (1993) for other details.

cAMP-activated Cl secretion was not impaired by these inhibitors. These findings on endogenously expressed CFTR are corroborated by studies of the exogenous expression of mutant CFTR in which the glycosylation sites have been deleted (Gregory *et al.*, 1991). Nonglycosylated recombinant CFTR also continues to function as a cAMP-activated Cl conductance. These findings have direct implications for the targeting hypothesis of CF, which has arisen from the finding that some disease-producing mutations of CFTR, when expressed inheterologous systems, do not produce the fully glycosylated form of the protein (Cheng *et al.*, 1990). This has been interpreted to indicate that mutant protein is degraded prior to its entry into compartments (i.e., the Golgi cisternae) where the glycosylation reactions occur. The finding that alterations in CFTR glycosylation lack functional consequences supports this conclusion and suggests that a failure to produce the fully glycosylated form of the protein would not, in itself, lead to the CF phenotype.

CELLULAR LOCALIZATION OF CFTR

To determine whether differentiation-dependent changes in the cellular location of CFTR account for the observed functional differences between polarized and nonpolarized cells, CFTR location was probed by immunofluorescence. Figure 4 compares the CFTR staining patterns present in the Cl.19A clone before and after polarization (in these experiments, at 3 and 12 days post-seeding). As in our comparisons of the polarized and nonpolarized HT-29 cell lines, there are no differences in CFTR mRNA or protein expression level in the Cl.19A cells before and after polarization

occurs. In addition, the unpolarized Cl.19A cells do not respond to cAMP with an increase in Cl conductance and, thus, both CFTR function and expression in these cells resemble that in the unpolarized parental HT-29 cell line, discussed above. For morphologic orientation, cell nuclei were stained with Bisbezidine. The cells were grown on filters; the filters were folded and examined at the folded edge so that the cells could be viewed in the apical-to-basal orientation. In the polarized cells (Fig. 4A), a band of CFTR fluorescence is present at the apical membrane domain, and little or no CFTR staining was detected below (basal to) this location. In subconfluent monolayers of the same cells (Fig. 4B), CFTR staining was perinuclear and little or no plasma membrane staining was present. Thus, the presence of a cAMP-dependent Cl conductance response in the polarized cells correlates with the presence of CFTR in the apical membrane domain. The absence of this response in the nonpolarized cells is explained by the failure of CFTR to traffic to the plasma membrane.

This correlation between CFTR location and function is further substantiated by studies of Cl.19A cells treated with the antifungal isoprenoid metabolite, Brefeldin A (BFA).Brefeldin A interferes with the outward migration of cytosolic membrane vesicles along the glycoprotein secretory pathway (Dorner and Kaufman, 1990; Takami *et al.*, 1990). Thus, BFA interferes with the targeting of glycoproteins to the cell surface. Treatment of polarized HT-29 cells with Brefeldin A results in a loss of apical membrane domain CFTR and a relocation of CFTR into large, supranuclear membrane vesicles (data not shown; see Morris *et al.*, 1993). With a timecourse similar to its effect on apical CFTR localization, BFA decreases cAMP-stimulated Cl secretion across polarized Cl.19A monolayers. Thus, the presence of CFTR in the apical membrane domain is required for the functional Cl conductance response. The effects of Brefeldin A on apical CFTR and Cl secretion are reversible. In addition, BFA did not affect the Ca-mediated stimulation of Cl secretion. Thus, BFA does not generally disrupt cellular functions. Rather, its effects are specific in inhibiting the outward migration of CFTR (and other glycoproteins) toward the apical membrane. This, together with the continued retrieval of CFTR from the apical domain, leads to a corresponding decrease in cAMP-stimulated Cl secretion.

During treatment with BFA, the 170-kDa CFTR band is progressively lost and a 145-kDa band appears. After ~24 h BFA treatment, both of these

forms of the protein are present. This is consistent with the retrieval and retention of the apically derived fully glycosylated form of CFTR into the internal membrane vesicles discussed above, together with the buildup of the high-mannose, newly synthesized form of the protein, which cannot be further glycosylated due to BFA-induced disruption of glycoprocessing reactions. If this is correct, then the time course of inhibition of the cAMP-induced I_{sc} in the presence of BFA reflects the inherent rate of CFTR degradation by these cells. In 12-day-old HT-29 monolayers the half time for BFA-induced inhibition of the cAMP-stimulated I_{sc} was $\sim 10\,h$. This estimate of CFTR half-life agrees well with that derived from pulse-labelling of CFTR protein (Cheng et al., 1990) and from phorbol ester-mediated inhibition of CFTR gene transcription (Yoshimura et al., 1991).

IMPLICATIONS FOR GLYCOPROTEIN TARGETING IN EPITHELIAL CELLS

These findings indicate that the activation of a plasma membrane Cl conductance in epithelial cells by cAMP does not develop until the cells polarize and form discrete apical and basolateral membrane domains. During this period when the cells are polarizing, the expression of CFTR at either the RNA or protein level does not vary. Rather, the cellular location of CFTR shifts from a perinuclear site to the apical membrane domain. The actions of Brefeldin A on CFTR targeting also indicate that the functional expression of the cAMP-activated Cl conductance response requires this apical localization of CFTR.

The intracellular site at which CFTR is retained until cell polarization occurs can be inferred from the immunoprecipitation experiments (Fig. 3B). These results show that both unpolarized and polarized HT-29 cells glycosylate CFTR to a similar extent. The similar actions of N-glycanase and endoglycosidase H also suggest that CFTR is similarly processed in the polarized and nonpolarized cells. Accordingly, unpolarized cells process CFTR through the Golgi cisternae, but in the absence of a discrete apical membrane, they do not further traffic this protein along the secretory pathway. Therefore, epithelial cells possess a glycoprotein targeting mechanism that lies at or beyond the level of the trans-Golgi network. This targeting mechanism is either activated or expressed when cells polarize. Our findings establish a novel apical membrane glycoprotein targeting mechanism in

epithelial cells which differs from that involved in basolateral membrane protein targeting. Studies of Na/K-ATPase have inferred that proteins destined for the basolateral membrane incorporate into the plasma membrane of nonpolarized cells (Mays & Nelson, 1992). Upon polarization, they are retrieved from the apical membrane, but are retained basolaterally. Our findings suggest that glycoproteins destined for the apical membrane are retained within the cell, probably at the level of the trans-Golgi network, and are released from this compartment only when cellular polarization occurs.

Finally, our findings also have implications for functional studies of CFTR expression and for the lack of function of CFTR mutants. First, CFTR is apparently handled differently by epithelial and nonepithelial cells. Nonepithelial cells, by definition, are not polarized. Yet it has been shown repeatedly that exogenous expression of CFTR generates a cAMP-activated Cl conductance in these cells. This suggests that there are mechanisms that retain CFTR within epithelial cells until polarization occurs, and that these mechanisms are not expressed by nonepithelial cells. Alternatively, the overexpression of CFTR in heterologous systems overwhelms such mechanisms. This implies that caution should be exercised in interpreting the results of CFTR expression studies in nonepithelial systems. Second, these findings have implications for the protein targeting hypothesis of CF (Cheng et al., 1990), which argues that CFTR mutants may not reach their appropriate cellular destination because a lack of proper protein folding leads to premature degradation of mutant CFTR. The rapid degeneration of $\Delta F508$ CFTR in the endoplasmic reticulum, which results in its unavailability for transit to the plasma membrane, has been suggested to result in the absence of a cAMP-activated Cl conductance in fibroblasts expressing this mutant variant of the protein. The presence of a distal targeting pathway, lying at or beyond the level of the trans-Golgi network, provides another site at which mutant protein could be arrested in its progression to the apical membrane domain of epithelial cells. One candidate for this peripheral trafficking step is cAMP-stimulated membrane insertion. Recent studies have suggested that CFTR expression confers cAMP-dependent exocytosis on epithelial cells (Bradbury et al., 1992). Accordingly, mutant CFTR could target as far as the early endosomal membrane pool but, due to errant protein structure or function, fail to make the final trafficking step into the apical membrane. This could

explain, for example, why the mis-sense mutation, G551D, produces a fully glycosylated protein when expressed in fibroblasts, but fails to function as a cAMP-activated Cl conductance in most expression systems. It will be interesting to determine whether this post-Golgi targeting mechanism is a site at which mutant forms of CFTR are impaired in their progression toward the apical surface.

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