LOCALIZATION OF GFP-KFCFTR TO THE PLASMA MEMBRANE OF MAMMALIAN EPITHELIAL CELLS

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Cystic fibrosis is a life-limiting lung disease that affects approximately 30,000 people in the United States. The disease is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR). The most common mutation, ΔF508, alters folding of the native protein such that CFTR no longer reaches its targeted location, the apical membrane, where it would otherwise mediate chloride secretion across airway epithelia (Mickle *et al.* Am J Hum Genet 66:1485-1495, 2000). Not surprisingly, many CF gene therapies aim to shuttle the ΔF508 mutant protein to the apical membrane so that function can be restored. However, the mechanisms that facilitate CFTR trafficking to this location are not clear. To elucidate these mechanisms we employed a comparative approach. CFTR has been identified in numerous species, the most divergent form is from killifish, *Fundulus heteroclitus*. Since killifish (kf)CFTR also localizes to the apical membrane of native epithelia (Mickle, *et al.*, MDIBL Bulletin 39:75-76, 2000; Lankowski *et al.* Submitted), we hypothesized that the trafficking mechanism is evolutionarily conserved. Accordingly, we expressed kfCFTR in polarized mammalian epithelial cells and assessed protein localization.

To distinguish kfCFTR expression from any trace of endogenous CFTR upon heterologous expression in mammalian epithelial cells, the cDNA of green fluorescent protein (GFP) (provided by N. Muzyczka, Zolotukhin *et al.*, J Virol 70:4646-4654, 1996) was conjoined to full-length kfCFTR cDNA (provided by T. Singer, Singer *et al.*, Am J Physiol 43:C715-C723, 1998). A successive subcloning strategy was utilized because suitable restriction sites were unavailable to readily subclone these two cDNAs in tandem. A PCR product that fused GFP cDNA to the first 21 nucleotides of kfCFTR cDNA was generated using oligonucleotide primers that incorporated an *Eco*RI and *Age*I restriction site onto the 5' and 3' termini, respectively. The product was then ligated into the TA cloning vector pCR2.1 (Invitrogen, Carlsbad, CA), and sequenced to verify fidelity. Once affirmed, the PCR insert was excised with *Eco*RI and *Age*I, size separated from vector DNA by gel electrophoresis, extracted and column purified for ligation to kfCFTR cDNA. Similar manipulation of the plasmid containing kfCFTR cDNA yielded two cDNA products: a 1.2 Kb fragment generated by *Age*I digestion and a 4.3 Kb segment cut by *Age*I on the 5' terminus and *Eco*RI on the 3' end. To foster ligation efficiency, a three-way ligation reaction was performed with the excised PCR insert, the 4.3 Kb fragment and the expression vector pcDNA3.1(-) (Invitrogen), which had been linearized with *Eco*RI and dephosphorylated to promote intermolecular assembly. Subsequent transformation and growth in DH5α subcloning efficiency cells (Invitrogen), plasmid DNA extraction, and restriction enzyme analyses confirmed proper construction. To complete the process, the three-way construct was linearized with *Age*I, dephosphorylated, ligated to the 1.2 Kb fragment, and transformed as previously described. Restriction analyses and DNA sequencing established that the final product was assembled properly.

To determine whether GFP-kfCFTR trafficks to the apical membrane of mammalian epithelial cells, the full-length GFP-kfCFTR cDNA construct was transiently expressed in MDCK II cells using standard transfection protocols (Lipofectamine Plus Reagent, Invitrogen). Cells were cultured for three days at 37°C and 5% CO_2 with supplemented media (DMEM + 10% FBS + 0.375% $NaHCO_3$, Invitrogen); then, fixed, permeabilized, and stained with dyes for F-actin (rhodamine phalloidin, Molecular Probes, Eugene, OR) and nuclei (DAPI, Sigma, St. Louis, MO). Fluorescent images were captured with a Zeiss LSM 410 confocal microscope. GFP alone is expressed throughout the cytoplasm, but GFP-kfCFTR colocalizes with F-actin to the plasma membrane (Figure 1), particularly at the apical and lateral surfaces. In contrast, GFP-human CFTR localization is primarily apical in MDCK cells (Moyer, *et al.*, J Clin Invest 104:1353-1361, 1999). This suggests that kfCFTR can interact sufficiently with mammalian orthologs for membrane localization, but that specific apical localization is precluded by differences in kfCFTR sequence. These differences are currently being exploited to identify key factors involved in apical targeting. To confirm that these initial observations reflect trafficking in a polarized system, the transepithelial resistance is also being measured and other membrane markers are also being used to localize kfCFTR in MDCK II cells. Support was provided by a MDIBL New Investigator Award to J.E.M.

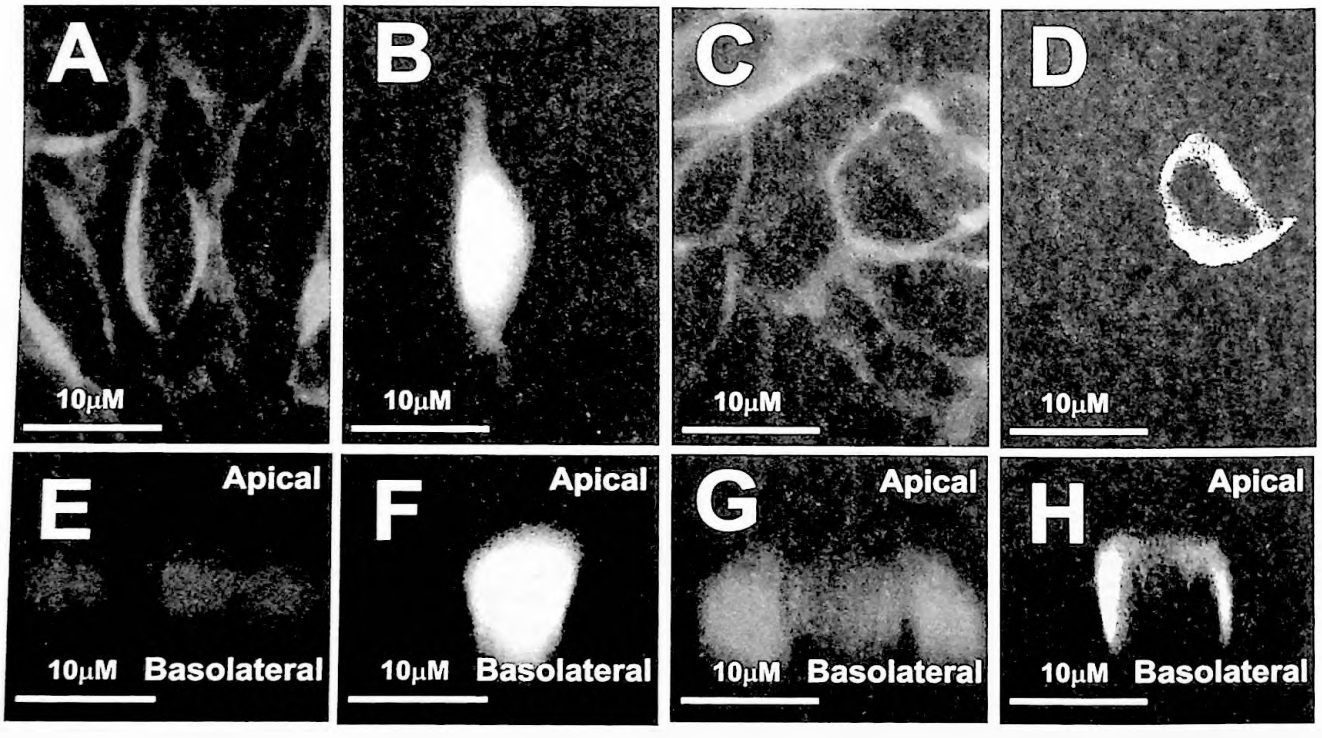

Figure 1. Heterologous expression of killifish CFTR in mammalian epithelial cells. Confocal imaging reveals that F-actin is concentrated at the plasma membrane (A) while GFP (B) is expressed throughout the cytoplasm in the same MDCK II cell. In contrast, F-actin (C) and GFP-kfCFTR (D) co-localize at the plasma membrane. Cross-sectional reconstructions of serial scans through the cells presented in panels A-D show nuclei (E) and cytoplasmic GFP expression (F). Likewise, nuclei are shown for comparison (G), but expression of GFP-kfCFTR (H) localizes to the apical and lateral membrane of MDCKII cells