CELLULAR TRAFFICKING SIGNALS AT THE CARBOXY-TERMINUS OF THE CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR ARE EVOLUTIONARILY CONSERVED

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Life-limiting pulmonary disease is a hallmark of the common genetic disorder cystic fibrosis (CF). The disorder is caused by dysfunction of the CF transmembrane conductance regulator (CFTR). In human airway epithelial cells CFTR is both a chloride channel and a regulator of several different ion channels. Both functions implicate CFTR as a key component in the coordination of ion movement across apical membranes of airway cells. CFTR is highly conserved among species, and is a critical component of osmoregulation in killifish, *Fundulus heteroclitus*, gill (Am. J. Physiol. 43:C715-C723, 1998) and dogfish, *Squalus acanthias*, rectal gland epithelia (J. Biol. Chem. 266:22749-22754, 1991). Thus, identification of CFTR regions that have conserved functional roles may elucidate integrative mechanisms of ion regulation and CF pathophysiology.

Our observation that patients with a carboxy-terminal truncation of 26 amino acids have sweat gland dysfunction in the absence of CF (Hum. Mol. Genet. 7:729-735, 1998) suggests that this region may have a specific function in sweat duct epithelia. Since this is the only tissue known where CFTR is localized to both apical and basolateral membranes (Nature Genet. 1:21-27, 1992) the presence of membrane targeting signals in the C-terminus could explain the consequences of some CFTR mutations. Accordingly, expression constructs were created to study evolutionarily conserved localization signals at the C-terminus. The region of the C-terminus selected started from a common reference point: the last aspartate acid of the conserved Walker B motif in the second nucleotide binding domain of CFTR. Fusion-PCR was used to link green fluorescent protein (GFP) cDNA with the C-terminus of CFTR cDNA from human (residues 1370-1480), dogfish (1380-1492), and killifish (1388-1503). Amplicons were cloned into the propagation plasmid pCR 2.1, and bacterial transformants were selected by α-complementation and antibiotic resistance. Plasmid DNA was extracted for restriction enzyme analysis and DNA sequencing of the insert. Accurate fusion constructs were subcloned into the expression plasmid pAVS6, and recombinants were characterized by restriction analysis to establish insert orientation. GFP cDNA was also subcloned into pAVS6.

To study localization, non-polarized human bronchial epithelial cells, IB3-1, were transfected with the pAVS6 constructs using lipofectin, fixed after 72 hours, and stained with DAPI. Images were captured a cooled charged coupling device (CCD) camera using epifluorescent microscopy. GFP is distributed throughout the cytoplasm while GFP-CFTR fusion proteins localize to the perinuclear region (figure 1). Perinuclear localization of the chimeric proteins also occurs in polarized epithelial cells (16HBE14o-, MDCK I, and MDCK II). Subsequent mutagenesis of the human CFTR C-terminus has revealed two localization motifs (figure 2). Apical localization is

influenced by residues 1478-1480 (TRL), motif A. This motif constitutes a PDZ-binding domain, and is being evaluated for protein-protein interactions. Perinuclear localization is mediated by residues 1395-1403, the B motif. Fusion proteins localized to this region appear to be confined within intracellular bodies. The structure of these intracellular bodies is presently being investigated by electron microscopy. These data indicate that signal sequences in the C-terminus of CFTR influence apical membrane trafficking, and suggests that localization is mediated by conserved protein-protein interactions.

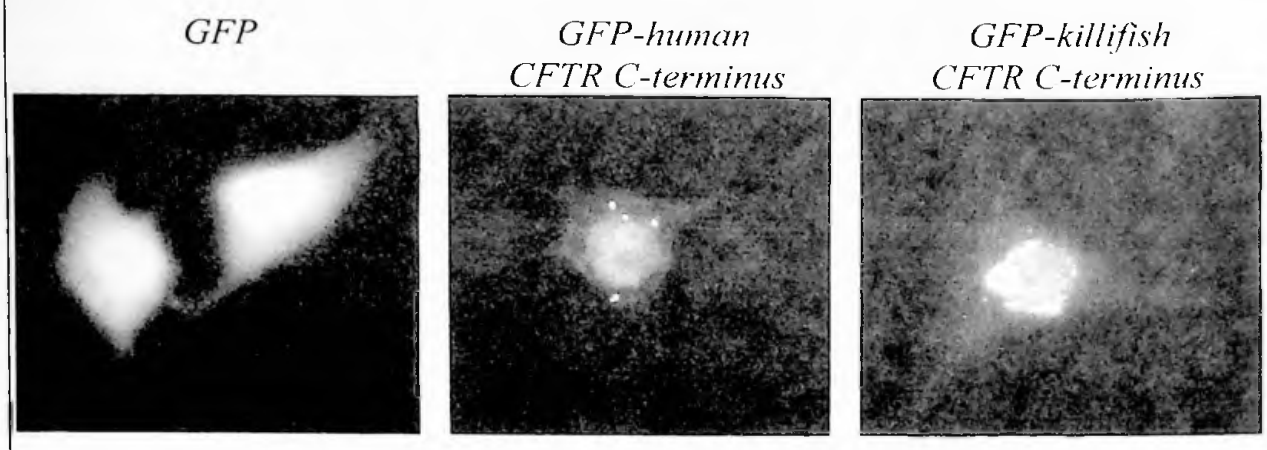

Figure 1. Human airway epithelial cells expressing GFP-CFTR chimeras. Cells expressing GFP are counter-stained with DAPI to indicate the nucleus. GFP is distributed throughout the cytoplasm while GFP-CFTR fusion proteins localize to the perinuclear region, punctate fluorescence. Epifluorescent images are DAPI/FITC composites at 400x magnification taken with a cooled CCD camera and shown in grayscale image contrast. Human CFTR C-terminus: residues 1370-1480. Killifish CFTR C-terminus: residues 1388-1503.

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                                                                B
Human       1370-1424   DEPSAHLDPVTYQIIRRTLKQAFADCTVILCEHRIEAMLECQQFLVIEENKVRQY
Dogfish     1380-1434   DEPTAHLDPVTFQIIRKTLKHTFSNCTVILSEHRVEALLECQQFLVIEGCSVKQF
Killifish   1388-1442   DEPSSYLDPITLQVLRKTLKQSFSGCTVILSEHKVEPLLECQSFLMIEKSSVKSY

                                                                          A
Human       1425-1480   DSIQKLLNERSLFRQAISPSDRVKLFP--HRNSSKCKSKP---QIAALKEETEEEVQDTRL
Dogfish     1435-1492   DALQKLLTEASLFKQVFGHLDRAKLFTAHRRNSSKRKTRPK---ISALQEEAEEDLQETRL
Killifish   1443-1503   DSIQKLMNEMSHLKQAISPADRLHLFPTPHRLNSIKRPQPQTTKISSLPEEAEDEIQDTRL
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Figure 2. Localization motifs in the C-terminus of CFTR. Signal sequences affecting localization are boxed in the alignment of human, dogfish and killifish CFTR C-termini. The sequence in box A influences trafficking to the apical membrane. Perinuclear localization within intracellular bodies is mediated by the B motif.

To investigate conserved protein-protein interactions, we also subcloned full-length killifish CFTR cDNA (courtesy of Dr. Thomas Singer, Vanderbilt University, Nashville, TN) into the expression plasmid pBK-RSV. Human CFTR regulates several other channels. Experiments are on-going to determine whether transfected killifish CFTR regulates endogenous outwardly rectified chloride channels in human airways cells. This research will provide insight into conserved protein-protein interactions affecting CFTR function.

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