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Research Paper

Zinc Deficiency via a Splice Switch in Zinc Importer ZIP2/SLC39A2 Causes Cystic Fibrosis-Associated MUC5AC Hypersecretion in Airway Epithelial Cells☆

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ABSTRACT

Airway mucus hyperproduction and fluid imbalance are important hallmarks of cystic fibrosis (CF), the most common life-shortening genetic disorder in Caucasians. Dysregulated expression and/or function of airway ion transporters, including cystic fibrosis transmembrane conductance regulator (CFTR) and epithelial sodium channel (ENaC), have been implicated as causes of CF-associated mucus hypersecretory phenotype. However, the contributory roles of other substances and transporters in the regulation of CF airway pathogenesis remain unelucidated. Here, we identified a novel connection between CFTR/ENaC expression and the intracellular Zn^{2+} concentration in the regulation of MUC5AC, a major secreted mucin that is highly expressed in CF airway. CFTR-defective and ENaC-hyperactive airway epithelial cells specifically and highly expressed a unique, alternative splice isoform of the zinc importer ZIP2/SLC39A2 (ΔC-ZIP2), which lacks the C-terminal domain. Importantly, ΔC-ZIP2 levels correlated inversely with wild-type ZIP2 and intracellular Zn^{2+} levels. Moreover, the splice switch to ΔC-ZIP2 as well as decreased expression of other ZIPs caused zinc deficiency, which is sufficient for induction of MUC5AC; while ΔC-ZIP2 expression *per se* induced ENaC expression and function. Thus, our findings demonstrate that the novel splicing switch contributes to CF lung pathology *via* the novel interplay of CFTR, ENaC, and ZIP2 transporters.

1. Introduction

Airway homeostasis of fluid volume, viscosity, and chemical components is essential for maintaining mucus clearance and keeping the lungs free from microbial infections, which is mainly regulated by epithelial ion transporters. One well-known and characterized transporter is the cystic fibrosis transmembrane conductance regulator (CFTR), a cyclic AMP-dependent Cl^- channel that controls fluid and ion transport across the lung epithelium. Consistently, CFTR dysfunction causes an imbalance between fluid absorption and secretion, which leads to the development of cystic fibrosis (CF), a genetic disease characterized by hyperviscoelastic mucus secretions in the lungs (Haq et al., 2016; Elborn, 2016). The epithelial sodium channel (ENaC) is also a major ion transporter that is expressed in the apical membrane of airway epithelial cells in the lungs. ENaC activation helps the generation of a concentration gradient of sodium ions, followed by water absorption by cells; however, its hyperactivation can result in airway mucus hyperproduction and dysregulated airway clearance (Almaça et al., 2013; Astrand et al., 2015; Haq et al., 2016). Indeed, the expression and

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function of ENaC were inversely associated with lung function in CF patients (Boucher, 2007). Moreover, we and others have reported that mice with airway-specific overexpression of βENaC (βENaC-transgenic [Tg] mice) exhibit the critical pulmonary phenotypes of CF, such as mucus obstructive, airway inflammatory, and emphysematous phenotypes (Mall et al., 2004; Johannesson et al., 2012; Shuto et al., 2016). These findings imply that CFTR and ENaC are essential ion transporters that control airway fluid homeostasis. Importantly, because an inverse relationship between CFTR and ENaC functions has been widely accepted in CF research fields (Mall and Galietta, 2015), hyperactive-ENaC condition is increasingly being recognized as a CF-like condition. Notably, despite the contribution of Cl^- and Na^+ imbalance in CF airway, little is known about the role of other substances and their transporters in the regulation of CF-associated airway phenotypes.

The zinc ion (Zn^{2+}) plays an essential role in many biological activities in different organisms by regulating the proper functions of zinc-finger proteins, which mainly control cellular transcription, and zinc-containing enzymes involved in the antioxidant defense system (Vallee and Falchuk, 1993; Andreini et al., 2006; Hara et al., 2017). Thus, zinc deficiency causes a variety of symptoms associated with oxidative stress, inflammation, aging, and other symptoms (Prasad, 2013; Fukada et al., 2011). One interesting research topic is how the zinc ion functions in transducing cellular signals (referred to as "zinc signaling"), like the calcium ion (Ca^{2+}) (Liang et al., 2016; Fukada et al., 2011). The importance of the zinc ion and its related signal pathways has been described for numerous aspects of, not only physiology, but also in the pathology of diseases, such as diabetes, Alzheimer's disease, and cancer (Miao et al., 2013; Duce et al., 2010; Grattan and Freake, 2012). Moreover, based on the facts that zinc levels are dysregulated (lower in blood and higher in sputum) in CF patients (Gray et al., 2010; Van Biervliet et al., 2007; Damphousse et al., 2014) and that zinc supplementation can improve CF phenotypes (respiratory function and the incidence of infection) (Van Biervliet et al., 2008; Abdulhamid et al., 2008), zinc dysregulation may also be a possible pathophysiological hallmark of CF. However, these findings could only explain the importance of zinc in airway in a nutritional but not as cellular and molecular aspects. Thus, how zinc levels are controlled under pathological CF conditions and exactly which molecules are responsible for zinc regulation in CF airway epithelial cells are interesting questions to be investigated.

In this study, we first showed decreased levels of intracellular zinc as a common feature in both CFTR-defective CF airway epithelial and ENaC-hyperactive, CF-like airway epithelial cells. We also determined the down-regulation of several zinc importer ZIPs (Zrt-, Irt-like proteins) in CF and CF-like airway cells. Importantly, electrophoretic analysis revealed that a novel and unexpected, pathologically relevant splicing switch in zinc importer ZIP2 (Zrt-, Irt-like protein 2)/SLC39A2 is dominantly involved in intracellular zinc depletion in CF and CF-like airway epithelial cells. This study shows that zinc deficiency via the unique splicing switch of ZIP2 is crucial for CF lung pathology, especially with respect to the induction of MUC5AC, a major secreted mucin that is highly expressed in CF airway, which is exerted by the novel interplay of CFTR, ENaC, and ZIP2 transporters.

2. Materials and Methods

2.1. Cell Culture

The human bronchial 16HBE14o- (Cozens et al., 1992), CFBE41o- (Cozens et al., 1994), βγENaC-stable 16HBE14o- (Caldwell et al., 2005), and WT or ΔF508 CFTR-stable CFBE41o- (Illek et al., 2008) cell lines were previously generated and grown in fibronectin/bovine serum albumin (BSA)-coated dishes from Invitrogen (Mizunoe et al., 2012). The CFBE41o- cell line was reported to be homozygous for the ΔF508 CFTR mutation (ΔF508/ΔF508) and retain many phenotypes of CF bronchial epithelial cells (Mizunoe et al., 2012). These cells were maintained in minimum essential medium. 293 T cells were maintained in Dulbecco's modified Eagle's medium and used for lentivirus transduction. These media were supplemented with 10% fetal bovine serum, 100 U/mL of penicillin, and 100 mg/mL of streptomycin. Primary normal (NHBE) and primary DHBE-CF cells were purchased from Takara and maintained as described previously (Mizunoe et al., 2012). All cells were cultured in a humidified incubator at 37 °C with 5% CO_2.

To induce intracellular zinc depletion, 16HBE14o- cells were treated with TPEN (Dojindo) or dimethyl sulfoxide (DMSO) alone for 2 or 6 h. Concurrent treatment with 20 μM $ZnCl_2$ (Wako) was performed to abrogate the zinc-depletion effect.

2.2. siRNA Transfections and Lentivirus Infections

For gene-knockdown experiments, ZIP2, ZIP4, ZIP8, ZIP10, and ZIP14 siRNA SMARTpools (GE Dharmacon) and control GL2 siRNA (Suico et al., 2014) were transfected using RNAiMAX (Thermo Fisher Scientific), following the manufacturer's recommended protocol.

For lentivirus production, the full-length human ΔC-ZIP2 cDNA sequence was generated and inserted into the *Eco*RI site of the pLVSIN-IRES-ZsGreen1 vector (Takara). The EF1α promoter and puromycin-resistance gene were additionally inserted into the 5′-*Not*I and 3′-*Bam*HI sites for drug selection. The splice site-mutated (IVS1 + T > C) and stop codon-inserted (K8X) ΔC-ZIP2 mutants were generated utilizing the QuikChange II Site-Directed Mutagenesis Kit (Agilent). 293 T cells were transfected with the pLVSIN, pMD2.G, and pCMVR8.74 vectors for 3 days. The supernatant was collected and incubated with Lenti-X Concentrator (Takara) for virus concentration. Virus-infected 16HBE14o- stable transductants overexpressing the genes of interest were established by selection in 4 μg/mL puromycin selection.

2.3. RNA Isolation, cDNA Synthesis, and PCR Analysis

Total RNA was isolated from cells and mouse lung tissues using the RNAiso Plus Kit (Takara) and cDNA synthesis was performed using the PrimeScript RT Regent Kit (Takara). Real-time quantitative RT-PCR analysis was performed as described previously (Shuto et al., 2016). α-, β-, and γ-ENaC-expression plasmids (pCMV-Tag5A) were produced previously (Sugahara et al., 2009) and used to measure the total amounts of mRNA. The sequences of primers used for real-time quantitative RT-PCR are shown in Table S1.

Semi-quantitative RT-PCR was conducted using the KOD-Plus-Neo Kit (Toyobo) according to manufacturer's protocol. PCR was performed for 35 cycles at 94 °C for 30 s, 62 °C for 30 s, and 72 °C for X s (X = 10 s for ZIP2 exons 1–2, or 60 s for ZIP2 exons 1–4), or for 25 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 10 s (for GAPDH). The sequences of primers used for semi-quantitative RT-PCR are shown in Table S1.

2.4. Western Blotting and Slot Blotting Analysis

The protocol used for western blotting was previously reported (Suico et al., 2014; Sato et al., 2012). Western blots were probed with antibodies against ZIP2 (#ab99071; Abcam) and Hsc70 (#ADI-SPA-815; Enzo Life Sciences), followed by incubation with appropriate horseradish peroxidase-conjugated secondary antibodies. The Amersham ECL Western Blotting Detection Reagent (GE Healthcare) was used to visualize the blots.

MUC5AC protein secretion into the medium was detected by slot blotting. The protocol used was described previously (Nishimoto et al., 2010). An MUC5AC antibody was purchased from Thermo Fisher Scientific (#MA1-38223).

2.5. Flow Cytometric Analysis

Intracellular zinc was detected using Newport Green PDX acetoxymethyl ether (Thermo Fisher Scientific). Harvested cells (3×10^5 cells/mL) were washed twice with PBS and loaded with

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