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The proteome speciation of an immortalized cystic fibrosis cell line: New perspectives on the pathophysiology of the disease

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ABSTRACT

Cystic Fibrosis (CF) is a recessively inherited disease caused by mutations in the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene. CFTR has a pivotal role in the onset of CF, and several proteins are involved in its homeostasis. To study CFTR interactors at protein species level, we used a functional proteomics approach combining 2D-DIGE, mass spectrometry and enrichment analysis. A human bronchial epithelial cell line with cystic fibrosis (CFBE41o-) and the control (16HBE14o-) were used for the comparison. 73 differentially abundant spots were identified and some validated by western-blot. Enrichment analysis highlighted molecular pathways in which ezrin, HSP70, endoplasmin and lamin A/C, in addition to CFTR, were considered central hubs in CFTR homeostasis. These proteins acquire different functions through post-translational modifications, emphasizing the importance of studying the CF proteome at protein species level. Moreover, serpin H1, prelamin A/C, protein-SET and cystatin-B were associated to CF, demonstrating the importance of heat shock response, cross-talk between the cytoskeleton and signal transduction, chronic inflammation and alteration of CFTR gating in the pathophysiology of the disease. These results open new perspectives for the understanding of the proteostasis network, characteristic of CF pathology, and could provide a springboard for new therapeutic strategies.

Biological significance: Homeostasis of CFTR is a dynamic process managed by multiple proteostatic pathways. The used gel-based proteomic approach and enrichment analysis pointed out protein species variations among Human Bronchial (16HBE14o-) and Cystic Fibrosis Bronchial Epithelial cell lines (CFBE41o-) and specific molecular mechanisms involved in CF. In particular, we have highlighted HSP70 (HSP7C), HSP90 (endoplasmin), ERM proteins (ezrin), and lamin-A/C as central hubs of the functional analysis. Moreover, for the first time we consider serpin H1, lamin A/C, protein-SET and cystatin-B important player in CF, affecting acute exacerbation, cytoskeleton reorganization, CFTR gating and chronic inflammation in CF. Due to the presence of different spots corresponding to the same protein, we focalize our attention on the idea that a “protein species discourse” is mandatory to well-define functional roles of proteins.

Our approach has permitted to pay attention to the molecular mechanisms which regulate pathways directly or indirectly involved with CFTR defects: heat shock response, cross-talk between cytoskeleton and signal transduction, chronic inflammation and alteration of CFTR gating. Our data could open new perspectives into the understanding of CF, identifying potential targets for drug treatments in order to alleviate Δ508CFTR membrane instability and consequently increase life expectancy for CF patients.

1. Introduction

Cystic Fibrosis (CF, OMIM #219700) is the most common, life threatening, recessively inherited disease in Caucasians. It affects 1 in 2000–3000 newborns in the EU and 1 in 3500 in the USA [1]. It is caused by mutations in the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene which was discovered and associated with the disease in 1989 [2]. Currently, > 1900 different mutations have been associated to the gene (www.genet.sickkids.on.ca). Among them is the ΔF508 mutation, which is characterized by the loss of a

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single phenylalanine in position 508. This mutation is the most prevalent, affecting 66–70% of CF patients (www.cftr2.org). The ΔF508CFTR gene encodes for an inefficiently folded CFTR protein which is initially properly inserted into the rough endoplasmic reticulum (ER) membrane, but later fails to reach its native state. The incorrectly folded protein is recognized by the ER quality control system and subsequently polyubiquitinated and degraded by the proteasome, resulting in failed delivery to the plasma membrane (PM) [3]. CFTR acts as a PKA-regulated chloride channel, with a direct or indirect influence in trans-epithelial fluid homeostasis. Loss of CFTR triggers the production of thick, viscous secretions which lead to three main systemic consequences: chronic airway obstruction with a related increase in infection and inflammation; formation of cysts on the pancreas which block the secretion of digestive enzymes leading to consequent malnutrition; male infertility [4]. Despite the great efforts of the research community in studying this disease, and the recent approval of different treatments such as Ivacaftor (Kalydeco) or a combination of Ivacaftor and Lumacaftor (Orkambi) [5], life expectancy is still 39 years [2]. This demonstrates the necessity for an effective cure for the most severe form of CF.

Most of the current research refers to the link between CFTR gene mutations and the pathophysiology of the disease. However, the role of potential CFTR interactors which could influence the onset and endurance of the disease or the effectiveness of a specific cure has not yet been carefully investigated. Indeed, proteins and their distribution in individual or connected pathways directly or indirectly linked to the pathology of CF play an important role in CFTR proteostasis. To evaluate this, a holistic approach, such as proteomics, has the advantage of considering all the perturbations that can be present, at least at protein level, due to the presence of CFTR mutations. Proteomics paves the way to explain some of the characteristics of the pathology by providing plentiful data, which could help to elucidate the pathophysiology of the disease from an amplified and different perspective [6,7]. It is well known that protein function is related to the protein species obtained after post-translational modifications (PTMs) of precursor proteins. This generates the so called “protein species discourse” (the speciation of the proteome), which is essential for obtaining biologically relevant information [6,7].

Proteomics has been used to study CF, both in bottom-up and top-down approaches. In recent years, Liquid Chromatography-Mass Spectrometry (LC-MS) based procedures have been used [6–8,9–12] in order to identify a large number of proteins involved in the disease. A Multidimensional Protein Identification Technology-Mass Spectrometry (MudPIT-MS) study performed by the Yates' group revealed differentially abundant peptides corresponding to 349 proteins, some of which were already known to be directly associated with CF pathogenesis [13]. Moreover, a new method to evaluate the interactome of CFTR was performed in a cell line containing a ΔF508CFTR mutation, which identified 638 CFTR interactors. Interestingly, most of these interactors may have a relevant role in the rescue of the disease [14]. SILAC based quantitative proteomics was also performed to study the secretome of CF (ΔF508/ΔF508) bronchialepithelial cells [10]. In this study, 666 proteins were identified and quantified, with 70 showing altered abundances in CF secretions. More recently, SILAC method was also used to study lipid raft upon TNF-α stimulation in HeLa cells expressing normal or ΔF508CFTR mutation [11,12]. Gel-free approaches have the advantage of obtaining more complete proteome coverage, identifying a high number of proteins involved in CF [13] but, also using specific PTM enrichment steps, do not allow to perform protein species characterization. To better assess the speciation of the proteome, gel-based approaches have been extensively used to investigate the global protein species pattern in CF. Two-Dimensional Gel Electrophoresis (2DE) was used to study protein changes in CF-IB3-1 lung epithelial cells [15,16], and to study the hyperinflammatory response, induced by mutations in CFTR, in human airway epithelial cells *in vivo* [17,18]. In addition, 2DE was used to show that correct cell surface delivery and expression of mutated CFTR could be rescued by lowering the cell culture temperature (26°–30 °C) [19], and to identify the proteins involved in this process [20]. Furthermore, this classical gel-based proteomic approach has been used to analyze CF protein changes *in vivo*, using sputum, as well as bronchial and nasal epithelial tissues from CF patients [21–23]. 2DE was also used in combination with LC-MS to compare serum of CF patients against healthy CF-carrier and non-CF carrier individuals [9]. Interestingly, due to the importance of PTMs in CF, some authors have also used a target proteomic approach (SRM) to investigate the phosphorylation status of wild type and ΔF508 CFTR in its regulatory domain, showing an impaired phosphorylation of Ser-660 in ΔF508 CFTR condition [24]. Other studies report that inhibition of histone deacetylase activity may partially restore CFTR stability and trafficking [25,26]. The latter demonstrates that PTMs can be extremely important for CF pathogenesis, thus confirming the importance of studying CF at protein species level. Therefore, we performed comparative gel-based proteomic analysis by two dimensional Differential In Gel Electrophoresis (2D-DIGE) to resolve the protein species pattern of bronchial epithelial cells obtained from control and CF patients. Matrix Assisted Laser Desorption/Ionization-Time of Flight Mass spectrometry (MALDI-ToF-MS) and LC-MS/MS were used to identify differentially abundant spots. In this study we used Human Bronchial Epithelial (16HBE14o-) and Cystic Fibrosis Bronchial Epithelial (CFBE41o-) cell lines, originated from the first bifurcation of normal and CF bronchus, and characterized by an endogenous and ΔF508/ΔF508 CFTR genotype. This CF cell line model was chosen as it represents patients with the most severe damage (chronic obstruction, infection and inflammation) in the airway tract.

Progressive degeneration of this anatomic region is considered the main cause of CF morbidity and mortality [27–29]. In this work we identified 73 protein species with a varied intensity which were used for network analysis by MetaCore™ software to highlight possible affected pathways. Functional analyses indicate that the Ezrin-Radixin-Moesin(ERM) proteins (ezrin), HSP70 (HSP7C), HSP90 (endoplasmin) and lamin A/C are central hubs, which are predominantly involved in the regulation of homeostasis in wild type and Δ508CFTR cell lines. Our results also uncover specific protein species of serpin H1 (HSP47), prelamin A/C, protein-SET and cystatin-B, that affect specific cellular pathways, providing new perspectives in molecular mechanisms associated with CF.

2. Experimental procedures

2.1. Cell culture and protein extraction

Human Bronchial Epithelial cells (16HBE14o-) and Cystic Fibrosis Bronchial Epithelial cells, with a ΔF508 mutation in the CFTR gene (CFBE41o-), were provided by Dieter Gruenert, University of California, San Francisco. Cells were seeded in plates previously incubated with coating solution (88% of LHC basal medium, 10% bovine serum albumin, 30 μg/ml bovine collagen type I and 1% human fibronectin). Cells were grown in minimal essential medium (MEM; Calbiochem, San Diego, CA) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 1% L-glutamine in a humid atmosphere (37 °C) with 5% CO_2. Three independent 16HBE14o- and CFBE41o- cell cultures were harvested by trypsinization and washed 3 times in PBS, with brief centrifugation (5 min. 1600 × *g*) after each wash. The cells were then re-suspended in 60 μl DIGE lysis buffer (7 M Urea, 2 M Thiourea, 4% (w/v) CHAPS and 25 mM Tris) to extract the whole protein content. Protein concentration was then estimated using a Bradford assay [30] before proceeding to fluorescent dye labelling (see below). All the sample aliquots were stored at − 80 °C until use.

2.2. Fluorescent dye labelling

Minimal protein labelling for 2D-DIGE was performed according to