Journal of Respiratory Medicine and Lung Disease

Research Article
Published: 16 Oct, 2017

Impaired Interaction between F508del-CFTR and Calumenin Is Cystic Fibrosis

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Abstract

Cystic fibrosis is a lethal autosomal recessive disease characterized by defects in epithelial ion transport. It is caused by mutations in both CFTR alleles encoding the cystic fibrosis transmembrane conductance regulator (CFTR). The most common CFTR mutation found in patients, F508del-CFTR, encodes a cAMP-regulated Cl- channel that is retained in the endoplasmic reticulum (ER). In a previous work we found that calumenin, a ER resident, directly interacts with the CFTR. Furthermore, because it is a chaperon, it can modulate F508del-CFTR expression. Our aim was to depict a possible interaction between calumenin and the F508del-CFTR protein. We show here, using surface plasmon resonance, that the interaction between F508del-CFTR and calumenin is impaired in cystic fibrosis, what is likely involved in the cellular physiopathology of CF.

Introduction

Cystic fibrosis (CF) is a lethal autosomal recessive disease characterized by defects in epithelial ion transport [1]. It is caused by mutations in both CFTR alleles encoding the cystic fibrosis transmembrane conductance regulator (CFTR), an ATP-binding cassette transporter functioning as a chloride (Cl^-) channel [1-4]. The CFTR protein comprises two hydrophobic core regions, two nucleotide-binding domains (NBDs) with ATP-binding activity and a regulatory domain (R domain) [5]. CFTR channel opening requires phosphorylation by cAMP-dependent protein kinases (PKA and CK2, [6,7]) and hydrolyzable MgATP [8,9]. CFTR's regulation is complex and involves dimerization of the protein [10,11] and interdomain interactions [12]. Syntaxin 1A, EBP50, E3KARP, the μ subunit of the endocytic clathrin adaptor complex, cysteine string proteins and annexin A5 are CFTR-binding proteins [2,13-18], but the extent to which CFTR channels are regulated by protein-protein interactions remains largely unknown.

The most common CFTR mutation found in patients, F508del-CFTR, encodes a cAMP-regulated Cl^- channel that is retained in the endoplasmic reticulum (ER) during translation and folding. It is targeted to the proteasome and prematurely degradated [19]. F508del-CFTR's folding is inefficient and more than 99% of the F508del-CFTR is targeted for proteasomal degradation. Nevertheless, when it is expressed in systems that permit its biosynthesis and trafficking to the membrane, it is partially functional [20,21]. Some conditions have been demonstrated to promote the partial rescue of F508del-CFTR from proteasomal degradation. They include reduced temperature [22,23], nitrosoglutathione [24], SERCA calcium pump inhibition [25], and chemical chaperones [26]. In physiological conditions, transient interactions between CFTR and molecular chaperones determine the early fate of the misfolded protein [27]. Therefore, it is important to search for F508del-CFTR's chaperones and to study their involvement upon F508del-CFTR release out of the ER. The identification of specific targets to correct the defective F508del-CFTR folding and in consequence its cellular processing to the membrane (correctors) is a strategy for therapy of CF. The best characterized molecular CFTR's chaperones localized in the ER lumen and in the cytosol are calnexin and Hsp70, respectively. They transiently interact with the immature form of CFTR [28-30]. The F508del-CFTR-Hsp70 and F508del-CFTR-calnexin complexes exhibit a longer half-life than the complexes formed with Wt-CFTR. Indeed, Hsp70 and calnexin may function in the quality control system which recognizes misfolded ER proteins. In the ER, aberrant chaperone binding is responsible for the F508del-CFTR retention. Some compounds such

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Received Date: *14 Sep 2017*
Accepted Date: *09 Oct 2017*
Published Date: *16 Oct 2017*

Citation:
Huguet F, Calvez ML, Benz N, Férec C, Kerbiriou M, Trouvé P. Impaired Interaction between F508del-CFTR and Calumenin Is Cystic Fibrosis. J Respir Med Lung Dis. 2017; 2(4): 1026.
ISSN: 2475-5761

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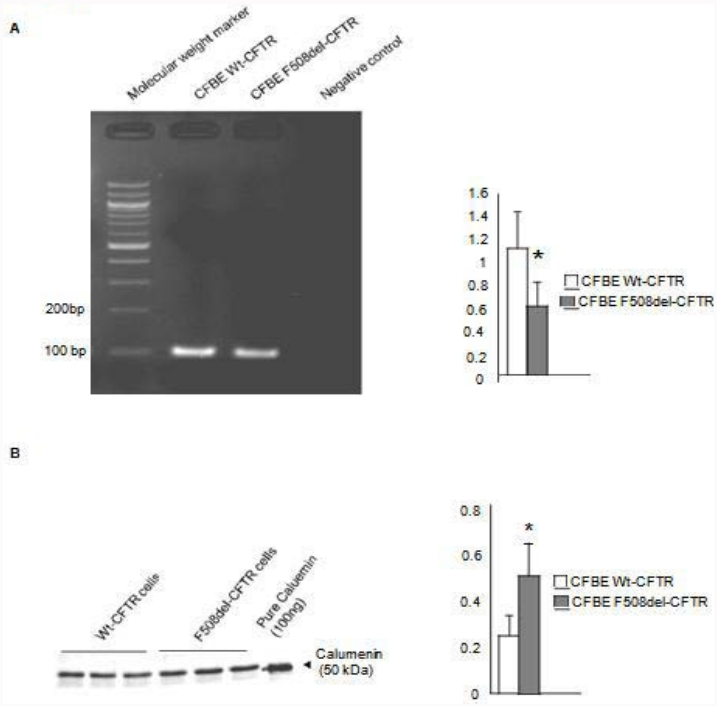

Figure 1: Detection and quantitation of calumenin in Wt-CFTR and F508del-CFTR expressing cells.
A) Gel showing the expression of the mRNA of calumenin in cells expressing Wt- and F508del-CFTR (left panel). A band is observed in both cell lines (100bp). The right panel shows the bar graph representation of the statistical analysis. The amount of mRNA is higher in in cells expressing F508del-CFTR. **B)** Example of immunoblot showing the presence of calumenin in CFBE41o_/Wt and CFBE41o_/F508del cells (left panel). 30 ng was loaded. Pure calumenin was loaded on the gel as a reference due to the low specificity of the anti-calumenin antibody. Histogram showing the quantitation of the expression of calumenin in Wt-CFTR and F508del-CFTR expressing cells (right panel). CFBE41o_/F508del cells (n=5) significantly express more calumenin than CFBE41o_/Wt (n=5).

as thapsigargin, curcumin and the alpha-1,2-glucosidase inhibitor miglustat (N-butyldeoxynojirimycin) that disrupts the F508del-CFTR-calnexin interaction and in consequence release F508del-CFTR, show the importance to look for ER chaperones [25,31-33]. In a previous work in which co-immunoprecipitated proteins with Wt-CFTR and G551D-CFTR were resolved by 2D-gel electrophoresis and analyzed by mass spectrometry, we found that calumenin directly interacts with the CFTR [34]. The calumenin-CFTR interaction was further confirmed by Surface Plasmon Resonance and computational analysis of the predicted calumenin's partners and localized in the ER of cells, by Immunofluorescence [34] Calumenin (315aa) belongs to a family of multiple EF-hand proteins that include reticulocalbin, ERC-55, and Cab45 [35-37]. It is ubiquitously expressed with a high expression in the heart in which it has mostly been studied [38]. Calumenin contains six EF-hands with possible conformational changes upon Ca^{2+} binding. The binding of Ca^{2+} to each individual of its EF-hands is estimated to occur with a Kd of 600 μM indicating a low affinity for Ca^{2+}. In the heart, Calumenin is known to interact and modulate the activity of SERCA2 and RyR2 and thus to regulate Ca^{2+} homeostasis by increasing Ca^{2+} storage in the ER within muscle cells and thereby muscle contraction [39]. Beside its characterized role in Ca^{2+} homeostasis due to direct interactions with SERCA2 and RyR2, Calumenin may have various functions in cells and out of cells. Indeed, it is not confined to the ER but is also found throughout the whole secretory pathway. Furthermore, the protein is also secreted, suggesting that the protein possesses extracellular as well as intracellular functions. Whereas it is poorly studied in the lung, it is suggested that Calumenin is involved in mechanisms that drive lung fibrogenesis [40]. Because it was suggested that Calumenin could modulate F508del-CFTR expression [41], because a Wt-CFTR-Calumenin interaction was confirmed [34] and because Calumenin is found in the ER of the cells and in the secretory pathway [34,42], our aim was to depict a possible interaction between calumenin and the F508del-CFTR protein. Indeed, we showed using bioinformatics that Calumenin is likely as a charged F508del-CFTR folding modulator [42]. Therefore, the aim of the present paper was to prove the F508del-CFTR interaction with Calumenin.

Methods

Cells

In order to use cell with the same genetic background, transduced CFBE41o_ cell lines (CFBE41o_/Wt and CFBE41o_/F508del) were used [43-46]. They were cultured as previously described [45]. Cell culture media and supplements were purchased from Lonza (Basel, Switzerland) and PAA (Pasching, Austria).

RNA Extraction and Quantitative Real-time PCR

Cultured medium was removed and cells were washed twice with phosphate-buffered saline (PBS). RNA was extracted using RNeasy Plus mini kit (QIAGEN) according to the manufacturer's instructions. Extracted RNA was eluted in RNase-free water and the concentration was determinated using a nanophotometer. Relative quantification of the transcripts was assessed in a two steps format (RT and qPCR). Real-Time PCR was performed using a Quanti Tect H SYBRH Green PCR kit (QIAGEN), according to the manufacturer's instructions. A Chromo 4TM System (Bio-Rad) was used to amplify cDNAs and detect emitted fluorescence. The following primers were used: Calumenin (99pb), forward GTTAGAGATGAGCGGAGGTT and reverse GTCATACTCCTCAGGGTGC; G3PDH (121pb), forward 59-CCCATGTTCGTCATGGGTGTGAAC-39 and reverse

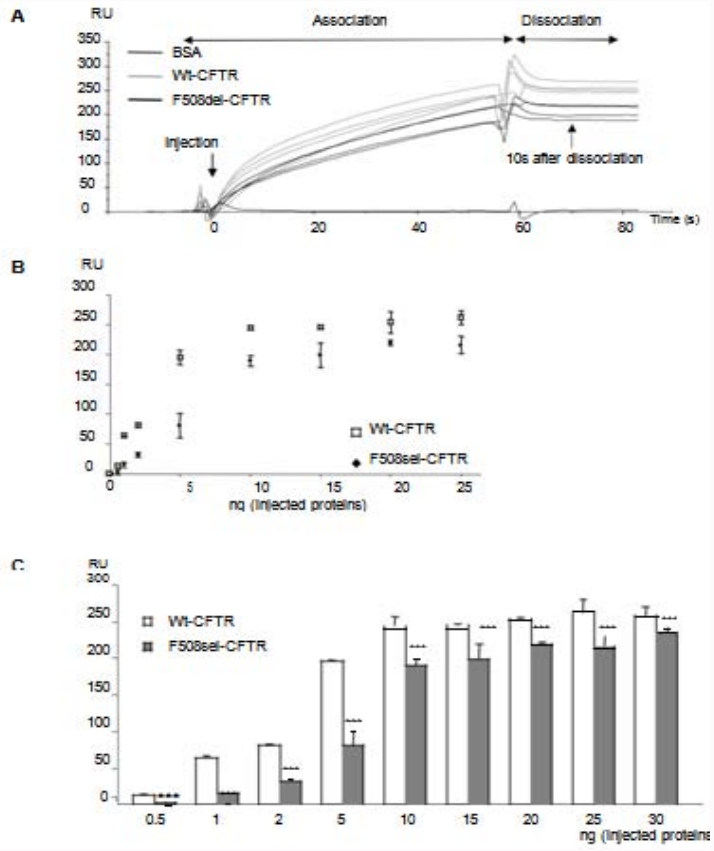

Figure 2: RPS analysis of the Wt-CFTR and F508del-CFTR interaction with calumenin.
A) Example of sensorgramms obaitend when 15, 20, 25 and 30 ng of pure Wt-CFTR and F508del-CFTR were injected over the immobilized pure calumenin. The graphs show that the binding of Wt-CFTR on calumenin is higher than that of F508del-CFTR. BSA was used as a negative control and showed no binding. **B)** The graph shows the response in RU at the end of the injection phase, in function of the quantity of injected Wt-CFTR and F508del-CFTR proteins (from 0 to 20 ng). A higher binding of Wt-CFTR on calumenin is observed when compared to that of F508del-CFTR. Each injection was performed from three to five times. **C)** Histrogram representing the statistical analysis of the bindings, RU values being taken 10s after the beginning of the dissociation phase (3<n<5). It can be observed that the amount of bound CFTR onto calumenin is significantly higher with Wt-CFTR than with F508del-CFTR for all quantities of injected proteins (0<ng<30; 3<n<5).

59 CAAAGTTGTCATGGATGACCTTGGC-39. Reactions were carried out with the following parameters: enzyme activation at 95°C for 15 min, denaturation at 95°C for 30 sec, annealing at 57°C for 30 sec, extension at 72°C for 30 sec and a final extension at 72°C for 10 min. 30 cycles were used for Calumenin. For negative controls (NTC), cDNA was replaced by sterile RNase free water.

Protein preparation and immunoblot analysis

Immunodetection of calumenin was performed as previously described [35]. In brief, cells were lysed on ice in RIPA buffer (25mM Tris-HCl pH 7.5, 150mM NaCl, 1% Triton X-100, 1% Na Deoxycholate, 0.1% SDS) with 10 mM iodoacetate and protease inhibitor (complete tablets-EDTA free, ROCHE, France). Samples were centrifugated (16,000 g, 15min, 4°C) and protein concentrations were determined by the method of Lowry, using bovine serum albumin as standard [47]. Equal amounts of protein (30 µg) were resolved on a 12% SDS-PAGE and transferred on a PVDF membrane. Membranes were probed with anti-Calumenin (D-19, Santa Cruz Biotechnology, 1/500) and with secondary antibodies anti-Goat (donkey anti-goat IgG-HRP: sc-2020, Santa Cruz Biotechnology 1/10000). The specific signals were detected using Luminata Forte Western HRP Substrate (Merck-Millipore). Densitometric analysis of the signals was performed using software Bio-1D (Vilber Lourmat) and each value was normalized to the total amount of loaded proteins per lane, which was estimated after Coomassie blue staining of the membranes and densitometric analysis. Because of the poor anti-calumenin specificity, pure recombinant human calumenin (from Prospec, Israel) was loaded to unsure the molecular weight of the quantified bands.

Surface Plasmon Resonance (SPR)

The SPR experiments were performed at the PurIProb core facility (Inserm, UMR1078, Brest), as previously described [34]. Pure recombinant human Calumenin was from Prospec (Israel). Purified full length Wt-CFTR and F508del-CFTR were generous gifts from Pr Robert Ford (University of Manchester, UK, [48]). CM5 Sensor chip, amine coupling kit (N-hydroxysuccinimide, (NHS), N-ethyl-N-(3-diethylaminopropyl) carbodiimide hydrochloride (EDC), ethanolamine (1 M, pH 8.5) and HBS-EP buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, and 0.005 % surfactant P20 at pH 7.4) were obtained from GE Healthcare Bio-Sciences AB. Real-time detection of the Calumenin - Wt-CFTR and Calumenin - F508del-CFTR interaction were performed using a Biacore system (Biacore 3000; GE Healthcare) and its Control Software version 3.2. All injections were performed at 25°C in HBS-EP 1 × running buffer (GE Healthcare). Sensorgrams were analyzed using the BIA evaluation software (GE Healthcare). For each sample the obtained RU value was the value on the active flow cell (FC) minus the value

of the reference FC, 10 seconds after the beginning of the dissociation phase. Immobilization of pure Calumenin was performed according to Biacore's recommendations on the CM5 sensor chips using an Amine Coupling Kit (GE Healthcare) to achieve about 3000 RU. The surfaces were then blocked with 1 M ethanolamine hydrochloride (pH 8.5). The reference channels were activated with equal volumes of NHS and EDC and immediately saturated with ethanolamine. Binding experiments were carried out at a flow rate of 10 µl/min using HBS-EP buffer pH 7.4 as running buffer, in duplicate, at 25°C. In between injections, the surfaces were regenerated by 50 mM NaOH pulses, which were suitable conditions for removing the bound analyte on the immobilized protein with a very low impairment of the ligands. The reverse experiments in which CFTR was bound were also performed (not shown) and Bovine Serum Albumin (BSA) was used as a negative control. Affinity constants were calculated using the Biacore 3000 Control Software's wizard (GE Healthcare). Affinity constants were calculated using the Biacore 3000 Control Software's wizard (GE Healthcare).

Statistics

Results are expressed as the means ± S.E.M. of n observations. Data were compared using the Student's t test analysis with STATGRAPHICS version 4.1 (SIGMA PLUS, Levallois-Perret, France) and differences were considered statistically significant for $P < 0.05$.

Results and Discussion

We searched for a possible differential expression of calumenin between cells expressing either Wt- or F508del-CFTR. Quantitative Real-Time PCR was first performed to assess the basal expression of Calumenin mRNA in these cells. As shown in (Figure 1A) (left panel), PCR bands were detected in both cell lines with an expected size of 100bp. The quantification was performed and is presented in (Figure 1A) (right panel). We observed a significant higher amount of calumenin mRNA in cells expressing F508del-CFTR than in Wt-CFTR cells. The protein expression was then assessed by western blot. As shown in (Figure 1B) (left panel), calumenin is detected in both cell lines. The statistical analysis revealed that the expression of the calumenin protein is significantly higher in cells expressing F508del-CFTR than in Wt-CFTR cells. These results are likely in contradiction with a previous work showing that calumenin expression is not changed in CF-cells compared to non-CF cells [48]. Nevertheless, this work was performed using homozygous cells expressing F508del-CFTR (CFBE41o-) and cells expressing Wt-CFTR (16HBE14o-) which have a different genetic background. In the present paper cells with the same genetic background were used, likely explaining these different results regarding calumenin expression. Whereas the interaction of calumenin with Wt-CFTR was previously shown [35], no data was available regarding the interaction between calumenin and F508del-CFTR. Therefore, we performed SPR experiments to assess the interaction of calumenin with F508del-CFTR and to compare the binding with that of Wt-CFTR [49,50].

In SPR experiments, one protein is linked to a sensor chip while the other protein is injected over it. The interaction is observed in real time, as a curve (sensorgram) showing association, equilibrium and dissociation phases in resonance units (RU) as a function of time. We first immobilized CFTR-His proteins on the NTA sensor chip and injected annexin V. We found that the RU values increased with the amount of injected annexin V. The specificity of the binding was checked by injection of an irrelevant protein (BSA), which did not induce a signal. We first immobilized calumenin and injected either Wt- and F508del-CFTR and compared sensorgrams (Figure 2A). We found that the RU values during the association and dissociation phases were higher when Wt-CFTR was injected than with F508del-CFTR, indicating that the calumenin - Wt-CFTR interaction is faster and stronger than with F508del-CFTR. The level of bound Wt- and F508del-CFTR (from 0 to 20 ng) that were bound onto calumenin were measured (in RU), at the end of the injection phase and a higher binding of Wt-CFTR on calumenin was observed, when compared to that of F508del-CFTR (Figure 2B). A statistical analysis of the bindings was performed using RU values 10s after the beginning of the dissociation phase and is presented as bar graphs (Figure 2C). The amount of bound CFT onto calumenin was significantly higher with Wt-CFTR than with F508del-CFTR for any quantities of injected proteins. Finally, KD values were calculated using the Biacore software and were found to be 2.5 10-11 M and 1.1 10-8 M for Wt-CFTR and F508del-CFTR, respectively. These experiments showed that as for Wt-CFTR, there is an interaction between F508del-CFTR and calumenin and that this interaction is weaker. According with what is known about calumenin, what could be the physiological relevance of its weaker interaction with f508del-CFTR than with Wt-CFTR? A decreased interaction between CFTR and calumenin, in cells expressing the mutated CFTR, was previously reported, despite it was in a different cell model than ours [48]. Therefore, previous and present results indicate that the folding state of F508del-CFTR is poorly in favor of an interaction with calumenin. On the other hand, a study demonstrated that calumenin is probably a chaperone, positively or negatively regulating the folding of F508del- CFTR [42]. Therefore, the loss of F508del-CFTR-calumenin interaction could partly explain the decreased presence of the mutated CFTR within membranes. We are aware that further experiments are needed to reinforce this hypothesis. In conclusion, we show here that the interaction between F508del-CFTR and calumenin is impaired and that it is likely involved in the cellular physiopathology of CF.

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