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CSN5 binds to misfolded CFTR and promotes its degradation

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Abstract

Cystic fibrosis is mainly caused by mutations that interfere with the biosynthetic folding of the cystic fibrosis transmembrane conductance regulator (CFTR) protein. The aim of this study was to find cellular proteins interacting with CFTR and regulating its processing. We have used a genetic screen in yeast to identify such proteins and identified CSN5 that interacted with the third cytoplasmic loop of CFTR. CSN5 is the 5th component of the COP9 signalosome, a complex of eight subunits that shares significant homologies to the lid subcomplex of the 26S proteasome and controls the stability of many proteins. The present study shows that CSN5 associates with the core-glycosylated form of CFTR and suggests that this association targets misfolded CFTR to the degradative pathway. Identifying CSN5 as a new component of the degradative pathway is an important step towards the goal of unraveling the sorting between misfolded and correctly folded CFTR proteins.

Keywords: CFTR; CSN5; COP9 signalosome; Interaction and degradation

1. Introduction

Cystic fibrosis (CF) is the most common severe autosomal recessive genetic disorder in Caucasians. It is caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene which encodes a cAMP-regulated Cl^- channel [1]. The most frequent mutation of CFTR, F508del (deletion of phenylalanine at codon 508) induces a defective processing (class II) where almost 100% of the newly synthesized F508del-CFTR is degraded due to improper folding. The misfolded protein is retained in the ER, retrotranslocated and then degraded by the ubiquitin proteasome pathway [2,3]. This process is called endoplasmic reticulum (ER)-associated degradation (ERAD) (reviewed in Ref. [4–6]). Decrease of class II mutant protein degradation by using proteasome inhibitors slow CFTR degradation, but maturation does not increase concomitantly [2,7], indicating that degradation and maturation are not in equilibrium [8]. This implies therefore the possibility of additional mechanisms involved in the degradation of CFTR such as proteasome-independent ERAD [9]. Hence, the strategies aiming to increasing the maturation of these mutants will require a better understanding of the underlying processes of their folding and intracellular processing.

Initially, F508del-CFTR was not detected at the apical cell membrane [10–12]; since then several studies have detected either cAMP chloride conductance or apical localization of the protein in CF patients [13–18]. More recent studies have shown that cAMP-dependent Cl^- secretion is absent from rectal tissues freshly excised from F508del homozygous CF patients [19] or from airway specimens freshly excised from F508del-CFTR homozygous subjects [20]. These data raise the question of the

Abbreviations: CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; CSN, COP9 signalosome; ERAD, ER-associated degradation; ERQC, ER-quality control

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variability of processing mutants, a plausible explanation being that these mutants are associated with variable protein partners that could modify the processing of CFTR during its course to the cell surface.

CSN5 was originally identified as a co-activator of the *c*-Jun transcription factor named Jun activation domain-binding protein 1 (Jab1) [21], and later as the fifth component of the COP9 signalosome (CSN), a complex of eight subunits that shares significant homologies to the 26S proteasome lid subcomplex [22]. This complex was first identified in *Arabidopsis* as a repressor of photomorphogenesis [23], and is an evolutionarily conserved protein complex that controls pleiotropic functions in various eukaryotes ranging from cell cycle progression and signal transduction to transcriptional regulation by regulating specific protein degradation events. CSN regulates protein degradation either through direct regulation of E3 ubiquitin ligases [24–26] or through regulation of the phosphorylation of specific substrates such as p53 and *c*-Jun [27,28]. We have chosen to name this protein CSN5 according to the nomenclature proposed by Deng although the HUGO name is COPS5 [29].

Many mutations in cytoplasmic loops impair CFTR processing, suggesting that the correct folding of these loops is important for proper maturation of the whole protein. The third cytoplasmic loop is highly conserved throughout CFTR evolution and among ABC transporters [30], and contains thoroughly investigated class II mutations [31,32]. The aim of this study was to identify intracellular proteins capable of interacting with CFTR and regulating its processing. For this purpose, a yeast two-hybrid screening was performed using the third cytoplasmic loop of CFTR as a bait. We have identified CSN5 as a binding partner and investigated the cellular consequences of its expression on CFTR processing and function. Here, we show that CFTR and CSN5 associate and that this direct or indirect interaction targets misfolded core-glycosylated CFTR to the ubiquitin proteasome system. Therefore, this study emphasizes on the CSN5-CFTR interaction as a novel step towards selection between misfolded and correctly folded CFTR proteins during their processing.

2. Materials and methods

2.1. Antibodies and reagents

The following antibodies were used: MAB25031 (clone 24-1, R and D systems) and MM13-4 (Upstate) monoclonal antibodies (mAb) for CFTR detection, mAb anti-V5 coupled with FITC for V5-tagged protein detection (Invitrogen), a mouse mAb for CSN5 detection (Abcam) and α-Tubulin and Lamin B1 antibodies (Abcam). Fluorescence-labeled antibodies used: Alexa Fluor™ 488 and 568-coupled goat anti-mouse $IgG2_a$ or $IgG2_b$ isotypes respectively (Molecular Probes). Horse radish peroxidase (HRP)-coupled secondary antibodies and piceatannol were purchased from Sigma-Aldrich.

2.2. Yeast two-hybrid screening

The sequence encoding the 52 amino acids corresponding to the 3rd cytoplasmic loop (CL3) of CFTR was PCR-amplified using the following oligonucleotide primers (5′-TGCATATGG*CCATGG*AGCTGGTGCATACTC-TAATC-3′ and 5′-TGCGGCCG*CTGCAG*TAAGAGGCAGAAGGTCATC-3′) and subcloned into *NcoI-PstI* sites of plasmid pGBKT7 (Clontech), generating the bait plasmid: pGBKT7-CL3wt. This bait plasmid was transformed into yeast strain AH109 Mat a. We used a pretransformed Human testis MATCHMAKER cDNA library in yeast strain Y187 Mat α (Clontech), containing 3.5×10^6 independent clones with an average length of 2 kb (0.4 to 4 kb). The library was screened by mating before spreading on synthetic dropout (SD) medium, lacking histidine, leucine, tryptophan and adenine, to select diploid clones. Positive interactions were further confirmed by the $LacZ^+$ phenotype. Absence of self-activation was verified by mating the bait with a control prey and selection on SD medium. To characterize the CL3-interacting proteins, plasmids were recovered from yeast strains showing positive interactions, and their identity was determined by DNA sequencing.

2.3. Plasmids constructs and site-directed mutagenesis

The entire coding sequences of the human CSN5 cDNA was PCR isolated from a human brain cDNA library and subcloned into the plasmid pcDNA3.1/V5-His Topo® (Invitrogen). CSN5 mutants (D151N and the four silent mutations: 5′-CA*ATCC*-3′ (CSN5 nucleotide 398 to 403; Q23Q and S24S) were constructed using the Gene-Editor kit (Promega) and the resulting mutants were fully sequenced. CFTR plasmids were constructed in the expression vector pTracer-CMV (Invitrogen) as already described [32–35].

2.4. Cell culture and transfection

HT-29 (ATCC, HTB-38), HEK (ATCC, CRL-1573), and stably transfected HeLa cells expressing wild-type CFTR (spTCF-wt), F508del-CFTR (spTCF-ΔF) or pTracer (spTracer) [36,37] were grown at 37 °C with 5% CO_2 in DMEM with Glutamax containing 10% fetal calf serum, 100 U/ml penicillin and 100 g/ml streptomycin (all from Invitrogen).

All cells were grown on 60 or 100 mm diameter dishes. Subconfluent cells were transfected by lipofection using Lipofectamine® and Plus reagent (Invitrogen) according to the manufacturer's instructions. Confluent monolayers were harvested and used 24 or 48 h post-transfection for immunoprecipitation, immunocytochemistry or functional assays.

2.5. Co-immunoprecipitation (Co-IP) and western blots

Cells grown on 100 mm diameter dishes were harvested 24 h after transfection in PBS 1×, pelleted at 2000 g at 4 °C and resuspended in 600 μL Co-IP buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$ 5% glycerol and 1% NP-40) supplemented with complete protease inhibitors (Roche Applied Sciences). After preclearing 1 to 5% of lysates were withdrawn for input assays and the remaining material was incubated with 0.8 μg MAB25031 (R and D System) or 0.8 μg anti-CSN5 (Abcam) overnight at 4 °C. Pansorbin cells were then added. The totality of the immunoprecipitated material was subjected to SDS-PAGE and analyzed by Western blotting. When endogenous Co-IPs were performed two 100 mm dishes were pooled before loading.

Equal amounts of proteins, assayed by the Bradford technique (Pierce) were loaded on 10% SDS-PAGE gels and then transferred to PVDF membrane (GE Healthcare). Proteins were immunodetected by monoclonal or polyclonal antibodies followed by HRP-conjugated anti-mouse or anti-rabbit IgGs, and visualized by chemiluminescence with the ECL+ kit (GE Healthcare), or West Dura kit (Pierce). Antibodies were used at the following dilutions: MM13-4, 1/2000; anti-CSN5, 1/5000; anti-Tubulin, 1/10000; V5-HRP, 1/5000; anti-mouse-HRP, 1/50000. Direct recording of the chemiluminescence was performed using the CCD camera of the GeneGnome analyzer and quantification using the GeneTools software (Syngene BioImaging Systems, Synoptics Ltd).

2.6. Immunoprecipitation/PKA assay and pulse–chase experiments

Cells grown on 60 mm diameter dishes were harvested 48 h after transfection. CFTR protein immunoprecipitation using MAB25031 (R and D System) was performed as already described [32–35]. Briefly, cell lysates were mixed with 0.4 μg of MAB25031 and Pansorbin cells. The resulting proteins were phosphorylated in vitro with 5 units of the catalytic subunit of PKA (Promega) and 10 μCi [γ-^{33}P] ATP (GE Healthcare), separated by 5% SDS-PAGE, dried and autoradiographed. Radioactivity was quantitated by radioanalytic scanning (Molecular Dynamics PhosphoImager).