Journal of Cystic Fibrosis 18 (2019) 244–250

Contents lists available at ScienceDirect

Journal of Cystic Fibrosis

journal homepage: www.elsevier.com/locate/jcf

SPX-101 is stable in and retains function after exposure to cystic fibrosis sputum

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ARTICLE INFO

Article history:
Received 15 February 2018
Revised 1 June 2018
Accepted 2 June 2018
Available online 20 June 2018

Keywords:
ENaC
Airway hydration
Mucus
SPX-101

ABSTRACT

Background: In healthy lungs, epithelial sodium channel (ENaC) is regulated by short, palate, lung, and nasal clone 1 (SPLUNC1). In cystic fibrosis (CF), ENaC is hyperactivated in part due to a loss of SPLUNC1 function. We have developed SPX-101 to replace the lost function of SPLUNC1 in the CF lung.
Methods: Expression of SPLUNC1 was determined in sputum from healthy and CF donors. Stability of SPLUNC1, S18 (the ENaC regulatory domain of SPLUNC1), and SPX-101 was determined in sputum from CF donors and towards neutrophil elastase. Activity of SPX-101 after exposure to CF sputum was determined in airway epithelial cells from CF donors and in the βENaC transgenic mouse model.
Results: SPLUNC1 protein expression is significantly reduced in CF as compared to healthy sputum. SPLUNC1 is rapidly degraded in CF sputum as well as by a number of individual proteases known to be found in the sputum. SPX-101, but not S18, is stable in CF sputum. Finally, SPX-101 retains its ability to internalize ENaC, regulate airway surface liquid height, and increase survival of βENaC mice after exposure to CF sputum.
Conclusions: Our results demonstrate that SPX-101, but not SPLUNC1 or S18, is stable in CF sputum. These results support the therapeutic development of SPX-101 for the treatment of cystic fibrosis.

1. Introduction

Cystic fibrosis (CF) is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, but is also characterized by increased activation of the epithelial sodium channel (ENaC) [1, 2]. Hyperactivation of ENaC results in increased sodium absorption. In the lung, the loss of chloride secretion and the increased absorption of sodium creates an osmotic gradient that draws fluid away from the airway surface. Dehydration of the airway leads to the accumulation of thick, static mucus, decreased mucociliary clearance, and consequently colonization with bacteria such as *Pseudomonas aeruginosa* and members of the *Burkholderia cepacia* complex [3–5].

Inhibition of ENaC has the potential for hydrating CF airways with the advantage of being agnostic to CFTR mutation. The initial observation that ENaC is hyperactivated in CF airways was made nearly 40 years ago [2, 6, 7]. Since then, airway-expressed ENaC has been targeted with the use of inhaled small molecule inhibitors such as amiloride and its derivatives. While these compounds are potent ENaC inhibitors in laboratory settings, clinical trials results have been disappointing in part due to dose-limiting diuresis and hyperkalemia [8–11].

In the healthy lung, ENaC is naturally regulated by short palate, lung, and nasal clone 1 (SPLUNC1) [12]. Unlike small molecule inhibitors of ENaC which target open probability of the channel, SPLUNC1 reduces the number of channels on the surface of cells [13, 14]. SPLUNC1-mediated regulation of ENaC is achieved through an 18 amino acid peptide (dubbed S18) [15, 16]. In CF, the SPLUNC1/ENaC axis has been disrupted due to airway acidification and loss of SPLUNC1 protein expression [15, 17]. Currently, this loss of SPLUNC1 protein expression is not well understood. To replace this lost function, we have developed SPX-101, a peptide promoter of ENaC internalization that mimics the actions of SPLUNC1/S18 to regulate airway hydration [18]. Here, we have produced a comprehensive examination of SPLUNC1, S18, and SPX-101 stability in CF sputum. We find that SPLUNC1 and S18 are degraded in CF sputum and are therefore unsuitable as therapeutic options. SPX-101 is stable in CF sputum, and retains normal activity after exposure to CF sputum, providing evidence that it can reduce ENaC activity in a diseased setting.

2. Methods

2.1. Reagents and peptides

Peptides were manufactured by Genscript (Piscataway, NJ) at >95% purity. The sequences are as follows; SPX-101 (aaLPIPLDQTaa),

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https://doi.org/10.1016/j.jcf.2018.06.002

ABC (aaDILLPPQTaa), S18 (GGLPVPLDQTLPLNVNPA) and 4031 (aaLPIPLDQTAAAVVRGRaa) where "a" implies D-Alanine. Recombinant human SPLUNC1 (BPIFA1) was purchased from LS Bio (Seattle, WA). All other reagents were from Sigma Aldrich (St. Louis, MO) unless otherwise noted.

2.2. Tissue procurement and cell culture

Primary human bronchial epithelial cells (HBECs) were obtained from the CF Canada Primary Airway Cell Biobank at McGill University. Cells were maintained at air liquid interface on transwell membranes (Corning; Corning, NY) for at least three weeks before experiments as previously described [18]. HEK293T cells were obtained from ATCC (Manassas, VA) and grown in DMEM containing 10% fetal bovine serum and penicillin/streptomycin solution (last three from Life Technology; Grand Island, NY). Cells were transfected using with αENaC-GFP, βENaC-FLAG, and γENaC using Lipofectamine 2000 (Life Technologies) according to the manufacturer's protocol.

2.3. Surface Biotinylation

Assays were carried out as previously described [18]. Briefly, HEK293T cells were treated with 10 μM peptide for 1 h and then labeled with 0.5 mg/ml Sulfo-NHS-SS-biotin solution according to the manufacturer (Thermo Fisher Scientific, #21331). Cells were lysed and incubated with Nutravidin beads (Thermo Fisher Scientific #29204) at 4 °C for 2 h with rotation. 10% of the lysate was reserved for input control. Nutravidin beads were washed 4× with lysis buffer and then boiled at 95 °C for 5 min.

2.4. Sputum samples

Sputum samples from CF patients and those without a diagnosed pulmonary disease (healthy) were obtained from Discovery Life Sciences (Los Osos, CA). All sputum samples were spontaneously collected except for six of the healthy subjects which were induced by inhalation of hypertonic saline. All samples were directly frozen at −80 without the addition of protease inhibitors. Samples were shipped on dry ice, thawed overnight at 4 °C, and all subsequent experiments were undertaken within 24 h of thawing.

2.5. SPLUNC1 stability assay

Recombinant SPLUNC1 was incubated in a 1:1 mixture of PBS:sputum at a final concentration of 40 μg/ml. Where indicated, the PBS:sputum mixture was prepared to contain ONO-4456 (sivelestat; 10 and 100 μM final concentration) or an EDTA-free protease inhibitor cocktail (1×, 5×, and 10× final concentration). For degradation in specific proteases, solutions were prepared as above but contain no enzyme, neutrophil elastase (0.1 mg/ml, Innovative Research, Inc), trypsin (0.025%, Life Technologies), prostasin (0.2 mg/ml), cathepsin G (0.2 mg/ml), matriptase (0.2 mg/ml), or cathepsin B (0.2 mg/ml, last four Novus Biologicals; Littleton, CO). Samples were collected at the indicated time points and all reactions stopped by incubation at 95 °C for 5 min.

2.6. Western blot analysis

Sputum samples were resolved for western blot analysis on 8–16% TGX gels (BioRad; Hercules, CA) and transferred to nitrocellulose membranes. For SPLUNC1 degradation studies, a total of 100 ng of recombinant SPLUNC1 was loaded into each well. For detection, samples were incubated with goat anti-SPLUNC1 (R&D Systems; Minneapolis, MN) overnight at 4 °C and a donkey anti-Goat RD680 secondary antibody (Licor; Lincoln, NE). Signals were detected using a Licor Odyssey imager. Surface biotinylation samples were resolved on 4–15% TGX gels (BioRad) and transferred to nitrocellulose membranes. Antibodies used were: GFP (Cell Signaling #2956), FLAG (Sigma #F1804), and γENaC (abcam ab3468). IRDye 680 and 800 secondary antibodies (LiCor) were used to label ENaC and IRDye 680 streptavidin (LiCor) was used to label total biotinylated protein. Western blots were scanned on a LiCor Odyssey and processed with Image Studio Lite v.5.2.

2.7. Experiments in βENaC transgenic mice

Studies assessing the survival of mice overexpressing the *Scnn1b* gene were conducted as previously described [18, 19]. Once daily intranasal instillation of peptides (50 mM solution, 1 μl/g bodyweight) beginning at post natal day (PND) 2 until PND14. Mice were housed in a pathogen-free facility maintained at Spyryx Biosciences, on a 12-h day/night cycle with ad libitum access to regular chow diet and water. Animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Spyryx Biosciences.

2.8. Protease activity assays

Total protease activity was determined by measuring cleavage of succinylated Casein (Pierce #23263) according to manufacturer's instructions. Neutrophil elastase activity was measured using a fluorimetric assay kit (abcam #ab204730) according to manufacturer's instructions. Experiments were performed in duplicate and average data is presented. For heat-inactivation, CF samples were diluted as above and incubated at 95 °C for 5 min.

2.9. ASL height measurements

ASL height was measured as previously described [15]. Briefly, CF HBECs which had been cultured at ALI for at least three weeks were washed with PBS. The following day Ringer's solution containing the indicated peptide and TRITC-Dextran (Invitrogen) were added to the apical surface of the cells. After six hours images were collected using a Leica SP8 confocal microscope.

2.10. Peptide stability in sputum samples

Sputum samples were first centrifuged at 2000 ×*g* for 10 min and then the supernatants were collected and re-centrifuged at 17,000 ×*g* for 10 min at 4 °C. These neat supernatants were collected and a 10-fold dilution of each sample were used to perform the assays. Peptides were added to the samples to a final concentration of 2.5 mM and incubated at 37 °C. At different time points during the assays, aliquots of the samples were collected, boiled for 5 min, iced and centrifuged at 17,000 *g* for 5 min. A 5-fold dilution of each sample was loaded and analyzed by HPLC using a C18 reversed phase column (YMC-Pack Pro C18, 150 mm × 3.0 mm, 3 μm, YMC America Inc., Allentown, PA, USA). Solvent A contained 0.1% trifluoroacetic acid (TFA) in water. Elution was achieved using a linear gradient of 15–30% of solvent B (0.1% TFA in acetonitrile).

2.11. Peptide stability in neutrophil elastase and DNAse1

Peptides (2.5 mM) were incubated in 0.1 mg/ml of Human Neutrophil Elastase (Innovative Research, Inc) or 0–10 mg/ml DNAse1 (Sigma Aldrich) at 37 °C. Aliquots were taken at the indicated times, boiled at 95 °C for 5 min, and analyzed by HPLC as described above.

2.12. Data analysis and statistics

All data were analyzed with GraphPad Prism (GraphPad, La Jolla, CA). Unless otherwise noted, all data are presented as means ± SEM for n experiments or samples as identified in the figure legend. Differences between means were tested for statistical significance using a

Mann-Whitney test or a Kruskal-Wallis test. Significance was considered as $p < 0.05$.

3. Results

3.1. SPLUNC1 is reduced or absent in CF sputum

There are conflicting reports on the expression of SPLUNC1 in CF sputum [15, 20, 21]. To clarify this discrepancy, we first determined if SPLUNC1 protein expression was altered between healthy and CF sputum (patient demographics are in Supplemental Table 1). SPLUNC1 protein was readily detected in healthy sputum samples. In contrast, only two of 25 CF sputum samples had appreciable levels of SPLUNC1 with undetectable levels of the protein observed in multiple samples (Fig. 1A/B). This data demonstrates that SPLUNC1 protein is significantly reduced, and frequently absent, in CF sputum samples.

3.2. SPLUNC1 is degraded by multiple proteases found in CF sputum

SPLUNC1 is known to be degraded by proteases [15, 22] and it is well established that protease levels are increased in CF sputum [23–27]. Total protease activity and neutrophil elastase activity was significantly increased in the CF sputum samples as compared to healthy. This activity could be abolished by heat inactivation of the CF sputum at 95 °C for 5 min (Fig. 2A/B). Several proteases enriched in CF sputum, including neutrophil elastase, cathepsin G, trypsin, and matriptase are all efficient at degrading SPLUNC1. In contrast, only a small amount of cleavage was observed with prostasin and no cleavage was detected with cathepsin B (Fig. 2C).

We next examined the stability of recombinant SPLUNC1 in healthy and CF sputum. Recombinant SPLUNC1 was stable in sputum from healthy donors with <10% degradation of the protein over the course of the experiment (Fig. 3A/B). In contrast, SPLUNC1 was rapidly degraded in sputum from CF patients in as little as 15 min (Fig. 3C/D). Incubation with CF sputum alone resulted in degradation of 89% of SPLUNC1, while boiling to heat-inactivate proteases prevented this degradation. Co-incubation with sivelestat (ONO-5546) had a marginal effect on degradation with the highest dose reducing total degradation to ~75%. Co-incubation with a broad-spectrum protease inhibitor cocktail (PIC) at 1×, 5×, or 10× resulted in 71, 45, and 15% degradation (Fig. 3E/F). This data demonstrates that SPLUNC1 is rapidly degraded in sputum from CF patients and that a broad spectrum of proteases are responsible for this degradation.

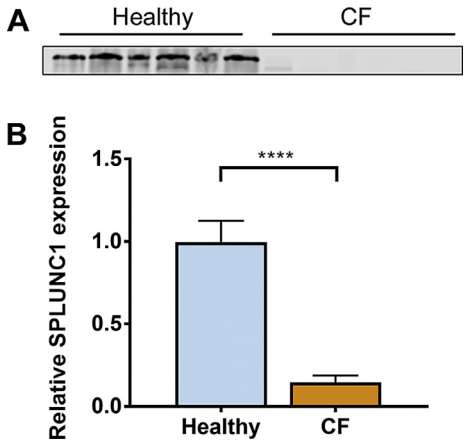

Fig. 1. SPLUNC1 is reduced or absent in CF sputum. Sputum collected for healthy or CF patients was subjected to western blot analysis for detection of SPLUNC1. Representative data are shown in A and data from all samples tested quantified in B. In total 18 healthy and 25 CF samples were tested. Data shown in B depicts mean ± SEM. ****indicates $p < 0.0001$ versus healthy sputum.

3.3. SPX-101 is stable in CF sputum and neutrophil elastase

SPX-101 was developed to replace the natural ENaC regulatory function of SPLUNC1 that is lost in the CF lung. SPX-101 resisted degradation in CF sputum with 92% of the peptide still present after 4 h and 67% present after 24 h of exposure (Fig. 4A). In contrast, S18 was degraded such that only 2% of the peptide remained after 24 h. Similarly, S18 was rapidly degraded in concentrated neutrophil elastase, with <50% remaining after 1 h of exposure and 10% remaining after 5 h. SPX-101 was significantly more stable in concentrated neutrophil elastase than S18, with 79% of SPX-101 intact after 5 h (Fig. 4B). In both studies a control peptide (4031) with a known neutrophil elastase cleavage site was included. We also examined the stability of SPX-101 towards human DNAse1 and no degradation was observed (Supplemental Fig. 1).

3.4. SPX-101 is functional after exposure to CF sputum

For SPX-101 to be efficacious in the CF lung it must retain its ENaC regulatory function in the presence of, and after exposure to, sputum proteases. We tested the ability of CF sputum-exposed SPX-101 to internalize ENaC (Fig. 5A/B), and of CF sputum exposed SPX-101 or S18 to regulate ASL height (Fig. 5B), and increase survival in a murine model of CF, the βENaC-Tg mice (Fig. 5C). We also included a control peptide (ABC peptide) in the animal studies to account for any osmotic effects. For these studies, SPX-101 and S18 were exposed to CF sputum for 4 h before the reaction was inhibited by heating the sample at 95 °C for 5 min. In all three assays, exposure to CF sputum did not alter the activity of SPX-101. In contrast, S18 was able to increase ASL height in CF HBEC, and to increase survival of βENaC-Tg mice, but these effects were lost after the peptide was exposed to CF sputum. Combined, these data demonstrate that SPX-101, but not S18, retains activity after being exposed to CF sputum.

4. Discussion

In healthy lungs, SPLUNC1 is secreted by airway epithelia and provides a natural allosteric inhibition of ENaC by reducing membrane concentration of the channel [12, 28, 29]. SPLUNC1 is reported to be absent in sputum from CF patients as well as those suffering from other chronic diseases such as COPD and asthma [15, 21, 22, 30]. While arising from different combinations of genetic and environmental causes, each of these diseases feature by high levels of sputum proteases. Our data herein demonstrates that SPLUNC1 is degraded by multiple proteases found in diseased sputum.

The discovery that a peptide derived from SPLUNC1's N-terminus, S18, retains ENaC regulatory activity provides the foundation for an optimized peptide therapeutic [17]. However, in examining the endogenous S18 sequence there is a canonical neutrophil elastase cleavage site at amino acid 5 (GGLP**V**PLxxx). Therefore both SPLUNC1 and the S18 peptide are unsuitable for therapeutic use. In creating SPX-101, the nutrophil elastase cleavage site was changed to create a peptide resistant to cleavage (LPIPLxxx). To this end, when exposed to neutrophil elastase, SPX-101 is resistant to degradation while S18 is rapidly degraded. This observation is further confirmed in that SPX-101, but not S18, is stable in CF sputum. Importantly, SPX-101, but not S18, that has been exposed to CF sputum retains normal activity.

Orthogonal approaches were used to test SPX-101 activity in these studies and this included test survival of the βENaC transgenic mouse [19]. This model develops a CF-like airway disease consisting of decreased percilliary liquid, increased mucus solids, and decreased mucus clearance. Approximately 50% of mice die with two week of birth due to these mucus clearance defects. Previously we have demonstrated that SPX-101, but not a control peptide that was also used as an

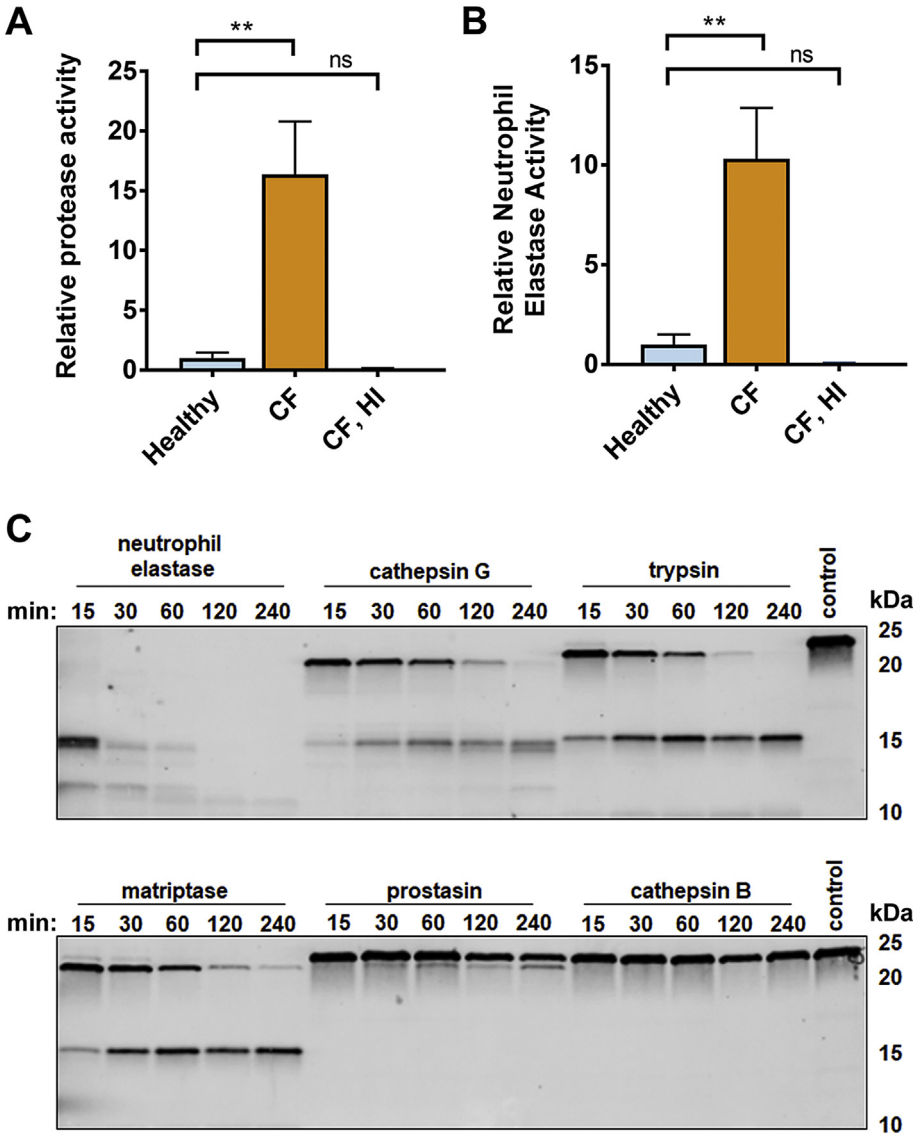

Fig. 2. SPLUNC1 is degraded by multiple proteases found in CF sputum. Total protease (A) and neutrophil elastase (B) were assessed in 10 healthy and 16 CF sputa samples. All graphs depict mean ± SEM. **indicates $p < 0.01$ versus healthy sputum. Degradation of recombinant SPLUNC1 by neutrophil elastase, trypsin, cathepsin G, prostasin, matriptase, and cathepsin B was determined by co-incubation and subsequent western blot analysis. Data in C are representative of at least three experiments per enzyme.

osmotic control in these studies, increases survival in this model [18]. When amiloride was used in this model survival was increased only with thrice-daily dosing. These animals also required supplemental saline injections to compensate for the diuretic effect of the small molecule [31]. SPX-101 increased survival in this model with once-daily dosing and did not require supplemental saline as mice did not lose weight during the study. In the current work we extend these findings to demonstrate that SPX-101, but not S18, exposed to CF-sputum can increase survival in this model. This key finding demonstrates that SPX-101 retains function in vivo even after prolonged exposure to protease-rich CF sputum. Additional future work is needed to investigate the effect of SPX-101 on lung histology and inflammation in this model but lies outside the scope of the current manuscript.

It has been reported that at acidic pH SPLUNC1 fails to regulate ENaC. However, the S18 peptide from SPLUNC1 retains ENaC regulatory function in an acidic environment [17]. This mild acidification in the ASL of HBEC cultures has been reported by multiple groups [32, 33] and work continues to understand why this acidification occurs [34]. Likewise, there have been multiple reports of acidification in the CF airway in humans and animal models of the disease [34–36]. However, a recent study suggested that there is no acidification of the CF airway or in the ASL of cells derived from CF donors [37]. Several factors could explain these conflicting observations, including age of the patients, microbiome status, medications being used by patients, growth conditions of cells for in vitro experiments, and assay technique. This area will certainly require more work in the coming years to fully understand the role that airway pH plays in CF lung disease.

Previous therapeutic approaches directed towards ENaC have induced hyperkalemia [38]. This is because these small molecule inhibitors of ENaC enter the systemic circulation where they engage ENaC in the kidney thereby inducing potassium-sparing diuresis. Nebulized SPX-101 was tested in toxicology trials in rats and dogs and found to have no impact on serum potassium concentrations and was rapidly removed from systemic circulation [18, 39]. Moreover, in clinical trials in healthy volunteers and adult patients with cystic fibrosis SPX-101 was not associated with any relevant changes in serum or urinary potassium (manuscripts in preparation). These findings supported the initiation of an ongoing CFTR mutation agnostic clinical trial of SPX-101 for the treatment of cystic fibrosis (NCT03229252).

The hyperactivation of ENaC observed in CF airways is the result of both an increase in channel number [40] combined with an increase in the open probability (Po) of the channel [41, 42]. ENaC can be

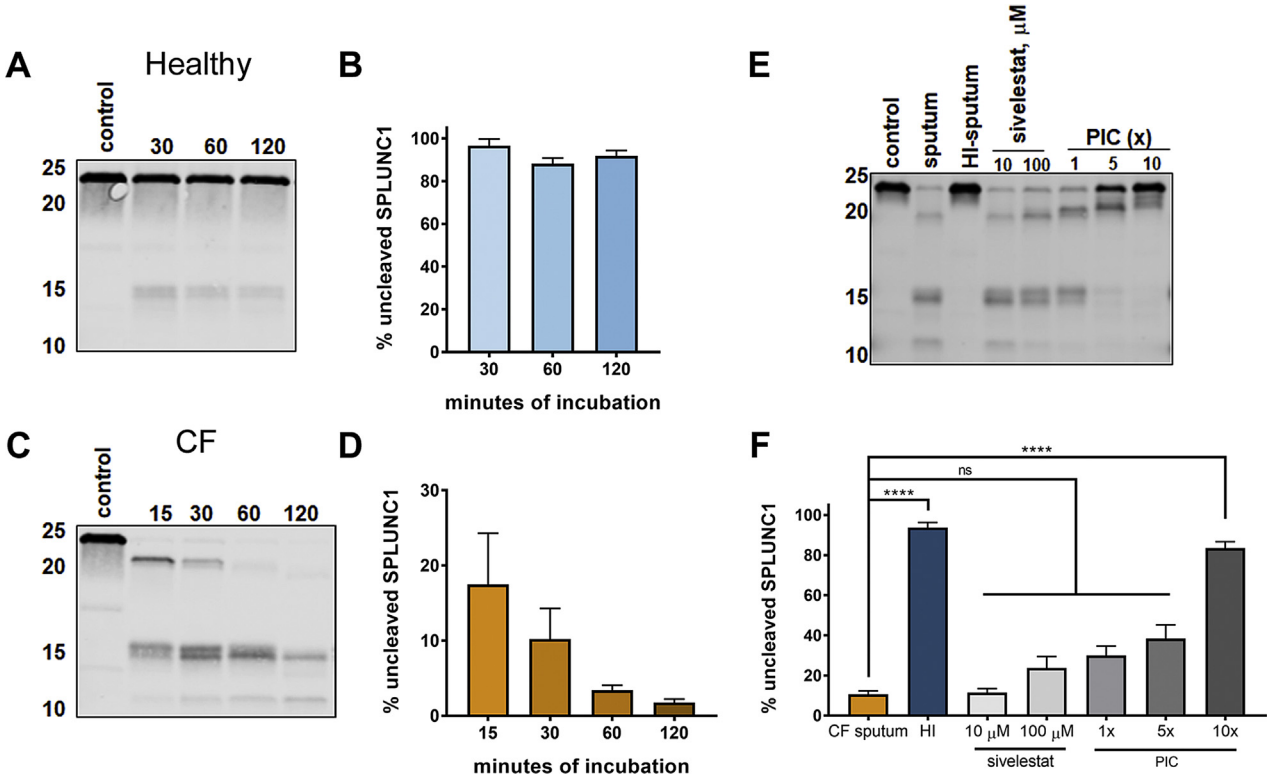

Fig. 3. Recombinant SPLUNC1 is rapidly degraded in CF sputum. Stability of recombinant SPLUNC1 in healthy sputum (5 individual pateint samples) or CF sputum (15 individual patient samples) was determined by co-incubation and western blot analysis. Representative blots are show in A and C and quantification of data in B and D. The ability of protease inhibitors to prevent degradation in CF samples in shown E and quantified in F (data are representative of 11 sputum samples). All graphs depict mean ± SEM. ****indicates $p < 0.001$ versus sputum alone.

regulated by a variety of proteases found in CF sputum [43]. Moreover, the expression level of ENaC and SPLUNC1 can be regulated by a number of hormones and soluble proteins [30, 44]. Small molecule inhibitors of ENaC such as amiloride and its derivatives work by reducing Po, but the channels remain on the surface of cells. As such, ENaC hyperactivation, and correspondingly sodium and fluid uptake, rapidly resumes when these inhibitors are washed away. This short duration of action could explain why these inhibitors have not generated positive results in clinical trials [16, 18, 31]. In contrast to small molecule inhibitors, SPX-101 provides a durable reduction of ENaC activity by removing the channels from the surface of cells [18].

The data presented herein has demonstrated that SPLUNC1 is susceptible to degradation by multiple proteases found in CF sputum while SPX-101 is not. Multiple attempts have been made to treat CF using anti-proteases and protease inhibitors in the hopes of reducing inflammation and improving lung function [45, 46]. To date, this approach has proven to be safe for patients, but has yet to have a positive impact on lung function. With regard to the SPLUNC1/ENaC axis, inhibition of a single protease, or even a family of proteases, would fail to protect SPLUNC1 from degradation. These therapeutic approaches might reduce inflammation but are unlikely to impact airway hydration, the key factor in mucus transport.

In summary, SPX-101 differentiates itself from SPLUNC1 and S18, as the peptide is stable in CF sputum and is, therefore, suitable for investigation as a therapeutic treatment. By restoring the lost SPLUNC1 regulatory function to the CF lung, it may be possible to increase airway

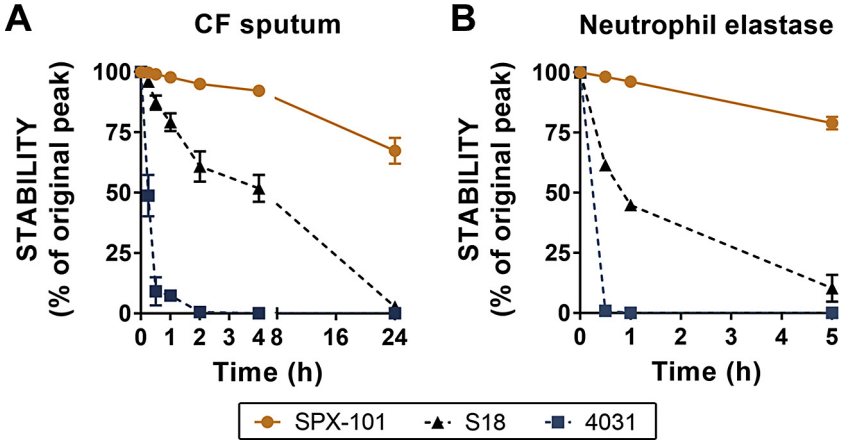

Fig. 4. SPX-101, but not S18, is stable in CF sputum. A) Stability of SPX-101, S18, and 4031 peptides in CF sputum was determined by HPLC analysis. Data represent the mean ± SEM of original peptide (time 0) as detected in 16–24 individual sputum samples. B) Stability of SPX-101, S18, and 4031 peptides in neutrophil elastase was determined by HPLC analysis. Data represent mean ± SEM of original peptide (time 0) from three separate experiments.

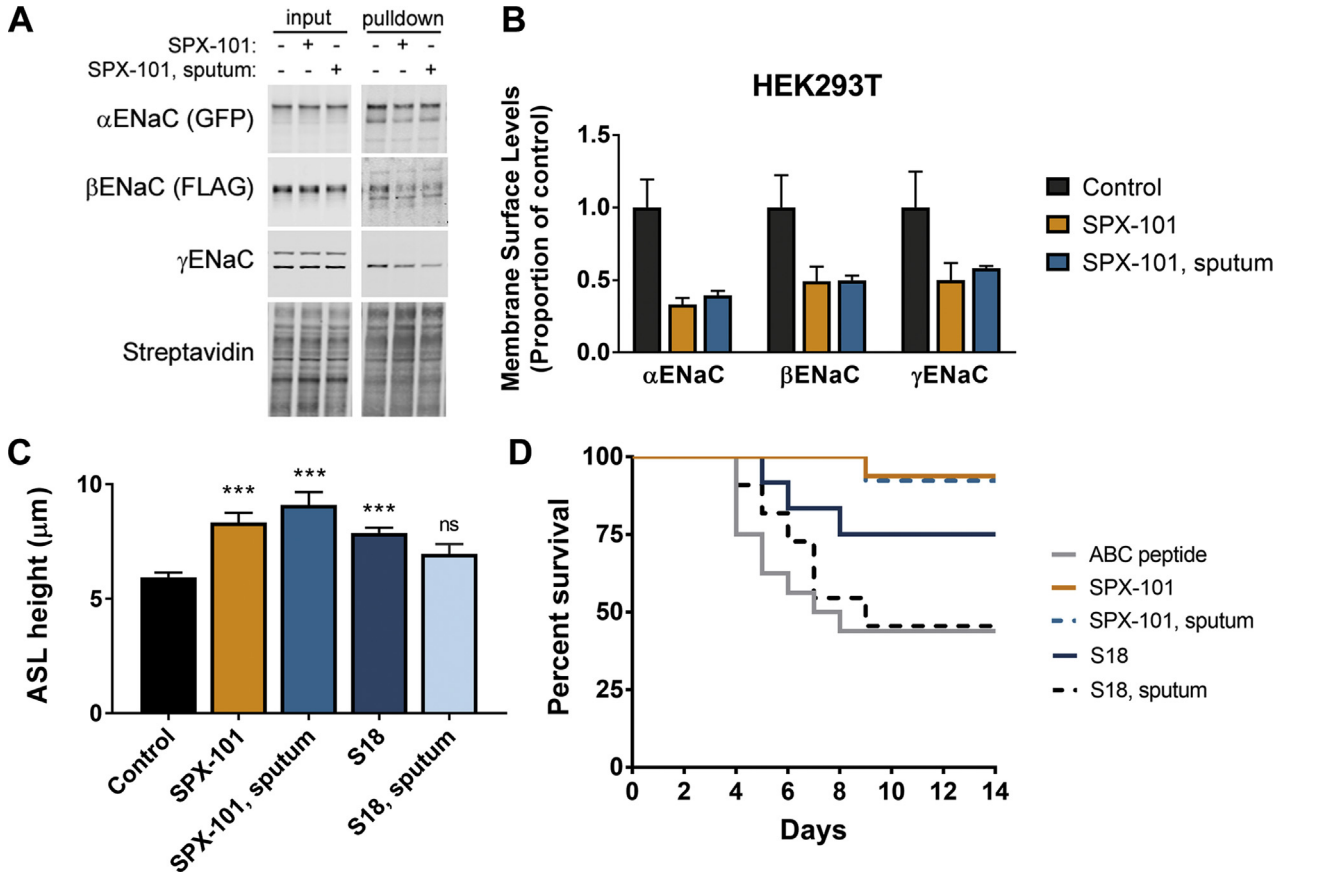

Fig. 5. SPX-101 retains functions after exposure to CF sputum. A/B) ENaC membrane density was determined by surface biotinylation and western blot analysis as described in methods. Data represents mean ± SEM from three experiments. 10 μM SPX-101 and 10 μM SPX-101 exposed to sputum both cause internalization of ENaC subunits, $p < 0.05$ versus control for each subunit C) Airway surface liquid (ASL) height was determined in cells exposed to Ringers solution or the buffer containing 10 μM SPX-101 or 10 μM SPX-101 that had been exposed to CF sputum, 10 μM S18 or 10 μM S18 that had been exposed to CF sputum, n = 8–12 per condition. ***indicates $p < 0.001$ versus control. D) Survival of βENaC-Tg mice was monitored in animal receiving ABC peptide (control peptide), SPX-101, SPX-101 exposed to CF sputum, S18, or S18 exposed to CF sputum beginning at post-natal day 2. Data include ABC (n = 16), SPX-101 (n = 16), SPX-101 exposed to CF sputum (n = 13), S18 (n = 12), and S18 exposed to CF sputum (n = 12).

hydration, promote mucocillary clearance, and recover lost lung function. Beyond CF, SPX-101's potential to increase airway hydration could prove to be beneficial in other diseases associated with mucociliary clearance defects and high levels of sputum proteases, such as non-CF bronchiectasis, COPD, and severe asthma. Currently, SPX-101 is in Phase 2 clinical development for the treatment of cystic fibrosis.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jcf.2018.06.002.

Declaration of interest and support

JIS, BW, TJS, and DWS are Spyryx Biosciences employees and this research is supported by Spyryx Bioscience.

Conflict of interest statement

All authors of the manuscript are employees and have equity interests in Spyryx Biosciences which is developing SPX-101 for the treatment of cystic fibrosis.

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