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Regulation of ENaC biogenesis by the stress response protein SERP1

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Abstract Cystic fibrosis lung disease is caused by reduced Cl^- secretion along with enhanced Na^+ absorption, leading to reduced airway surface liquid and compromised mucociliary clearance. Therapeutic strategies have been developed to activate cystic fibrosis transmembrane conductance regulator (CFTR) or to overcome enhanced Na^+ absorption by the epithelial Na^+ channel (ENaC). In a split-ubiquitin-based two-hybrid screening, we identified stress-associated ER protein 1 (SERP1)/ribosome-associated membrane protein 4 as a novel interacting partner for the ENaC β-subunit. SERP1 is induced during cell stress and interacts with the molecular chaperone calnexin, thus controlling early biogenesis of membrane proteins. ENaC activity was measured in the human airway epithelial cell lines H441 and A549 and in voltage clamp experiments with ENaC-overexpressing *Xenopus* oocytes. We found that expression of SERP1 strongly inhibits amiloride-sensitive Na^+ transport. SERP1 coimmunoprecipitated and colocalized with βENaC in the endoplasmic reticulum, together with the chaperone calnexin. In contrast to the inhibitory effects on ENaC, SERP1 appears to promote expression of CFTR. Taken together, SERP1 is a novel cochaperone and regulator of ENaC expression.

Keywords ENaC · SERP1 · CFTR · Cystic fibrosis transmembrane conductance regulator · CFTR · RAMP4 · Calnexin

Abbreviations

ENaC	Epithelial sodium channel
SERP1	Stress-associated endoplasmic reticulum protein 1
ER	Endoplasmic reticulum
CFTR	Cystic fibrosis transmembrane conductance regulator
amil	Amiloride

Introduction

Cystic fibrosis (CF) is characterized by reduced Cl^- secretion due to mutated cystic fibrosis transmembrane conductance regulator (CFTR) and enhanced Na^+ hyperabsorption through amiloride-sensitive epithelial sodium channels (ENaC). A balance between Na^+ absorption and Cl^- secretion is crucial to maintain an adequate level of airway surface liquid (ASL), necessary for effective mucociliary clearance of the lungs. Pharmacological strategies to restore function of mutant CFTR or to modulate ENaC expression and/or activity should therefore be beneficial for treatment

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of the CF lung disease. Strategies are on the way to identify proteins that control folding and maturation of CFTR during its transition from the endoplasmic reticulum (ER) to the Golgi, in order to find ways to increase plasma membrane expression of misfolded F508del-CFTR, which otherwise remains in the ER [2, 35]. Moreover small molecule strategies aim to rescue F508del-CFTR to the plasma membrane [2, 23].

It is believed that airway Na^+ absorption by ENaC is a major factor that controls airway surface liquid and mucociliary clearance [4, 14, 27, 28]. However, little is known about the mechanisms that control intracellular maturation of ENaC [1, 7, 16]. In order to identify novel interacting proteins that could serve as potential targets for pharmacotherapy of the apparent Na^+ hyperabsorption in CF, we employed the split-ubiquitin two-hybrid system in yeast [25, 33]. We identified the ubiquitous ER-localized stress-associated protein 1, SERP1, as a binding partner of βENaC. SERP1, also known as ribosome-associated membrane protein 4 (RAMP4), is homologous to yeast suppressor of SecY 6 protein (YSY6p) [37], which suggests a role in pathways controlling membrane protein biogenesis at the ER level. Expression of SERP1 is enhanced during cellular stress, causing accumulation of unfolded proteins in the ER. By interaction with the molecular chaperone calnexin, SERP1/RAMP4 controls biogenesis of membrane proteins [37]. Since Sec61alpha and Sec61beta but not SERP1 associate with newly synthesized integral membrane proteins under stress, it was concluded that stabilization of membrane proteins in response to stress involves the concerted action of SERP1, molecular chaperones, and other components of the translocon [37]. Our results suggest SERP1 as a novel binding partner of the β-subunit of ENaC, which dramatically inhibits ENaC expression and function. This inhibitory effect of SERP1 on ENaC appears to be selective, since it did not suppress CFTR.

Material and methods

Split-ubiquitin assay and protein identification The split-ubiquitin system (DUALmembrane technology, Dualsystems Biotech, Schlieren, Switzerland) was applied to screen βENaC against a human lung cDNA library. An oligo(dT) primed cDNA library was constructed from total human lung RNA and cloned into the prey vector pPR3-N (average insert size of 1.6 kbp, 4.8×106 independent clones). Approximately 2.3×10^6 transformants were screened for colonies growing on selective medium. For more details, see Supplement 5.

Cell line production Three cellular systems expressing fluorescently labeled βENaC, wtCFTR, or F508del-CFTR after induction with doxycycline (DOX) were created. For this, βENaC and CFTR were each fused in the N-terminus to mCherry, a fluorescent protein obtained from DsRed by changing the chromophore environment. Additionally, a FLAG tag (octapeptide: DYKDDDDK) was inserted by mutagenesis PCR. Cells were stably transfected using pLenti4-V5 (Invitrogen) with CMV promoter and pLenti with TetON DOX-sensitive promoter. For more details, see Supplement 5.

Cell culture H441 and A549 cells were grown in RPMI-1640 and DMEM media, respectively, substituted with 1 % Pen/Strep, 1 % insulin-transferrin-selenium, 200 nM dexamethasone, 2 % Ultrosa G in a humidified CO_2 incubator at 37°C. CFBE wtCFTR cells [3], which were derived originally from CFBE41o − cells [6] and Calu-3 cells were grown in MEM media supplemented with 10 % FBS and 1 % Pen/Strep. A549 inducible mCherry-FLAG-βENaC and mCherry-FLAG-CFTR cells were incubated with doxycycline at 100 ng/ml for 16 h for induction.

RNA extraction, reverse transcription, and real-time PCR Total RNA was extracted and reversed transcribed. Primers used for PCR reactions are listed in Supplement 5 (detailed material and methods). All relevant expression data were normalized using expression of the housekeeping gene large ribosomal protein RPLP0 or β-actin.

Immunofluorescence SERP1 and calnexin were visualized by immunofluorescence, and colocalization between the signals in the red and green channels was quantified in confocal images using Spearman's rank correlation coefficient using ImageJ PlugIn PSC-colocalization after background subtraction [13]. For more details, see Supplement 5.

Western blot, coimmunoprecipitation, and siRNA Expression of CFTR, βENaC, and SERP1 was quantified by Western blotting. Expression of SERP1 was suppressed using specific siRNAs (Silencer Select from Ambion (s25992, s25993)). As positive controls, cells were transfected with siRNA for CFTR (s2945, s2947) or βENaC (s12546, s12547). For coimmunoprecipitation, cells were lysed in 45 mM Tris pH 7.2, 135 mM NaCl, 1 mM Na_3VO_4, 5 mM EDTA, 5 mM $MgCl_2$, 1 mM EGTA, and 30 mM NaF and incubated overnight with the first Ab. Protein G agarose beads were added, and incubation continued for a further 4-h period.

Microscopic FMP assay Cells were loaded by continuous bath perfusion with FLIPR® Membrane Potential dye (FMP). FMP-quenching by amiloride (FMP-Amil) indicated ENaC conductance. We examined potential effects on cell volume using the well-established calcein fluorescence method. ENaC-expressing A549 cells as well as nonexpressing

cells were loaded with calcein and then exposed to 30 μM amiloride or FMP (20 μM). No significant changes were observed suggesting no effects of amiloride or FMP on cell volume. FMP excitation wavelength was 544 nm, and emission was measured at 590 nm. For more details, see Supplement 5.

Ussing chamber H441 cells were grown to confluence on permeable supports (12-mm diameter; Millipore, Schwalbach, Germany) for 10 days and were measured in a perfused micro-Ussing chamber as described earlier [1]. For more details, see Supplement 5.

cRNAs, double-electrode voltage clamp, and chemiluminescence Oocytes were injected with cRNA (10 ng, 47 nl double-distilled water) encoding the human SERP1 plus wt ENaC or wtCFTR and measured by DEVC as described earlier [18]. Membrane expression of βENaC in *Xenopus* oocytes was measured by chemiluminescence [24]. All methods are described in more detail in Supplement 5. All data are presented as mean values ± SEM. p values <0.05 are regarded as significant. The data of the different experimental series were normally distributed, and paired or unpaired t tests were used where appropriate.

Results

SERP1 interacts and colocalizes with βENaC in airway cells The yeast-based split-ubiquitin system was applied to screen for proteins that interact with βENaC. This technique detects interaction of integral membrane proteins in both plasma and intracellular membranes. It allows to use full-length integral membrane proteins as baits to hunt for partner proteins [25, 33]. We decided to use βENaC as a bait, since it is a highly regulated subunit of ENaC [22, 24]. The βENaC bait was tested by pairwise interaction with the two other ENaC subunits, αENaC and γENaC, which both interacted with βENaC in the split-ubiquitin system (Fig. 1a, left panel). Screening of a human lung cDNA library with βENaC as the bait identified the ER protein SERP1. Using SERP1 as the prey, we examined pairwise interaction using αENaC, βENaC, and γENaC as baits. SERP1 interacted with βENaC but showed only weak interaction with αENaC and γENaC (Fig. 1a, right panel).

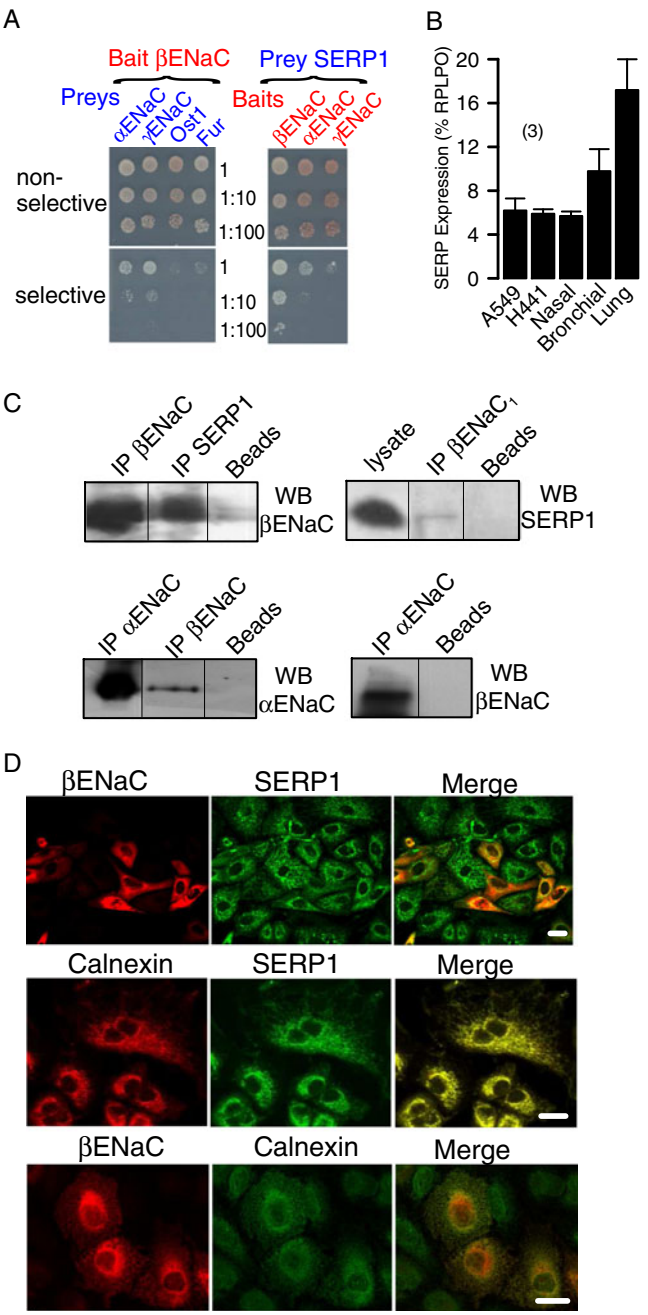
SERP1 is a broadly expressed chaperone present in human alveolar (A549) and bronchial (H441) epithelial cell lines, as well as nasal, bronchial and alveolar epithelial cells, as shown by real-time RT-PCR (Fig. 1b) and semiquantitative RT-PCR using β-actin as a reference (Supplement 1A).

Further evidence for direct interaction of SERP1 and βENaC in A549 cells was obtained by coimmunoprecipitation of both proteins. Using D3-βENaC antibody, βENaC was immunoprecipitated and was detected in Western blots using the H190-βENaC antibody. βENaC could be coimmunoprecipitated with SERP1 (Fig. 1b, upper left panels; Supplement 2A). Only small amounts of SERP1 were coimmunoprecipitated when βENaC was pulled down by the D3-βENaC, while no protein was detected when only beads were used in COIPs (Fig. 1c, upper right panels). Moreover, αENaC and βENaC could be coimmunoprecipitated by each other, suggesting that SERP1 and αβγENaC form a complex (Fig. 1c, lower panels).

We further examined colocalization of βENaC with SERP1 and the chaperone calnexin in A549 cells using a mCherry-fusion protein (βENaC) and specific antibodies for SERP1 and calnexin. A predominant portion of overexpressed βENaC was localized intracellularly, putatively within the ER, as demonstrated by colocalization with the ER-resident protein calnexin (Fig. 1d). The Spearman rank correlation coefficient (Spearman R) was 0.46±0.03 (n=11). Colocalization was also found for SERP1 and ENaC (Spearman R=0.29±0.03, n=16 cells) or SERP1 and calnexin (Spearman R=0.57±0.11, n=13). Similarly, colocalization of SERP1 was also found for αENaC and γENaC (data not shown).

SERP1 regulates ENaC The present results suggested regulation of βENaC expression by SERP1. To further examine this regulatory relationship, we knocked down expression of SERP1 in A549 cells by siRNA. Significant knockdown of SERP1 was demonstrated by Western blotting (Fig. 2a, b) and immunofluorescence (Fig. 2c) (p<0.05). Viability of transfected cells was assessed using trypan blue and MTS assays and demonstrated no reduced cell viability due to transfection with scrambled RNA or knockdown of SERP1 (data not shown). A second batch of siRNA also downregulated the expression of SERP1 significantly by about 50 % (n=3). We found that knockdown of low baseline SERP1 levels in A549 cells had no clear effects on the expression of βENaC (Fig. 2d, e). Similar results were found with another batch of siRNA-SERP1 (Supplement 1B–I). This result is in line with earlier reports indicating that SERP1 is expressed at low levels in alveolar and airway epithelial cells under control conditions but is largely upregulated under cell stress such as hypoxia [37].

Using the voltage-sensitive fluorescence dye FMP, we assessed amiloride-induced hyperpolarization which is proportional to the Na^+ transport in A549 cells. Upon application of FMP to A549 cells, the fluorescence was activated and was reduced subsequently by application of amiloride, which specifically blocks ENaC channels and thus hyperpolarizes the membrane voltage (Fig. 2f). Knockdown of SERP1 increased significantly the amiloride sensitive Na^+ transport in A549 alveolar epithelial cells (p<0.05), when measured as amiloride-inhibitable FMP fluorescence (FMP-



◀ **Fig. 1** SERP1 interacts with βENaC and is expressed in airways and alveolar cells. **a** Yeast was cotransformed with the bait p-BT3-N-βENaC and the preys NubG-αENaC and NubG-γENaC and the negative control preys Ost1-NubG and Fur4-NubG (*left panel*). The prey pPR3-N-SERP1 was transformed together with the baits pBT3-N-βENaC, pBT3-N-αENaC, and pBT3-N-γENaC (*right panel*). Dilutions were spotted onto nonselective medium and medium selective for protein–protein interaction. The data indicate interaction of the ENaC-subunits and βENaC with SERP1. **b** Quantitative real-time RT-PCR analysis of endogenous SERP1 expression in tissues and cell lines, normalized to the level of expression of the housekeeping gene RPLP0. Experiments were performed in triplicates. **c** Immunoprecipitation of βENaC by D3-anti-βENaC and coimmunoprecipitation of βENaC by SERP1-AB in A549 cells overexpressing mCherry-FLAG-βENaC. βENaC was detected by M2-anti-βENaC AB (*left lanes*). SERP1 was detected in A549 cell lysates. Only small amounts of SERP1 were coimmunoprecipitated when βENaC was pulled down by the D3-βENaC, while no protein was detected when only beads were used in COIPs (*upper right panels*). αENaC (endogenous) and βENaC could be coimmunoprecipitated by each other (Fig. 1c, *lower panels*). **d** Colocalization of SERP1, calnexin, and ENaC in A549 cells. βENaC (mCherry fluorescence, *red*) and SERP1 (Alexa 488, *green*) showed partial overlap (*upper panel*). ER-located calnexin (*red*) and SERP1 (*green*) demonstrated strong colocalization (*middle panel*). βENaC (*red*) and calnexin (Cy5, *green*) demonstrated partial overlap (*lower panel*) (*bar*=20 μm). *Numeral within parentheses* indicates the number of experiments

Amil) (Fig. 2f, g; Supplement 1). Effects of amiloride on FMP fluorescence were due to specific inhibition of ENaC since [1] effects were observed at low ENaC-specific concentrations of amiloride and [2] no effects of amiloride on FMP fluorescence were seen in the absence of ENaC expression (siRNA knockdown of ENaC) (Supplement J, K). siRNA knockdown of SERP1 by two different siRNAs batches was also performed in H441 human airway epithelial cells, which only marginally increased βENaC expression and slightly enhanced amiloride-sensitive Na^+ transport (data not shown). We also examined whether additional expression of SERP1 inhibits ENaC in A549 cells. To that end, we compared amiloride-sensitive FMP fluorescence in mock-transfected cells with that in SERP1-overexpressing cells and found a significant ($p<0.05$) inhibition of amiloride-sensitive Na^+ transport (FMP-Amil) by SERP1 (Fig. 3a, b). Although we did not assess potential direct effects of SERP1 on the open probability of ENaC, the present experiments strongly suggest that SERP1 has a pronounced inhibitory effect on amiloride-sensitive Na^+ transport probably by affecting early biogenesis of ENaC.

SERP1 inhibits trafficking of ENaC Although unlikely, ENaC may be inhibited by SERP1 through enhanced dynamin-dependent endocytosis and retrieval from the plasma membrane. We therefore examined the effects of siRNA in the absence or presence of the dynamin inhibitor dynasore [19]. As expected, dynasore enhanced amiloride-sensitive transport significantly, due to inhibition of endocytosis of ENaC. However, in the presence of dynasore, siRNA knockdown of SERP1 still upregulated ENaC-dependent transport (Fig. 3c). These results suggest that SERP1 does not operate through activation of endocytic pathways. Hypoxia has been shown to inhibit ENaC in alveolar epithelial cells, probably due to activation of chaperones and reduced membrane expression [8, 17, 36]. Indeed, hypoxia significantly inhibited FMP-Amil, and this negative effect of hypoxia on Na^+ transport was significantly ($p<0.05$) antagonized by knockdown of SERP1 (Fig. 3d). SERP1 could therefore have a role in hypoxia-induced inhibition of ENaC [8, 17, 36].

In order to examine whether a similar SERP1-dependent regulation of Na^+ absorption occurs in airway epithelial cells and to see whether this also takes place under polarized

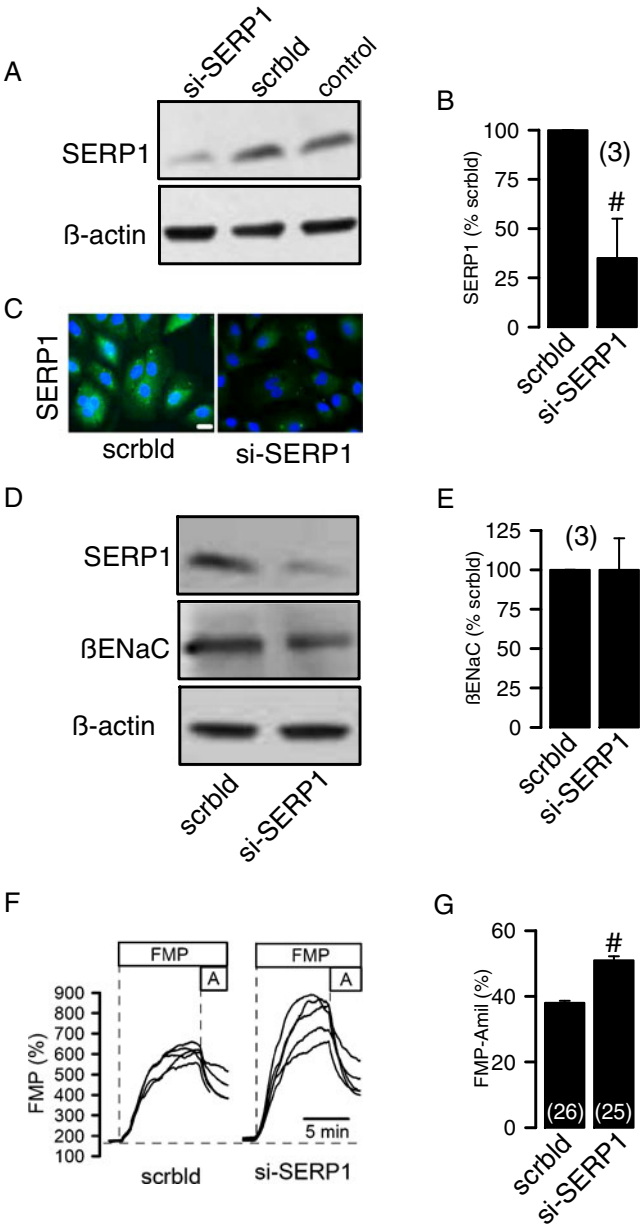


Fig. 2 Regulation of biogenesis of ENaC by SERP1. **a** Expression of SERP1 (Western) in A549 cells and downregulation by siRNA. β-actin is shown as a loading control. **b** Densitometric analysis of downregulation of SERP1 by 60 nM siRNA (relative to SERP1 expression in cells treated with scrambled RNA). **c** Immunocytochemistry of SERP1 expressed in A549 cells treated with siRNA-SERP1 or scrambled RNA (*bar* 10 μm). **d** Expression (Western) of SERP1, βENaC (stably overexpressed), and β-actin in A549 cells treated with siRNA-SERP1 or scrambled RNA. **e** Densitometric analysis of βENaC expression in A549 cells treated with siRNA-SERP1 (relative to scrambled), normalized to β-actin (loading control). **f** Original recordings of fluorescence generated by the voltage-sensitive dye FMP and effects of amiloride in A549 cells treated with scrambled RNA or siRNA for SERP1. Perfusion of the bath with FMP induced fluorescence. Fluorescence quenching by application of amiloride (*A*, 10 μM) due to inhibition of ENaC and hyperpolarization of the membrane voltage, which is a measure for ENaC conductance. **g** Summary of the effects of siRNA knockdown of SERP1 and scrambled RNA on amiloride-inhibited FMP. FMP-Amil (%) reflects the inhibition of FMP fluorescence induced by amiloride *Numeral within parentheses* indicates the number of experiments. *Number sign* indicates significant difference (unpaired *t* test)

conditions, we examined amiloride-sensitive transport in filter-grown H441 airway epithelial cells in a perfused Ussing chamber under open circuit conditions. In fact, knockdown of SERP1 enhanced the lumen-negative transepithelial voltage (V_{te}) that was produced by amiloride-sensitive Na^+ channels (Fig. 3e). From amiloride-induced voltage deflections, we calculated the Na^+ transport by ENaC (Isc-Amil) and found a significant upregulation of ENaC by siRNA knockdown of SERP1 (Fig. 3f, $p<0.05$).

Nedd4-2-independent inhibition of ENaC We also examined regulation of ENaC by SERP1 when expressed in *Xenopus* oocytes using double-electrode voltage clamp, which is a powerful system to study functional interactions of proteins [18]. Expression of all three ENaC subunits in *Xenopus laevis* oocytes generated a large Na^+ current that was strongly inhibited by amiloride (A) (Fig. 4a, left tracing—con). Coexpression of ENaC with SERP1 abolished $p<0.05$) amiloride-sensitive membrane currents and whole-cell conductance (Fig. 4a, b). SERP1 not only inhibited amiloride-sensitive membrane currents but also reduced membrane expression of ENaC, as measured by chemiluminescence, using a βENaC subunit that was FLAG-tagged in the extracellular epitope in combination with peroxidase-coupled antibodies (Fig. 4e, $p<0.05$). The ubiquitin ligase Nedd4-2 is a distal regulator of plasma membrane expression of ENaC [10, 21, 32]. Truncation of the C-terminus of αENaC (H648X-αENaC) has been shown to interfere with regulation of ENaC by Nedd4-2 [18]. If SERP1 would inhibit ENaC via a Nedd4-2-dependent mechanism, ENaC channel formed by H648X-αENaCβ,γ should be largely independent of SERP1. As expected, expression of H648X-αENaCβ,γ produced largely augmented amiloride-sensitive whole-cell conductances (G= 121 ± 34 μS, n=18), when compared to wt-αENaCβ,γ (G= 32 ± 4.3 μS, n=10) (Fig. 4c). However, the fraction of H648X-αENaCβ,γ inhibited by coexpression of SERP1 was almost identical to that of wt-αENaCβ,γ (Fig. 4d). This suggests that SERP1 inhibits ENaC in a Nedd4-2-independent fashion and probably at an early stage during biogenesis. Also, chemiluminescence of FLAG-tagged alpha-ENaC-H648X was changed by coexpression of SERP1 from 2,100 ±190 to 710±96 rel.U. (n=6). Moreover, immunocytochemistry suggested reduced expression of βENaC after overexpression of SERP1, while suppression of low baseline SERP1 levels by siRNA only slightly increased expression of βENaC (Supplement 2B). Taken together, the results show that enhanced expression of SERP1 inhibits expression of ENaC, probably at an early stage of biogenesis.

SERP1 does not suppress expression of CFTR We examined whether the effects of SERP1 on ENaC are specific or

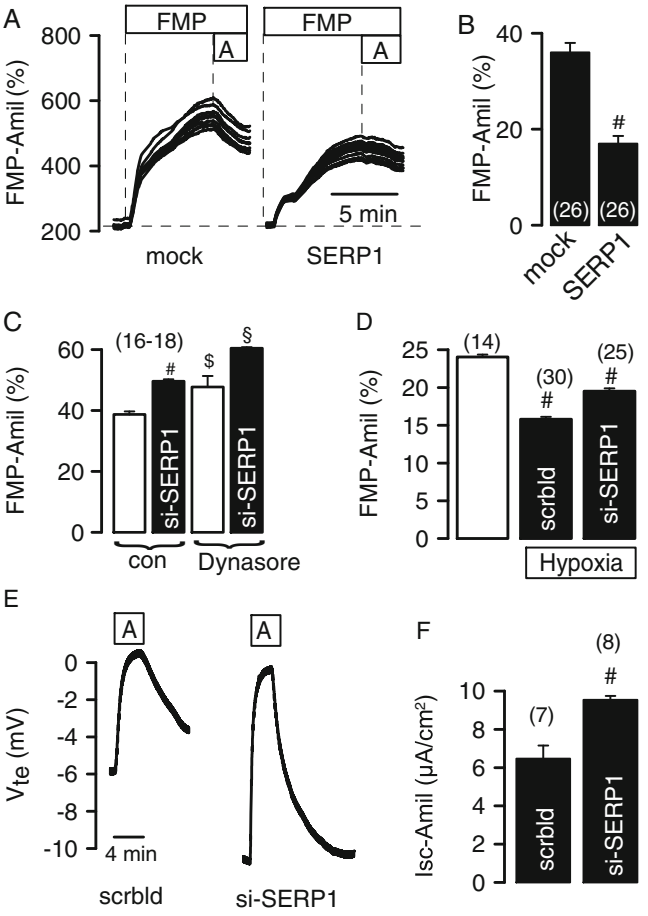


Fig. 3 Mechanism of regulation of endogenous ENaC by SERP1 and effects of hypoxia. **a** Original recordings of FMP fluorescence in A549 cells and effects of overexpression of SERP1 or mock transfection (empty plasmid). Fluorescence quenching by application of amiloride (*A*, 10 μM) due to inhibition of endogenous ENaC and hyperpolarization of the membrane voltage. **b** Summary of the effects of overexpression of SERP1 or mock transfection on FMP-Amil. FMP-Amil (%) reflects the inhibition of FMP fluorescence induced by amiloride. **c** Summary of the effects of siRNA knockdown of SERP1 on FMP-Amil in the absence or presence of dynasore, which inhibits dynamin-dependent endocytosis. **d** Summary of the effects of hypoxia (2 %) and siRNA knockdown of SERP1 (60 nM) on FMP-Amil, which attenuated significantly the inhibitory effect of hypoxia on ENaC. **e** Original recordings of Ussing chamber experiments in polarized H441 cells, grown on permeable supports. Na^+ absorption causes a lumen-negative transepithelial voltage (V_{te}), which is abolished by inhibition of ENaC with amiloride (*A*, 30 μM). **f** Summary of the calculated equivalent short-circuit currents inhibited by amiloride ($I_{sc\text{-}Amil}$) in cells treated with scrambled RNA or SERP1-siRNA. *Numeral within parentheses* indicates the number of experiments. All functional measurements were performed immediately after removal of the cells from hypoxia. *Number sign* indicates significant difference (unpaired *t* test). *Dollar and section* signs indicate significant differences when compared to control and dynasore, respectively (unpaired *t* test)

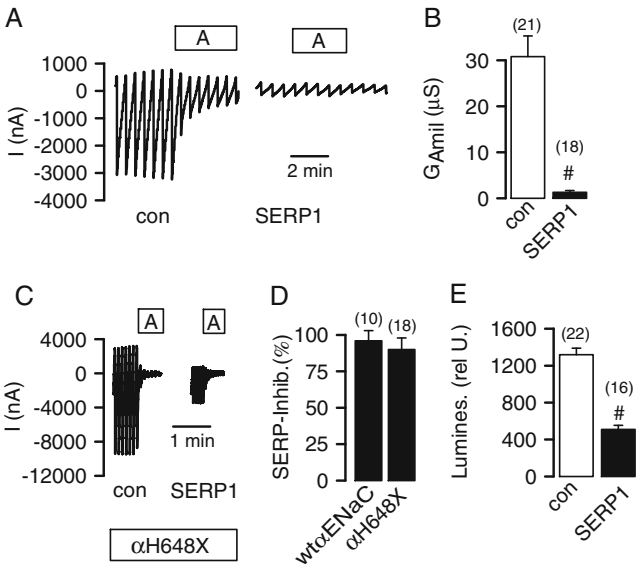


Fig. 4 SERP1 inhibits ENaC in Xenopus oocytes. **a** Original tracings of whole-cell currents measured in oocytes expressing three ($\alpha\beta\gamma$) ENaC subunits in the absence or presence of coexpressed SERP1. **b** Summary of the calculated amiloride-sensitive whole-cell conductances. **c** Original tracings of whole-cell currents measured in oocytes expressing H648H-αENaC together with wt βγENaC subunits in the absence or presence of coexpressed SERP1. **d** Summary of the relative inhibition of amiloride-sensitive currents generated by wild-type $\alpha\beta\gamma$ENaC or H648H-α,βγENaC. **e** Expression of βENaC in oocyte membranes as detected by chemiluminescence and inhibition of membrane expression by coexpressed SERP1. *Numeral within parentheses* indicates the number of experiments. *Number sign* indicates significant difference (unpaired *t* test)

whether it also inhibits other epithelial ion channels, such as CFTR. We examined the effects of siRNA knockdown of SERP1 on CFTR in the two human airway epithelial cell lines Calu-3 and CFBE/wtCFTR. To our surprise, expression of CFTR was not augmented but was significantly ($p<0.05$) reduced by SERP1-siRNA, suggesting SERP1 as a potent positive regulator of CFTR expression (Fig. 5a, b; Supplement 3). Notably, knockdown of SERP1 largely reduced expression of CFTR-Band C in airway cells expressing CFTR endogenously (Calu-3) or overexpressing CFTR (CFBE/wtCFTR), indicating that the fully mature, membrane-localized form of CFTR is reduced in these cells. This was further confirmed by increased levels of polyubiquitinated CFTR under downregulation of SERP1 (data not shown). Thus, SERP1 interaction is not unique to ENaC but is also demonstrated for CFTR (also shown in Supplement 3). CFTR function was examined upon expression in *Xenopus* oocytes and after activation of CFTR by stimulation with IBMX and forskolin (I/F). Notably, coexpression of CFTR together with SERP1 augmented CFTR currents significantly ($p<0.05$), when compared with solely expression of CFTR (Fig. 5c, d). However, in contrast to wtCFTR, residual Cl^- currents generated by the most frequent CFTR mutant F508del-CFTR were not augmented by SERP1. Expression of SERP1 alone did not change the properties of oocytes (data not shown). Taken together, the present results indicate a specific inhibitory effect of the newly identified cochaperone SERP1 on expression of ENaC with some differential positive effects on CFTR expression. Thus, SERP1

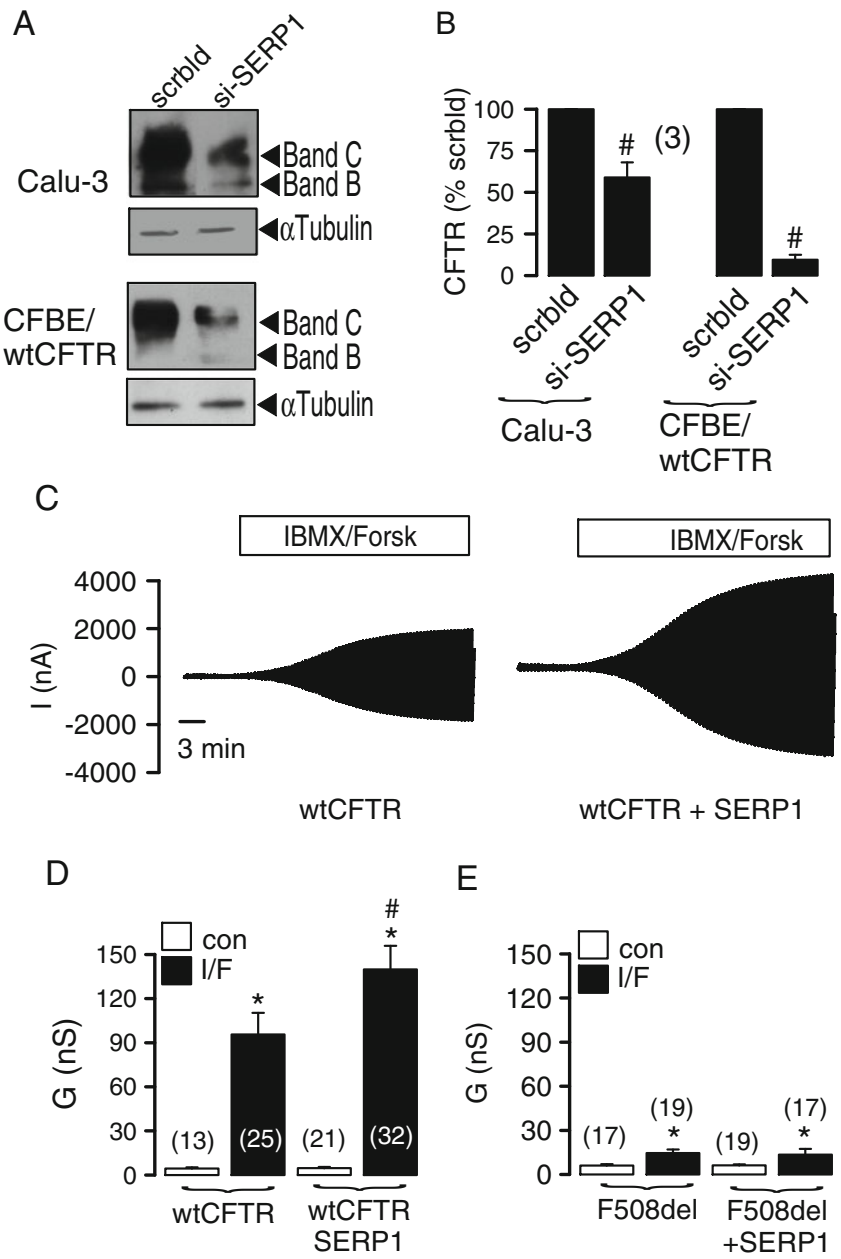


Fig. 5 No inhibition of CFTR by SERP1. **a** Expression (Western) of CFTR in Calu-3 (endogenous) cells and CFBE cells stably expressing wtCFTR (exogenous) in the absence or presence of SERP1-siRNA. **b** Densitometric analysis of Western blots indicates significant inhibition of CFTR expression by siRNA knockdown of SERP1 (60 nM), when compared with the treatment by scrambled RNA. **c** Original tracings of whole-cell currents measured in CFTR-expressing *Xenopus* oocytes, when activated by application of IBMX and forskolin (1 mM/2 μM). Currents were larger in oocytes coexpressing SERP1. Summary of the calculated whole-cell conductances in oocytes expressing wtCFTR (**d**) or the most common CFTR mutant F508del-CFTR (**e**) with or without coexpression of SERP1. *Numeral within parentheses* indicates the number of experiments. *Number sign* indicates significant difference (unpaired *t* test). *Asterisk* indicates significant activation by I/F (paired *t* test)

could be a pharmacological target to inhibit excessive airway Na^+ absorption in cystic fibrosis or may be a useful target to counteract lung edema during left heart failure, ARDS, or high-altitude breathing.

Discussion

SERP1 inhibits biogenesis of ENaC The present study identified SERP1 as a novel regulator of ENaC expression in airway and alveolar epithelial cells. SERP1 was identified in a yeast-based split-ubiquitin screening using the ENaC β-subunit as a bait. The pronounced inhibitory effect of SERP1 appears to be rather specific for ENaC, since another ion channel, CFTR, often coexpressed in epithelial cells together with ENaC, was not inhibited by SERP1. SERP1 even appears to be necessary for proper expression of CFTR. SERP1 is also known as RAMP4, which is a small tail-anchored membrane protein that exposes its N-terminus to the cytoplasm and its C-terminus to the luminal side of the ER membrane [12]. It is recruited to the translocon complex when the transmembrane segment of the nascent chain of a membrane protein is present in the ribosomal exit tunnel. There it interacts with Sec61α and Sec61β. Thus, SERP1 has been implicated in stabilizing newly synthesized membrane proteins and regulating N-linked glycosylation [30].

Notably, Sec61α and Sec61β, but not SERP1, were found to associate with newly synthesized integral membrane proteins under stress conditions, suggesting that stabilization of

membrane proteins in response to stress is due to other members of the translocon, as well as ER-localized chaperons. However, SERP1 may serve as a cochaperone since it interacts with the chaperone calnexin [37]. Notably, we did not find an additive effect of siRNA knockdown of SERP1 and calnexin on amiloride-sensitive Na^+ transport (data not shown). SERP1 may interact directly with target proteins or may indirectly regulate integral membrane proteins during biosynthesis, such as RAGE and CD8 [37]. The effects of inhibiting (siRNA) and increasing (overexpression) SERP1 expression on ENaC activity were consistent among the different cell lines and in oocytes. Probably, due to very efficient overexpression, the inhibitory effect of SERP1 on ENaC was very pronounced (95 %) in *Xenopus* oocytes. Although we did not examine potential inhibitory effects of SERP1 on the open probability of ENaC in patch clamp experiments, the present results suggest a dominant inhibitory effect on the biogenesis of ENaC.

Hypoxic inhibition of ENaC Hypoxia is well known to induce cell stress and to downregulate ENaC activity as demonstrated recently [20, 29, 34, 36]. Inhibition of ENaC is due to compromised trafficking of ENaC to the cell membrane [5]. However, Bouvry and collaborators demonstrated that hypoxia also disrupts the cytoskeleton as well as the tight junctions in alveolar epithelial cells [5]. This probably contributes to hypoxia-induced decrease in Na^+ transport. Moreover, the team demonstrated that reduced anterograde trafficking under hypoxia is reversed by simultaneous stimulation of the cells with beta-2-receptor agonists [29]. Since hypoxia has also been demonstrated to enhance expression of SERP1, it is likely that at least parts of the hypoxic effects on ENaC are caused by upregulation of SERP1 [37]. In fact, we detected upregulation of SERP1 protein and mRNA by hypoxia in both A549 and H441 cells (Supplement 4). Using semiquantitative RT-PCR, increased expression of SERP1 was related to expression of the housekeeping protein RPLP0 (Supplement 4C) or β-actin (0.21 ± 0.05 vs. 0.53 ± 0.06, $n=3$). The effects of hypoxia on ENaC were completely revoked by dexamethasone, which is known to antagonize hypoxia-induced inhibition of protein synthesis, as reported earlier for A549 cells [17, 26]. Also, in our studies, we found complete blockage of hypoxic inhibition of ENaC in A549 cells by 0.1 μM dexamethasone (Supplement 4D). Taken together, the present experiments provide some evidence for the role of SERP1 for hypoxic inhibition of ENaC and alveolar Na^+ absorption, which is a severe problem in left heart failure, ARDS, and during high-altitude breathing [17].

SERP1 activates CFTR We examined the effects of SERP1 on maturation of endogenous CFTR (Calu-3 cells) and overexpressed CFTR (CFBE/CFTR cells). The data indicate significant inhibition of expression of CFTR by knockdown of SERP1. We found that activation of wtCFTR, but not F508del-CFTR, a trafficking (class II) mutant which is mostly retained at the ER, was enhanced by SERP1. Class II mutations, including the most prevalent F508del mutation, cause retention of misfolded protein in the ER and subsequent degradation by the proteasome [2]. This somewhat surprising effect of SERP1 on CFTR might be explained by the fact that SERP1 acts in a calnexin-dependent manner. Accordingly, while calnexin has been shown to be required for correct folding and processing of wtCFTR [9, 15, 31], F508del-CFTR is targeted to degradation at an earlier folding checkpoint during protein synthesis, involving the Hsp70 chaperone machinery and mostly independent of calnexin [11]. So far, we have no evidence for enhanced expression of SERP1 in epithelial cells from CF patients. Nevertheless, activation of SERP1 could be beneficial in CF, to counteract hyperabsorption of Na^+ and to promote secretion which both ought to improve mucociliary clearance and lung function [28].

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