Mutations linked to interstitial lung disease can abrogate anti-amyloid function of prosurfactant protein C

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INTRODUCTION

Human lung surfactant protein C (SP-C), a hydrophobic 35-residue acylated transmembrane peptide, is synthesized as a 197-residue proprotein [proSP-C (prosurfactant protein C)] that undergoes multiple proteolytic cleavages [1]. The mature SP-C peptide corresponds to residues 24–58 of proSP-C (see Figures 1 and 7) and is flanked by an N-terminal propeptide that is important for trafficking and stability of the proprotein in the secretory pathway [2,3], and a C-terminal domain with unknown function. In the lung, proSP-C is expressed only in alveolar type II epithelial cells and is anchored in the ER (endoplasmic reticulum) membrane in a type II orientation (the C-terminus extends into the ER lumen) [4,5]. SP-C, together with SP-B and phospholipids, are secreted into the alveoli and are responsible for lowering surface tension at the air/liquid interface, thereby preventing alveolar collapse at end expiration [6].

SP-C, and hence also proSP-C, contains a polyvaline stretch that forms a transmembrane α-helix. This helix is composed of amino acids with a high propensity to form β-strands [7]. The SP-C helix is consequently metastable in solution and can spontaneously convert into β-sheet aggregates and amyloid fibrils [8,9]. SP-C fibrils have been observed in the alveoli of PAP (pulmonary alveolar proteinosis) patients, but not in healthy controls [9]. Replacement of the SP-C polyvaline segment with polyleucine results in a stable CTC–proSP-C[Δ380] complex, increased proSP-C[Δ380] half-life and reduced formation of Congo Red-positive deposits; (iii) replacement of the metastable polyvaline transmembrane segment with a stable polyleucine transmembrane segment likewise prevents formation of amyloid-like proSP-C[Δ380] aggregates; and (iv) binding of recombinant CTC to non-helical SP-C blocks SP-C amyloid fibril formation. These results suggest that CTC can prevent the polyvaline segment of proSP-C from promoting formation of amyloid-like deposits during biosynthesis, by binding to non-helical conformations. Mutations in the Brichos domain of proSP-C may lead to ILD via loss of CTC chaperone function.

Key words: amyloid, Brichos domain, chaperone, interstitial lung disease, prosurfactant protein C (proSP-C), protein folding.

The newly synthesized proSP-C (surfactant protein C precursor) is an integral ER (endoplasmic reticulum) membrane protein with a single metastable polyvaline α-helical transmembrane domain that comprises two-thirds of the mature peptide. More than 20 mutations in the ER-luminal CTC (C-terminal domain of proSP-C), are associated with ILD (interstitial lung disease), and some of the mutations cause intracellular accumulation of cytotoxic protein aggregates and a corresponding decrease in mature SP-C. In the present study, we showed that: (i) human embryonic kidney cells expressing the ILD-associated mutants proSP-C[Δ380] and proSP-C[ΔC-terminal] accumulate Congo Red-positive amyloid-like inclusions, whereas cells transfected with the mutant proSP-C[Δ77S] do not; (ii) transfection of CTC into cells expressing proSP-C[Δ380] results in a stable CTC–proSP-C[Δ380] complex, increased...
localized in a region between the Birchos domain and the transmembrane domain, were associated with altered intracellular trafficking, but not aggregation [18,19].

Protein folding is a complex process further complicated by the crowded intracellular milieu. The importance of molecular chaperones, such as heat-shock proteins, in the folding process is well known and has been studied extensively [20,21]. In addition to chaperones, a number of proteins use part of their pro-sequence as a scaffold during folding. The importance of pro-sequence-assisted folding has been established for several proteins, e.g. serine proteases [22] and human nerve growth factor [23]. A recent study from our group showed that recombinant CTC binds to unfolded SP-C, but not to α-helical SP-C [24]. These findings, together with the phenotypes associated with mutations in proSP-C, led us to investigate the hypothesis that CTC promotes stability during biosynthesis of proSP-C by preventing misfolding and aggregation of the polyvaline transmembrane domain.

EXPERIMENTAL

Analysis of aggregated and soluble proSP-C in HEK-293 cells

The generation and culture of HEK-293 cell lines stably expressing human wild-type proSP-C, proSP-C L188Q and proSP-C A180D have been described previously [24,25]; proSP-C C37T was constructed in a similar manner. A construct encoding the signal peptide from proSP-B, CTC and the ER retention signal KDEL (Lys-Asp-Glu-Leu) (CTC–KDEL) was cloned into pcDNA3.1 (Invitrogen) and constructs encoding proSP-C, proSP-C L188Q, proSP-C(Leu) or proSP-C(Leu)L188Q (Figure 1) were cloned into pIRE2-EGFP (BD Bioscience). Addition of a KDEL tag (which is not found in proSP-C) does not alter the effect of CTC on wild-type proSP-C, i.e. CTC does not interact with wild-type proSP-C (see Figure 3), and CTC expression is not altered by inclusion of the KDEL tag as observed by pulse–chase experiments (results not shown). A C-terminal HA (haemagglutinin) tag (YPYDVPDYA) was added to all full-length constructs used in transient transfection experiments. Cells were cultured until reaching 70% confluence and thereafter removed from the dish and replated on culture slides at 1.5 × 10⁵ cells/slide. Cells transiently transfected with proSP-C(Leu)L188Q or proSP-C(Leu)L188Q as described above, were replated on culture slides at 3.5 × 10⁵ cells/slide 24 h after transfection. After 24 or 48 h, the cells were washed three times with PBS, fixed with 4% paraformaldehyde and permeabilized with 0.5% (v/v) Triton X-100. The cells were then incubated in 80% ethanol with saturated NaCl and 1% NaOH for 10 min, followed by 10 min in the same solution with 0.02% Congo Red. Finally, the slides were rinsed in ethanol for 5 s before dehydration and mounting. The cells were analysed and photographed using a Zeiss LSM 510 Meta confocal microscope. Excitation wavelength was 543 nm, and a normal light image was used as background to show the cell-surface structure. For each low magnification spot suspected to represent intracellular protein aggregates, a Z-stack tool for visualization of different layers at higher magnification was used to differentiate it from condensed nuclear material. The arrows seen in Figure 2 mark spots concluded to represent protein aggregates by this procedure.

Congo Red fluorescence

HEK-293 cells stably expressing human wild-type proSP-C, proSP-C(Leu)L188Q, proSP-C A180D or proSP-C C37T were cultured until reaching 70% confluence and thereafter removed from the dish and replated on culture slides at 1.5 × 10⁵ cells/slide. Cells transiently transfected with proSP-C(Leu)L188Q or proSP-C(Leu)L188Q as described above, were replated on culture slides at 3.5 × 10⁵ cells/slide 24 h after transfection. After 24 or 48 h, the cells were washed three times with PBS, fixed with 4% paraformaldehyde and permeabilized with 0.5% (v/v) Triton X-100. The cells were then incubated in 80% ethanol with saturated NaCl and 1% NaOH for 10 min, followed by 10 min in the same solution with 0.02% Congo Red. Finally, the slides were rinsed in ethanol for 5 s before dehydration and mounting. The cells were analysed and photographed using a Zeiss LSM 510 Meta confocal microscope. Excitation wavelength was 543 nm, and a normal light image was used as background to show the cell-surface structure. For each low magnification spot suspected to represent intracellular protein aggregates, a Z-stack tool for visualization of different layers at higher magnification was used to differentiate it from condensed nuclear material. The arrows seen in Figure 2 mark spots concluded to represent protein aggregates by this procedure.

Pulse–chase and immunoprecipitation experiments

HEK-293 cells were incubated for 30 min in methionine- and cysteine-free DMEM (Dulbecco’s modified Eagle’s medium).
(Invitrogen) supplemented with 10 % dialysed fetal bovine serum. Cells were then pulse-labelled with 0.5 mCi/ml [35S]methionine/cysteine (MP Biomedicals) for 30 min and chased in complete medium (fresh DMEM containing 95 mM non-radio labelled methionine/cysteine) for 1, 2 or 4 h. Immunoprecipitations were performed from equivalent amounts of cell lysates with anti proSP-C and Protein G beads (Invitrogen) at 4°C overnight on a rotating wheel. Captured immunocomplexes were washed six times and then incubated at 100°C for 4 min with electrophoresis sample buffer. Immunoprecipitated proteins were analysed by SDS/PAGE (10–20 % Tricine gels) under reducing electrophoretic conditions followed by autoradiography.

Co-precipitation experiments
Stably transfected HEK-293 cells expressing wild-type proSP-C or proSP-C<sub>Exon4</sub> (2.5 × 10<sup>8</sup>) were transiently transfected with 10 μg of pcDNA3.1 + (Invitrogen) or pcDNA3.1+–CTC–KDEL and 18 μl of Lipofectamine<sup>TM</sup> 2000 (Invitrogen) in lysine-coated six-well plates. At 48 h, cells were washed twice with 1 × PBS and harvested in 500 μl of lysis buffer [20 mM Hepes (pH 7.6), 125 mM NaCl, 10 % (v/v) glycerol, 1 % (v/v) Triton X-100, 1 mM EDTA, 1 mM EGTA, 1 mM DT (dithiothreitol) and Sigma #P8340 protease inhibitor cocktail]. After 2 h of rotation at 4°C, cells were briefly sonicated and centrifuged at 9300 g for 10 min at 4°C. Supernatants were incubated with or without 5 μl of N-terminal proSP-C antibody overnight at 4°C. Samples were rotated with 50 μl of recombinant Protein A–Sepharose 4B (Zymed Laboratories) at room temperature for 2 h. Four 1 ml washes (20 mM Hepes, pH 7.6, 500 mM NaCl and 1 % Triton X-100) were followed by elution with 50 μl of reducing Laemmlli buffer. Eluates were heated at 100°C for 4 min and subjected to Western blot analysis with C-terminal anti-proSP-C antibody using GenScript one-step IP-Western kit.

CTC expression and purification
CTC was expressed and purified as described recently [24]. Briefly, a fragment covering residues 59–197 of human proSP-C (Figure 1) was expressed as a fusion protein with thioredoxin, His<sub>6</sub> and S-tags in Escherichia coli. The protein was purified using immobilized metal affinity and ion-exchange chromatography. Thrombin was used for removal of thioredoxin and His<sub>6</sub> and S-tags in C (Figure 1) was expressed as a fusion protein with thioredoxin, CTC and the individual peptides were mixed at a 1:1 molar ratio and 18 μl of the suspended SDS-insoluble pellets were aliquoted for 5 min at 4°C. Supernatants were incubated with or without 5 μl of recombinant Protein A–Sepharose 4B (Zymed Laboratories) at room temperature for 2 h. Four 1 ml washes (20 mM Hepes, pH 7.6, 500 mM NaCl and 1 % Triton X-100) were followed by elution with 50 μl of reducing Laemmli buffer. Eluates were heated at 100°C for 4 min and subjected to Western blot analysis with C-terminal anti-proSP-C antibody using GenScript one-step IP-Western kit.

SP-C amyloid fibril formation
Curosurf and SP-C33 (IIPSPVHLKRKLLLLLLIIILLI-LGALLMGL) surfactant were provided by Chiesi Farmaceutici SpA. Curosurf is prepared from porcine lung and contains 98 % (w/w) phospholipids and 1–2 % (w/w) of SP-B and SP-C [28]. SP-C33 surfactant is fully synthetic and contains 98 % (w/w) phospholipids and 2 % (w/w) of an SP-C analogue in which the polyvaline sequence of native SP-C is replaced by a poly-leucine stretch [29]. Curosurf or SP-C33 surfactant were diluted to 5 mg/ml in 150 mM NaCl with 0.02 % sodium azide to a final volume of 4.5 ml and incubated at 22°C with shaking, with or without 7.5 μM CTC. This corresponds to a CTC-SP-C/SP-C33 molar ratio of 0.5–1. After 0, 7 or 14 days, suspensions were centrifuged at 33,000 rev./min for 30 min at 22°C in a T:70 rotor, the supernatants were removed, and the pellets were suspended in 1 % (w/v) SDS. This procedure was repeated four times. The final, SDS-insoluble, pellets were suspended in 200 μl of water and bath-sonicated. Aliquots of 2 μl were removed for electron microscopy as described below.

Transmission electron microscopy
Aliquots of 2 μl of the suspended SDS-insoluble pellets were adsorbed for 1 min on 200-mesh copper grids and stained with 2 % uranyl acetate for 30 s before being examined and photographed using a Hitachi H7100 microscope operated at 75 or 100 kV.

MALDI-MS
SP-C33 and a synthetic non-helical SP-C analogue (LRIPSSPVNLKRLVVVVVVVVVVVVVVGALLMGL) [30] were dissolved to a concentration of 4.5 mM in ethanol or methanoic acid respectively, and diluted 10-fold in 30 % (v/v) acetonitrile and 0.1 % TFA (trifluoroacetic acid). CTC in 20 mM sodium phosphate buffer (pH 7.4) and 30 mM NaCl was diluted 10-fold to a concentration of 120 μM in 30 % (v/v) acetonitrile and 0.1 % TFA. CTC and the individual peptides were mixed at a 1:1 molar ratio and diluted 100-fold further in 30 % (v/v) acetonitrile and 0.1 % TFA. Aliquots of 0.5 μl of the CTC/peptide mixtures were spotted on to a pre-prepared thin layer of 30 mg/ml sinapinic acid (Bruker Daltonics) in acetone and covered with another 0.5 μl of 30 mg/ml sinapinic acid in 50 % (v/v) acetonitrile and 0.1 % TFA. MALDI-MS was performed using a Bruker Autoflex instrument (Bruker Daltonics) in linear mode.

RESULTS
Aggregation behaviour of proSP-C<sub>Exon4</sub>, proSP-C<sub>Exon4</sub> and proSP-C<sub>Exon4</sub> in HEK-293 cells
To study effects of the L188Q, ΔExon4 and I73T mutations respectively on proSP-C aggregation, HEK-293 cells stably transfected with wild-type proSP-C or mutant proSP-C were used. Congo Red staining, a classical method for detection of amyloid [31], revealed red spots in the cells expressing proSP-C<sub>Exon4</sub> or proSP-C<sub>ΔExon4</sub> (Figure 2A). In the L188Q cells, the spots were brighter than in ΔExon4 cells. Cells expressing proSP-C<sub>ΔExon4</sub> showed <10 % red spots compared with proSP-C<sub>Exon4</sub> and proSP-C<sub>ΔExon4</sub> (results not shown), and in cells expressing wild-type proSP-C, no Congo Red spots could be found (Figure 2A). To examine further the effect of the mutations, SDS-insoluble, but methanoic acid-soluble, protein (a feature typical of amyloid [32]) from HEK-293 cells expressing either wild-type or mutant proSP-C was analysed using Western blotting (Figure 2B). In the mutant proSP-C cells, large (> 210 kDa) immunoreactive aggregates were found, which were not present in the cells expressing wild-type proSP-C. The relative amounts of these aggregates were L188Q > ΔExon4 > I73T (Figure 2B). Together, these findings indicate that the L188Q mutation promotes aggregation into Congo Red-positive deposits in HEK-293 cells, and the same phenomenon, although somewhat less pronounced, is seen with the ΔExon4 mutation. The I73T mutation does not produce Congo Red-positive inclusions in HEK-293 cells, and gives rise to smaller amounts of SDS-insoluble protein. Further experiments were performed using cells expressing proSP-C<sub>Exon4</sub>, since these showed the clearest differences compared with wild-type cells.
ProSP-CL188Q aggregation after transfection with CTC

HEK-293 cells stably expressing proSP-CL188Q were transiently transfected with CTC–KDEL (Figure 1) and were analysed for Congo Red inclusions and amounts of aggregated and soluble proSP-CL188Q. An increase in SDS-soluble proSP-CL188Q levels was observed (results not shown), and was more pronounced than found previously after transfection with CTC lacking KDEL [24], as expected since the KDEL tag holds CTC in the ER. More importantly, transfection of CTC inhibited formation of Congo Red-positive inclusions (Figure 3A) and SDS-insoluble/methanoic acid-soluble aggregates (Figure 3B). Moreover, co-precipitation experiments showed that CTC binds to proSP-CL188Q (in the absence of any cross-linking reagent), while only marginally binding to wild-type proSP-C (Figure 3C). Pulse-chase experiments showed a pronounced increase in proSP-CL188Q half-life upon CTC transfection and confirmed that CTC forms a complex with proSP-CL188Q (Figure 3D). Formation of CTC–proSP-CL188Q complex also increased the stability of CTC (Figure 3D). These results indicate that CTC added in trans exerts effects in the ER, forms a complex with proSP-CL188Q and prevents aggregation of proSP-CL188Q.

Recombinant CTCCL188Q does not aggregate in vitro [24], which suggests that the proSP-CL188Q inclusions seen in HEK-293 cells (Figure 2) may be mediated by aggregation of a region(s) outside CTC. Furthermore, recombinant CTCCL188Q, in contrast with wild-type CTC, does not bind to non-helical SP-C [24]. It is thus possible that the polyvaline segment mediates proSP-CL188Q aggregation, and that, in the CTC–proSP-CL188Q complex, CTC binds to the polyvaline region of proSP-C. In order to test these hypotheses, we next studied the effects of CTC on SP-C amyloid fibril formation (a process that requires unfolding of its α-helix [8]), CTC binding to non-helical SP-C, and the effects of replacing the polyvaline region of proSP-CL188Q with a polyleucine sequence.

Effects of recombinant CTC on SP-C amyloid fibril formation and CTC binding to SP-C

In order to study SP-C in its natural phospholipid environment, a lung surfactant preparation containing phospholipids, SP-C and SP-B, was incubated at room temperature for 7 or 14 days and thereafter subjected to an SDS extraction procedure used previously for isolation of fibrillar amyloid β-peptide from brain tissue of Alzheimer’s disease patients [32], or SP-C fibrils from PAP patients [9]. At zero time, no aggregates were observed, but, after 7 or 14 days SP-C had aggregated into amyloid-like fibrils, as seen by electron microscopy (Figures 4A and 4B). MALDIMS confirmed the presence of SP-C in the aggregates (results not shown). In the presence of CTC at a molar ratio of approx. 0.5–1, SP-C fibril formation was fully prevented after 7 days (Figure 4C) and after 14 days incubation (results not shown).
ProSP-C self-guards against amyloid formation

A surfactant preparation containing phospholipids and an SP-C analogue with a polyleucine stretch replacing the polyvaline part [33] was incubated in the same manner as described above. In this case, no amyloid fibrils were observed by electron microscopy (Figure 4D), and the presence of CTC had no effect (results not shown). This is relevant with regard to the experiments with polyleucine-substituted proSP-C described below.

MALDI-MS (Figure 5) shows that recombinant CTC binds to synthetic SP-C which is non-helical [30], but not to a polyleucine analogue which is helical [34] under all conditions analysed. Complexes between CTC and one, two or three SP-C molecules were observed. Recombinant CTC on its own forms dimers and trimers (Figure 5A) [35], but binding to non-helical SP-C seems to reduce CTC oligomerization (compare the amounts of CTC dimers and trimers in Figures 5A and 5B).

These results show that, in the presence of phospholipids, SP-C, but not a polyvaline → polyleucine analogue, can assemble into amyloid fibrils, that fibril formation is prevented by the presence of CTC, and that recombinant CTC binds to non-helical SP-C, as shown previously for the polyleucine peptide K2V7K2 [24].
Figure 4  CTC prevents SP-C amyloid fibril formation

Negatively stained transmission electron micrographs of material from SDS-insoluble pellets of (A) SP-C incubated for 7 days, (B) SP-C incubated for 14 days, (C) SP-C incubated for 7 days in the presence of CTC, and (D) polyleucine-substituted SP-C incubated for 14 days. (B) is enlarged to show the fibrillar appearance of SP-C aggregates. Scale bars, 100 nm.

Figure 5  CTC binds non-helical SP-C

MALDI mass spectra of (A) a mixture of 11 pmol of polyleucine-substituted SP-C and 3 pmol of CTC, and (B) a mixture of 11 pmol of non-helical SP-C analogue and 3 pmol of CTC. In (A), peaks corresponding to singly charged monomer of CTC, doubly charged trimer of CTC, singly charged dimer of CTC and singly charged trimer of CTC are labelled. In (B), peaks corresponding to complexes between a singly charged monomeric CTC and one, two or three SP-C, and a singly charged dimer of CTC and one or two SP-C are labelled.

Effects on proSP-C\textsuperscript{L188Q} aggregation by modulation of its transmembrane amino acid sequence

The data above imply that the polyvaline region of (pro)SP-C is unstable and can convert from α-helix into β-sheet aggregates, and that CTC prevents it from aggregating. Therefore, we analysed how replacement of polyvaline with a less aggregation-prone polyleucine sequence (cf. Figure 4) affects aggregation of proSP-C\textsuperscript{L188Q}. Transient expression of proSP-C(Leu) in HEK-293 cells resulted in processing of the proprotein to the mature peptide, an event that occurs in the lysosome. The level of proprotein and mature peptide was similar to wild-type proSP-C (Figure 6A, lanes 1 and 2) and the rate of processing (estimated from pulse-chase experiments, not shown) was likewise similar for the two proproteins. Thus substitution of polyleucine for the polyvaline region of proSP-C (Figure 1) did not affect expression, ER export or processing of proSP-C. To determine whether polyleucine substitution would promote ER export and processing of mutant proSP-C, the L188Q mutation was introduced into proSP-C(Leu) to generate proSP-C(Leu)\textsuperscript{L188Q}. Mutant proSP-C accumulated in HEK-293 cells (Figure 6A, lanes 3 and 4) compared with cells expressing wild-type proSP-C (Figure 6A, lanes 1 and 2) regardless of the presence (Figure 6A, lane 4) or absence (Figure 6A, lane 3) of the polyleucine substitution. The mutant proprotein was not processed to mature peptide, consistent with retention in the ER (Figure 6A, upper panel). Importantly, upon transient transfection of proSP-C\textsuperscript{C\textsubscript{138Q}} or proSP-C(Leu)\textsuperscript{C\textsubscript{138Q}} into HEK-293 cells, only the former resulted in Congo Red-positive inclusions (results not shown) and formation of large SDS-insoluble methanoic acid-soluble proSP-C aggregates (Figure 6B). The Congo Red-positive spots obtained after transient expression of proSP-C\textsuperscript{C\textsubscript{138Q}} were not as bright as those found after stable expression (results not shown). The presence of SDS-insoluble monomeric proSP-C(Leu)\textsuperscript{L188Q} (Figure 6B) is compatible with aggregation without formation of amyloid-like deposits. In line
with decreased levels of aggregated protein, proSP-C(Leu)L188Q transfection resulted in increased levels of SDS-soluble protein relative to the polyvaline proSP-C(Leu)188Q (Figure 6A, lanes 3 and 4). However, the half-lives of soluble proSP-C(Leu)188Q and proSP-C(Leu)L188Q are similar, as determined from pulse–chase experiments (results not shown).

These experiments indicate that formation of Congo Red-positive inclusions and large SDS-insoluble aggregates of proSP-C carrying the mutation L188Q requires a polyvaline transmembrane region. ProSP-C(Leu)L188Q, like proSP-C(Leu)188Q, was not processed to a 4 kDa mature peptide (Figure 6A), and both proteins were degraded with similar kinetics. This indicates that substitution of polyleucine for the polyvaline transmembrane domain prevents aggregation, but does not allow proSP-C(Leu)188Q to escape ER quality control.

**DISCUSSION**

The present study shows that two proSP-C mutations associated with familial forms of ILD in humans, L188Q and ΔExon4, give rise to intracellular Congo Red-positive inclusions when expressed in HEK-293 cells. The L188Q mutation appears to give rise to such inclusions more efficiently than the ΔExon4 mutation. The data also suggest that the L188Q mutation gives rise to the formation of amyloid-like inclusions by an indirect mechanism, since it requires the presence of a polyvaline transmembrane segment in proSP-C. These data are of interest with regard to ILD pathogenesis and anti-amyloid mechanisms.

The C-terminal part of proSP-C contains a recently discovered Brichos domain. Sequence alignments indicate that the Brichos domain of proSP-C covers residues 94–197 and this is supported by limited proteolysis experiments [35]. One of several suggested functions of the Brichos domain is a chaperone-like role [10], but this has so far not been shown experimentally. Mutations in the SFTPC gene that are associated with ILD are found in three different regions of proSP-C. First, a majority of the mutations are found within the Brichos domain, and these appear to lead to a more severe phenotype, with decreased or no mature SP-C and formation of cytotoxic protein aggregates [11]. Secondly, mutations localized in the region between the Brichos domain and the transmembrane domain are mainly associated with altered intracellular trafficking [18]. Thirdly, one mutation has been found in the mature peptide and the phenotype associated with it has not been well characterized, but ER retention has been suggested [11]. HEK-293 cells that stably express proSP-C(Leu)L188Q and proSP-CΔExon4 (located in the Brichos domain), but not proSP-CΔExon4 (located between the Brichos domain and the transmembrane domain) show Congo Red-positive depositions and abundant SDS-insoluble aggregates (Figure 2). This supports the hypothesis that different mechanisms are involved in pathogenesis of ILD linked to proSP-C mutations.

After transfection of HEK-293 cells stably expressing proSP-C(Leu)L188Q with a CTC-KDEL construct, Congo Red-positive inclusions and SDS-insoluble/methanolic acid-soluble aggregates are reduced, a bimolecular complex is formed, and an increased half-life of the SDS-soluble form of proSP-C(Leu)L188Q, as well as CTC, is seen (Figure 3). This shows that CTC can prevent aggregation of mutant proSP-C in the ER and that CTC can function in trans, consistent with a chaperone function. Moreover, SP-C can aggregate into amyloid fibrils, but this is prevented by CTC (Figure 4). CTC binds to monomeric and oligomeric non-helical SP-C in solution (Figure 5), and in the presence of phospholipids [24], but recombinant CTC(ΔExon4) does not bind non-helical SP-C [24]. These data imply that CTC binds to unfolded SP-C and that a function of CTC may be to prevent unfolded polyvaline segments of proSP-C from aggregating into amyloid-like deposits during biosynthesis (Figure 7). They suggest further that loss of this chaperone function by the ILD-associated mutation L188Q may contribute to formation of amyloid-like intracellular inclusions.

Mechanisms that prevent protein aggregation reduce ER stress and promote cell survival. Examples of intramolecular features include proline residues localized N-terminally of transmembrane helices, which promote helix formation by disfavouring alternative intermolecular hydrogen-bonding [36], localization of charged residues in edge β-strands to prevent intermolecular β-sheet formation [37] and structural ‘gatekeepers’ (e.g. charged side chains) that prevent unfavourable interactions by interrupting contiguous stretches of hydrophobic residues [38]. In addition to these mechanisms, molecular chaperones afford an intermolecular mechanism for prevention of aggregation [20]. Molecular chaperones include several families of proteins that prevent misfolding by interacting with folding intermediates, thereby preventing aggregation and allowing the proteins to fold correctly. Chaperones have little apparent substrate specificity and are, as a rule, promiscuous, recognizing common features of unfolded proteins such as exposed hydrophobic patches. The extracellular protein clusterin, also known as apolipoprotein J, can prevent amyloid formation by binding to prefibrillar species, and it may thus confer protection to extracellular amyloid formation [39,40]. SP-C, by design, has a high tendency to misfold and aggregate into amyloid fibrils, mediated by its polyvaline domain [41]. The polyvaline domain constitutes a regular α-helix in native SP-C,
but is predicted to form a β-strand by algorithms that are based on information largely from water-soluble proteins [7]. The efficiency of folding of transmembrane helices correlates with their helical propensity in aqueous solution; polyleucine segments are folded into compact, possibly helical, structures already in the translocon, whereas polyvaline has a more extended conformation [42]. SP-C is highly inefficient in forming helical structures, in contrast with polyvaline→polyleucine substituted variants [8,30,33,34]. It is therefore likely that formation of the proSP-C polyvaline transmembrane helix is comparatively inefficient [24]. In support of this suggestion, the L188Q mutation of the proSP-C polyvaline transmembrane helix is comparatively ineffective [8,30,33,34]. It is therefore likely that formation of amyloid-like deposits of proSP-Clec, iso is mediated by the polyvaline segment localized approx. 140 residues N-terminal to the mutation.

The concept of pro-sequence-assisted protein folding was first demonstrated for serine proteases, but has been found in a number of proteins, including proinsulin and certain growth factors [43]. These pro-sequences are highly substrate-specific and, in many cases, act like true catalysts for folding. The segments vary in length ranging from ten residues up to 800, and are typically cleaved off after folding has occurred [43]. Covalent linkage of the pro-sequences to the mature part is not essential for correct folding; some pro-sequences function in trans when added as separate polypeptide chains [44]. ProSP-C appears to represent an example of pro-sequence-assisted folding, and it remains to be clarified whether it works intra- and/or inter-molecularly. To elucidate whether CTC can catalyse formation of the proSP-C transmembrane helix or whether it only prevents aggregation of non-folded variants also warrants further studies.

The issue of interactions between wild-type and mutant protein is of interest in relation to amyloid disease. Tissue deposits containing fibrils from patients with systemic lysozyme amyloidosis contain only mutant lysozyme, even though all patients analysed have been heterozygotic, with wild-type as well as amyloidogenic mutants in serum [45]. In contrast, proSP-C mutations, at least those in the Brichos domain, give rise to a dominant-negative phenotype, as all mutations so far characterized are found on only one allele and are frequently associated with reduced levels of mature SP-C in the alveoli, consistent with intra-cellular trapping of wild-type protein [46]. The indirect mechanism now proposed for amyloid formation by proSP-C Brichos mutations is compatible with non-mutant protein being incorporated into fibrils, as the sites of mutation and aggregation are suggested to be separate.

In conclusion, this work suggests that the CTC, particularly the Brichos domain, has chaperone-like properties that prevent the transmembrane part of proSP-C from aggregating before it has attained helical conformation. Such an in-built protective mechanism for prevention of aggregation and amyloid fibril formation has not been described previously. Whether this CTC function is required because of the apparently unique polyvaline region of proSP-C, or whether similar cases exist, remains to be investigated. The results presented here raise questions as to whether the effects of CTC are applicable to amyloidogenic proteins other than proSP-C, and whether they represent a function also present in other proteins containing a Brichos domain.

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